UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
F. EDWARD HÉBERT SCHOOL OF MEDICINE
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

GRADUATE AND CONTINUING EDUCATION

TEACHING HOSPITALS
WALTER REED ARMY MEDICAL CENTER
NAVAL HOSPITAL, BETHESDA
MALCOLM GROW AIR FORCE MEDICAL CENTER
WILFORD HALL AIR FORCE MEDICAL CENTER

APPROVAL SHEET

Title of Thesis: "Structural organization and strain variation in the genome of Varicella Zoster Virus"

Name of Candidate: Thomas A. Casey
Doctor of Philosophy Degree
October 23, 1984

Thesis and Abstract Approved:

Paul D Rick Committee Chairperson	10/23/84 Date
Kathryn V Holmes Committee Member	10/23/84 Date
James J Kenney Committee Member	10/23/84 Date
William T. Ruyechan Committee Member	10/23/84 Date
[signature] Committee Member	10-23-84 Date

The author hereby certifies that the use of any copyrighted material in the dissertation manuscript entitled:

"Structural organization and strain variation in the genome of Varicella Zoster Virus"

beyond brief excerpts is with the permission of the copyright owner, and will save and hold harmless the Uniformed Services University of the Health Sciences from any damage which may arise from such copyright violations.

Thomas A. Casey
Department of Microbiology
Uniformed Services University
of the Health Sciences

ABSTRACT

Title of Dissertation: Structural Organization and Strain Variation in the Genome of Varicella Zoster Virus

Thomas A. Casey, Doctor of Philosophy, 1984

Dissertation directed by: John Hay, Ph.D., Professor and Vice-chairman, Department of Microbiology.

The molecular organization of varicella zoster virus (VZV) DNA was examined using restriction endonuclease analysis and physical mapping techniques. Digestion of VZV DNA by EcoRI or BglII results in four fragments which are present in 0.5 M amounts while the remaining restriction fragments are 1 M. Three terminal fragments were identified for both restriction enzymes using exonuclease III digestion. The results suggest that VZV DNA contains a terminal region which inverts in orientation. Electron microscopic examination shows that this terminal invertible segment is approximately 13.5 Mdal in size and consists of 4.3 Mdal of unique sequence DNA that is bound by 4.6 Mdal inverted repeat sequences. Thus VZV DNA can be divided into two covalently linked segments. The short segment is located at one terminus, can invert in orientation relative to the long segment and is bounded by inverted repeats.

Examination of VZV DNA isolated from lesion fluids of patients with chickenpox or shingles or from virus grown in vitro all show strain

specific differences in the migration of several restriction fragments which are stable in vitro. One difference appeared to map to the inverted repeats, because restriction fragments which contain these repeats increase or decrease in size by approximately the same amount relative to the size of the same fragments in other strains. Two additional variable regions were identified which mapped to the middle of the long unique segment. To characterize these strain variations, all the EcoRI restriction endonuclease fragments from four independent isolates of VZV were molecularly cloned. Recombinants containing variable fragments from each of the three regions in all four strains were initially identified by colony hybridization and were confirmed by Southern blots using specific probes. Restriction endonuclease mapping and electron microscopic heteroduplex analysis of the variable regions suggest that they all result from insertion or deletion at a single specific site in each variable region. The EcoRI G strain difference, which was unstable in the recombinant, was mapped to a 1200 to 1500 bp region located near one end of the G fragment. The EcoRI P strain difference was mapped to a region near the middle of the P fragment and results from a different copy number of a 35 bp tandemly-repeated DNA sequence. The cloned VZV DNA fragments which contain the repeat sequence variation (EcoRI E) were further subcloned and the DNA sequence of this variation determined. A 27 base pair repeat sequence was found which is tandemly repeated a characteristic number of times for each strain (from 6 to 14 in the strains examined).

These strain-specific VZV DNA variations can be, and have been, used in studies of the epidemiology of varicella and zoster infections.

STRUCTURAL ORGANIZATION AND STRAIN VARIATION IN THE GENOME OF VARICELLA ZOSTER VIRUS

by

Thomas A. Casey

Dissertation submitted to the Faculty of the Department of Microbiology
Graduate Program of the Uniformed Services University of the
Health Sciences in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy 1984

TO DEBBIE

AND NATHAN

WITH LOVE

ACKNOWLEDGEMENTS

I would like to thank the members of the Department of Microbiology and my commmittee for their encouragement, help and patience during the preparation of this disseration.

Special thanks go to my collaborators: Dr. Steve Straus, for providing strains and advice, Dr. Bill Ruyechan and Jim Remenick for collaboration in the electron microscopy studies, Dr. P.R. Kinchington, for collaboration on the EcoRI G fragment, to Mike Flora and Mark Wellman for their technical assistance and to Drs. Mike Bartkoski, John McGowan and Mike Wathen for many enlightening discussions.

To Dr. Jack Wohlhieter, for encouraging me to enter graduate school.

To Dr. Iain Hay, for the training, advice, guidance and friendship he has given me.

Finally, to my family, for their patience, understanding and love.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Varicella zoster virus (VZV), one of the five human herpesviruses, causes two clinically distinct diseases. Primary infection results in the common childhood disease varicella or, as it is more commonly known, chickenpox. This is currently the second most frequently reported infectious disease in the United States (Annual Summary, Morbid. Mortal. Weekly Rep. 29:3, 1980). More than 95% of the population has been infected before reaching adulthood (Gershon et al., 1976). Following the inital infection, the virus becomes latent and after a generally long and variable time can recur and cause a very painful condition known as shingles or zoster.

Varicella remains the only major childhood disease for which a vaccine is not generally available. Interest in VZV has recently increased because of the development of a live attenuated VZV vaccine by Takahashi et al. (1975). This vaccine has been successful in preventing varicella in clinical trials with normal and immunocompromised individuals (reviewed by Gershon, 1980). This is an important development, particularly for immunocompromised childen who are at great risk of life-threating complications of chickenpox and zoster infections (Dolin et al., 1978). There remain several important questions regarding the use of this vaccine, however, such as the ability of the vaccine virus to reactivate, the nature and duration of protective immunity and the possible oncogenicity of VZV, all of which should be answered before routine immunization against VZV can be advocated.

The clinical and epidemiological aspects of VZV infections are well characterized. Although VZV was successfully grown in tissue

culture in 1953 by Weller, genetics and molecular studies have lagged far behind those of other herpesviruses, because of the difficulty of growing VZV in vitro. Thus, many basic questions concerning the pathogenesis, immunology and molecular biology of VZV remain unanswered.

Varicella

The origin of the term "chickenpox" is not clear. Scott-Wilson (1978) suggests that it may have been named for the itching associated with the chickenpox rash. The old English word for itch is "gican" (with a soft G) which sounds very much like the word for chicken "ciecen" and perhaps these two words became confused. "Chickenpox" might also have been derived from the French "chiche" and the Latin "cicer" which refers to a plant, the chick-pea, whose seeds resemble the skin vesicles which are characterisic of varicella infection (Jeul-Jensen and MacCallum,1972; Taylor-Robinson and Caunt, 1972). "Varicella" is derived from the Latin "varius" meaning various or mottled (Taylor-Robinson and Caunt, 1972). The same Latin root is also the origin of variola (smallpox). Varicella, the irregular diminutive of variola, may have been used to distinguish the mild chickenpox from the more deadly smallpox; these two diseases were, and still are, occasionally confused (CDC, Morbid. Mortal. Weekly Rep. 29:193, 1980; ibid. 32:490-491, 1983).

Varicella was not clinically distinguishable from smallpox until Heberden's work in 1767 (Gordon 1962). The distinction was based on differences in the distribution of the vesicles in varicella and smallpox. The vesicles in chickenpox are more numerous on the trunk of the body while smallpox lesions are more numerous on the extremities.

Tyzzer (1906) was able to distinguish between smallpox and chickenpox based on histological examination of chickenpox vesicles. Tyzzer identified eosinophilic inclusions in the nucleus and cytoplasm and multi-nucleated giant cells in vesicles from cases of chickenpox. These are not seen in smallpox vesicles. In addition, Tyzzer could not produce characteristic smallpox lesions by inoculation of varicella vesicle fluids into monkeys or in the rabbit cornea, both experimental models of smallpox. More recently, electron microscopic examination of vesicle fluid yielded the definitive differentation between the brick-shaped poxvirus and the VZV herpesvirus particle (Cruickshank et al., 1966).

Chickenpox is a highly infectious systemic viral disease. The incubation period can range from 7 to 23 days but is usually about 14 days (Juel-Jensen and MacCallum, 1972). The attack rate is very high in susceptibles (90 to 95%), particularly household contacts and playmates (Ross et al., 1962). There may be prodromal symptoms such as fever but they are often so mild that they are not noticed; this is followed by a maculopapular rash which quickly vesciculates and then scabs over in 3 to 4 days (Gordon, 1962). The infection is passed from person to person by direct contact or droplet nuclei, and there is evidence that individuals incubating varicella may be infectious before the rash develops (Gordon and Meaders, 1929; Takahashi, 1983). There is a seasonal difference in the number of chickenpox infections with most cases occurring during the winter and spring (Preblud and D'Angello, 1979).

Typically, varicella infection occurs in children and is generally mild and self-limiting. Chickenpox can be more severe in

neonates (Overall and Glasgow, 1970), adults (Preblud, 1981) and immunocompromised children (Feldman et al., 1975), and complications occur most frequently in these groups. These complications, such as pneumonia, hepatitis and meningitis, can be serious enough to be life-threatening (Brunell, 1979). Congenital varicella infections, which occur during the first six months of pregnancy, have also been reported to have severe consequences, such as malformed limbs and central nervous system damage (Weller, 1983b).

Data suggest that nearly all children in the United States seroconvert before adolescence (Gershon et al., 1976; Schydlower et al., 1984). However, this is not true in less developed or tropical countries. In West Bengal, Somalia and Israel, epidemological studies show that chickenpox frequently occurs in young adults (Singha, 1976; Jezek et al., 1981; Leventon-Kriss et al., 1978). The reason for this difference is not known. The shift in age distribution places immigrants to temperate, developed countries, particularly pregnant woman and their fetuses, at great risk of varicella-related complications (Gershon et al., 1976; Lenventon-Kriss et al., 1978).

Hope-Simpson (1965) proposed that the pathogenesis of varicella infection is similar to the mousepox model (Fenner, 1948). The virus invades and replicates locally in the respiratory tract. This is followed by viremic spread and replication in reticuloendothelial cells, and then a second or multiple viremias occur with invasion of the skin, local replication and vesicle formation. Although the epidemiological evidence is suggestive, this pathogenesis model of primary VZV infection has not been proved; VZV is very infrequently isolated from the blood of chickenpox patients (Feldman and Epp, 1976). The infection is thought

to be spread by the airborne route (Thomson and Aberd, 1916; Leclair et al. 1980) however, virus is infrequently isolated from the respiratory tract (Nelson and Geme, 1966) and has only been reported to be isolated from throat washings on one occasion (Gold, 1966). This inability to isolate VZV from the blood or throat of patients with chickenpox may be due to difficulties in detecting small numbers of infectious virus in these specimens using the inefficient cell culture systems which are available for VZV.

Infection with VZV is thought to produce life-long immunity to reinfection so that second cases of chickenpox do not occur. A recent report suggests this may not be entirely true. Gershon et al. (1984) have described several cases of what appeared to be varicella which may have been due to exogenous reinfection. This group found 8 patients who had detectable antibody titers 3 days to 6 months prior to onset of a second episode of chickenpox. One patient, examined in detail, was a child with leukemia who had been vaccinated with the live attenuated VZV vaccine 10 months before developing a varicella-form rash. VZV DNA isolated from this case was digested with restriction endonucleases and compared to digestion patterns of the vaccine strain and wild type VZV DNA. It was concluded that this isolate was a wild type strain of VZV and that, therefore, this patient had a second case of chickenpox. However, close examination of the restriction endonuclease data shows a missing BglI fragment (BglI A). This fragment was identified by Martin et al. (1982) as being specifically missing in only the vaccine strain. Consequently, there would seem to be some doubt whether this is really exogenous reinfection or if recurrence of zoster is due to the VZV vaccine virus latent in this patient. Perhaps this case represented

atypical disseminated zoster which occurred because of this patient's immunocompromised condition. Thus the possiblity of chickenpox occurring because of exogenous reinfection remains unresolved.

Zoster

The recurrent infection of VZV is called zoster or shingles. The name "zoster" comes from the Greek word "zona" which means "a girdle;" "shingles" is from the Latin "cingere" which also means "girdle" (Taylor-Robinson and Caunt, 1972; Jeul-Jensen and MacCallum, 1972). Both terms refer to the typical dermatomal distribution of zoster vesicles which often surround the trunk of the body like a girdle. The outbreak of vesicles is usually limited to one dermatome and is frequently very painful (Taylor-Robinson and Caunt, 1972). A prerequisite for developing a zoster infection is a previous encounter with VZV in a chickenpox infection (Hope-Simpson, 1965), and zoster generally occurs later in life. Hope-Simpson (1965) reported that in his general medical practice the attack rate increased sharply with age, and that, for those over 80 years old, it was 14 times higher than for those under 10 years old.

The relationship between zoster and chickenpox was first suggested by von Bokay (1909) who noticed that in a family where zoster had occurred, there frequently were cases of chickenpox shortly after in young susceptibles. Kundratitz (1925) and Bruusgaard (1932) confirmed the relationship by inoculating children with zoster vesicle fluid. These children subsequently developed chickenpox which could be naturally transmitted, as chickenpox, to other susceptible children, but not to older children, or those with a history of previous chickenpox

infection. Previous attempts by others to carry out similar experiments had variable results, perhaps because the children had previous exposure and immunity (reviewed by Tyzzer, 1906; Bruusgaard, 1932). A common etiology of varicella and zoster was confirmed, based on common tissue culture characteristics of VZV isolated from zoster or chickenpox cases (Weller et al., 1958a) by neutralization (Weller et al., 1958b), complement fixation (Weller, 1953) and immunoflouresence (Weller and Coons, 1954), using convalescent antisera from patients recovering from either disease. In addition, the restriction endonuclease patterns of VZV DNA isolated from a case of zoster and chickenpox in the same patient are identical (Straus et al., 1984) and DNAs from unrelated cases of chickenpox and zoster are very similar (Iltis et al. 1977; Oakes et al. 1977; Richards et al. 1979; Straus et al. 1981, 1983; this dissertation).

Garland (1943) and Hope-Simpson (1954) first suggested that zoster was a recurrent form of chickenpox and that, like herpes simplex virus, varicella zoster virus could become latent and later reactivate to produce a distinct disease. Hope-Simpson (1954, 1965) provided support for this hypothesis based on epidemiological data from 16 years in his general medical practice and from the course of an epidemic of chickenpox on an isolated island. He was able to show that cases of zoster were not caused by chickenpox cases and also that zoster could not be caused by another case of zoster. He also noted a seasonal distribution of varicella, peaking in the spring, while zoster occurred with equal, low frequency throughout the year. In addition, in years that had large numbers of cases of varicella there was no increase in the cases of zoster. Miller and Brunell (1970) found that an anamnestic

anti-VZV antibody response following zoster infection, suggesting previous experience with VZV is a prerequsite for the occurrence of zoster.

Before zoster was known to be caused by an infectious agent, the effects of the disease were well known. Head and Campbell (1900) presented a detailed description of the neuropathology and histopathology of zoster lesions in a large number of cases, and used the dermatomal distribution of zoster vesicles to map the innervation of regions of the skin by specific nerve ganglia. Using the distribution of the zoster rash they were able to relate abnormalities of specific nerves and ganglia in these patients at death. These studies suggested that VZV remains latent in ganglia prior to reactivation.

Explantation of normal ganglia at autopsy and co-cultivation with susceptible cells in vitro have not resulted in the activation of latent VZV virus (Meurisse, 1969; Plotkin et al., 1977), a method which has been successful with HSV (Bastian et al., 1972). In acute cases, where the the patient had zoster at the time of death, VZV has been shown in ganglia and satellite cells by electron microsopy, immunofloresence and co-cultivation (Shibuta et al., 1974; Bastian et al., 1974; Esiri and Tomlinson, 1972). Recent experiments have shown viral DNA in ganglia from an indivual who, at death, did not have the symptoms of zoster (Gilden et al. 1983). Hyman et al. (1983) have found VZV RNA by in situ hybridization, confined to neurons from the trigeminal ganglia taken at autopsy from an individual who also did not have zoster at the time of death.

It is not clear exactly which cell type in ganglia contains the latent VZV nor is it known what the physical state of the viral genome

is in ganglion cells. Gilden et al. (1978) have shown that growth of VZV in tissue culture cells derived from brain or ganglia is not more restricted than in other commonly used cell lines, although these cultures did not contain neurons.

It is not clear how the virus is maintained in a latent state nor is it known exactly what causes it to reactivate. In addition, it is not clear if the VZV RNA detected by Hyman et al., (1983) represents specific viral transcription which might be required for the maintenance of lantency in ganglion cells. An assortment of precipitating factors have been proposed. These include arsenic, X-irradiation, trauma and immunosuppressive therapy (Juel-Jensen and MacCallum, 1972; Rifkind, 1966). It has been shown that despite the presence of specific antibodies against VZV, zoster can still occur (Miller and Brunell, 1970; Brunell el al., 1975), and depression of cellular immunity has been suggested as the primary reason for zoster recurrence (Steele, 1982; Weller, 1983a).

Growth of VZV in tissue culture

VZV was first cultured in a classic series of experiments by Weller in 1953. He inoculated monolayers of human embryonic cells with vesicle fluids isolated from patients with chickenpox or zoster. A typically focal cytopathic effect (CPE) in these cultures could be seen in 6 to 8 days. He also found (Weller, 1953) that the infection could only be passed to uninfected tissue cultures using trypsinized cells and not cell-free supernates from VZV-infected cultures. Eosinophilic nuclear inclusions were noted in VZV-infected tissue culture cells which were similar to the inclusions seen by Tyzzer (1906) in biopsies of

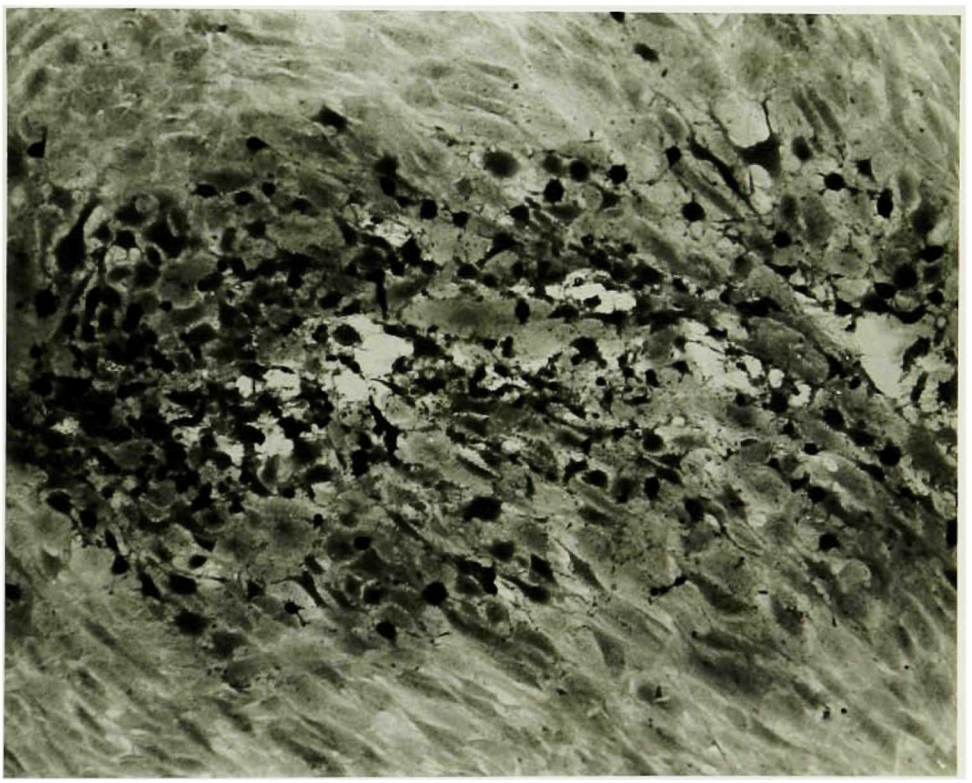
vesicles from varicella patients.

A typical focus of VZV-infected human foreskin fibroblast cells in tissue culture is shown in Figure 1. The infected cells characteristically appear as rounded, refractile cells which, as they die, detach from the monolayer and float away. These plaques form slowly, with the first signs of viral CPE seen after approximately 1 week. The plaques slowly increase in size, apparently by the spread of the virus to uninfected cells in close contact with infected cells and not by release of virus into the culture medium. Thus, it appears that VZV in culture remains cell-associated, which may be one reason why its growth in vitro is so slow. This is in direct contrast to the situation of VZV infection in people, where there are large quantities of virus present in vesicle fluids which are infectious both for tissue culture cells and for other humans (Weller, 1953; Bruusgaard, 1932).

The reason for the lack of infectivity of VZV grown in cell culture is not clear. Cook and Stevens (1968) have suggested that it may be because of an extremely labile "coat" which is degraded during passage through the cell cytoplasm from the nucleus where it is assembled. By examining infected cells by electron microscopy, these authors compared HSV-infected cells with VZV-infected cells, both by viral particle counts and by a determination of particle "intactness" in different subcellular compartments. They found that VZV looked like a normal herpesvirus in the nucleus but, that following the acquisition of the viral envelope at the nuclear membrane, and during the migration through the cytoplasm, the virions appeared to deteriorate in that they seemed to lose their core and began to be penetrated by the negative stain. By the time VZV reached the plasma membrane there were almost no

FIGURE 1

VZV-infected cell plaque. This is a photomicrograph of human foreskin fibroblasts (HFF) infected with VZV strain Oka (ATCC # VZ-795) stained with crystal violet. The infected cells have become rounded, refractile and are begining to detatch from the monolayer near the center of the plaque. The infection will spread slowly to adjacent cells which are in contact with infected cells but not to distant parts of the cell monolayer by release of virus into the medium. The magnification is approximately 900X. Micrograph provided by C.R. Roberts.



particles that contained a core. This is in direct contrast to HSV infection, where they found large quantities of completed virions in all the cellular compartments examined and in the extracellular spaces, where VZV particles were not detected. Cook and Stevens (1968) interpret this to mean that VZV is much more sensitive to some cellular degradative process than is HSV. This notion is supported by data from Shiraki and Takahashi (1972) who found large numbers of virions in infected cell supernates, penetrated by negative stain, with no infectivity, and calculated a particle to infectivity ratio of 1-$2X10^6$. This is again in contrast to HSV, which releases much more infectious virus into the culture supernates and has a particle to infectivity ratio of approximately 50 (Shiraki and Takahashi, 1972).

An alternative possibility, suggested by Achong and Meurisse (1968), is that in tissue culture, VZV is blocked in its release from the surface of infected cells. These authors observed that the surface of infected cells seem to have large numbers of VZV virions attached. These virus particles appeared to be normal and presumably infectious. This idea is supported by several experiments. Becker et al. (1965) also saw large numbers of mature, apparently intact, VZV particles on the surface of infected cells. Caunt (1963) found that small amounts of infectious VZV could be released from an infected human thyroid cell line by brief sonication, suggesting that the virions in infected cells are not degraded or defective and that the block is in viral release, although the data did not prove that these virions were those originally located on the membrane. Additional evidence, from Dumas et al. (1980), showed that infectious virus could be released from infected cells by gentle trypsin treatment and suggested that VZV might require this

treatment to be released from the surface of the infected cells. The release by trypsin treatment (Dumas et al. 1980) suggests that it may normally require a protease activity in the virus particle or at the cell surface. The cell culture systems may lack this protease which in the host could facilitate the virion release as suggested with other viral systems (Choppin and Scheid, 1980).

Abrahamsen and Jerkofsky (1981) have shown that the pesticide, carbaryl, enhances the replication of VZV in culture approximately 10 to 50 fold. This suggests that the treatment may cause an increased release of infectious virus because of an interaction between the lipid-soluble pesticide and the cellular membrane, implying that lipids in the cell membrane may also be contributing to the prevention of viral release.

It is possible that all of these explanations are correct, because the amount of virus released by trypsin treatment, sonication of infected cells or treatment with the pesticide is still very low. It may be that VZV virions are sensitive to some cellular degradative enzyme and are also blocked in release from the surface of infected cells perhaps because of a defect at the membrane in tissue culture cells.

Growth of VZV in cell culture is limited to a small number of tissue culture cell lines. These are primarily derived from human tissue, but other non-human cell culture systems have also been shown to support the growth of this virus (Takahashi, 1983). In these different cell culture systems, VZV remains cell-associated with little released virus in the culture fluids. Also, changes in cell culture conditions such as incubation temperature or medium composition do not

significantly increase the amount of released infectious VZV (Vaczi et al. 1962; Hondo et al. 1976; Schmidt and Lennette 1976; Grose et al. 1979).

Recently, Meyers et al. (1980), reported the first sucessful experimental VZV infection in an animal model. Weanling guinea pigs, inoculated intranasally or subcutaneously, seroconverted to VZV and could transmit the infection to other guinea pigs. However, these animals do not develop the vesicular rash characteristic of the human infection and it is not known if VZV can remain latent in these animals. Thus, this model may not be relevant to the pathogenesis of VZV in human infections.

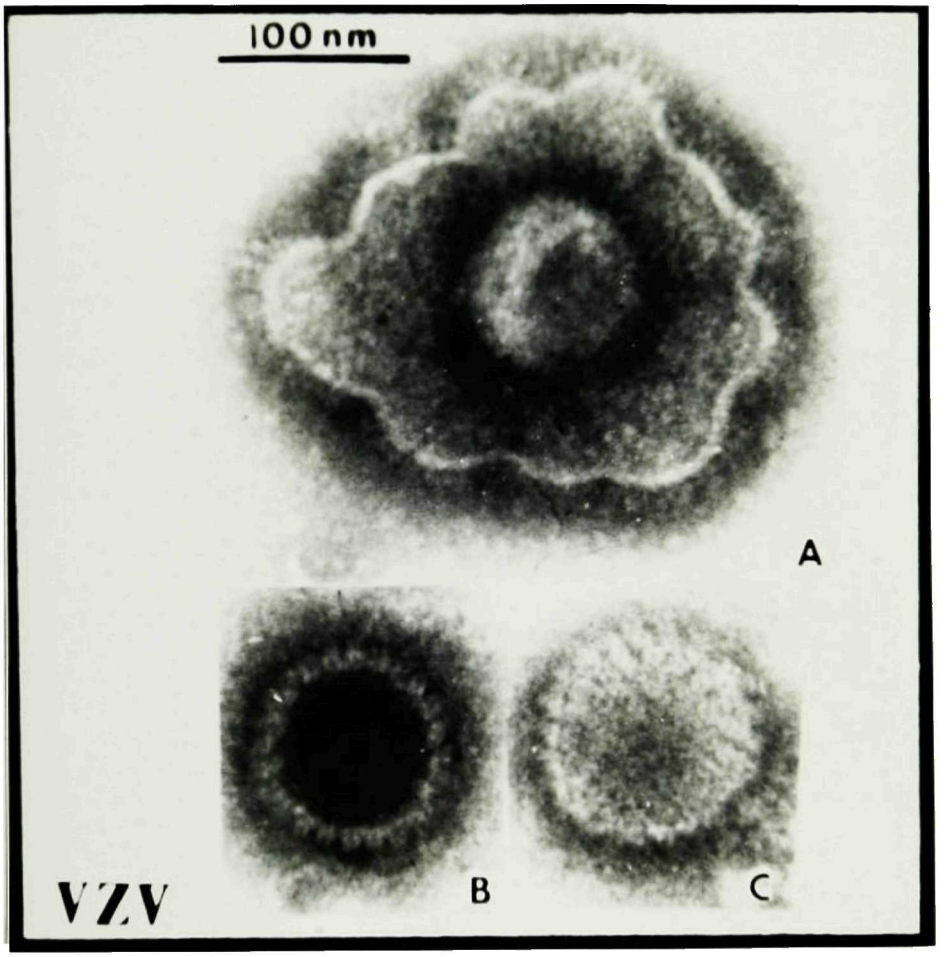
Structure and proteins of VZV

VZV has the characteristic morphology of all the members of the herpesvirus group (Almeida et al. 1962). Electron micrographs which show several of these features are shown in Figure 2. VZV has an icosahedral nucleocapsid and is surrounded by a lipid envelope which is derived from the nuclear membrane by budding (Cook and Stevens, 1968, 1970; Achong and Meurisse, 1968). The nucleocapsid is composed of 162 hollow capsomers arranged in 20 equilateral triangular faces in an icosahedron with five-fold symmetry which is similar to HSV nucleocapsids (Almeida et al. 1962; Wildy et al., 1960).

The structural proteins of VZV virions have been examined by several groups (Wolff, 1978; Asano and Takahashi, 1979, 1980; Shemer et al., 1980; Zweerink and Neff, 1981; Shiraki et al., 1982; Grose 1980; Grose et al., 1981, 1983; Grose and Friedrichs, 1982; Okuno et al., 1983; Roberts, 1984). These studies are generally difficult to

FIGURE 2

Electron micrographs of VZV virion and nucleocapsids. These are negatively stained preparations of purified virus and nucleocapsids which show the characteristic morphology of herpesviruses. VZV, like other members of the herpesvirus group, has an icosahedral nucleocapsid surounded by an envelope as shown in panel A. Panels B and C are VZV nucleocapsids, one of which has been penetrated by the stain (panel B). The magnifiction is approximately 250,000X. Micrographs provided by W.T. Ruyechan.


100 nm
A
B
C
VZV

interpret except in the last case, because of the potential contamination of the VZV virion preparations with cellular proteins released by the sonication procedures used, and because cellular protein synthesis is not turned off during VZV infection (Grose and Friedrichs, 1982). This problem was avoided by some investigators who relied on multi-step purifications which resulted in decreased yields or used immunoprecipitation with anti-VZV sera which, however, may lack specific antibodies to some of the structural components.

Despite these problems, there appears to be a consensus that approximately 30 polypeptides exist in VZV virions, which can be labeled with ^{35}S-methionine and which range in size from 12K daltons to greater than 200K daltons as determined by SDS-polyacrylamide gel electrophresis of disrupted purified virions (Wolff, 1978; Shemer et al., 1980; Shiraki et al., 1982; Roberts, 1984). Only 14 to 16 of these polypeptides can be immunoprecipitated with human convalescent VZV antiserm or hyperimmune horse serum against VZV (Wolff, 1978; Zweerink and Neff, 1981; Grose and Friedrichs, 1982). The predominant polypeptide species seems to be about 155K (range: 145K to 180K) daltons and, by analogy to other herpesviruses, it seems that this is the major nucleocapsid protein (Shemer et al., 1980; Grose et al., 1981; Shiraki et al., 1982; Roberts, 1984).

VZV glycoproteins have been identified by labeling infected cells with ^{14}C-glucosamine, followed by isolation of enveloped virions. These studies suggest that there are at least 5 glycoproteins which range in size from 45K to 140K (Shemer et al., 1980; Shiraki et al. 1982; Roberts, 1984). A subset of these glycoproteins (3 or 4) can be immunoprecipitated with anti-VZV serum (Grose, 1980; Grose et al. 1981;

Grose and Friedrichs, 1982) and are thought to be the major VZV glycoproteins. The sizes of these major VZV glycoproteins are approximately 118K, 98K, 62K and 45K (Grose et al., 1983). Okuno et al. (1983) reported similar molecular weights for these glycoproteins (115K, 100-80K, 64K and 55K). Asano and Takahashi (1979) found 13 glycoproteins which were immunoprecipitated from VZV infected cells with anti-VZV antisera raised in monkeys or guinea pigs. This larger number of glycoproteins from infected cells may contain precursors which differ in amounts of glycosylation (Okuno et al., 1983).

Shiraki et al. (1982) also noted a possible immunological relationship between VZV and HSV. Although there appears to be no cross-neutralization between VZV and HSV, they found some cross-reactivity by immunofluorescent staining with heterologous antiserm, primarily in nuclei of VZV or HSV-infected cells. They were also able to immunoprecipitate the 115K, 64K and 55K glycoproteins from VZV-infected cells with anti-HSV serum raised in rabbits. In addition, they immunoprecipitated 115-120K and 53-55K glycoproteins from HSV-infected cells with anti-VZV serum and suggested that these are the VZV equivalents of the HSV gA/gB and gD respectively.

Very few functional studies of VZV nonstructural proteins have been successful. VZV infection has been shown to induce novel (viral coded?) enzymatic activities which are similar to activities induced by other herpesviruses. Thus, a virus specific DNA polymerase (Mar et al., 1978), DNase (Cheng et al.,1979) and thymidine kinase (Dobersen et al., 1976; Ogino et al., 1977; Hackstadt and Mallavia, 1978; Cheng et al., 1979) have been identified. These enzymes have similar activities, and may have properties similar to their counterparts in HSV, but they have

not been characterized adequately. Roberts (1984) has identified several DNA-binding proteins in VZV-infected cells. The functions of these proteins are not known, but by analogy with proteins of similar size and binding activity in HSV-infected cells, they may have important roles in viral replication.

Attempts at locating the genes for VZV proteins have not yet been successful using techniques such as isolation of temperature-sensitive mutants and marker rescue, which have been successfully used to map proteins in HSV (Stow et al., 1978). DNA sequence analysis has yielded some information on potental locations for several VZV proteins (Davison 1983, personal communication) but final proof of gene assignments awaits either RNA transcript mapping and in vitro translation or the use of mutants and marker rescue.

Structure of HSV DNA

Sheldrick and Berthelot (1974) first identified inverted repeat sequences in the HSV genome by electron microscopic examination of denatured and renatured HSV DNA. They found that intact single-stranded HSV DNA could form structures which consisted of two single-stranded circles of different size joined together by double-stranded DNA. The conclusion from these observations is that the two single-stranded circles consist of unique sequence DNA which are called U_L (for unique long region) and U_S (unique short region). The double-stranded DNA that joins the two circles forms because there are inverted repeats at the ends of both the U_L and U_S sequences. That is, at both ends of the DNA are sequences which are repeated internally in an inverted orientation. These repeated sequences are called TR_L and IR_L (terminal and internal

repeat sequences of the L region) and TR_S and IR_S (terminal and internal repeat sequeces of the S region).

HSV DNA was also found to be terminally redundant (Sheldrick and Berthelot, 1974; Grafstrom et al., 1974). This means that there is a sequence which is directly repeated at both ends of HSV DNA. The terminally redundant sequence was shown by limited digestion with DNA exonuclease, which removes bases progressively from only one strand at the ends of DNA molecules. Treatment of HSV DNA with this enzyme uncovers nucleotide sequences at one end of the DNA which are complementary to sequences on the other strand at the opposite terminus. Following exonuclease treatment, these complementary sequences at the termini can base-pair and allow HSV DNA to form circular molecules which can be seen by electron microscopy. Sheldrick and Berthelot (1974) mistakenly concluded that all four of the inverted repeat sequences were identical, except for their relative orientation, and that the entire repeat sequences were represented by the terminal redundancy. In fact, the terminal redundancy was, at that time, estimated to be between 1 and 3% ($1\text{-}3X10^6$ daltons) (Grafstrom et al., 1974) and was too small to represent the entire TR_L/IR_L and TR_S/IR_S sequences estimated to be about $4.5\ X10^6$ daltons. (Sheldrich and Berthelot, 1974). Using restriction endonuclease mapping of the terminal and internal repeat sequences, Wagner and Summers (1978) have found that the terminally redundant sequences, which they determined, more precisely, to be approximately 280 bp in size, are also repeated internally in HSV DNA, between the repeat sequences at the junction of U_L and U_S, in an inverted orientation and can occur one or two times in this location. This sequence has been termed the "a" sequence. Recent

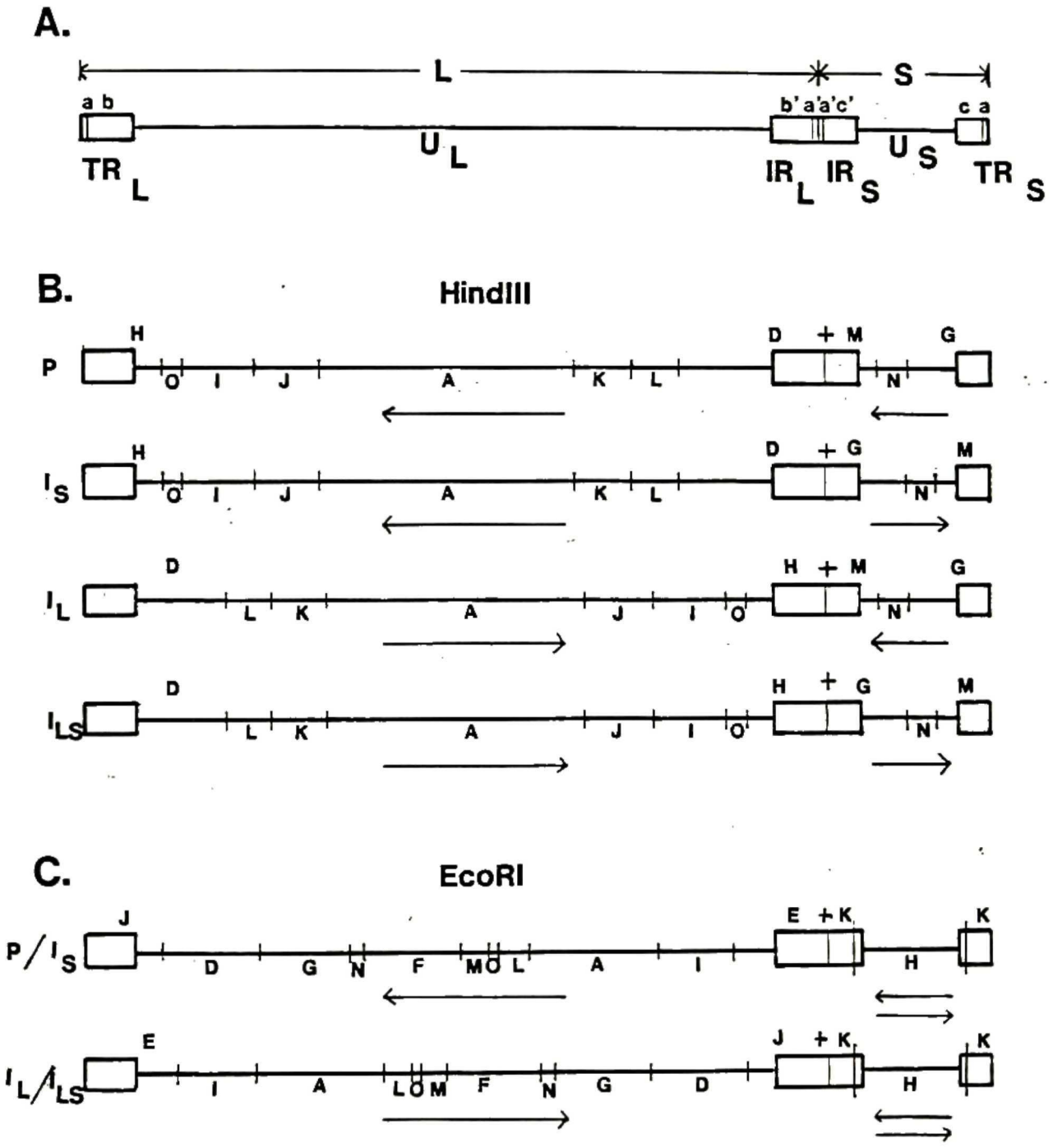
nucleotide sequencing of the "a" sequence suggests that it is actually 400 bp in length but can vary in different isolates of HSV (Davison and Wilkie, 1981; Mocarski and Roizman, 1981).

The presence of inverted repeat sequences in HSV DNA was confirmed by Wadsworth et al. (1975) who extended the original observations to show that all the inverted repeats were not identical, but only the repeats which bracket either U_L or U_S. They also found that the terminal redundancy ("a") was a subset of the sequences in each of the inverted repeats. These conclusions were based on electron microscopic measurements of double-stranded regions of intact denatured and self-renatured HSV DNA which formed structures consisting not only of two single-stranded circles joined by a double-stranded region, as seen by Sheldrick and Berthelot (1974), but also of molecules which had only one single-stranded circle per molecule. These molecules fell into two size classes and are formed by the intramolecular hybridization of the TR and IR of either the L or S segment. These could be distinguished based on the size of the single-stranded loop formed by intramolecular hybridization between either TR_L/IR_L or TR_S/IR_S which form the double-stranded regions. The double-stranded DNA stem in these molecules was not the same size in molecules with different sizes of single-stranded loops and, thus, it was concluded that the inverted repeats bounding U_L and U_S were not identical. These authors labeled the repeat sequences which bound U_L "ab" and "a'b'" (the primed letters indicate the inverted sequence). The sequences which bound U_S are labeled "a'c'" and "ca". The HSV DNA molecule can therefore be schematically represented as "ab-U_L-b'a'a'c'-U_S-ca" (Figure 3, A).

Based on the presence of two sets of inverted repeat sequences

FIGURE 3

Organization of the genome and restriction endonuclease maps of the four isomers of HSV 1 DNA. A. The sequence arrangement of HSV is shown. HSV DNA is divided into two regions, long (L) and short (S). Each of these regions consist of unique sequences (U_L and U_S) which are bounded by terminal (TR_L and TR_S) and internal (IR_L and IR_S) inverted repeat sequences. TR_L and IR_L are further subdivided into "a" and "b" and their inverted complement "a'" and "b'". The TR_S and IR_S are subdivided into "c" and "a" their inverted complement sequences "c'" and "a'". B. The HindIII restriction endonuclease maps of the four isomers of HSV are shown. The four isomers are labeled prototype (P), inverted L (I_L), inverted S (I_S) and inverted L and S (I_{LS}). As described in the text, the 0.5 M fragments are terminal (H and D at the L end and M and G at the S end) and are present in half the molecules. The 0.25 M fragments are located at the L/S junction and each is present in one isomer (D+M, D+G, H+M, or H+G). C. EcoRI restriction map of HSV. This enzyme cleaves in IR_S and TR_S and does not produce 0.25 M fragments. The 0.5 M fragments are J and E which are terminal and the L/S junction fragments J+K and E+K. The maps were redrawn from Roizman (1979).


A.
L
S
a b
b' a' a' c'
c a
U L
U S
TR L
IR L
IR S
TR S
B.
HindIII
P
I S
I L
I LS
C.
EcoRI
P / I S
I L / I LS

in HSV DNA, Sheldrick and Berthelot (1974) suggested that the two segments of DNA which are bound by these sequences could invert in orientation. This could occur by intramolecular recombination between these repeat sequences and form four isomers which differ only in the orientation of the U_L and U_S DNA segments.

This inversion of HSV genomic segments was confirmed by Delius and Clements (1976) and Hayward et al. (1975b). Both groups examined partially-denatured HSV DNA molecules by electron microscopy. This technique relies on the property of DNA to be preferentially denatured, for example by heat or alkali, at specific regions which are rich in A+T base pairs. Using denaturing conditions which do not completely separate the two DNA strands, molecules can be seen which contain single-stranded loops at characteristic and reproducible positions (high in A+T) in predominantly double-stranded DNA molecules. The positions in these molecules which remain double-stranded are relatively G+C-rich regions. Alignment of the loops and double-stranded regions in these molecules defines the distribution of A+T and G+C in a DNA molecule. Hayward et al.(1975b) examined 10 partially-denatured HSV DNA molecules and found that a histogram of the distribution of denatured sites between the repeated sequences (TR and IR), which are high G+C and remain double-stranded, was symmetrical between the repeated sequences. This is the expected result for a DNA molecule which contains sequences which invert if all isomers of the inverted molecules are present in equal amounts. Delius and Clements (1976) examined 30 partially-denatured HSV DNA molecules and found that when the molecules were aligned with each other at the high G+C rich repeated regions of IR_S and TR_S, the denatured regions of all the molecules could be divided

into four classes which occur at equal frequency. These classes differed solely in the orientation of the long and short A+T-rich regions between the high G+C-rich repeat sequences. Each of these denatured classes represents one of the four possible orientation isomers of HSV DNA. The isomers have been arbitrarily labeled as prototype (P), inverted L (I_L), inverted S (I_S) and inverted L and S (I_{LS}) (Hayward et al., 1975b).

Hayward et al. (1975b) and Clements et al. (1976) showed that HSV DNA segments invert in orientation by using restriction endonuclease digestion. Restriction enzymes which do not cut in the repeated sequences, such as HindIII in the case of HSV 1, produce different fragments depending on the orientation of the unique sequences (Figure 3, B). These fragments are those located at the termini and those which map across the IR_L/IR_S junction. These terminal and junction restriction fragments would not be present in all four of the HSV DNA isomers. The P and I_S isomers have the same terminal fragment at the L end and the I_L and I_{LS} isomers have a different terminal fragment at the L terminus due to L inversion. If each isomer is equally present in the population, then each of the L terminal fragments will be present in only half the molecules (P and I_S or I_L and I_{LS}). The same is true at the end of the S segment. The P and I_L isomers have the same terminus at the S end of the DNA molecule and the I_S and I_{LS} have a different fragment at this terminus. These two S terminal fragments, like the L terminal fragments, are only present in half the DNA molecules in the population and are seen to be 0.5 M in relation to fragments which map in entirely unique sequence DNA, and which are 1 M. Restriction fragments which map across the IR_L /IR_S junction are different in each

of the four isomers and are 0.25 M in relation to fragments from the unique sequences. These fragments can be thought of as fusions of fragments which, in other isomers, are located at the termini. Inversion of either L or S changes at least one terminal fragment and results in a new fusion fragment at the junction.

Hayward et al. (1975b) found that HindIII digestion of HSV DNA results in 15 fragments. They determined fragment molarity based on the molecular weight and the relative proportion of radioactivity for each HindIII fragment separated by gel electrophoresis. Similar results were obtained by Clements et al. (1976) using both radioactivity determinations and area measurements of the peaks for each band in densitometer tracings of stained gel negatives. These procedures show that four of the HindIII fragments are 0.5 M, four other fragments are 0.25 M and the remaining 7 fragments are 1 M. The 0.5 M fragments were found to be terminal by exonuclease digestion prior to digestion by HindIII (Hayward et al., 1975b). The four 0.25 M fragments were found to map across the IR_L/IR_S junction as predicted by the model described above (Hayward et al., 1975b; Clements et al. 1976).

Restriction endonucleases which have cleavage sites in only one of the repeat sequences of HSV 1 DNA, either in those which bound U_L or U_S but not in both, such as EcoRI (Hayward et al., 1975b) or HpaI (Clements et al., 1976) produce no 0.25 M fragments, but only four 0.5 M and several 1 M fragments. These results can be most easily explained using a specific example (Figure 3, B). The restriction endonuclease EcoRI cleaves HSV 1 DNA in the inverted repeats which bracket U_S (i.e. TR_S/IR_S). The fragments produced by EcoRI at the S terminus (EcoRI K) are the same in all four isomers regardless of the orientation of U_S and

are therefore 1 M. At the L terminus, two 0.5 M fragments (EcoRI J or E) result from the inversion of the L region as described above. The remaining two 0.5 M fragments are located at the joint (S/L) region. These are produced from the fusion of the inverted copy of the terminal EcoRI K fragment in the internal repeat, which is the same size in all four isomers, with inverted copies of either the terminal EcoRI J (in the P or I_S isomers) or EcoRI E (in the I_L or I_{SL} isomers) fragments, depending on the orientation of U_L. Similar results (no 0.25 M fragments and four 0.5 M fragments) result with a restriction enzyme such as HpaI (Clements et al., 1976) which cleaves the repeated sequences which bound U_L. In both cases, the remaining fragments are all 1 M and occur by digestion at two sites entirely within unique sequences as described above.

A restriction enzyme, such as SalI, which cleaves only once in inverted repeat sequences of both L and S (TR_L/IR_L and TR_S/IR_S) would produce only 1 M fragments (Locker and Frenkel, 1979). The terminal and junction fragments from this digest would be identical in each isomer regardless of the orientation of the unique sequences. Enzymes which cleave more than once in either of the inverted repeats, such as BamHI (Locker and Frenkel, 1979) produce 2 M fragments (one copy from each repeat) which are independent of sequence orientation.

Classification of herpesviruses based on DNA structure

Other herpesviruses were subsequently also shown to have repeated sequences and to have genomic segments which invert in orientation. The repeated sequences or orientational isomers in the DNAs of these viruses were identified by procedures analogous to those

described above for HSV. Electron microscopic and restriction endonuclease analyses have identified, and can distinguish between, herpesvirus DNA molecules which have two sets of inverted repeats, and two invertible regions, resulting in four isomers, and those which have a single set of inverted repeats and one invertible region producing two isomers. These methods can also be used to identify and position direct repeat or tandemly-duplicated sequences. Honess and Watson (1977) have divided the known herpesviruses into three classes based on the positions and polarity of the repeated sequences and the number of isomers of DNA found for each virus.

The first class has only one isomer. The DNA of this class does not invert but contains directly-repeated sequences at either end which may be tandemly-duplicated. Examples of this group are Herpesvirus ateles, Herpesvirus saimiri, channel catfish virus and Epstein-Barr virus. H. saimiri and H. ateles consist of a single long unique region, $70X10^6$ daltons in size, which is A+T rich and called the L region (meaning light); it is bound at the termini by tandemly-duplicated direct repeat sequences which are high in G+C and are called the H (heavy) region (Bornkamm et al.,1976; Kaschka-Dierich et al., 1982). The size of the repeated sequences at each end of the molecule varies between 1 and $34X10^6$ daltons because of different numbers of the monomer repeat units, each of which are approximately 1.25 Kb (Bornkamm et al. 1976). Channel catfish virus has terminally-repetitive sequences approximately $12X10^6$ daltons in size, with direct polarity, which bracket $62X10^6$ daltons of unique sequences (Chousterman et al., 1979). Epstein-Barr Virus (EBV) has tandemly-duplicated direct repeats at the termini (TR) and a large tandemly-duplicated direct repeat sequence

which is internal (IR). The TR repeat unit is approximately 530 bp and the IR repeat unit is approximately 3 Kb (Baer et al., 1984). Additional smaller repeated regions have been found throughout the EBV genome based on nucleotide sequence data (Baer et al., 1984). EBV can therefore be divided into unique long and short regions (U_L and U_S) which are bound by non-identical tandemly-duplicated direct repeats (TR and IR); U_L and U_S and do not invert in orientation.

The second class has one set of inverted repeat sequences which divide the genome into U_L and U_S seqments. The inverted repeat sequences bracket the U_S region in all such herpesvirus DNA molecules examined thus far. These inverted repeats are called IR_S/TR_S and can be identified by electron microscopic examination of denatured and self-reannealed DNA molecules which then appear as small single-stranded loops (U_S), a short double-stranded region (IR_S/TR_S) and a long single-stranded end (U_L) (e.g. Stevely, 1977). The short segment inverts in orientation resulting in two isomers. Restriction endonuclease digestion with an enzyme which does not cleave in the inverted repeats results in four 0.5 M fragments, due to inversion of U_S, and the remaining fragments are all 1 M. Two of the 0.5 M fragments are located at the S terminus and each is present in only one orientational isomer. The terminal fragments can be identified by examination of DNA which has been exonuclease-treated prior to digestion with the restriction enzyme and has lost the 0.5 M fragments (Whalley et al., 1981). The remaining two 0.5 M fragments are internal and map across IR_S sequences. Examples of viruses in this class are equine herpesvirus type 1 (EHV 1; Whalley et al., 1981; Ruyechan et al., 1982) and pseudorabies virus (PRV; Stevely, 1977; Ben-Porat et al., 1979).

Herpes simplex virus types 1 and 2 (HSV 1, HSV 2) are the prototypes of the third class of herpesviruses. These DNAs contain two sets of inverted repeats which divide the genome into two segments, both of which invert to produce four different isomers as described above. Additional examples of this class are bovine mammillitis virus (BMV; Buchman and Roizman 1978a, b), Marek's disease virus (MDV; Cebrian et al., 1982) and human cytomegalovirus (HCMV; Westrate et al., 1980).

Strain variation in herpesvirus DNA

Strain variability in the DNA restriction endonuclease digestion patterns have been seen in many different herpesviruses including HSV 1 and 2 (Skare et al., 1975; Hayward et al., 1975b; Locker and Frenkel, 1979; Lonsdale et al., 1979, 1980; Smith et al., 1981; Channey et al., 1983), HCMV (Kilpatrick et al., 1976; Huang and Huong, 1980; Pritchett, 1980), EBV (Raab-Traub et al., 1978; Dambaugh et al., 1980; Bornkamm et al., 1980; Heller et al., 1981) H. saimiri (Desrosiers and Falk, 1982) EHV 1 (Allen et al., 1983) and PRV (Ben-Porat et al., 1984; Lominiczi et al., 1984).

Two types of strain variation can be identified by restriction endonuclease analysis of herpesvirus DNA (Buchman et al. 1980). The first is the gain or loss of a restriction endonuclease cleavage or recognition site which would cause the fusion of two fragments (loss) or produce two new fragments (gain). The other type of strain variation is the increase or decrease in size of one or more specific restriction endonuclease fragments. It has been hypothesized, but not proved, that the change in mobilities of specific fragments is the result of insertion or deletion of nucleotide sequences at specific locations in

the genome (Lonsdale et al. 1980; Allen et al. 1983). Both types of DNA strain variation have been observed in all of the herpesviruses.

In HSV, new restriction sites have been used in molecular epidemiology (Buchman et al., 1978; 1979; 1980; Linnemann et al., 1978; Halperin et al., 1980; Sakaoka et al., 1984). The gain or loss of restriction sites were used in these studies because these differences appeared, in HSV, to be very stable. For example, Roizman and Tognon (1982) were unable to find a single example of the gain or loss of a restriction endonuclease site during growth of HSV in tissue culture. Alterations in specific restriction fragment mobilities, however, were common in culture and they suggested that only the gain or loss of a site is useful for molecular epidemiology of HSV infection. These studies showed that all epidemiologically-related strains were identical while unrelated strains were different, and could be distinguished using only one enzyme (Buchman et al. 1980). There are at least 14 different sites in HSV 1 DNA that have gained or lost a restriction site based on 50 different clinical isolates examined by Buchman et al. (1978).

Insertion or deletion-type strain differences are not useful in molecular epidemiology of HSV infections because they occur very frequently in culture or even in separate cloned viral isolates from the same patient (Lonsdale et al., 1980). However, they have been found to be limited to small areas of the HSV genome including the inverted repeat sequences and two or three additional regions in unique regions (Locker and Frenkel, 1979; Lonsdale et al., 1980; Roizman and Tognon, 1982).

EBV has also been shown to have insertion/deletion-type strain variations. These strain differences in EBV appear to result from

increases or decreases in the number of the tandemly duplicated sequences particularly in IR and TR (Heller et al., 1981; Dambaugh et al., 1980; Bornkamm et al., 1980).

Insertion/deletion strain variations appear to be the predominant type of variability in the genomes of PRV (Wathan and Pirtle, 1984; Ben-Porat et al., 1984) and EHV 1 (Allen et al., 1983). These strain differences are stable in culture and occur in limited regions of the unique sequences and in the inverted repeats of these viruses. The gain or loss of restriction sites apparently does not occur in EHV 1 (Allen et al., 1983) and occurs infrequently in PRV (Ben-Porat et al., 1984)

VZV DNA

VZV DNA has been purified by several different procedures. All require large numbers of infected cells, because of the poor growth characteristics of VZV in vitro. In addition, the DNA is frequently contaminated by cellular sequences because of the cell-association of VZV in tissue culture. VZV DNA has been isolated from virions prepared from infected cells by sonication (Ludwig et al., 1972), trypsin treatment (Dumas et al., 1980) and by Triton X-100 cytoplasmic extractions (Gilden et al., 1982a), followed by gradient centrifugation. These procedures have the advantage of producing highly purified VZV DNA but suffer from poor yields. VZV DNA has also been isolated from the "Hirt supernantes" of VZV infected cells (Hirt, 1967; Rapp et al., 1977; Iltis et al., 1977; Oakes et al., 1977; Richards et al., 1979) followed by sucrose gradient centrifugation. This procedure results in greater yields of VZV DNA which are, however, often

contaminated by cellular DNA. Isolation of VZV DNA from nucleocapsids purified by lysis of infected cells, treatment with DNase and glycerol gradient centrifugation prior to release of the VZV DNA by proteolytic digestion and phenol extraction, is a compromise which results in sufficient amounts of DNA and minimial cellular contamination (Straus et al., 1981).

The buoyant density of VZV DNA has been determined to be 1.705 gm/cm^3 corresponding to a G+C content of 46% by Ludwig et al. (1972) using VZV DNA isolated from virions by sonication. Rapp et al. (1977) reported a similar G+C content (47%) of VZV DNA isolated for Hirt supernates. Using thermal elution profiles, Straus et al. (1981) determined a Tm of 89.0°C for VZV DNA isolated from nucleocapsids, corresponding to a G+C of 47.8%, in good agreement with the previous determinations.

Iltis et al. (1977) observed that labeled VZV DNA from Hirt supernates of strains isolated from cases of zoster reproducibly had a slightly higher buoyant density than strains isolated from cases of varicella. Ludwig et al. (1972) had used unlabeled DNA from varicella and zoster strains and did not see differences in buoyant densities. Richards et al. (1979) showed that the difference in buoyant densities of varicella and zoster strains seen by Iltis et al. (1977) were due to an isotope effect and were not caused by changes in base composition of VZV DNA.

The molecular weight of VZV DNA was determined by Rapp et al. (1977) and Iltis et al. (1977), as 92 to $110X10^6$ daltons based on sedimentation of VZV DNA isolated from "Hirt supernates" in sucrose gradients. Smaller molecular weights of $76X10^6$ and $80X10^6$ were

determined by electron microscopic contour length measurements of VZV isolated from virions (Dumas et al., 1980) and nucleocapsids (Straus et al., 1981), respectively. The difference in molecular weights of VZV DNA estimated by these procedures is probably due to the relative inaccuracy of the sucrose gradient method and perhaps to contamination by cellular sequences in the Hirt supernates. In any case, the lower molecular weight values would appear to be the correct ones.

Oakes et al. (1977) and Richards et al. (1979), first examined VZV DNA using restriction endonucleases. These authors identified major and minor bands in these digestions but were unable to determine precise molarities because of poor resolution of fragments with similar molecular weights. They cautioned against interpreting these minor bands as an indication of sequence rearrangement. Passage of other herpesviruses at continually high MOI (e.g. HSV; Frenkel et al., 1975, 1976) does generate defective DNA with altered restriction endonuclease patterns. Oakes et al. (1977) suggested that these minor bands might result from the accumulation of defective VZV DNA because of the uncontrolled high MOI passage of VZV in culture. Actually, the MOI of VZV cannot be determined because VZV can only be passaged in culture using infected cells which contain unknown numbers of infectious virus.

Richards et al., (1979) also noted that some of the restriction fragments varied in mobility in different VZV strains while the majority of fragments did not. These variable fragments appeared to be stable during passage in culture, suggesting that they might be useful in molecular epidemiological studies of VZV infection similar to studies using DNA variation in HSV (e.g. Buchman et al., 1980)

Specific aims

When the present work was initated (1980), there was no reliable information available on the basic structure of VZV DNA; nor was the size of the DNA known with any degree of certainty. No internal structure had been described and no reliable restriction endonuclease analysis had been reported. An understanding of the structure of the genome of VZV is a necessary prelude to further work on its replication and pathogensis and, in view of its role as a human pathogen, has important medical implications.

The purpose of this work was to determine the structural organization of VZV DNA. Using restriction endonuclease and electron microscopic methods, inverted repeat sequences and sub-molar restriction endonuclease fragments were identified in VZV DNA. A comparison of these data with similar data obtained from other herpesviruses suggests that VZV DNA is divided into two regions, one of which is bounded by inverted repeats and inverts in orientation to produce two isomers. These two isomers are present in equal amounts in populations of VZV DNA molecules isolated from virus paricles.

Additional experiments were performed to identify and characterize strain-specific DNA sequence variation in the VZV genome. The results show that altered mobilites of VZV restriction endonuclease fragments occur at three discrete locations. These strain variations are stable in culture and can be useful in VZV molecular epidemiology.

The strain-variable regions were further characterized by molecular cloning of restriction fragments from these variable regions in different VZV strains, followed by analysis with restriction endonucleases, electron microscopy and DNA sequencing. The results

suggest that the DNA variations are all located at single locations and, at least in two cases, are due to variable numbers of tandemly duplicated DNA sequences. The nucleotide sequence was determined for one of these regions and was compared to similar sequences in HSV.

MATERIALS AND METHODS

Cells and Viruses

Human foreskin fibroblasts (HFF; strain USU 184 or USU 521) and VZV strain Scott were obtained from Dr. Gerald Fischer (Department of Pediatrics, USUHS). VZV strains Ellen, Oka and Webster were obtained from the American Type Culture Collection. Strain Ellen (ATCC VR-586; Brunell and Casey, 1964) was originally isolated from the vesicle fluid of a child with chickenpox in Atlanta, GA. VZV strain Oka (ATCC VR-795; Takahashi et al., 1975) was isolated in Japan from a 3 year old boy with chickenpox and is presently in clinical trials as a live attenuated vaccine (Weibel et al., 1984). The VZV strain Webster (ATCC VR-916) was isolated by S. Plotkin and is also being considered for use as a vaccine strain. Other VZV strains were obtained as clinical specimens of vesicle fluids of patients with zoster or chickenpox from Dr. Stephen Straus (Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, Bethesda, MD).

Cultures of HFFs were grown to monolayers in 32 oz. prescription bottles (100 cm^2) or in 850 cm^2 roller bottles with Eagle's minimal essential medium supplemented with 10% v/v fetal calf serum (Flow Laboratories, McLean, VA) and 50 µg/ml gentamycin (United States Biochemical Corp., Cleveland, OH) at 37°C in 5% CO_2. Confluent monolayers from either flasks or roller bottles were dispersed into a cell suspension using trypsin-versene (0.175% w/v trypsin, 0.5% w/v ethylenediamine tetraacetic acid (EDTA) (tetra-sodium salt) and these cells were used to seed up to 6 new flasks or roller bottles.

VZV was generally passed in culture as infected cells. VZV-

infected monolayers showing greater than 90% cytopathic effect (CPE) were trypsinized and used to infect fresh cells at a 1 to 6 ratio. The first signs of CPE due to VZV infection under these conditions could be seen after five days and complete destruction of the monolayer occurred approximately two weeks post-infection.

Isolation of Virus

VZV was isolated from infected cells by a modification of a procedure published by Dumas et al. (1980). HFF monolayers were harvested when 90 to 100% of the cells showed CPE. The medium was removed and the infected monolayer was washed with trypsin-versene and then incubated at 37°C in a small amount of the trypsin-versene solution until the cell sheet was removed from the culture vessel. The cells were spun at 4000 rpm for 10 minutes in an IEC refrigerated centrifuge using an 8 place swinging bucket rotor. Cell pellets were saved, at -70°C, for preparation of nucleocapsids. The supernate was pooled with the culture media and spun in a SW 27 Beckman rotor at 20,000 rpm for 1 hour. The supernate was discarded and the virus pellet was resuspended in a small amount of Tris-buffered saline (TBS; 0.05M Tris (hydroxymethyl) aminomethane hydrochloride, 0.15M NaCl, pH 7.4) and layered onto a 5 to 55% sucrose gradient in TBS and spun at 35,000 rpm for 3 hours in a Beckman SW 40 rotor. These gradients generally contained two viral bands. The lower band contained primarily enveloped virions while the upper band was unenveloped as determined by negatively stained aliquots examined by electron microscopy. Both bands were pooled and stored at -70°C, and used for the preparation of VZV DNA.

Isolation of VZV nucleocapsids

VZV nucleocapsids were isolated using a modification of a method developed for herpes simplex virus by Denniston et al. (1981). Infected cell pellets and isolated infected nuclei were resuspended in 0.25 ml TE buffer (10 mM Tris, 1 mM EDTA, pH 8.1) per 32 oz. flask of cells and were dispersed in a bath sonicator (Heat Systems Ultrasonics, Plainview, NY) until a uniform suspension was obtained. To this suspension an equal volume of 2X lysis buffer was added (1X: 0.5% v/v nonidet P40, 3.6 mM $CaCl_2$, 5 mM $(CH_3COO)_2Mg$, 125 mM KCl, 0.5 mM EDTA, 6 mM 2-mercaptoethanol and 0.5% w/v deoxycholate). Then 25 µg DNase I and 25 µg RNase (Sigma Chemical Co., St. Louis, MO) were added to each ml of the lysate and incubated for 30 minutes at 37°C. The cell lysate was then extracted with 0.5 volumes of genesolv-D (trichloro-trifloroethane, (Freon-TF), Du Pont Chemical Company, Wilmington, DE) by shaking one minute, and then spinning for 10 minutes at 1000 rpm, 4°C, in an IEC centrifuge. The aqueous phase was removed and layered on a discontinous 5 to 40% glycerol gradient made in 1X lysis buffer and spun at 40,000 rpm for 40 minutes in an SW 40 rotor. Nucleocapsids were found in the pellet and were used as a source of VZV DNA.

Isolation of VZV DNA

VZV nucleocapsids or virions were incubated at 37°C in 1% w/v SDS, 1mg/ml proteinase K, overnight. The DNA was extracted two times with phenol saturated with TE, one time with phenol-chloroform (1:1) and then two times with chloroform. The DNA was precipitated by adding 0.1 volumes of either 3 M CH_3COONa or 2.5 M CH_3COOLi, 0.5 M $(CH_3COO)_2Mg$, pH 4.9 and two volumes of ethanol at -70°C for one hour to overnight. The

DNA precipitate was pelleted by centrifugation in a microfuge (Beckman model B) for 5 minutes. The alcohol was removed, DNA pellets were washed in 70% ethanol to remove excess salt and were centrifuged again. All traces of ethanol were removed under vacuum and the DNA was resuspended in a small amount of TE. This method typically yielded 1 to 5 µg of VZV DNA per roller bottle of infected cells. VZV DNA was also isolated from patient vesicle fluids by dilution with TE buffer (to at least 250 µl), addition of SDS to 1% w/v, proteinase K to 1 mg/ml, and extraction with phenol-chloroform and alcohol-precipitated as described above. Yields of VZV DNA from vesicle fluids prepared in this way were highly variable and seemed to depend on the age of the vesicle from which the sample was taken. Older vesicles seemed to contain lower amounts of VZV DNA than vesicles which were 1 or 2 days old, and most of the DNA from the older vesicles seemed to be more degraded than DNA in the younger vesicles.

Isolation of plasmid DNA

Two methods were used for the isolation of plasmid DNA. The first method was used primarily for screening VZV-plasmid recombinant clones for the presence of specific VZV restriction endonuclease fragments using 5 ml cultures. This method was also found to be useful for preparation of larger quantities of DNA (50 ml cultures) used in the restriction endonuclease mapping studies. The method is based on a procedure published by Birnboim and Doly (1979) and modified by Maniatis et al. (1982). Individual colonies containing plasmids were picked and grown in 10 ml of L broth (10 gm tryptone, 5 gm NaCl, 5 gm yeast extract / 1 H_2O, pH 7.4) containing the appropriate selective antibiotic

(generally ampicillin at a concentration of 100 µg/ml or tetracycline at 20 µg/ml) overnight with shaking at 37°C. The bacteria were pelleted at 3600 rpm in an IEC centrifuge for 10 minutes and resuspended in 4 ml of sterile 10 mM $MgCl_2$. Half of this bacterial suspension was diluted in 2 ml sterile 80% glycerol and stored at -70°C to be used as a stock. The remainder of the suspension was repelleted as above and resuspended in 200 µl of lysozyme solution (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0 and 2 mg/ml lysozyme) and incubated on ice for 30 minutes. Then 400 µl of the lysis solution (0.2 N NaOH, 1% w/v SDS) was added. The suspension was gently mixed to lyse the cells and allowed to sit on ice 5 minutes. Then 300 µl of 3 M CH_3COONa was added and incubated for an additional 60 minutes on ice. The lysate was centrifuged in a microfuge for 5 minutes to remove large chromosomal DNA. The supernate was carefully removed (approximately 750 µl) and the DNA was precipitated by the addition of 450 µl of isopropanol. The DNA pellet was resuspended in 200 µl 0.5 M NaCl, 0.05 M Tris pH 8.0 and reprecipitated with ethanol at -70°C. This DNA pellet was dissolved in TE and could be used for restriction digests. This method was also scaled up 10 fold for 50 ml cultures, with only a slight problem of contamination by RNA.

Larger quantities of plasmid DNA which were free of contaminating RNA for use in DNA sequencing were prepared from cleared bacterial lysates by CsCl-ethidium bromide density gradient centrifugation by a modification of the method of Clewell and Helenski (1969). Bacterial cultures were grown to an OD_{600} of 0.5 to 0.6 and plasmids were amplified by the addition of chloramphenicol (170 µg/ml, Clewell and Helinski, 1972). Incubation was continued overnight with vigorous shaking. The bacteria were harvested at 10,000 rpm in the

Sorvall SS-34 rotor for 10 minutes and washed one time in TE. The cell pellet was resuspended in 20 ml of cold 25% w/v sucrose, 0.05M Tris-HCl pH 8.0, 5mg/ml lysozyme, and 50 µg/ml pancreatic RNase and incubated on ice for 15 minutes. Then 8 ml of 0.25M EDTA was added and the incubation on ice continued for an additional 15 minutes. The bacteria were lysed by the addition of 28 ml 10% Triton X-100 in 0.05M Tris pH 8, 0.0625 M EDTA. The lysate was spun at 20,000 rpm and the supernate was removed. It was adjusted to a refractive index of 1.3975 to 1.3980 with CsCl and ethidium bromide was added to a final concentration of 1 mg/ml. This was centrifuged at 44,000 rpm for 40 hours. The DNA bands were visualized with ultraviolet light and the lower plasmid band was collected through the side of the tube using a syringe with an 18 gauge needle. The ethidium bromide was extracted with isopropanol equilibrated with CsCl-saturated TE and the DNA was dialyzed to remove the CsCl. DNA prepared in this way is free of contaminating RNA and is suitable for use in sequencing.

Isolation of Bacteriophage Lambda DNA

Lambda gtWES cloning vectors containing VZV DNA restriction endonuclease fragments (Straus et al., 1982) were grown in E. coli strain LE392 (F^-, SupF, SupE, hsdR, trpR, metB, LacY). Phages were plaque-purified by diluting existing stocks and plating on a lawn of LE392 in a tryptone broth agar overlay (TB; 10 gm tryptone, 5 gm NaCl, /1 H_2O, pH 7.4) on TB agar plates. The LE392 used in the overlay was from an overnight culture grown with 0.2% w/v maltose to maximize expression of the maltose permease which is the lambda receptor. These plaques were used to produce high-titer lysates in 5ml cultures. These

liquid culture lysates were titered and used to inoculate fresh LE392 in a 1 liter culture. About $5x10^8$ phages were used to inoculate $2.5x10^{10}$ cells in 1 liter of tryptone broth and incubated, with shaking, for 5 to 8 hours at 38 to 39°C. After lysis of the culture, NaCl was added to 0.5M and then 0.2 ml of $CHCl_3$ was added. The lysate was clarified at 4000 rpm in a Sorvall RC-5B centrifuge (GSA rotor) for 15 minutes. The pellet was discarded and polyethylene glycol 6000 (PEG) was added to the supernatant solution to give a final concentration of 10% w/v, and the mixture was shaken at room temperature until the PEG dissolved. After the PEG dissolved the lysate was placed in ice water for 1 hour. The PEG pellet was collected by centrifugation at 6000 rpm in the GSA rotor for 5 minutes. This pellet was redissolved in 10 ml of 50mM Tris HCl, 10mM $Mg(SO_4)_2$ pH 7.4 (TM buffer) and extracted with an equal volume of $CHCl_3$ by shaking at room temperature. The aqueous phase was collected after spinning at 4000 rpm for 10 minutes in a Sorvall SS-34 rotor. The aqueous phase was layered on a 5 to 40% glycerol-TM step gradient and centrifuged in a Beckman SW41 rotor at 35,000 rpm for 60 minutes at 4°C. This pellet was resuspended in 1 ml of TM buffer. The phage suspension was then treated with RNase at 50 µg/ml and DNase at 1 µg/ml for 30 minutes at 37°C. Then SDS was added to 0.1% w/v, Tris-HCl pH 7.5 to 10mM, EDTA to 0.1M and proteinase K to 1mg/ml and incubation continued at 50°C for 15 minutes. This mixture was extracted twice with TE-saturated phenol, twice with $CHCl_3$ and dialyzed against TE in the cold (3 to 4 changes of 1 liter). This procedure yielded large amounts of purified lambda containing VZV DNA on the order of 10 mg/l of culture and was free of detectable contaminating cellular DNA and RNA. It was used at this point for sub-cloning and as probes to detect specific VZV

plasmid clones.

Restriction endonuclease digestion of DNA

Viral and recombinant plasmid DNAs at a concentration of 1 to 2 µg per reaction were digested with various restriction endonucleases (Bethesda Research Labs, Gaithersburg, MD or Boehringer Mannheim Biochemicals, Indianapolis, IN) generally in a buffer recommended by the manufacturer or in one of four buffers, depending on the enzyme, as described by Maniatis et al., 1982. Reactions were generally done at 37°C except for the enzyme TaqI which was incubated at 65°C. Reactions were terminated after approximately 1 hour by the addition of EDTA to a final concentration of 5mM. Longer incubation times were used if large amounts of DNA (greater that 1 µg) were being digested. Restriction endonuclease mapping studies frequently required digestion of DNA with more than one enzyme. These double digestions were done simultaneously if the specific enzymes used had identical or similar reaction buffers. If the reaction conditions were incompatible, the enzyme with the lower NaCl requirement was used first followed by dilution into the reaction buffer of the second enzyme and an additional 1 hour incubation at 37°C was carried out with the second enzyme.

Radiolabeling DNA by Nick-Translation.

Viral, plasmid or bacteriophage DNAs were radiolabeled using alpha-^{32}P-deoxynucleoside triphosphates (either dATP or, more frequently dCTP) by "nick-translation" (Rigby et al., 1977). Radioactive ^{32}P-dNTPs were purchased from ICN Chemicals (Irvine, CA) at specific activities of > 3000 Ci/mmol. Between 10ng and 1µg of DNA was generally labeled in a

reaction volume of 50 to 100 µl. The DNA was diluted in 50mM Tris-HCl pH 7.8, 5mM $MgCl_2$, 10mM 2-mercaptoethanol, 50 µg/ml nuclease-free BSA, 2µM of dATP, dGTP, dTTP, 5ul of 10^{-9}M dCTP and 1 to 5 µl of the alpha-^{32}P-dCTP. The reaction was started by the addition of 1 to 2 units of E. coli. DNA polymerase I (Bethesda Research Labs, or Boehringer Mannheim) and incubated at 15°C for 1 hour. The reaction was terminated by the addition of EDTA, pH 8, to a final concentration of 10 mM. Pancreatic DNaseI was not included in the nick-translation reaction of whole VZV DNA, because single-stranded nicks are already present in these DNA preparations. However, it was included in nick-translations of plasmid or bacteriophage DNA to introduce nicks which are the substrate for E. coli DNA polymerase I. DNase I, (10ng/ml) was added to these DNAs in reaction buffer and incubated at 15°C for 15 minutes prior to addition of DNA polymerase I. Incorporation of ^{32}P into DNA was measured by TCA-precipitation or Cherenkov counting in a liquid scintillation counter (LS 9000, Beckman). Using this procedure, specific activities of between 1 and 5 x10^7 cpm/µg were consistently obtained.

Radiolabeling of DNA with polynucleotide kinase

Specific cloned VZV DNA fragments were end-labeled using T_4 polynucleotide kinase for use in restriction endonuclease mapping studies and for DNA sequencing. The 5' terminal phosphates were removed using calf intestine alkaline phosphatase (CIP, Boehringer Mannheim). The DNA was resuspended in CIP reaction buffer (50 mM Tris-HCl pH 9, 1 mM $MgCl_2$, 1mM $ZnCl_2$, and 1 mM spermidine) and incubated at 37°C for 30 minutes with 10 units of CIP. The reaction was continued for an

additional 30 minutes with an additional 10 units of CIP. The reaction was terminated by heating to 70°C for 15 minutes before phenol and phenol-chloroform extraction. Free phosphates, which inhibit subsequent reactions, were removed by spin dialysis chromatography (Maniatis et al., 1982) on sephadex G-50 columns and by alcohol precipitation. The dephosphorylated DNA was resuspended in kinase buffer (50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine and 0.1 mM EDTA) and incubated with 20 units polynucleotide kinase and 200 μCi of gamma-^{32}P-ATP (ICN) at 37°C for 30 minutes. The reaction was stopped by phenol extraction and the unincorporated ^{32}P was removed by spin dialysis.

Gel Electrophoresis

Whole DNA or DNA restriction endonuclease fragments were separated by electrophoresis in different agarose or polyacrylamide gel concentrations depending on the size of the DNA. Larger DNA fragments (between 100 Kb and 5 Kb) were separated on agarose gels (0.7 to 1.5% w/v) either horizontally (Bethesda Research Labs. Model H1 or H4) or vertically (Hoeffer Scientific Instruments, San Francisco, CA. Model SE500 or 520) using Tris-borate-EDTA running buffer (0.089 M Tris, 0.089 M boric acid and 2 mM EDTA). The horizontal gels were run at 50 V overnight and the vertical gels at 20 V for approximately 48 hours.

Polyacrylamide gels were used to separate DNA fragments between 2000 and 20 base pairs with acrylamide concentrations of between 5 and 20% w/v. Polyacrylamide gels were bis cross-linked (N,N' methylene-bis-acrylamide) at a ratio of 40:1 at the required concentration in Tris-borate-EDTA running buffer. The polymerization reaction was initated by

the addition of TEMED (N,N,N',N' tetramethylethylenediamine; 25 to 50 µl for 100 ml of acrylamide) and ammonium persulfate (0.8 ml of a 10% w/v solution per 100 ml of acrylamide) prior to pouring the mixture between the glass plates. These gels were run at 250 to 500 V until one of the running dyes (bromophenol blue or xylene cyanol) reached the bottom of the gel.

The DNA bands in either agarose or polyacrylamide gels were visualized by autoradiography or by staining with 1 µg/ml ethidium bromide for 15 minutes and photographed using a Polaroid MP-4 camera with a 254nm ultraviolet light source and Polaroid type 55 positive-negative film.

The molecular weight of each fragment was determined by comparison with restriction endonuclease fragments of known molecular weight run in the same gel. The standard fragments used in most of this work include: lambda EcoRI fragments (21.76, 7.55, 5.88, 5.54, 4.85, 3.43 Kb; Thomas and Davis 1975), adenovirus type 2 EcoRI fragments (20.54, 4.08, 3.47, 2.57, 2.11, 1.66 Kb; Mulder et al., 1974), SV40 HindIII fragments (1768, 1169, 1101, 526, 447, 215 bp; BRL), ΦX174 HaeIII fragments (1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72 bp; BRL), a 123 bp ladder (BRL) and several different double digestions of pBR325.

The molarities of restriction endonuclease fragments of whole VZV DNA were determined by calculation of the area under each peak of a densitometer tracing, in relation to the molecular weight of that fragment. The densitometer tracings were done with a Joyce-Loebl scanning densitometer provided by J.A. Wohlhieter, WRAIR.

Southern Blots

The blotting procedure used to identify cloned VZV restriction endonuclease fragments was a modification of the method of Southern (1975). Following electrophoresis, the gel was stained with ethidium bromide (1 μg/ml) and photographed. The gel was treated with 0.25M HCl for 5 minutes to depurinate the DNA and facilitate transfer of large fragments (Wahl et al., 1979) and then rinsed in water. The DNA was denatured with 0.5M NaOH for 45 minutes, neutralized with 1M Tris pH 7.4, 0.6M NaCl for 45 minutes and transferred to nitrocellulose paper (BA 85, Schleicher and Schull, Keene,NH) with 6x SSC (1X: 0.15M NaCl, 0.01M Na citrate pH 7) overnight. The blot was rinsed in 2x SSC and baked at 80°C for 2 hours in a vacuum oven. The baked blot was prehybridized at 55°C in 30% formamide, 6xSSC, 1X Denhart's (1966) solution (0.02% w/v Ficoll type 400, 0.02% w/v polyvinylpyrrolidone, 0.02% w/v bovine serum albumin), 0.1% w/v SDS and 50 μg/ml denatured calf thymus DNA for 4 hours to overnight. This prehybridization solution was removed and replaced with fresh hybridization solution which, in addition, contained the ^{32}P-labeled probe DNA. The blots were hybridized overnight at 55°C. The probe was removed and the blot was washed in 6 changes of 2x SSC at 55°C (15 minutes each wash) before drying and autoradiography on XAR-5 film (Eastman Kodak Co., Rochester, NY)

Extraction of DNA from gels

DNA fragments greater then 2 Kb in size were extracted from 1% w/v low melting temperature agarose (Sea-Plaque, FMC Corp., Rockland, ME) using the method of Langridge et al. (1980). Following

electrophoresis, the band was located (EtBr, ultraviolet light) and cut out of the gel with a razor blade. The gel fragment was melted at 70°C and cooled to 37°C. To this, 0.5 volumes of butanol-saturated water and 0.5 volumes of water-saturated butanol containing 1% w/v hexadecyl trimethylammonium bromide (CTAB, Sigma) was added and mixed by inversion 50 to 100 times keeping the temperature at 37°C to prevent the agarose from hardening. The phases were separated by low speed centrifugation at 37°C. The aqueous lower phase was re-extracted with butanol-CTAB two more times. The butanol phases were combined and 0.25 volumes of 0.2M NaCl was added and mixed by inversion 50 to 100 times. This was repeated two more times and the aqueous phases, containing the DNA, were combined. The CTAB was precipitated from the DNA solution by the dropwise addition of an equal volume of chloroform and incubation on ice for 0.5 hours. The DNA was then concentrated by the addition of butanol or ethanol precipitation. Excess salt was removed by spin dialysis.

Smaller DNA fragments (less than 2 Kb) were eluted from 5% polyacrylamide gels (40:1 Bis) using a procedure published by Maxam and Gilbert (1980). The DNA fragment was located in the gel (as above) and cut out with a razor blade. The gel was crushed into a paste using a teflon pestle, mixed with 1 ml of elution buffer (500mM CH_3COONH_4, 10 mM $(CH_3COO)_2Mg$, 1mM EDTA and 0.1% w/v SDS) and rotated overnight at 37°C. The samples were centrifuged, the supernate was saved and the acrylamide was re-eluted with extraction buffer. The supernates were combined and centrifuged through a glass wool column to remove small particles of acrylamide; the DNA was concentrated with butanol and the salt and SDS were removed by spin dialysis.

Molecular Cloning of VZV DNA

VZV DNA restriction endonuclease fragments were cloned into the plasmid vector pBR325 (Bolivar, 1978). This vector was chosen because there is an EcoRI restriction site within a gene coding for resistance to the antibiotic chloramphenicol. Insertion into this site causes inactivation of the gene and allows screening of transformants for insertion of foreign DNA by chloramphencol sensitivity.

Plasmid DNA was digested with EcoRI and treated with alkaline phosphatase to remove 5' phosphates and reduce the background of uninserted plasmid clones (Ullrich et al., 1977). VZV DNA digested with EcoRI was mixed with the EcoRI-digested, phosphatase treated pBR325 DNA at several different DNA concentrations and vector-to-insert ratios, to maximize the chance of having the correct condition to form singly inserted clones in the absence of accurate VZV DNA concentrations (Dugaiczyk et al., 1975). This DNA mix was then treated with DNA ligase (Bethesda Research Labs or Collaborative Research Inc., Waltham, MA) in ligation buffer (66 mM Tris-HCl pH 7.6, 5 mM $MgCl_2$, 5 mM dithiothreitol and 1 mM ATP) for 24 hours.

Bacterial Transformation

E. coli strain HB101 ($hsdR^-$, $hsdM^-$, recA, lacZ4, leuB6, supE4, proA2, Gal^-, str^R, thi^-1; Boyer and Roulland-Dussoix, 1969) was made competent to take up foreign DNA by a modification of the method of Cohen et al., 1973. An overnight culture of HB101 was diluted into 50ml of fresh LB broth and grown at 37°C with shaking to an OD_{600} of 0.5 to 0.6. The cells were pelleted at 4000 rpm for 10 minutes, washed once in 50 ml of TE and recentrifuged at the same speed and time. The cells

were then resuspended in 25ml of cold 0.1M $CaCl_2$, 0.05M $MgCl_2$ and incubated for 10 minutes on ice. The cells were again pelleted and resuspended in 2.5ml of 0.1M $CaCl_2$, 0.05M $MgCl_2$ and kept on ice until used. Plasmid DNA was added to 0.3 ml of these competent cells in a volume up to 50 µl and incubated an additional 20 minutes on ice. The cells were then heat-pulsed at 42°C for 5 minutes, diluted with 3.7ml of fresh pre-warmed LB broth containing 0.2% w/v glucose and incubated with shaking at 37°C for 90 minutes in order to express antibiotic resistance. The transformed bacterial cultures were then plated out on selective media (LB plus ampillicin, 200 µg/ml) in duplicate amounts of 0.1ml. The remaining cells were pelleted (4000 rpm for 10 mimutes), resuspended in 0.2ml of LB broth and 0.1ml was plated in duplicate and incubated at 37°C.

Identification of plasmid-VZV recombinants

Ampicillin-resistant transformants were screened for insertion of VZV DNA fragments by chloramphenicol-sensitivity. Ampicillin-resistant, chloramphenicol-sensitive clones were then tested for insertion of specific VZV fragments with the colony hybridization method of Grunstein and Hogness (1975). Bacterial colonies were grown on nitrocellulose filter paper (BA 85) in a petri dish on TB agar. Following overnight incubation at 37°C, the nitrocellulose paper, with the colonies on it, was peeled off the plate and placed on top of two, 17 chroma 80 mm circles (Whatman) in the lid of a petri dish soaked with 0.5M NaOH for 10 minutes to allow the solution to soak upwards into the colonies on the nitrocellulose, lyse the bacteria and denature the DNA. These blots were then transferred to four successive changes of

filter paper circles soaked with 1M Tris-HCl, pH 7.4, at one minute intervals to neutralize the NaOH and then were finally soaked with 1M Tris-HCl pH 7.4, 1.5M NaCl for 10 minutes. The blots were then baked at 80°C in a vacuum oven for 2 hours and were hybridized with specific nick-translated VZV DNA restriction fragments. Following washing, positive clones were identified by autoradiography. The presence of specific VZV fragments was confirmed by comigration of the fragments inserted in the plasmid and by Southern blot hybridization.

Electron Microscopy of VZV DNA

Viral DNA or isolated cloned VZV restriction endonuclease fragments were mixed with recrystallized formamide to a final concentration of 80% (v/v) and denatured by heating to 60°C for 10 minutes. The formamide concentration was lowered to 66% by the addition of 0.2M Tris-HCl, 0.1mM EDTA pH 8.5, and the DNA was allowed to reanneal or self-hybridize for 2-3 hours to overnight. SV40 and ΦX174 or fd phage DNA were added as double and single-stranded DNA size standards. Cytochrome C was added to a concentration of 0.03 mg/ml. The DNA was picked up from a buffered 10% v/v formamide hypophase, stained with uranyl acetate and rotary-shadowed with gold palladium in an Edwards evaporator. The DNA was examined with a Zeiss 10A electron microscope and photographed. The sizes of single and double-stranded regions of the DNA molecules were determined by measurement of projected lengths with a map measurer and comparison with standards on the same grid.

DNA sequencing

The base sequence of specific VZV DNA fragments was determined

by the base-specific chemical cleavage methods of Maxam and Gilbert (1977, 1980). Kits containing all the required reagents were purchased from New England Nuclear (#NEK-010). DNA fragments were labeled at 5' position by procedures already described (see kinase end labeling, above). The labeled ends of each fragment were separated by second restriction endonuclease digestion or by denaturation. Denatured single strand or second site cut, end-labeled DNA were separated in neutral 5% polyacryamide gels (see above), and the appropriate bands were cut from the gel and extracted by elution from the acrylamide. These DNA fragments were then treated for sequencing as published by Maxam and Gilbert (1977, 1980) and as modified by the kit manufacturer (New England Nuclear).

The basis for the method is the partial cleavage of singly end-labeled DNA in four separate reactions that are specific for one or two bases resulting in a population of molecules differing in length from the labeled terminus to all possible positions for that particular base or bases. In the four different reactions therefore there are bands representing all possible bases.

Sequencing gels were run on a BRL model S0 gel apparatus which has a gel size of 34x40cm, 0.4mm thick spacers and either a 32 tooth Delrin comb or a "shark's tooth" comb (BRL). The gel composition was 8% w/v acrylamide (20:1 Bis), 8M urea and standard Tris-borate-EDTA 1X electrophoresis buffer was used (see above). The acrylamide solution was heated slightly to get the urea into solution and filtered through a 0.45 micron filter before addition of ammonium persulfate and TEMED. This was then carefully poured between the glass plates which had been carefully cleaned, rinsed in deionized water, rubbed with 95% alcohol,

then wiped with "windex" and taped together with 2 inch wide waterproof tape. Gels were allow to set overnight and pre-run at 1200-1500 volts for 1 to 2 hours. When the gel surface reached a temperature of approximately 50°C, samples were boiled for 30 seconds and quickly chilled on ice and loaded on the gel. Several sample loadings were done for each sample so that more bases could be read from a single gel. When the marker dye (bromophenol blue) from the first load reached the bottom of the gel, the power was turned off, the wells rinsed clear of urea and the second set of the same samples were loaded in different wells and electrophoresis was continued. This was again repeated when the second load dye front reached the bottom of the gel. The run was ended when the third load bromophenyl blue approached the gel bottom.

After electrophoresis, the glass plates were carefully separated to prevent gel tearing, and the gel remaining on one of the plates was marked with a fluorescent pen (New England Nuclear) for orientation and running dye position. It was then covered with "Saran wrap" (Dow) and exposed using Kodak X-Omat RP film (35x43cm) with a DuPont Cronex Lightning Plus intensifying screen at -70°C overnight to several days.

RESULTS

Restriction endounclease digestion of VZV DNA

The major focus of this dissertation is the analysis of VZV DNA with restriction enzymes and the characterization of the structures found using these methods. These studies provide a base of information which can be used in future examinations of VZV replication and gene expression. Important first steps in these studies are to identify and locate the distinctive structural features of VZV DNA, and then to construct physical maps of the genome which can be used as a framework for subsequent experiments.

The first series of experiments undertaken was cleavage of purified VZV DNA with a number of restriction enzymes, chosen on the expectation (by extrapolation from their action on other herpesvirus DNAs), that a workable number of fragments (10-30) would result from cleavage. In fact, a total of ten enzymes was tested and, from that testing, three, EcoRI, BglII, and BamHI were selected for further study.

Densitometer tracings of restriction enzyme digestion patterns of VZV nucleocapsid DNA produced with EcoRI, BglII and BamHI are shown in Figures 4, 5, and 6 respectively. The molecular weight of each fragment is shown under the corresponding peak for each band and is listed in Table 1. These molecular weights were calculated based on the known sizes of EcoRI DNA fragments of phage lambda (see Materials and Methods) run in adjacent lanes on the same gel. The total molecular weight of all bands in each restriction digest is also listed in Table 1. The molarity of each of the fragments was calculated based on the area under each peak and its molecular weight, as described in Materials

and Methods and is shown in Table 2.

EcoRI Digests. Digestion of VZV DNA with the restriction endonuclease EcoRI results in 17 fragments (Figure 4). These fragments range in size from 10.8 to less than $0.5X10^6$ daltons. However, the sum of the molecular weights of all the fragments ($95.45X10^6$ daltons; Table 1) is greater than the molecular weight of VZV DNA determined by contour length measurements in the electron microscope ($80X10^6$ daltons; Dumas et al., 1980; Straus et al., 1981), by about $15x10^6$ daltons.

Several of the bands in the EcoRI digest appear less intense than they should for their molecular weight. Integration of the area under each peak is a measure of the amount of DNA present in each particular band. Considering the molecular weight of each band and the area under the peak, the relative amount of each DNA restriction fragment in the population can be calculated. If the DNA is homogenous and contains only unique sequences, then each fragment should be present in equal quantities. That is, all the fragments should have a molarity of 1. Visually this means that the peaks in a densitometer tracing of restriction fragments from DNA which is unique should decrease uniformly in area as molecular weight decreases. This is clearly not the case for the EcoRI restriction endonuclease digestion of VZV DNA (Figure 4). There are four EcoRI fragments which appear to be underrepresented in the population (A, E, F and J). The bands for these fragments are less intense than other fragments which have lower molecular weights. Calculation of the molar ratios of VZV DNA restriction endonuclease fragments (Table 2) shows that these four fragments, A, E, F and J, are present in 0.5 M amounts.

The presence of the 0.5 M fragments suggests that these four

fragments are not present in all VZV DNA molecules in the population. Each of these four 0.5 M fragments must only be present in half the molecules of VZV DNA; two of the fragments should be in half of the molecules and the other two are present in the other half.

The presence of four 0.5 M fragments and the finding that the sum of all the restriction fragments is larger than the molecular weight of VZV DNA determined by electron microscopy, can be explained by the presence of an invertible segment in VZV DNA, as has been described for other herpesviruses (see Introduction). This invertible segment would exist in two orientations which, from the restriction data, must be present in equal amounts. Therefore the sum of the molecular weights of two of the half molar fragments should equal the sum of the other two half molar fragments which should also equal the amount which is in excess of the molecular weight determined by electron microscopy. Thus, molecular weights of the EcoRI A fragment plus the J fragment (10.8 + 5.15 = 15.95) equals the molecular weights of the E plus F fragments (7.95 + 7.7 = 15.65). This is also equal to the difference between the sum of the molecular weights of all the EcoRI restriction fragments and the electron microscopic determination of VZV DNA molecular weight (95.45 - 80 = 15.45).

BglII digests. Similar results are obtained by examination of VZV DNA digested with the BglII restriction endonuclease (Figure 5, Table 1 and 2). Digestion of VZV DNA with BglII results in 20 fragments which range in size from 12.5 to $0.47X10^6$ daltons. The total molecular weight for all the BglII fragments is $93.88X10^6$ daltons, and is about $14X10^6$ daltons greater than the contour length measurements. Four of the fragments, BglII C, D, G and J, appear to be 0.5 M based on the

densitometer tracing (Figure 5; Table 2). The sum of two of these (C + J; 7.9 + 5.7 = 13.6) equals the sum of the other two (D + G; 7.6 + 6.0 = 13.6). Again, this also equals the difference between the sum of the molecular weights of all the BglII restriction fragments and the VZV DNA molecular weight determined by contour length (93.88 - 80 = 13.88).

BamHI digests. Digestion with BamHI produces a large number of VZV restriction fragments. There are approximately 27 bands resolved in this 0.5% agarose gel (Figure 6). None of these bands appears to be present in less than molar amounts based on the densitometer tracings (Figure 6 and Table 2). One of the bands (BamHI J) does appear, however, to be present in greater amounts than it should for its size. This could be due to comigration of 2 or more DNA fragments which are very close in molecular weight or it could be due to the presence of repeated sequences in VZV DNA which are cut by this restriction enzyme more than once. As an aside, there is some difficulty in the determination of molarity of fragments which are very small (less than $1.5X10^6$ daltons) such as the VZV BamHI fragments from S to AA . This is probably due to the non-linear response of the film used to detect the DNA fragments by autoradiography (Clements et al., 1976). It could also be due to an increase in the background due to DNA degradation introduced during isolation or which is present in the original DNA preparation. This increase in background makes it difficult to position the baseline for integration of smaller peaks and therefore these small fragments have a calculated molarity value which may be higher than it actually is (Table 2).

Southern blotting of DNA fragments. Examination of Southern hybridization blots using VZV DNA EcoRI, BglII and BamHI restriction

FIGURE 4

Densitometer tracing of the bands resulting from digestion of VZV DNA with EcoRI. VZV Scott DNA was labeled with ^{32}P-dCTP by nick-translation, digested with EcoRI and electrophoresed in a 0.5% agarose slab gel at 20 V for 48 hours. The gel was dried, autoradiographed and the autoradiogram scanned with a Joyce-Loebl densitometer. The molecular weights of each fragment were calculated based on DNA molecular weight standards run in the same gel and are listed in Table 1. The molarities of each fragment were determined by measuring both the area under each peak and that fragment's molecular weight, and are listed in Table 2. VZV EcoRI fragments are labeled A through R in order of decreasing molecular weight. EcoRI fragments P, Q and R are very small and are not seen in this tracing. The molarities of the small fragments could not be determined because they cannot be clearly identified in the densitometer tracings.

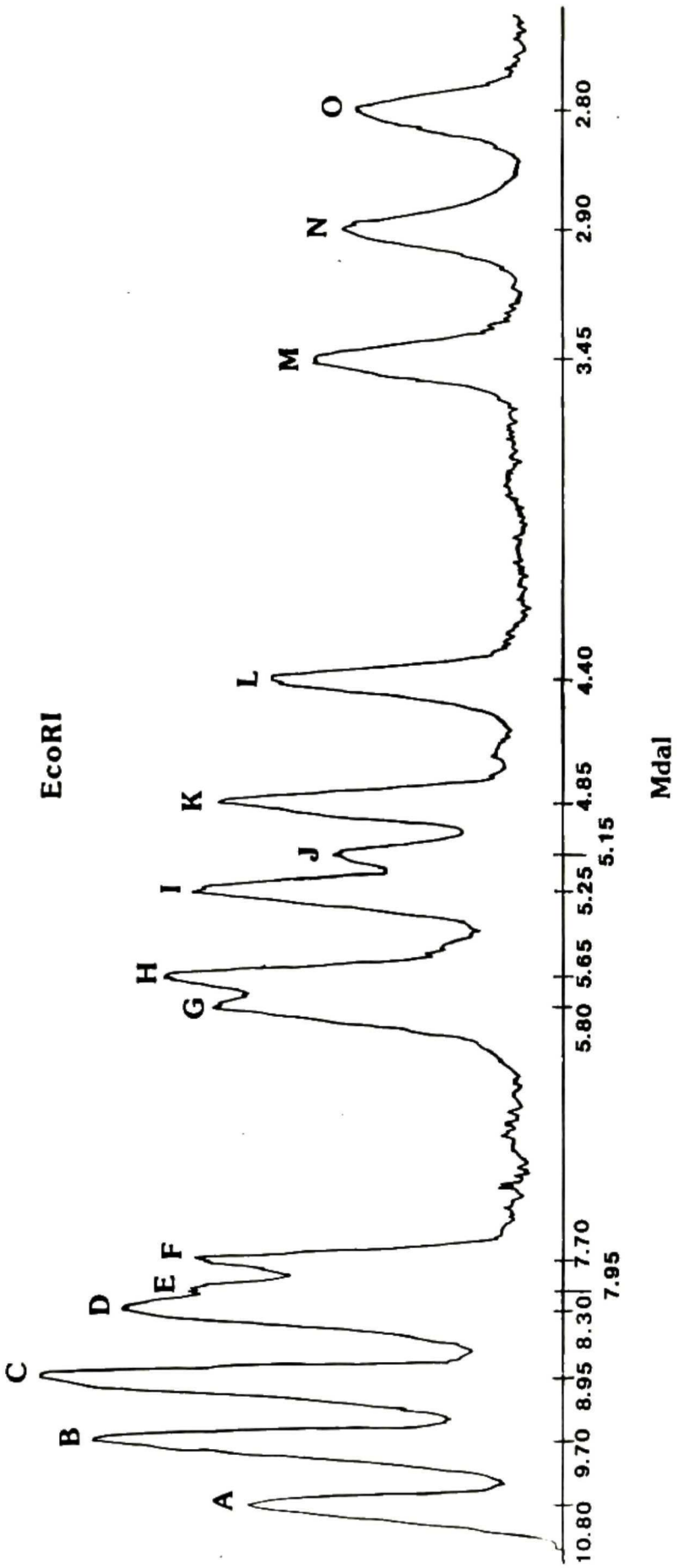

EcoRI
A
B
C
D
E F
G
H
I
J
K
L
M
N
O
10.80 9.70 8.95 8.30 7.70 7.95 5.80 5.65 5.25 5.15 4.85 4.40 3.45 2.90 2.80
Mdal

FIGURE 5

Densitometer tracing of the bands resulting from digestion of VZV DNA with BglII. VZV Scott DNA was labeled with ^{32}P-dCTP by nick-translation, digested with BglII and electrophoresed in a 0.5% agarose slab gel at 20 V for 48 hours. The gel was dried, autoradiographed and the autoradiogram scanned with a Joyce-Loebl densitometer. The molecular weights of each fragment were calculated based on DNA molecular weight standards run in the same gel and are listed in Table 1. The molarities of each fragment were determined by both measuring the area under each peak and that fragment's molecular weight and are listed in Table 2. VZV BglII fragments are labeled A through T in order of decreasing molecular weight. BglII fragments R, S and T are very small and are not seen in this tracing. The molarities of the small fragments could not be determined because they cannot be clearly identified in the densitometer tracings.

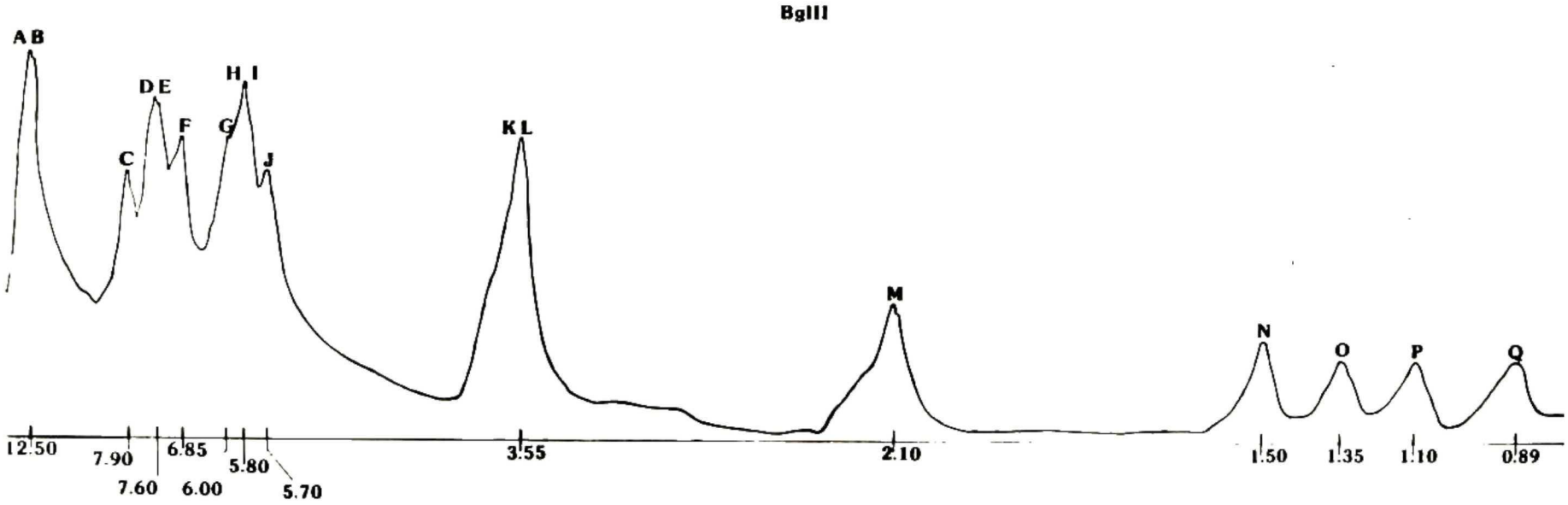

BglII
AB
C
DE
F
G
HI
J
KL
M
N
O
P
Q
12.50
7.90
7.60
6.85
6.00
5.80
5.70
3.55
2.10
1.50
1.35
1.10
0.89
Mdal

FIGURE 6

Densitometer tracing of the bands resulting from digestion of VZV DNA with BamHI. VZV Scott DNA was labeled with ^{32}P-dCTP by nick-translation, digested with BamHI and electrophoresed in a 0.5% agarose slab gel at 20 V for 48 hours. The gel was dried, autoradiographed and the autoradiogram scaned with a Joyce-Loebl densitometer. The molecular weights of each fragment were calculated based on DNA molecular weight standards run in the same gel and are listed in Table 1. The molarities of each fragment were determined by measuring both the area under each peak and that fragment's molecular weight and are listed in Table 2. VZV BamHI fragments are labeled A through AA in order of decreasing molecular weight. There may be additional small BamHI fragments which cannot be resolved by this type of electrophoresis. BamHI fragments Z and AA are too small to be seen in this tracing. The molarities of the small fragments could not be determined because they cannot be clearly identified in the densitometer tracings.

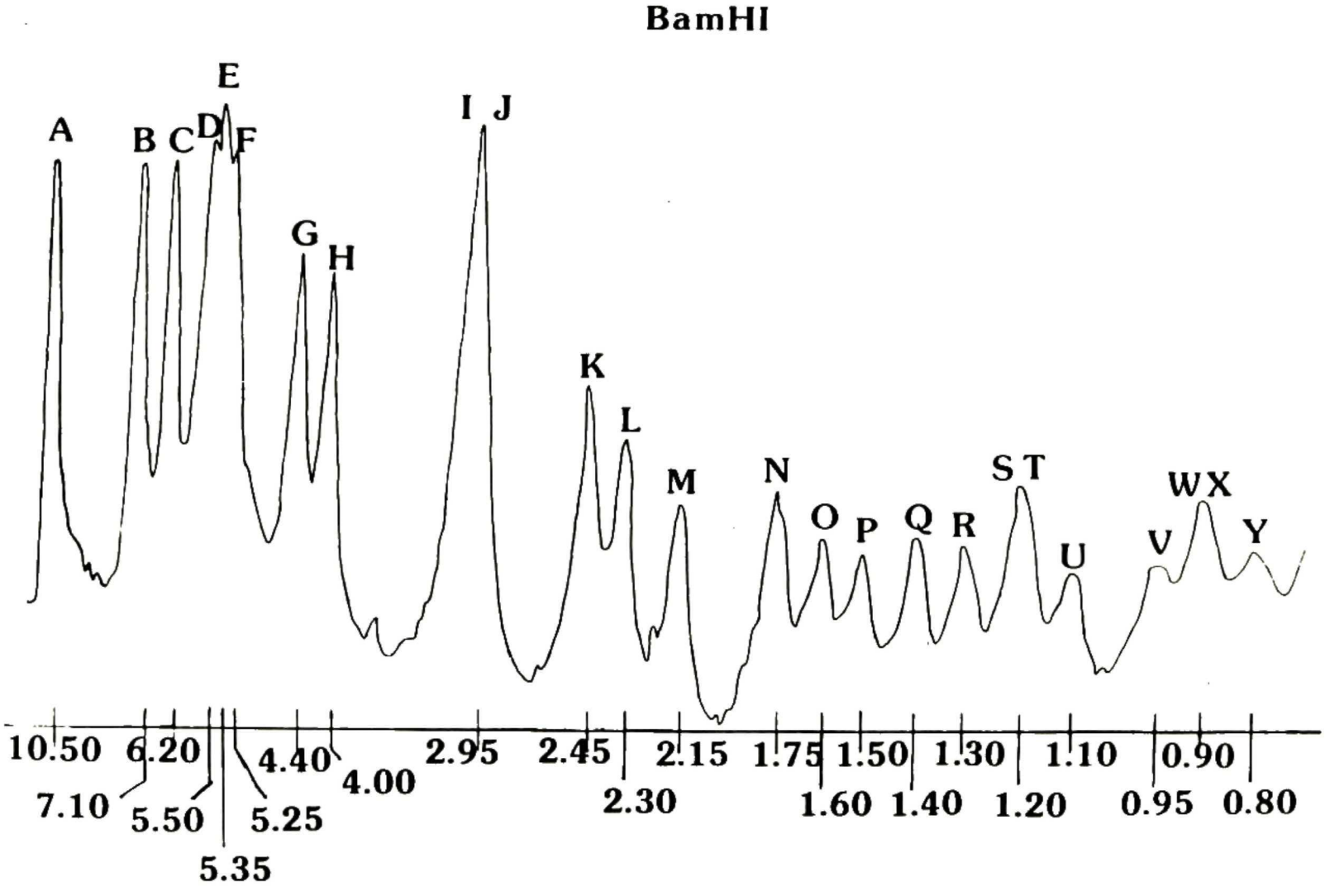

BamHI
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
Y
10.50
7.10
6.20
5.50
5.35
5.25
4.40
4.00
2.95
2.45
2.30
2.15
1.75
1.60
1.50
1.40
1.30
1.20
1.10
0.95
0.90
0.80
Mdal

Table 1. Estimations of molecular weights of VZV Scott DNA restriction endonuclease fragments.

Fragment	EcoRI (Mdal)	BamHI (Mdal)	BglII (Mdal)
A	10.80	10.50	12.50
B	9.70	7.10	12.50
C	8.95	6.20	7.90
D	8.30	5.50	7.60
E	7.95	5.35	7.60
F	7.70	5.25	6.85
G	5.80	4.40	6.00
H	5.65	4.00	5.80
I	5.25	2.95	5.80
J	5.15	2.95	5.70
K	4.85	2.45	3.55
L	4.40	2.30	3.55
M	3.45	2.15	2.10
N	2.90	1.75	1.50
O	2.80	1.60	1.35
P	1.10	1.50	1.10
Q	0.70	1.40	0.89
R	--[a]	1.30	0.60
S	--[b]	1.20	0.52
T	--	1.20	0.47
U	--	1.10	--
V	--	0.95	--
W	--	0.90	--
X	--	0.90	--
Y	--	0.80	--
Z	--	0.74	--
AA	--	0.67	--
TOTAL	95.45	77.11	93.88
Sum of 0.5 M fragments[c]	A+J=10.8+5.15 E+F=7.95+7.7 15.95 or 15.65		C+J=7.9+5.7 D+G=7.6+6.0 13.6
VZV mol. wt.[d]	79.65	79.71	80.58

[a] fragment not seen.
[b] no corresponding fragment.
[c] molarities values from Table 2
[d] approximate molecular weight based on the weights of 0.5M and 2M fragments (Table 2) and the proposed structure of VZV DNA (see text).

Table 2. Molar ratios of VZV Scott DNA restriction endonuclease fragments.

Frag.	EcoRI		Frag.	BamHI		Frag.	BglII	
	Observed[1]	Suggested[2]		Observed	Suggested		Observed	Suggested
A	0.53	0.5	A	0.82	1.0	A	1.86[3]	1.0
B	0.93	1.0	B	0.90	1.0	B		1.0
C	1.18	1.0	C	0.99	1.0	C	0.51	0.5
D	1.02	1.0	D	1.08	1.0	D	1.62	0.5
E	0.55	0.5	E	1.15	1.0	E		1.0
F	0.66	0.5	F	0.98	1.0	F	1.00	1.0
G	1.01	1.0	G	1.10	1.0	G	0.53	0.5
H	1.18	1.0	H	1.07	1.0	H	1.80	1.0
I	1.10	1.0	I	3.15	1.0	I		1.0
J	0.51	0.5	J		2.0	J	0.59	0.5
K	1.03	1.0	K	1.31	1.0	K	2.54	1.0
L	1.18	1.0	L	1.43	1.0	L		1.0
M	0.99	1.0	M	1.27	1.0	M	0.85	1.0
N	1.02	1.0	N	1.28	1.0	N	1.04	1.0
O	0.98	1.0	O	1.45	1.0	O	0.85	1.0
P	ND[4]	1.0	P	1.03	1.0	P	0.99	1.0
Q	ND	1.0	Q	1.08	1.0	Q	1.27	1.0
R	ND	1.0	R	1.30	1.0	R	ND	1.0
			S	2.43	1.0			
			T		1.0			
			U	1.22	1.0			
			V	1.41	1.0			
			W	2.38	1.0			
			X		1.0			
			Y	1.43	1.0			
			Z		1.0			
			AA	ND	1.0			

[1] Observed molarity determined from desitometer tracings of the band for each fragment

[2] Suggested molarity

[3] Observed molarity of two restriction fragments which comigrate are shown between the letter names for those fragments

[4] ND not determined

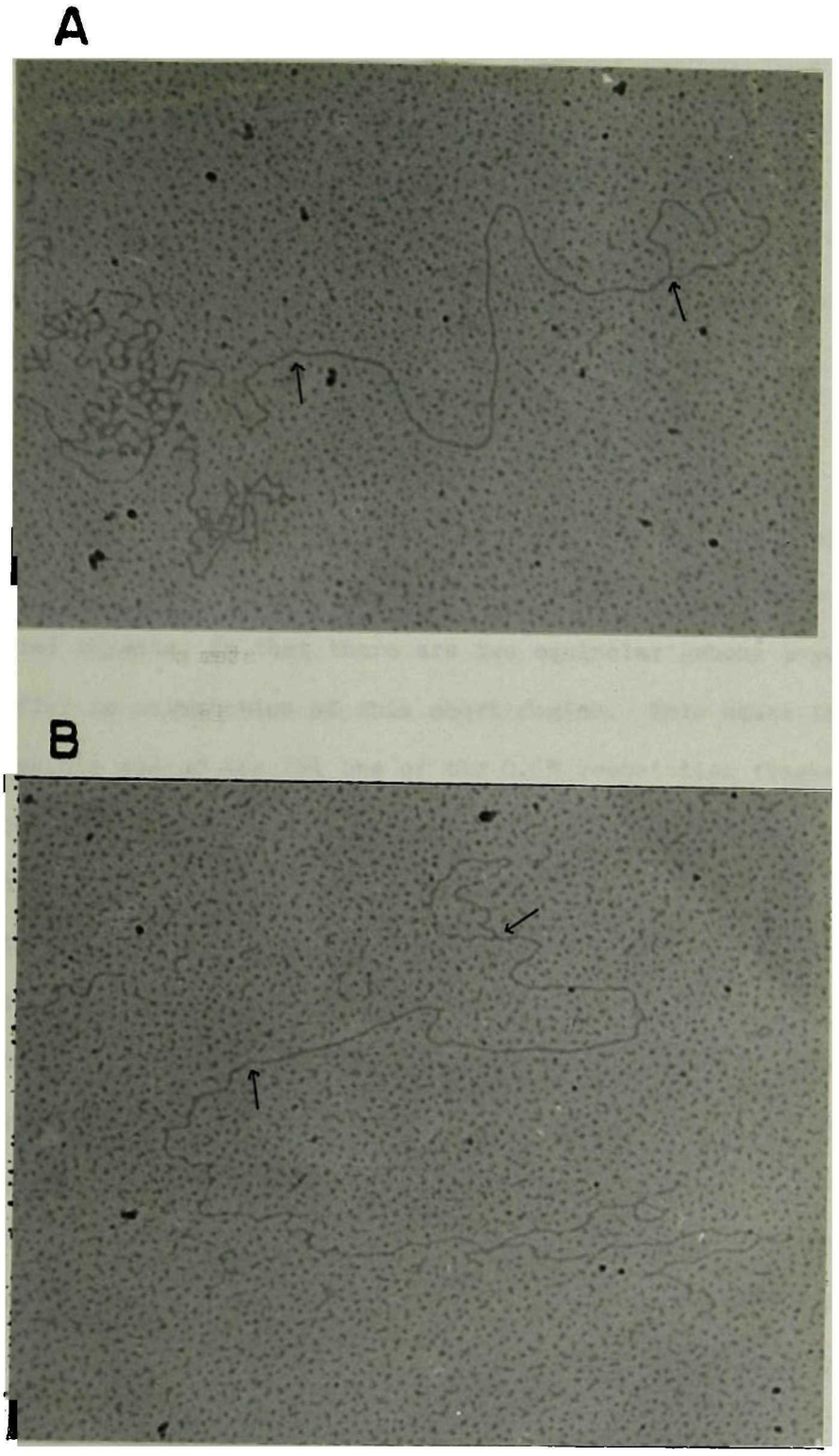
fragments (Dumas et al., 1981; Straus et al., 1982) indicated that the 0.5M fragments from the EcoRI and BglII digests are homologous with each other and with the BamHI J fragment which appears to be 2M. For example, using one of the 0.5M fragments as a probe, it will not only hybridize with itself on a homologous blot, but will also hybridize to the three other 0.5M fragents in that digestion as well as all four 0.5M fragments in other digestions and the 2M J fragment in the BamHI digests (Straus et al., 1982). This data suggested that VZV DNA contained repeated sequences which are located in the 0.5 M EcoRI and BglII fragments and that the 2 M BamHI J fragment was also located in these repeated sequences.

Intramolecular DNA homology identified by electron microscopy

The presence of repeated sequences in VZV based on the Southern blotting experiments (Dumas et al.,1981; Straus et al., 1982) and generation of 0.5 M restriction endonuclease fragments suggested that, like other herpesviruses (see Introduction), VZV DNA might contain inverted repeat sequences which bracket an invertible region. The possibility was investigated by examination of denatured and self-reannealed VZV DNA by electron microscopy. VZV strain Scott DNA was denatured and allowed to reanneal in dilute solution before spreading and examination in the electron microscope. Figure 7 shows two examples of VZV DNA prepared and examined in this way. The majority of the DNA is single-stranded which can be distinguished by its "kinky" appearance relative to that of double-stranded DNA. At one end, these DNA molecules contain a stem and loop structure. The loop is single-stranded DNA which is positioned between two homologous DNA sequences

FIGURE 7

Electron micrographs of the snap-back structure found at one end of VZV DNA. Panels A and B are two examples of self-hybridized VZV Scott DNA which show the stem and loop structure found at one end of VZV DNA. The arrows mark the double stranded region of the stem which are the inverted repeat sequences. One inverted repeat is terminal and is separated from the internal inverted repeat by unique sequence DNA which forms the loop. The sizes of the stem and loop were found to be 4.6 ± 0.3 MDal and 4.3 ± 0.9 MDal, respectively, based on single and double-stranded DNA standards on the same grid. The magnification is approximately 40,000X.


A
B

which are in an inverted orientation. One of these repeat sequences, which is terminal, has base-paired to an inverted internal homologous sequence to form the stem of this structure. The molecular weights of the stem and loop of VZV were calculated, based on single and double stranded DNA standards (fd phage and SV40 respectivly) present on the same grid. The single-stranded loop is 4.3×10^6 daltons separating inverted repeat sequences of approximately 4.6×10^6 daltons.

Determination of VZV terminal restriction fragments

A consequence of the evidence presented above for a short invertible region located at one end of the VZV genome which is bounded by inverted repeats, is that there are two equimolar genome populations which differ in orientation of this short region. This means that at the invertible end of the DNA one of the 0.5M restriction fragments should be terminal in half the molecules and in the other half a different 0.5M fragment should be terminal. The other end of the DNA is constant, so the same restriction fragment should be terminal at this position in both isomers. Based on the sizes of the EcoRI 0.5M fragments and the estimation of the size of the invertible segment by electron microscopy, the EcoRI A fragment is too large to be terminal. Thus EcoRI A must be internal to EcoRI J which is terminal. The other two 0.5M fragments, EcoRI E and F, could not be orientated in the same way because they are very close in molecular weight. The procedure chosen to determine which of the VZV DNA restriction endonuclease fragments are terminal was digestion with exonuclease III (exoIII) followed by digestion with a restriction enzyme. ExoIII removes nucleotides progressively in a 5' to 3' direction only from the DNA ends

and, therefore, fragments at the ends of the DNA molecule will be missing following this treatment. The resulting fragments were separated by agarose gel electophoresis and the patterns examined for the loss of restriction fragments. The results of exoIII digestion followed by digestion with the restriction enzymes EcoRI, BglII and BamHI and shown in Figures 8, 9 and 10, respectively.

EcoRI terminal fragments. The 0.5M EcoRI VZV fragments F and J are clearly not present in the lane treated with exoIII (Figure 8) and the 1M fragment EcoRI C is also missing in this lane. Thus, at the invertible end, in one orientation, the F fragment is terminal and in the other orientation the J fragment is terminal. Therefore the other two 0.5M EcoRI fragments E and A must be internal and contain the internal inverted repeat sequence. The 1M C fragment must be located at the unique end of VZV DNA.

BglII terminal fragments. Similar results are seen in Figure 9, for the terminal BglII fragments which were identified by exoIII digestion followed by BglII digestion and agarose gel electrophoresis. The BglII 0.5M fragments J and D are are both located at the invertible end, while the L fragment is located at the unique end. The other two 0.5M BglII fragments must therefore be internal. When the invertible region is in the orientation which places the J fragment at the terminus, the C fragment is internal and in the opposite orientation; when the D fragment is terminal, the G fragment is internal.

BamHI terminal fragments. The BamHI digestion after exoIII treatment (Figure 10) is more complex. This enzyme cleaves VZV DNA in the inverted repeat sequences which results in a 2M fragment (BamHI J). The BamHI J fragment is not present in the exoIII-digested lane,

FIGURE 8

Identification of the terminal EcoRI fragments by Exonuclease III digestion. Intact VZV Scott DNA was treated with exonuclease III for 10 minutes and the reaction stopped at 70°C for 15 minutes. The DNA was digested with EcoRI and labeled with ^{32}P-dCTP by nick translation. Control reactions, without exonuclease III, were carried out in parallel. The resulting bands were separated in adjacent lanes by electrophoresis in a 0.5% agarose slab gel at 20 V for 48 hours. The fragments marked (EcoRI C, F and J) are not present in the exonuclease III-treated lane.

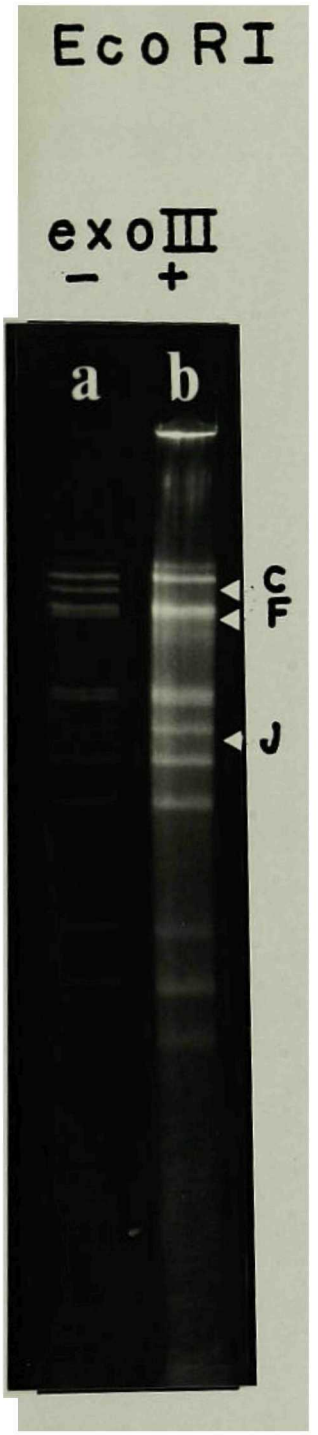

EcoRI
exoIII
−
+
a
b
C
F
J

FIGURE 9

Identification of the terminal BglII fragments by Exonuclease III digestion. Intact VZV Scott DNA was treated with exonuclease III for 10 minutes and the reaction stopped at 70°C for 15 minutes. The DNA was digested with BglII and labeled with ^{32}P-dCTP by nick translation. Control reactions, without exonuclease III, were carried out in parallel. The resulting bands were separated in adjacent lanes by electrophoresis in a 0.5% agarose slab gel at 20 V for 48 hours. The fragments marked (BglII D, J and L) are not present, or are reduced in relative intensity in the exonuclease III-treated lane.

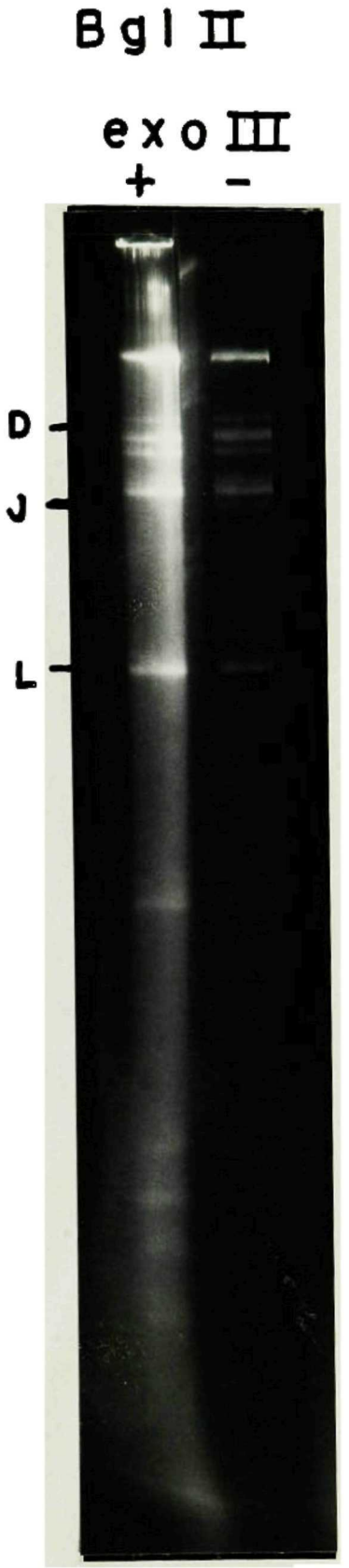
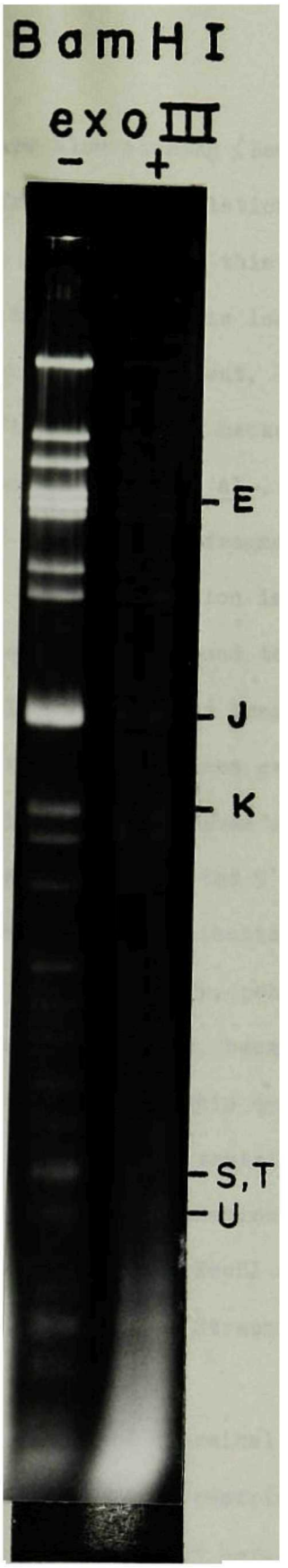

Bgl II
exo III
+
-
D
J
L

FIGURE 10

Identification of the terminal BamHI fragments by Exonuclease III digestion. Intact VZV Scott DNA was treated with exonuclease III for 10 minutes and the reaction stopped at 70°C for 15 minutes. The DNA was digested with BamHI and labeled with ^{32}P-dCTP by nick translation. Control reactions, without exonuclease III, were carried out in parallel. The resulting bands were separated in adjacent lanes by electrophoresis in a 0.5% agarose slab gel at 20 V for 48 hours. The fragments marked (BamHI E, J, K, S, T and U) are not present or are reduced in relative intensity in the exonuclease III-treated lane.


BamHI
exo III
–
+
E
J
K
S,T
U

and additional small bands are also missing (BamHI S, T and U). The relative position of these fragments in relationship to each other and to the DNA termini cannot be determined by this method, because of their small size and because more than one band is lost at this invertible end by exoIII digestion. However the 1M fragment, located at the constant end of VZV, could be identified as BamHI E because it is not homologous to the inverted repeat sequence (Straus et al., 1982).

The determination of the terminal fragments for these three restriction endonucleases by exoIII digestion is not necessarily definitive. For example, the fragments found to be terminal by this procedure may be sub-terminal, with smaller fragments which are truly terminal and are either not resolved on these gels or are removed very rapidly by exoIII. Alternative determinations of terminal restriction fragments, which involved the labeling of the 5' or 3' ends with polynucleotide kinase or terminal deoxynucleotide transferase respectively, were attempted unsuccessfully, pehaps because of an unusual terminal structure or, more likely, because insufficient quantities of VZV DNA were available for this type of analysis.

The determination of the terminal restriction endonuclease fragments for EcoRI has led to the determination of a complete EcoRI map in a collaborative effort using BamHI and EcoRI double digestions and hybridizations with cloned VZV fragments (Straus et al., 1982; see Figure 19).

Figure 11 shows a model for the terminal invertible region of VZV DNA and includes the positions of the restriction fragments which map to this region using the data presented here and by hybridization and sub-digestion of cloned VZV DNA fragments (Straus et al., 1982).

The map positions for the restriction endonucleases EcoRI, Bgl II and BamHI are shown for both isomers of VZV DNA. The thick black bars represent the inverted repeat sequences identified by electron microscopy. The names of the BamHI fragments shown in this figure are for strain Ellen (Straus et al., 1982). These designations were used here because the position of the BamHI fragments for strain Scott used in these analyses could not be definitively determined and because Scott DNA contains strain-specific fragments (see below) which leads to altered fragments compared to strain Ellen.

The model in Figure 11 shows that the change in orientation of the sequences between the repeats moves the position of the restriction sites between the repeats in the short unique region while the sites outside the repeat are constant in either orientation. The BamHI J fragment is 2M because it is present internally in each of the inverted repeat sequences.

In summary, VZV DNA is organized into two unique segments, a long unique sequence (U_L) and a short unique sequence (U_S). The U_S sequence is bound by inverted repeat sequences, one terminal (TR_S) and the other internal (IR_S) (Figure 11). Thus, VZV DNA exists as two isomers which are present in equal amounts in a population of DNA molecules.

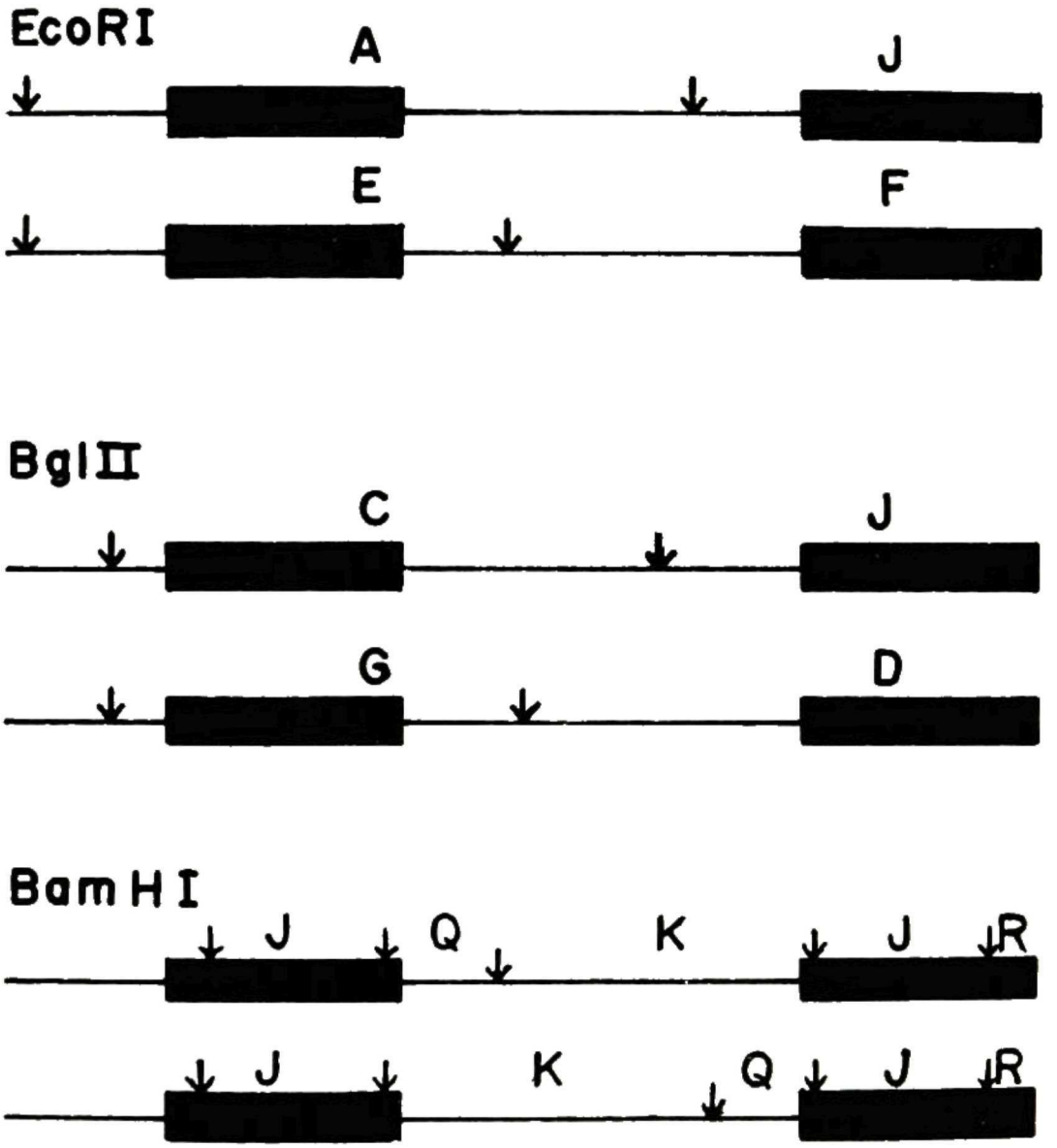
VZV DNA strain variations

Several different VZV strains were examined by restriction endonucleases during the mapping studies described above. These included DNA from tissue culture-grown virus, as well as DNA isolated directly from virions in lesion fluids of patients with chickenpox or

FIGURE 11

A model of the VZV invertible region. The positions of the cleavage sites for EcoRI, BglII and BamHI and the invertible terminus of VZV DNA are shown for both orientations. The black bars represent the internal and terminal inverted repeats (IR_S and TR_S) that flank the short genome segment (U_S). The fragments which were found to map to this region by exonuclease digestion and molarity determinations are shown for both orientations. The fragments shown for BamHI are for strain Ellen, and were determined by hybridization and double-digestion; VZV strain Scott contains two additional BamHI fragments which are strain specific and because of their size, alter the BamHI fragment designations for this strain although the terminal fragments are identical in both strains (see text and Figure 18).

MODEL OF VZV INVERTIBLE REGION



zoster. Careful examination of the DNA patterns produced for these different strains using several different restriction enzymes showed that there were always consistent small changes in the migration of a few fragments for each of the enzymes used.

As described in the Introduction, there are two different types of strain vaiation which can be detected by restriction endonucleases. The first is the gain or loss of a recognition site for a particular restriction enzyme. This can be caused by a single base change in the DNA sequence resulting in either a fusion of two fragments or the production of two new fragments. This has been infrequently seen with VZV DNA. The second, more common type of strain variation in VZV DNA involves a change in migration of a single restriction fragment or several unrelated fragments.

The studies decribed below were designed to determine the nature of strain variation in VZV DNA. The first step was to identify the regions of the DNA where the variation occurs and to estimate the number of regions involved. It was also of interest to determine if the variations could be consistently mapped to similar physical locations and if there was a relationship between the magnitude of the variation in different restriction endonuclease fragments. These experiments were performed using 6 different restriction endonucleases by comparison of the restriction endonuclease map position of the variable fragments from a variety of different VZV strains. Additional experiments were carried out to determine the potential usefulness of these DNA strain variations in molecular epidemiology of VZV infections as has been described for other herpesviruses (see Introduction). The required properties include the stability of the variation in tissue culture and the ability to

distinguish epidemiologically-related and unrelated strains.

EcoRI digests. An EcoRI digestion of DNA from 3 different VZV strains is shown in Figure 12. Lane 1 shows the EcoRI pattern with VZV strain Ellen, and lanes 2 and 3 are VZV strain KM. Lane 2 is KM isolated directly from the vesicle fluid of a patient with zoster and lane 3 is the same strain which has been passed in tissue culture 6 times before the DNA was extracted and digested with EcoRI. Lane 4 is the DNA from VZV strain Scott cut with EcoRI. The brackets on this figure mark the regions in the gels where the variable restriction fragments migrate. The EcoRI fragments J and G are clearly seen to migrate in different positions in each of these strains. In addition, it appears that if the EcoRI J fragment increased in size then the EcoRI fragments E and F increase by approximately the same amount (compare lane 2 with lane 1). The EcoRI A fragment may also increase in size, but it is difficult to see this small increase in size in such a large fragment. This suggested that this strain difference mapped to IR_S/TR_S which are common to these four EcoRI fragments (A, E, F and J). The size of this DNA variation in VZV is difficult to measure but was estimated to be up to approximately 200 bp in the strains examined.

Figure 12 also shows that these strain differences are stable in tissue culture. There is no difference in these variable fragments between the KM DNA isolated directly from vesicle fluid (lane 2) and KM DNA that was extracted from virus grown in culture (lane 3). VZV strain Ellen has been passed in our laboratory more than 50 times without a detectable change in migration of any restriction fragment (not shown). In addition, variation does not occur during a single recurrence of zoster, that is, the DNA is the same in virus from two

different vesicles from the same patient (lane 1 and 2, Figure 13), nor does it occur in a single person to person transmission of chickenpox based on comparisons of digestions with four different enzymes (Figure 14). Thus, the increase or decrease in the sizes of restriction fragments in VZV DNA satisfies critera for use as a marker for epidemiological studies.

Additional strains of VZV DNA were examined to determine the relationship, if any, between the variable EcoRI fragments. As mentioned previously, it appears that one strain variation mapped to the inverted repeat-containing fragments (EcoRI A, E, F and J). This can also be seen in Figure 15. In addition, Figure 15 shows that there is another variable fragment present and that this variation (in the EcoRI G fragment) is independent of the variation in the fragments which contain the VZV inverted repeats. Lane 1 contains VZV strain Ellen, lane 2 VZV strain Long, a clinical isolate, and lane 3 contains strain KM. The EcoRI J fragment, representing the strain variation which occurs in the inverted repeats, is very similar in the Long and KM strains, but the G difference between these two strains is large. It therefore appears that there is no relationship between these two strain variations. Further work along the same lines shows that there is also variation in EcoRI P (Figure 16) which is also independent of the repeat variation and of the EcoRI G variation. Thus, VZV DNA contains three independently variable DNA regions, in EcoRI A, E, F, J, in EcoRI G and in EcoRI P.

Mapping VZV DNA strain differences. Four strains of VZV were chosen to further characterize these strain differences by comparison of the variable fragments with the restriction maps of VZV DNA. The

FIGURE 12

Identification of VZV EcoRI fragments which vary in different strains. VZV DNA was labeled with ^{32}P-dCTP by nick translation, digested with EcoRI and the fragments were separated by electrophoresis in a 0.7% agarose gel at 20 V for 48 hours. The gel was dried and autoradiographed. Lane 1, strain Ellen; lane 2, strain KM DNA isolated directly from vesicle fluid; lane 3, strain KM DNA which had been passed in tissue culture six times; lane 4, strain Scott. The EcoRI fragments P, Q, and R are not seen in this gel. The variable fragments are marked along the right side.

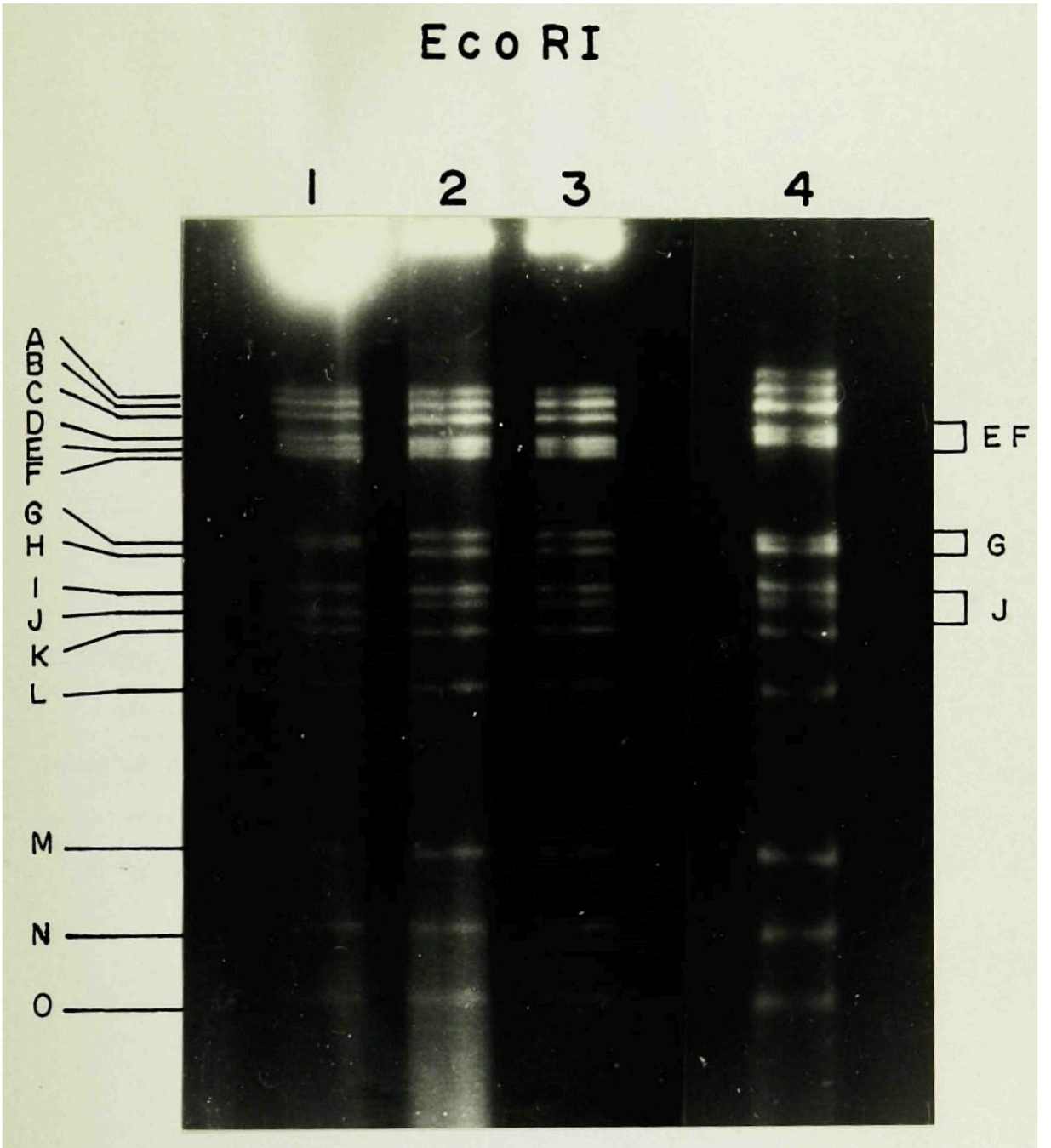
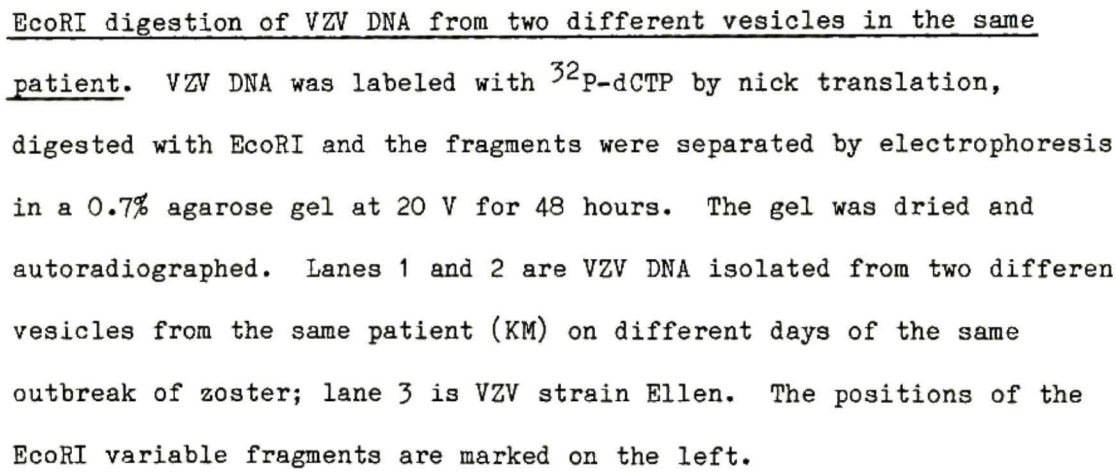

EcoRI
1
2
3
4
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
E F
G
J

FIGURE 13



EcoRI digestion of VZV DNA from two different vesicles in the same patient. VZV DNA was labeled with ^{32}P-dCTP by nick translation, digested with EcoRI and the fragments were separated by electrophoresis in a 0.7% agarose gel at 20 V for 48 hours. The gel was dried and autoradiographed. Lanes 1 and 2 are VZV DNA isolated from two different vesicles from the same patient (KM) on different days of the same outbreak of zoster; lane 3 is VZV strain Ellen. The positions of the EcoRI variable fragments are marked on the left.

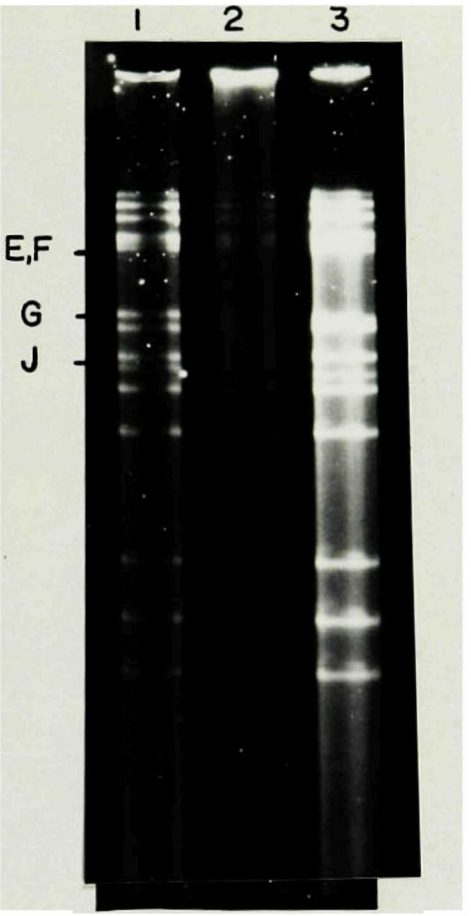

1
2
3
E,F
G
J

FIGURE 14

Comparison of VZV DNA from two epidemiologically-related cases of varicella. VZV was isolated from vesicle fluids and passed four times in tissue culture. VZV DNA was prepared from nucleocapsids isolated from infected tissue culture cells. The DNA was digested with restriction endonucleases, labeled with ^{32}P-dCTP by nick translation and the bands were separated by electrophoresis in a 0.5% agarose slab gel at 50 V for 18 hours. This is the resulting autoradiogram of VZV DNA from two epidemiologically related cases of varicella (AL and ML).

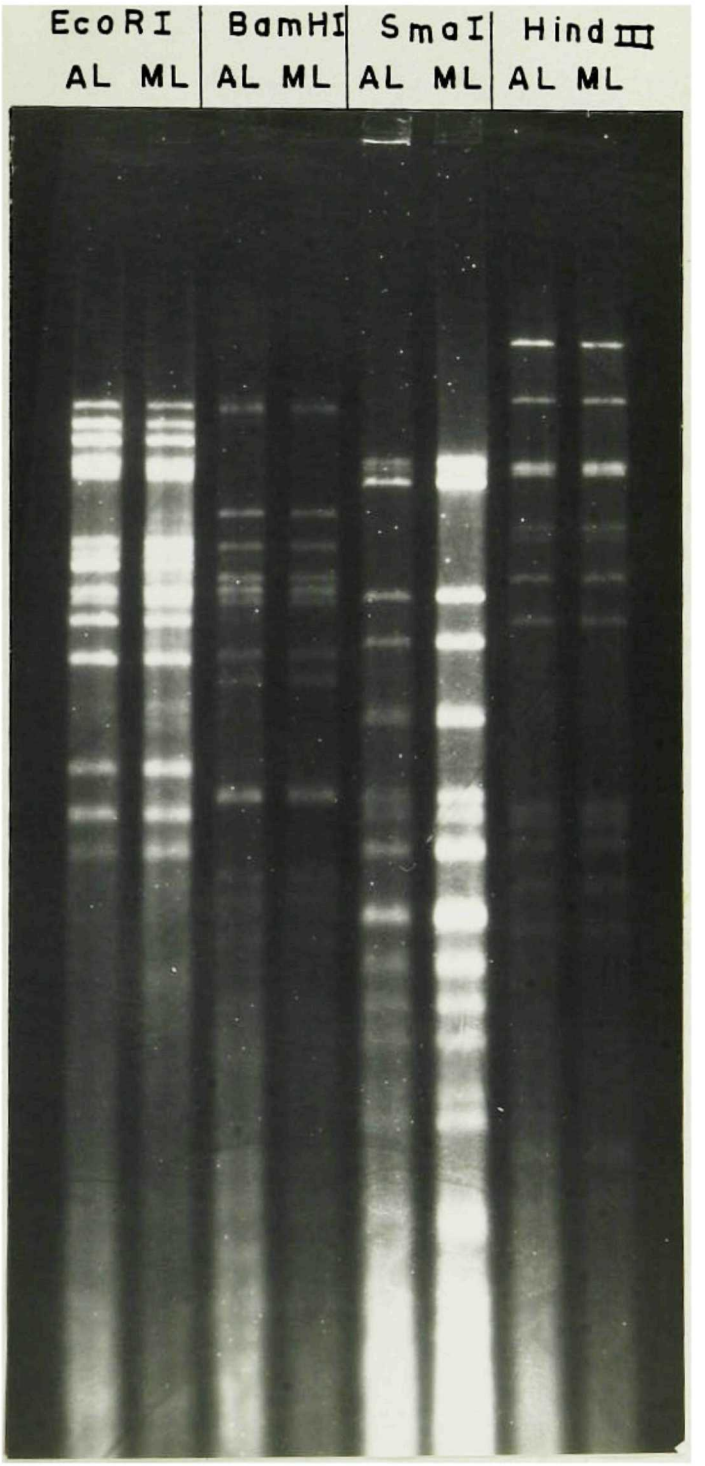
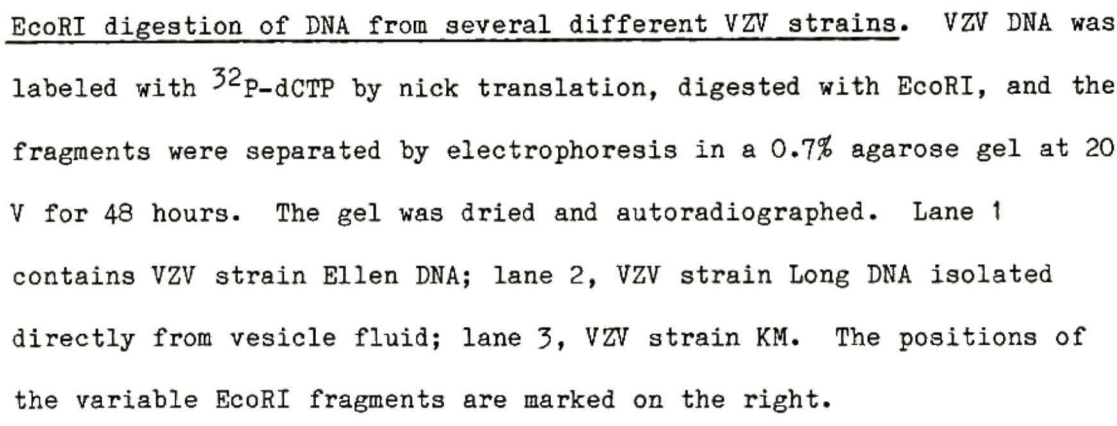
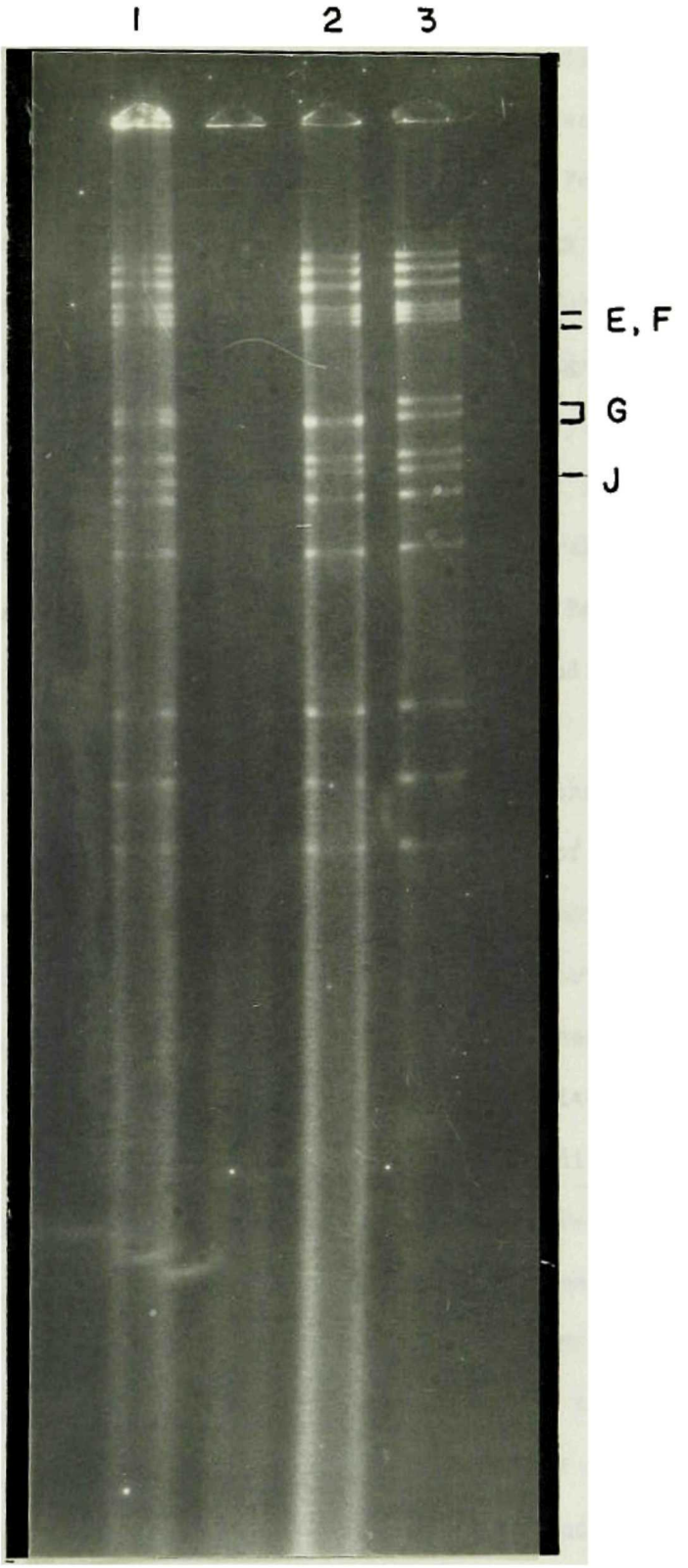

EcoRI
AL ML
BamHI
AL ML
SmaI
AL ML
Hind III
AL ML

FIGURE 15



EcoRI digestion of DNA from several different VZV strains. VZV DNA was labeled with ^{32}P-dCTP by nick translation, digested with EcoRI, and the fragments were separated by electrophoresis in a 0.7% agarose gel at 20 V for 48 hours. The gel was dried and autoradiographed. Lane 1 contains VZV strain Ellen DNA; lane 2, VZV strain Long DNA isolated directly from vesicle fluid; lane 3, VZV strain KM. The positions of the variable EcoRI fragments are marked on the right.


1
2
3
E, F
G
J

strains used were VZV Oka, Ellen, Webster and Scott, all of which we have been able to grow in culture in sufficient quantities for these studies. Figures 16, 17 and 18 show these strains digested with the enzymes HindIII, EcoRI, BglII, SmaI, PstI and BamHI. For each of these enzymes there is a small number of fragments which vary in size among the strains. In the HindIII digestion in Figure 16, for example, the C and E fragments vary. This figure also shows EcoRI digestion of these four strains and the E,F,G,J and P fragments which vary. Figure 17 shows the digestion patterns of these four strains cut with BglII and SmaI. The BglII framents which vary are C,D,G and J; the SmaI fragments which vary are A and G. Figure 18 shows the PstI and BamHI digestions; BamHI fragments C and J and the PstI fragments A, C and E are variable.

In the BamHI pattern shown in Figure 18 the other type of strain variation is also seen. The BamHI digestion pattern of VZV strain Oka contains several new restriction fragments which are not seen in the other strains. BamHI digestion of strain Scott results in two new fragments which are not seen in the other strains. These new fragments are probably due to the gain or loss of restriction sites which are unique to these two strains and are unrelated to the differences in migration of fragments with which we are concerned here.

A comparison of the restriction fragments which vary for each of these enzymes and the published restriction maps of VZV DNA (Dumas et al.,1981; Ecker and Hyman, 1982; Straus et al., 1983; Ruyechan et al., 1984) is shown on Figure 19. The black bars represent those fragments which were seen to vary for each of the restriction endonucleases used (Figures 16-18). The hatched bars represent fragments which should vary

based on their map position, but which could not be unambiguously determined to have differences in migration either because they were large or because they co-migrated with other fragments in that particular digest. For example, PstI A is too large to see a small variation such as the one seen in the region where it maps (i.e. the repeats, IR_S/TR_S) and the BglII fragments A and B are too large and, in addition, they co-migrate. In the lower part of Figure 19 is the model for VZV DNA showing the invertible region and the positions of the regions of strain difference as determined by restriction endonuclease analysis. For all the enzymes the strain differences appear to map to three locations. One region is in the inverted repeats (IR_S/TR_S), and the other two regions map in the long unique region (U_L).

At this stage in the project, work from other laboratories began to appear which increased our interest in these strain variations. First, it became clear that many herpesviruses exibited DNA strain variations, which remained uncharacterized and, second, areas of strain variation in other DNA viruses appeared to be associated with important transcriptional controlling regions. Thus, it was decided to examine these VZV variation in detail and, if possible, obtain the sequence of one of them.

Cloning of variable regions. In order to examine these strain-variable fragments in more detail, the EcoRI frgments P, G and E, which represent the three regions of VZV DNA which vary in different strains, were molecularly cloned. These fragments could then be isolated in large amounts and could be examined by electron microscopic heteroduplex analysis and mapped in detail, to precisely determine the location and the nature of these strain differences.

FIGURE 16

Hind III and EcoRI digestions of DNA from four different VZV strains. VZV DNAs from strains Ellen (E), Oka (O), Scott (S) and Webster (W) were labeled with ^{32}P-dCTP by nick translation and digested with either Hind III or EcoRI. The fragments were separated by electrophoresis in a 0.5% agarose gel at 50 V for 18 hours. The restriction fragments which had altered mobilities in the different strains are marked.

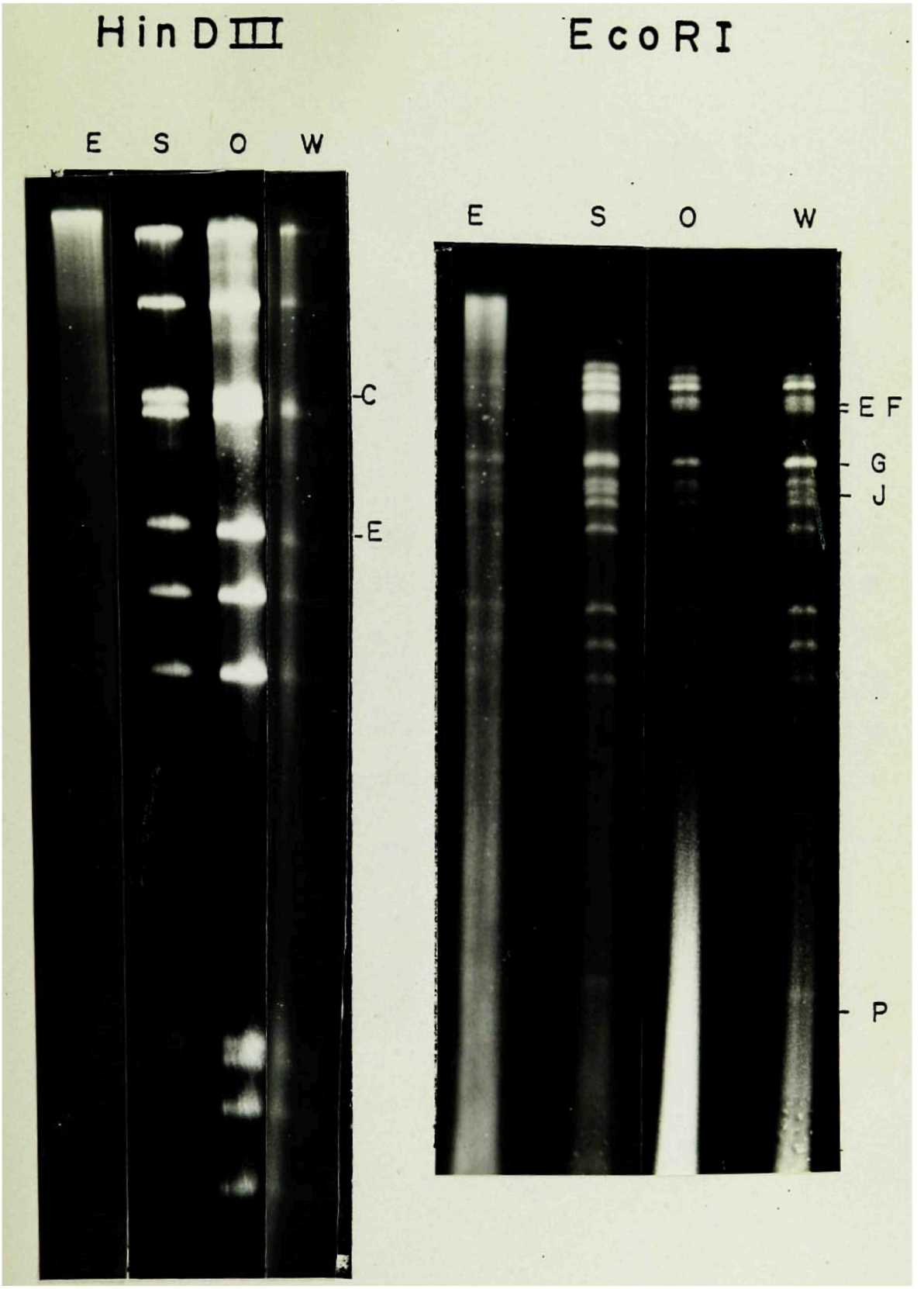

Hin D III
E S O W
C
E
EcoR I
E S O W
E F
G
J
P

FIGURE 17

BglII and SmaI digestions of DNA from four different VZV strains. VZV DNAs from strains Ellen (E), Oka (O), Scott (S) and Webster (W) were labeled with ^{32}P-dCTP by nick translation and digested with either BglII or SmaI. The fragments were separated by electrophoresis in a 0.7% agarose gel at 20 V for 48 hours. The restriction fragments which have altered mobilities in the different strains are marked.

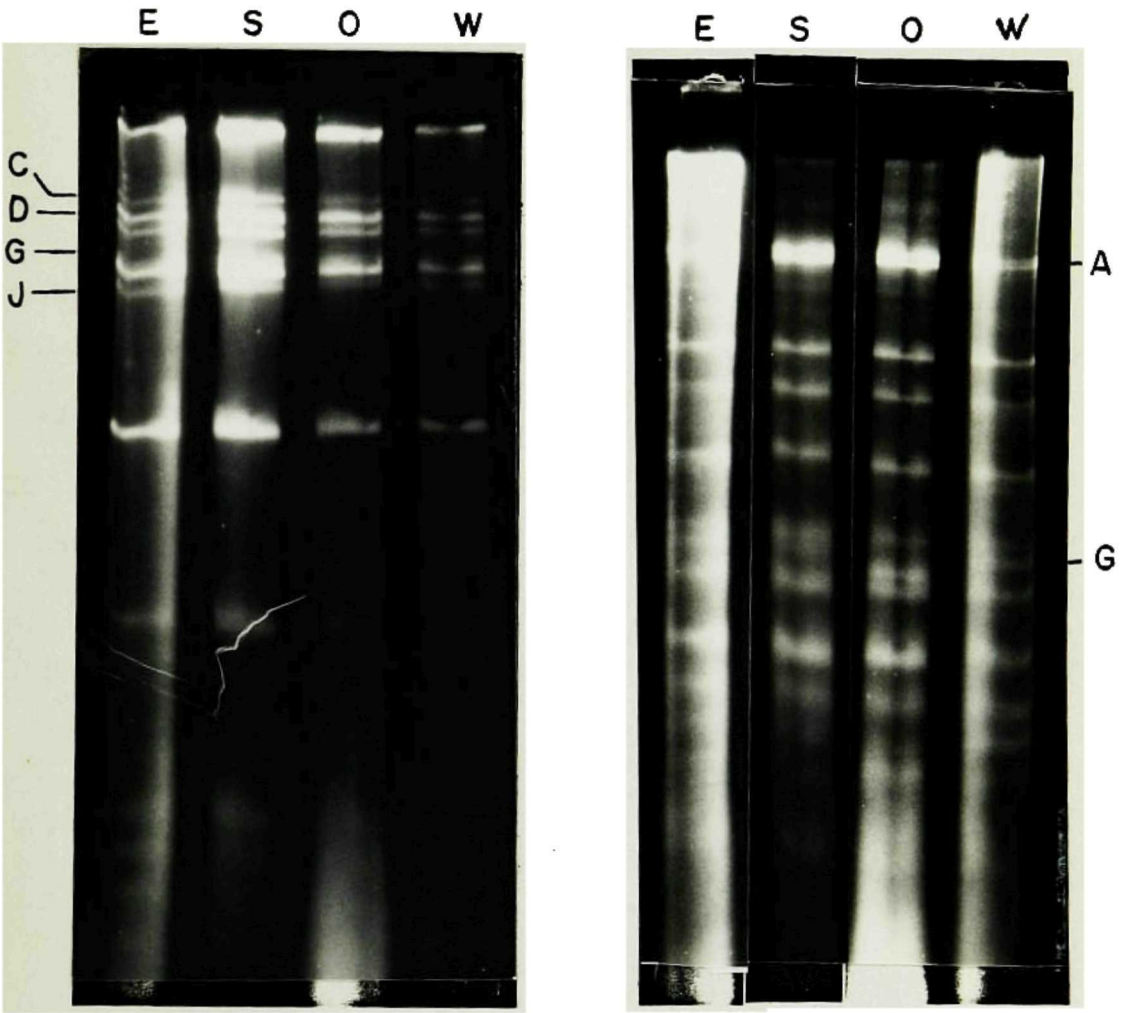

BglII
SmaI
E
S
O
W
C
D
G
J
E
S
O
W
A
G

FIGURE 18

PstI and BamHI digestions of DNA from four different VZV strains. VZV DNAs from strains Ellen (E), Oka (O), Scott (S) and Webster (W) were labeled with ^{32}P-dCTP by nick translation and digested with either Pst I or BamHI. The fragments were separated by electrophoresis in a 0.5% agarose gel at 50 V for 18 hours. The restriction fragments which have altered mobilities in the different strains are marked. Additional BamHI fragments, which are seen only in either VZV strain Oka or Scott and not in the other strains are also marked.

PstI BamHI

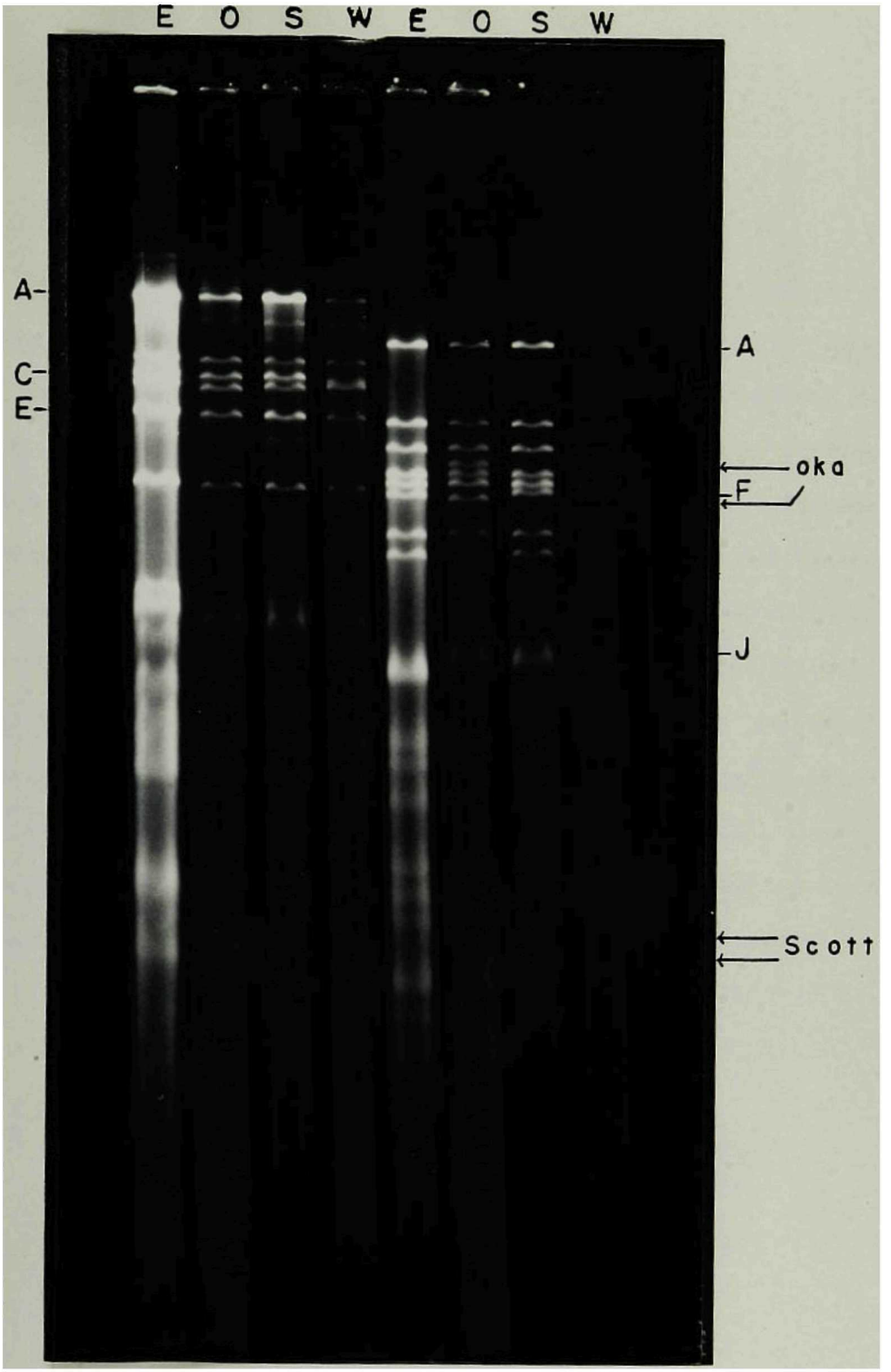
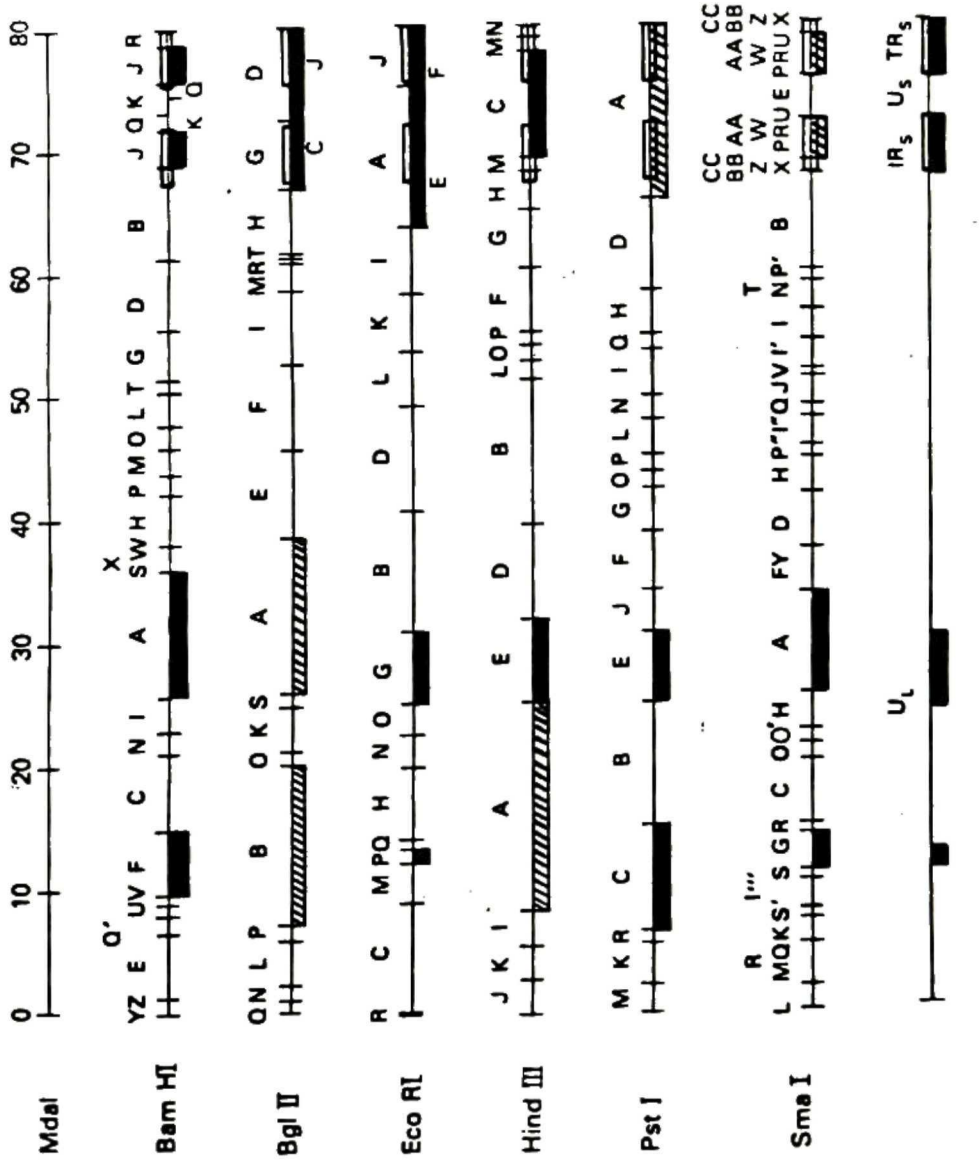


FIGURE 19

Locations of the strain variable fragments on the EcoRI, BamHI, SmaI, BglII, HindIII and PstI restriction maps of VZV DNA. The map position of VZV DNA restricion endonuclease fragments which have altered mobilities in different strains as seen in Figures 16, 17 and 18 are marked in black. The fragments which are hatched, might also vary but are either too large to see this small change or are too small to be resolved in the gels. At the bottom, the three regions of VZV which vary are marked on the model of the genomic organization of VZV DNA. There are two variable regions in the long unique segment (U_L) and the third variable region is present twice, once in each of the inverted repeats (IR_S and TR_S) which bound the short unique segment (U_S). The maps are redrawn from Dumas et al., (1981), for PstI and BglII; Ecker and Hyman, (1982), for HindIII; Straus et al., (1982, 1983), for EcoRI and BamHI; and Ruychan et al., (1984), for SmaI.


MdaI
0
10
20
30
40
50
60
70
80
Bam HI
Bgl II
Eco RI
Hind III
Pst I
Sma I
IR_S U_S TR_S
U_L

The plasmid cloning vector pBR325 was chosen because the EcoRI site in this vector is in the structural gene for chloramphenicol resistance. Thus, insertion of DNA into this site results in the inactivation of this gene and allows the simple screening of clones for inserted VZV DNA by chloramphenicol sensitivity. The basic strategy for these cloning experiments is digestion of the VZV DNA with EcoRI and ligation of these fragments to EcoRI-digested, alkaline phosphatase-treated pBR325. This DNA was used to transform E. coli strain HB101, selecting for presence of the plasmid by ampicillin resistance and screening for inserted DNA by chloramphenicol sensitivity. Approximately 800 transformants were screened to obtain 100 to 150 Amp^R, Cm^S clones for each of the VZV strains (Ellen, Oka, Scott and Webster). The Amp^R, Cm^s colonies were then hybridized with specific VZV probes for the presence of the strain-variable fragments EcoRI P, G and E, using colony hybidization as described in Materials and Methods. The autoradiogram in Figure 20 is an example of this type of colony blotting.

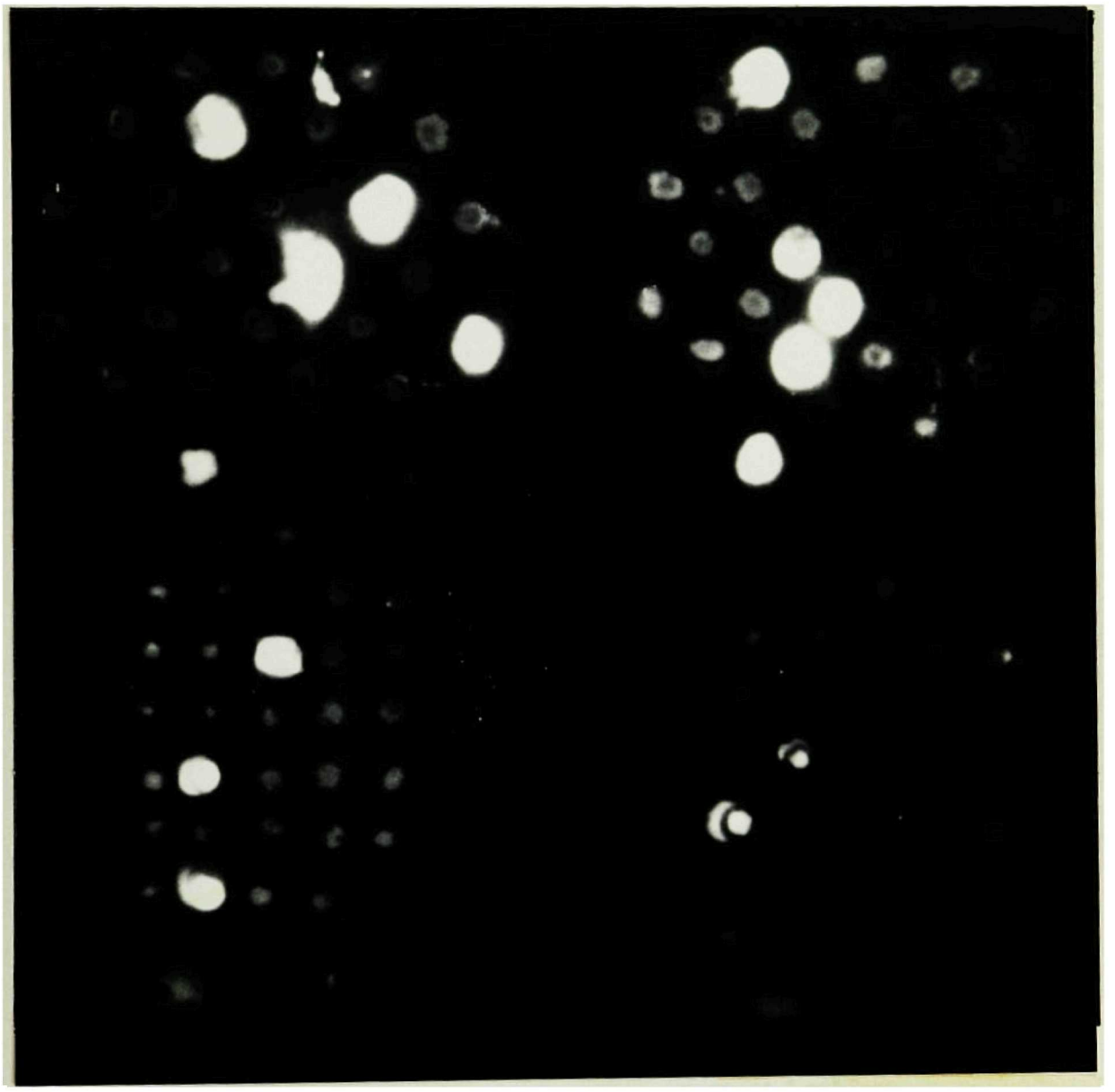
Positive colonies for each of the variable regions were grown up and their plasmid DNA was extracted as described in Materials and Methods. The DNAs from clones representing each variable region were examined by heteroduplex analysis, detailed restriction endonuclease mapping and, in the case of the difference which maps to the inverted repeats (IR_S/TR_S), DNA sequencing. Each of the three regions of strain variation will be discussed individually in detail.

Restriction endonuclease mapping methods

The remainder of the results section is primarily a detailed

FIGURE 20

Identification of colonies containing cloned VZV restriction endonuclease fragments by colony hybridization. VZV DNA was cut with EcoRI, ligated to EcoRI digested pBR325 and used to transform E. coli strain HB101. Transformants were selected by ampicillin resistance and screened for insertion of VZV DNA by chloramphenicol sensitivity. The Am^R, Cm^S colonies were then screened for insertion of specific VZV EcoRI fragments by colony hybridization (Grunstein and Hogness 1975; see Materials and Methods). This is a sample autoradiogram of colony hybridization. Colonies which contain homologous inserted VZV DNA (in this case the EcoRI E fragment from VZV strain Scott) appear in this figure as bright spots while negative colonies are faint.



description of the construction of restriction endonuclease maps for the variable EcoRI restriction fragments of VZV DNA. The process of constructing these maps is complex and difficult to explain. The following description of some of the thought processes and methods used for constructing these maps will hopefully make the procedure more intelligible.

The thought processes used in restriction map construction are similar to those used in puzzles. First, the sizes and number of all the fragments in a given digest must be carefully determined. Clearly the sum of all the fragments in a particular digestion must equal the size of the uncut fragment. It is easiest to determine the terminal fragments first and then, by trial and error, position the remaining fragments on the map in relation to sites already determined. These positions must then be consistent with all data from all the subsequent single and double restriction digestions.

Some of the experimental procedures used for restriction mapping are shown in Figure 21. These are not the only methods available and not all of these were used in this project. In this figure, the data that would be obtained by a particular method is shown on the right, and the maps constructed from these data are shown on the left.

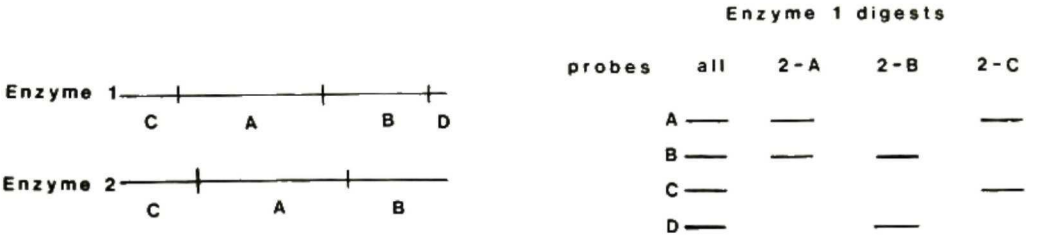
The hybridization method of restriction enzyme site mapping was not used here but has been used by us and others to construct restriction maps for whole VZV DNA (Straus et al., 1982; Dumas et al. 1981). This procedure identifies restriction fragments that are located next to each other, by hybridization with a fragment produced by a second restriction enzyme which overlaps the two adjacent fragments in the first enzyme digest. In the example in Figure 21, the DNA is

FIGURE 21

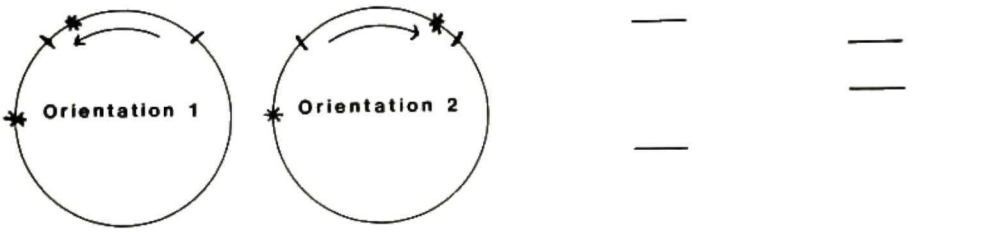
Selected methods of restriction endonuclease mapping. This figure illustrates four methods which are used to construct restriction endonuclease maps and are explained in the text. On the left side of this figure are the fragment maps which can be constructed from the schematic data presented on the right. The orientation, end-labeling / nick translation and double digestion procedures are used extensivly in the remainder of this dissertation.

RESTRICTION ENDONUCLEASE MAPPING

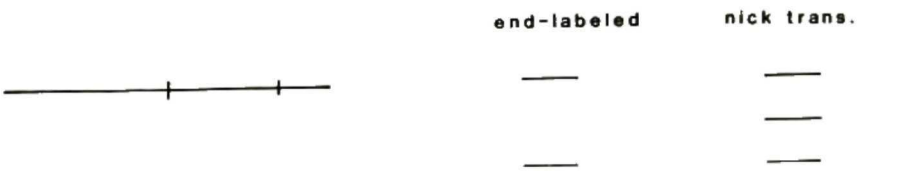
Hybridization



Orientation



End-Labeling / Nick Translation



Double digests

digested with enzyme 1. The fragments are separated by gel electrophoresis and then transferred to nitrocellulose and probed with an isolated restriction fragment produced by enzyme 2. Thus, in this example, the A fragment probe of enzyme 2 hybridizes to the enzyme 1 A and B fragments. These two fragments must therefore be next to each other on the physical map for enzyme 1. By determining which enzyme 1 fragments hybridize with each enzyme 2 probe a map can be constructed for enzyme 1. The map for enzyme 2 can be produced by probing enzyme 2 blots with restriction fragments from enzyme 1. If one of the probes hybridizes with only one or more than two restriction fragments, the site locations must be determined by another method (eg. further digestions with other enzymes).

The orientation and end-labeling / nick-translation methods are both used to determine the position of a small number (1 or 2) of restriction enzyme sites in either a cloned fragment (orientation) or in an isolated fragment (end-labeling / nick-translation). Both of these procedures are used in this dissertation.

The orientation method depends on obtaining the inserted fragment in both possible orientations in a specific vector, such as pBR325, and finding only 1 or 2 sites in both the insert and vector for a particular restriction enzyme. The positions of the restriction sites useful for mapping are already known for most vectors and can be used to locate the site(s) in the inserted fragment. Digestion of clones with an enzyme, other than the one used in the cloning, produces different fragment sizes, depending on the orientation of the inserted fragment in relation to the known position of the restriction site in the vector. Thus, in Figure 21, digestion with the enzyme (*), not used in the

cloning, results in two different fragments for each orientation. Digestion of either of these clones with both enzymes would produce the same four fragments for both orientation (two from the vector and two from the insert). The size of one of the vector fragments plus the size of one of the insert fragments and the size of the other plasmid fragment plus the other insert fragment from this double digestion will equal the sizes of the two fragments in one of the single (*) digestions. Adding the sizes of opposite plasmid and insert fragments will give the size of two fragments seen in the single (*) digestion of the other orientiation. The position of the restriction site in the insert can be determined by comparison of the sizes of fragments in single (*) digestions of clones in both orientations with the fragments produced by double digestion of either orientation clone. This procedure is also useful in mapping two sites in the insert. A fragment which is present in a single (*) digestion of clones in both orientations must be between the two terminal fragments which are identified as described above. All double digestions, with enzymes other than those used in the cloning, must be carried out for both orientations to prevent confusion due to different fragment sizes in clones with different orientations.

The end-labeling / nick-translation method is very similar to the orientation method described above. The DNA fragment is end-labeled or nick-translated and then digested with a restriction enzyme. The terminal fragments are identified because they are the only ones seen in the end-labeled lane. The nick-translated lane contains other fragments, in addition to the terminal fragments, which must be internal. If there is more than one internal fragment their absolute

positions must be determined by another method.

The final mapping method shown in Figure 21 uses double-digestion with two restriction enzymes. In this procedure, restriction sites are located by comparing single-digested and double-digested DNA. This procedure is particularly useful in mapping the position of a restriction fragment in relation to a known site determined by another procedure. In this example (Figure 21), it is assumed that the enzyme 2 site is known. The largest fragment, in the single digestion, is missing in the double digestion and two new fragments are seen. The sum of the sizes of the new fragments equals the size of the missing fragment. Therefore the enzyme 2 site is in the largest enzyme 1 fragment and must be located at the left end of this fragment. The exact postion of the enzyme 2 site cannot be determined in this example but it could be positioned using the end-labeling / nick-translation or the orientation methods (see above) in comparison with double-digestion.

In the course of this project, it was found most useful first to map restriction sites which cut the fragment of interest once or twice, by either orientation or end-labeling / nick-translation, and then to map the sites which occur more frequently by double-digestion in relationship to the infrequent sites.

VZV EcoRI G fragment strain variation

The variable region which maps near the middle of U_L (Figure 19) was examined by further restriction endonuclease mapping to precisely define the position of this variation. The physical map produced from these studies will also be useful in future analysis of VZV gene expression from this region which, because of detectable sequence

homology with HSV and the co-linear gene order of both these viruses (Davison and Wilkie, 1983), may contain genes with important functions which have previously been characterized in HSV.

The VZV EcoRI G fragment, which contains this variable region, was molecularly cloned into pBR235 from a lambda clone of G from the strain Ellen (Straus et al., 1982) and from whole VZV DNA isolated from the strains Scott, Oka and Webster (see above). Transformants containing the cloned EcoRI G fragment were identified by colony blot hybridization as described in Materials and Methods. The recombinant plasmid DNA from positive colonies was extracted, digested with EcoRI and the fragments were separated by agarose gel electrophoresis. The presence of the EcoRI G fragment from all four strains in these recombinant DNAs was confirmed by comigration and hybridization with the EcoRI G fragment from the lambda clone of strain Ellen. Figure 22 is the result of this experiment. The gel in panel A shows two representative recombinant DNAs from each strain (in lanes 2 to 9), each of which contains two bands. The larger band in each lane comigrates with the Ellen G fragment in the lambda clone (lanes 1 and 10). The other band is the plasmid cloning vector which migrates at a position characteristic of its molecular weight (6 Kb). The DNA fragments in this gel were transferred to nitrocellulose and hybridized to radiolabelled lambda Ellen EcoRI G. This blot is shown in panel B. The only band in lanes 2 to 9 which hybridized was the band that migrated at the same position as G in the lambda clone. Lanes 1 and 10 are the homologous controls in which the lambda arms also hybridize, because they are present in the probe.

The small variation in the molecular weight of the EcoRI G

FIGURE 22

Identification of recombinant plasmids that contain the EcoRI G fragment. Plasmid DNA was extracted from transformants which, on colony blots, were positive for VZV EcoRI G. The DNA from two representative isolates for each of the four VZV strains was digested with EcoRI and the fragments were separated by electrophoresis in a 0.7% agarose gel at 20 V for 18 hours. The gel was stained with ethidium bromide and photographed with ultraviolet light. The resulting gel is shown in panel A. Lanes 1 and 10 contain a lambda clone of VZV Ellen EcoRI G (Straus et al. 1982); lanes 2 to 9 are pBR325 clones of VZV EcoRI G from strain Ellen (lane 2 and 3); Scott (lane 4 and 5); Webster (lanes 6 and 7); and Oka (lanes 8 and 9). The DNA in this gel was transferred to nitrocellulose (Southern, 1975) and hybridized with ^{32}P-labelled lambda VZV Ellen EcoRI G. The autoradiogram is shown in panel B. The positions of the plasmid, EcoRI G and lambda bands on this gel and blot are marked.

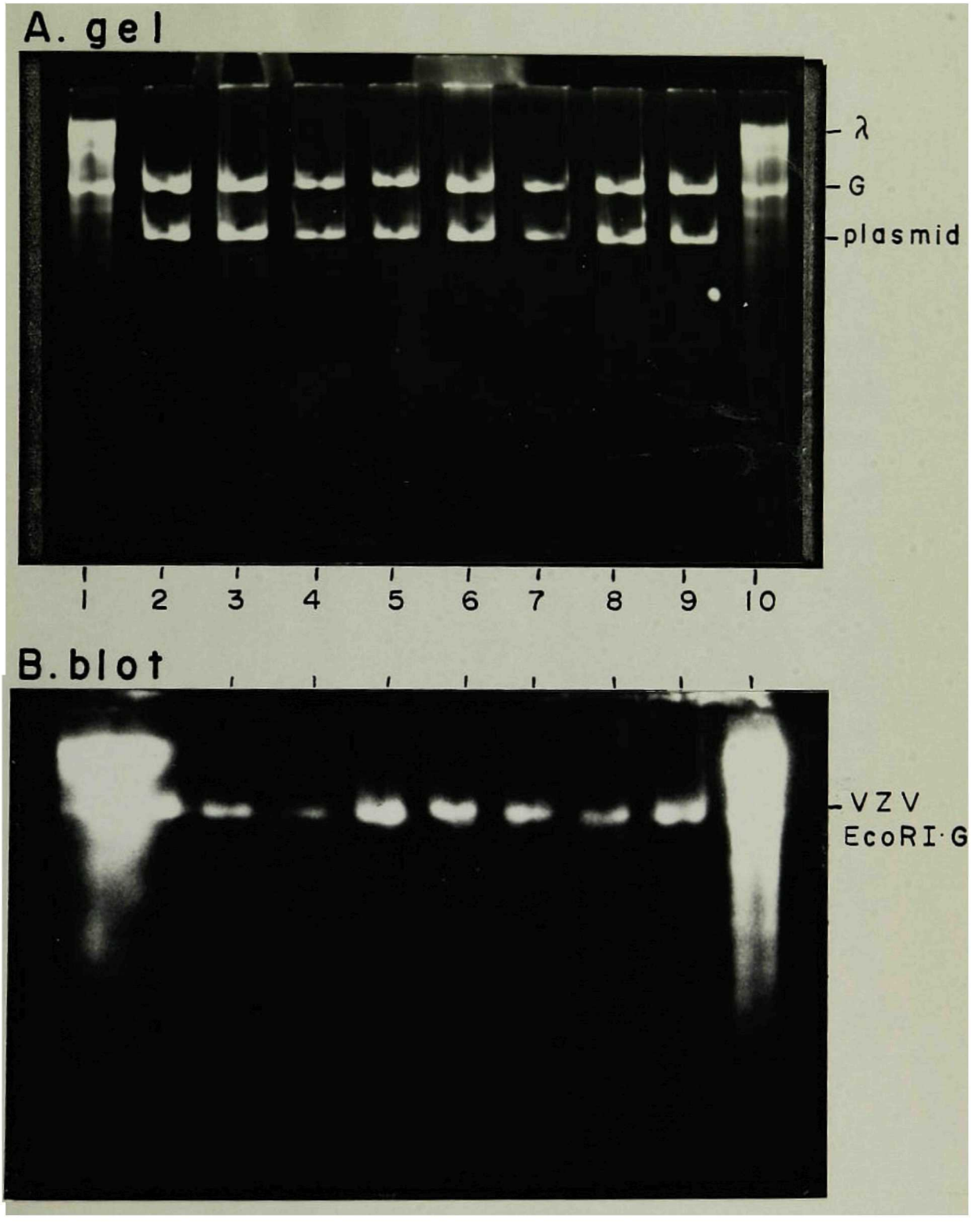
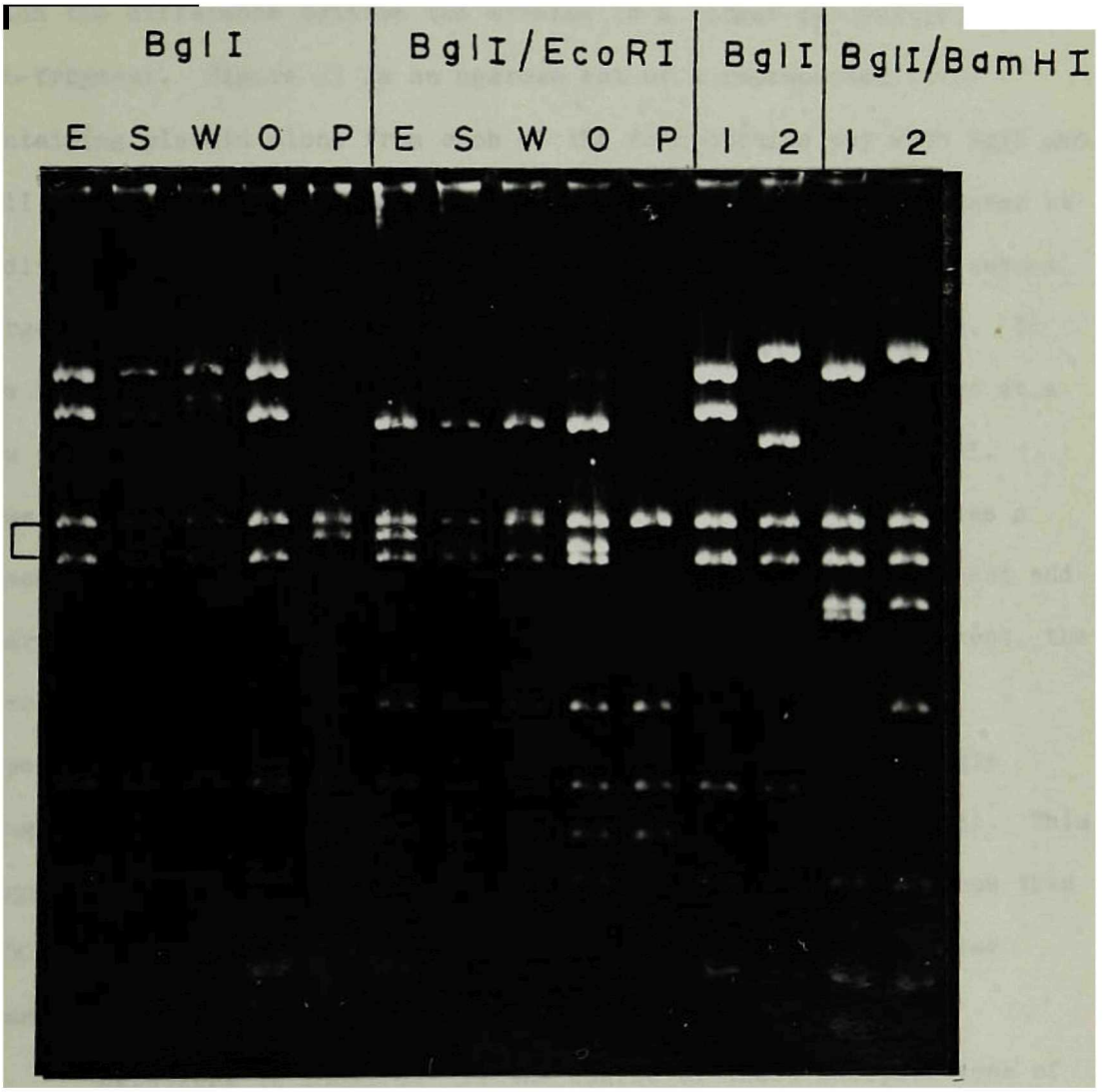

A. gel
λ
G
plasmid
1
2
3
4
5
6
7
8
9
10
B. blot
VZV
EcoRI·G

FIGURE 23

Characterization of the EcoRI G strain variation by BglI and BglI / EcoRI double digestions. Recombinant plasmid DNA containing the EcoRI G fragment from VZV strains Ellen (E), Scott (S), Webster(W) and Oka (O) were digested with BglI or BglI / EcoRI and the band were separated by electrophoresis in a 1.5% agarose gel at 50 V for 18 hours. The gel was stained with ethidium bromide and photographed with ultraviolet light. BglI and Bgl I / EcoRI-digested pBR325 DNA (P) was included as a control. The position of a variable BglI / EcoRI fragment is marked by a bracket. This gel also contains BglI and BglI / BamHI-digested plasmid clones of either orientation (1 or 2) of the Ellen EcoRI recombinants used in restriction site mapping (see text).


BglI
BglI/EcoRI
BglI
BglI/BamHI
E S W O P E S W O P 1 2 1 2

fragment in different strains of VZV cannot be seen in Figure 22 because it is such a small percentage of the total size of G (2%). However, digestion of these clones with restriction endonucleases which cleave this DNA more frequently produce fragments which are smaller and in which the difference between the strains is a larger percentage of the sub-fragment. Figure 23 is an agarose gel of a representative G-containing plasmid clone from each of the four strains cut with BglI and BglI plus EcoRI. In both sets of digestions, a single band migrates at a different position in each strain. In the BglI digestion the second largest band (approximately 3.2-3.5 Kb) is the variable fragment. In the BglI / EcoRI double digestion, the variable fragment migrates at a new position (2-2.3 Kb) because it has also been cleaved by EcoRI. Thus, the variation must be between one EcoRI site, which definies a plasmid-insert junction, and the first Bgl I site in the G fragment and therefore must be within 2.0 to 2.3 Kb of the end of the G fragment, the size of this junction fragment from each strain. In separate experiments, using 5 different restriction endonucleases, a single fragment in each case varied between the strains (data not shown). This suggests that the variation occurs at a small defined region, less than 1200 bp., (see below) and is not the result of a number of smaller changes scattered throughout the G fragment.

Deletions in EcoRI G. In the course of these manipulations of the EcoRI G fragment, unusual properties of this DNA appeared, which have made subsequent experiments difficult to interpret. Clones (both lambda and pBR) containing the G fragment rapidly delete sequences in the variable region and adjacent sequences. This has not been seen with other cloned VZV fragments. The variable BglI / EcoRI fragments in

FIGURE 24

Analysis of recombinant plasmids containing the EcoRI G fragment from different VZV strains. Plasmids containing the EcoRI G fragment from four different VZV strains were double-digested with BglI / EcoRI or SalI / EcoRI, and the bands were separated in a 1.5% agarose gel at 50 V for 18 hours. The positions of the plasmid bands in these double digestions are marked (P). The positions the the variable sub-fragments are also marked. Lanes 1 and 2 are Ellen EcoRI G; lanes 3 and 4, Scott; lanes 5 and 6, Webster; and lanes 7 and 8 are Oka.

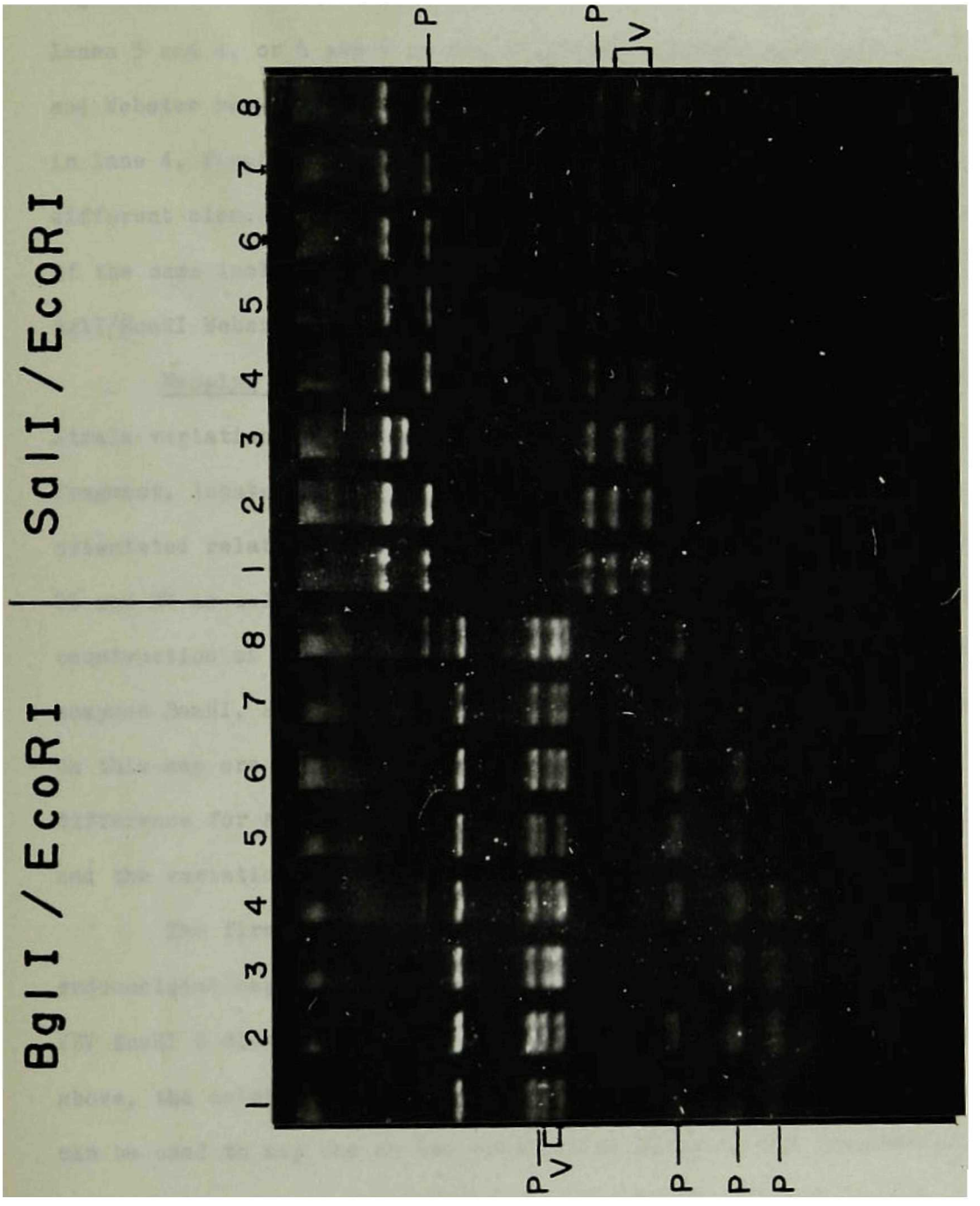

BglI/EcoRI
SalI/EcoRI
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
P
P
V
P
V
P
P
P

Figure 23 from strains Scott and Webster are blurred because of these deletions. Figure 24 contains BglI / EcoRI and SalI / EcoRI double digestion of two clones from each strain. In several cases, (compare lanes 3 and 4, or 5 and 6 in the SalI/EcoRI digests from strain Scott and Webster respectively) deletions, (or a mixed population, SalI/EcoRI in lane 4, Figure 24) have occurred. These deletions occur not only in different clones from the same strain but also in different preparations of the same isolate grown from a single colony (for example Figure 23, BglI/EcoRI Webster and lane 6 in Figure 24 are the same isolate).

Mapping the EcoRI G difference. Despite these deletions, the strain variation in VZV EcoRI G has been mapped to a SalI / BamHI fragment, located near one end of G, and the variation has been orientated relative to restriction maps of whole VZV DNA. Figures 25, 26 and 27 as well as lanes 13, 14, 15 and 16 in Figure 23 detail the construction of the restriction enzyme map shown in Figure 28 for the enzymes BamHI, HindIII, BglI, SalI and XbaI. The sizes of the fragments on this map are for strain Ellen but, as mentioned previously, the difference for all these enzymes was always traced to a single fragment and the variation was mapped to the same area for each enzyme.

The first step in the construction of this restriction endonuclease map was the determination of the orientation of each of the VZV EcoRI G clones in relation to the plasmid vector. As described above, the orientation of the inserted fragment in plasmid recombinants can be used to map one or two restriction sites in the fragment and must be known in order to interpret subsequent double digestions with enzymes not used for the cloning. Digestion by BamHI was used to determine orientation and the results are shown in Figure 25. Digestion of these

clones with BamHI results in two fragments the size of which depends on the orientation of the inserted EcoRI G fragment. The arrows on this figure mark the position of the bands for the two different orientations. All comparisons between strains used in the mapping experiments were done with cloned DNA from different VZV strains in the same orientation. Additional mapping experiments were done using cloned DNA in both orientations from the same strain.

BamHI and HindIII digests. The positions of the single BamHI site and the single HindIII site were determined from the digestions shown in Figure 26. This gel contains digestions of both orientations of the EcoRI G clone from strain Ellen. Digestion of these two clones with BamHI (lanes 5 and 6) results in two bands which are different because of the different orientation as described above. Digestion of either of these clones with BamHI and EcoRI should result in the same four bands, two from the plasmid and two from G, because there is a single BamHI site in G and in the plasmid, and there are two EcoRI sites which define the junction between the G fragment and the plasmid. The BamHI / EcoRI double digestions of these two clones are in lanes 7 and 8 and are identical. The fourth band expected in this digestion has run off the gel because of its small size but its size can be calculated from the sizes of the fragment remaining from G, the plasmid fragments (compare lanes 7 and 8 with pBR325 digested with BamHI / EcoRI in lane 9) and the sizes of the BamHI single-digested clones. One BamHI /EcoRI plasmid fragment plus the remaining G fragment and the other plasmid fragment plus the missing fragment from G equals the sizes of the two fragments of one of the orientations digested with BamHI alone. Switching the additions, that is, the other plasmid band plus the only

visible G band and the missing G band plus the first plasmid band, will equal the sizes of the two bands resulting from BamHI digestion of this orientation. Thus, the difference between the small band (2.3 Kb) in the orientation digested with BamHI in lane 6 and the small plasmid band (smallest band in lanes 7, 8 and 9; 1.8 Kb) is approximately 500 bp and must be the size of the missing BamHI / EcoRI fragment from G. Similar analysis was used to locate the HindIII site in EcoRI G. Lanes 10 and 11 are HindIII digestions of the same clones used in lanes 5 and 6 respectively. Lane 12 is the HindIII / EcoRI double digestion and the smallest band has again run off the gel. Its size was calculated, based on the difference between the small band in lane 10 and the small plasmid band in the double digest in lane 12, to be approximately 200 bp. In addition, these digestions indicate that the HindIII site is located at the other end of the EcoRI G fragment from the BamHI site. Comparison of HindIII and BamHI digestions of the identically oriented clone (lane 6 with lane 10 and lane 7 with lane 11) shows that one enzyme produces bands which are close in size and the other results in bands that are far apart for the same orientation. The opposite would occur if the BamHI and HindIII sites were located at the same end of the EcoRI G fragment, because the BamHI and HindIII sites are known to be near each other in pBR325 (Bolivar 1978). Using the location of the BamHI and HindIII sites in EcoRI G, the map can be orientated with respect to the maps for whole VZV DNA using these three enzymes (see Figure 19). Based on this comparison, the BamHI site is located at the left end and the HindIII site at the right end of EcoRI G as seen in Figure 28.

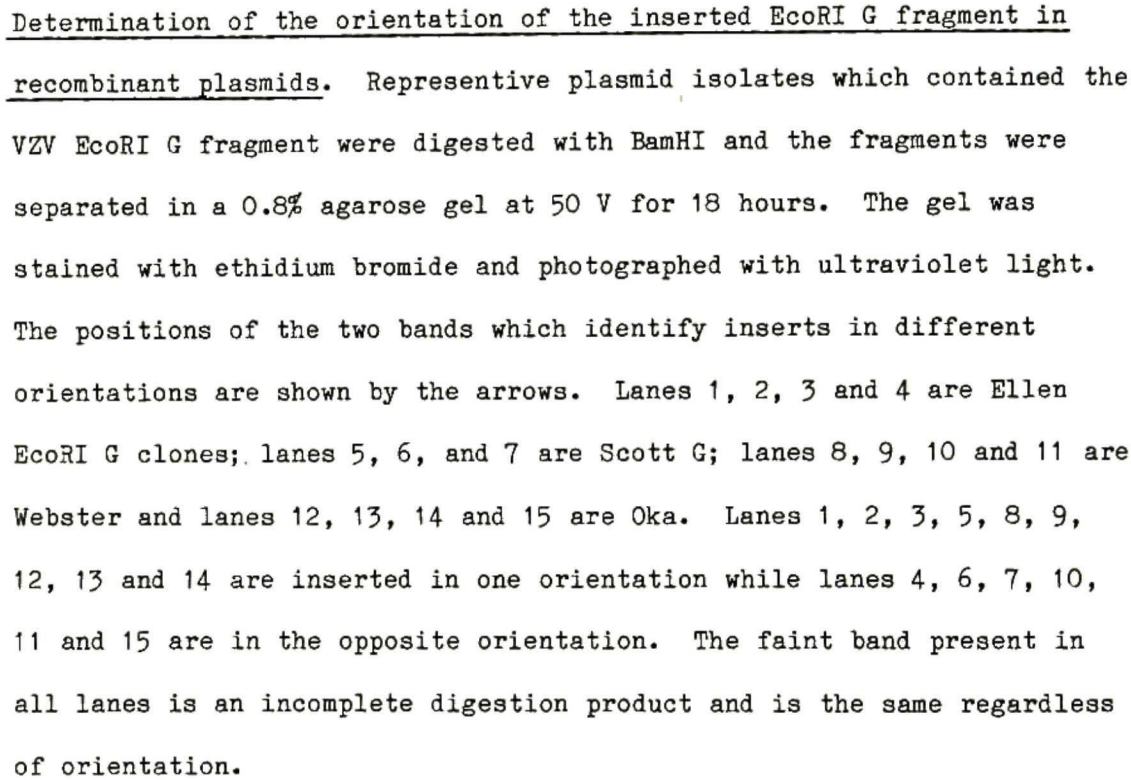
SalI digests. The locations of the two SalI sites in VZV EcoRI

G were determined in a similar manner. Lanes 14 and 15 in Figure 26 are the SalI digestions of both orientations (the DNA for one orientation in lane 14 has not been completely digested in this gel, however, a complete digestion of the clone in this orientation is shown in Figure 27, lane 15); lane 16 is the SalI / EcoRI double digestion and lane 17 is the plasmid control digested with SalI / EcoRI. The two new small fragments in the SalI / EcoRI double digestion in lane 16 are the junction fragments from EcoRI G. Based on the known orientation of the clone used in lane 15 and the known position of the SalI site in pBR325, the larger SalI / EcoRI junction fragment, which is variable (1.8 Kb in strain Ellen) maps at the left end of EcoRI G and the other junction fragment (1.5 Kb) is at the right end. The large SalI fragment (5.5 Kb) is internal to these junction fragments, because this fragment is present in the SalI digestion (comigration with the large plasmid-G junction fragment) in lane 15 and is at the same position in the SalI / EcoRI double digestion in lane 16.

BglI and XbaI digests. Mapping data for BglI and XbaI in relationship to the HindIII and SalI sites in EcoRI G are shown in Figure 27. The BglI sites were also mapped in relation to the BamHI site in Figure 23 (BglI and BglI/BamHI double-digestions for orientation 1 and 2).

There is a single XbaI site in EcoRI G and no sites in the plasmid. EcoRI / XbaI double digestion results in three fragments, one which is the plasmid, and two fragments (5.2 and 3.6 Kb) from G (not shown). XbaI cleaves the 5.5 Kb internal SalI fragment of EcoRI G (Figure 27, lanes 14 and 15) into 2 Kb and 3.5 Kb fragments and it also cleaves the 2 Kb BglI internal fragment into 1.8 Kb and 200 bp fragments

FIGURE 25



Determination of the orientation of the inserted EcoRI G fragment in recombinant plasmids. Representive plasmid isolates which contained the VZV EcoRI G fragment were digested with BamHI and the fragments were separated in a 0.8% agarose gel at 50 V for 18 hours. The gel was stained with ethidium bromide and photographed with ultraviolet light. The positions of the two bands which identify inserts in different orientations are shown by the arrows. Lanes 1, 2, 3 and 4 are Ellen EcoRI G clones; lanes 5, 6, and 7 are Scott G; lanes 8, 9, 10 and 11 are Webster and lanes 12, 13, 14 and 15 are Oka. Lanes 1, 2, 3, 5, 8, 9, 12, 13 and 14 are inserted in one orientation while lanes 4, 6, 7, 10, 11 and 15 are in the opposite orientation. The faint band present in all lanes is an incomplete digestion product and is the same regardless of orientation.

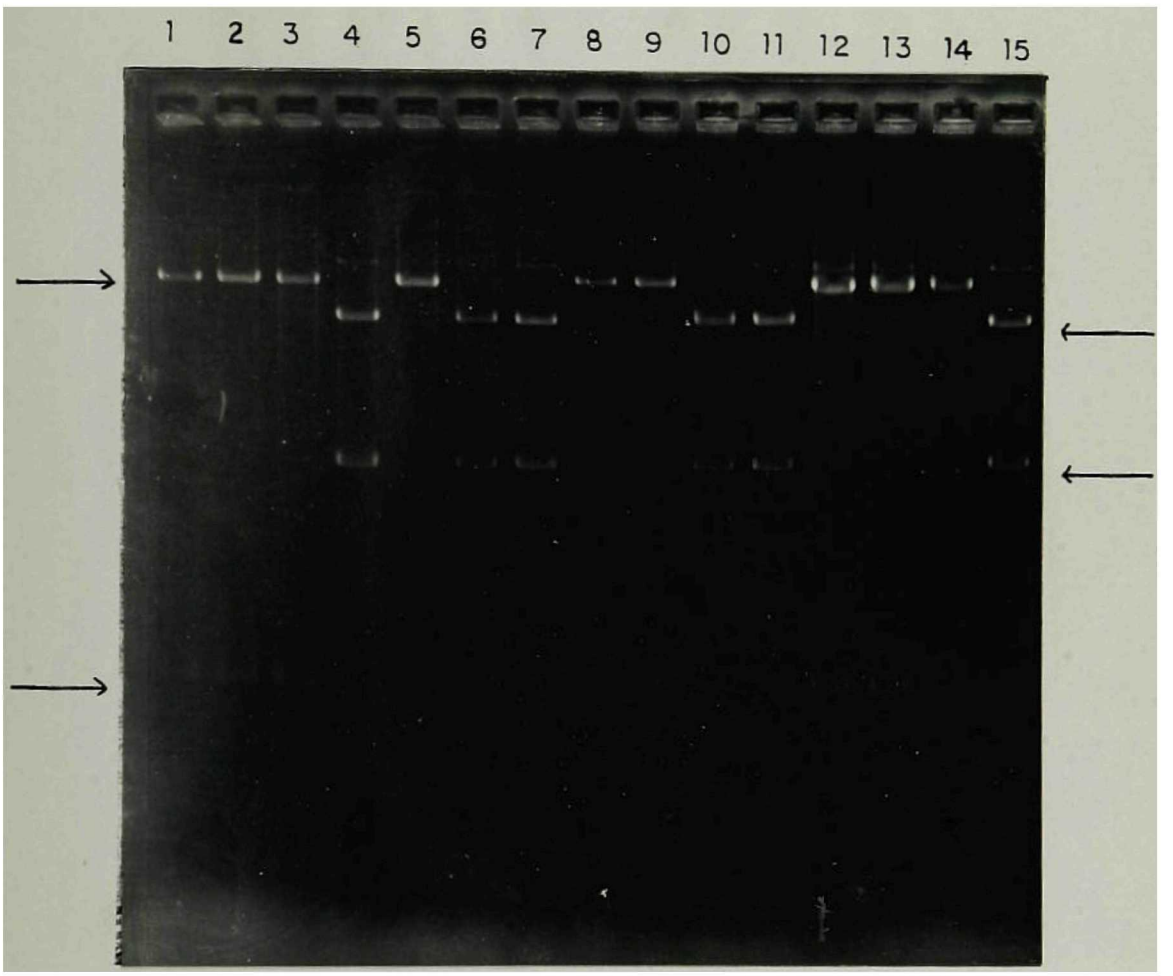

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

FIGURE 26

Restriction endonuclease mapping of the EcoRI G fragment for BamHI, HindIII, XbaI and SalI. Plasmids containing either orientation of the Ellen EcoRI G fragment were single or double-digested and the fragments were separated in a 1% agarose gel at 50 V for 18 hours. The gel was stained with ethidium bromide and photographed with ultraviolet light. Lane 1 contains HaeIII-digested ΦX174 DNA and lane 18 is HindIII-digested lambda DNA as molecular weight standards. Lane 2 and 3 are EcoRI digestions of orientation 1 and 2 respectively; lane 4 is pBR325 digested with EcoRI as a control. Lane 5 is orientation 1 and lane 6 is orientation 2 digested with BamHI; lane 7 and 8 are BamHI / EcoRI double digestions of these two orientations and lane 9 is pBR325 cut with BamHI and EcoRI. Lane 10 is orientation 1 and lane 11 is orientation 2 digested with HindIII; lane 12 is orientation 1 double-digested with HindIII / EcoRI and lane 13 is pBR325 cut with HindIII. Lanes 14 and 15 are SalI digestions of orientation 1 and 2 respectively. Lane 16 is a SalI / EcoRI double digestion of orientation 2 and lane 17 is pBR325 double-digested with SalI / EcoRI.

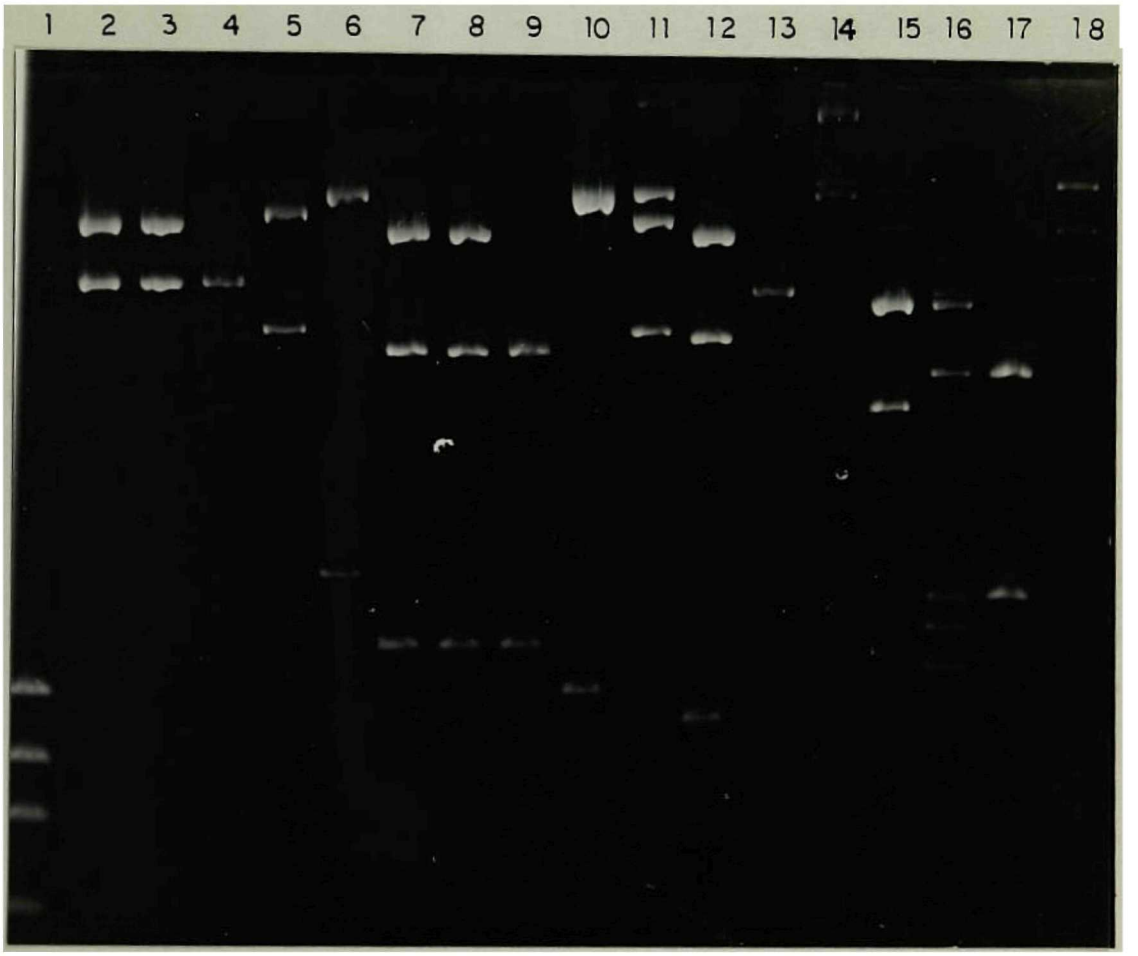

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

FIGURE 27

BglI mapping of the EcoRI G fragment. Plasmids containing the Ellen EcoRI G fragment in either orientation (A or B in this figure) were digested with BglI, BglI / XbaI, BglI / SalI, BglI / HindIII, XbaI / SalI and XbaI / HindIII and the fragments were separated in a 1.5% agarose gel at 50 V for 18 hours. The gel was stained with ethidium bromide and photographed with ultraviolet light. Lane 1 contains lambda EcoRI fragments; lane 2 is ØX174 HaeIII fragments; lane 18 is lambda HindIII fragments; lane 19 is EcoRI digested adenovirus type 2 and lane 20 is SV40 HindIII fragments which are included as molecular weight markers. pBR325 DNA was also double-digested with these same enzymes as controls (P). Lanes 3, 4 and 5 are BglI; lanes 6 and 7 are BglI / XbaI; lanes 8, 9 and 10 are BglI / SalI; lanes 11,12 and 13 are BglI / HindIII; lanes 14 and 15 are XbaI / SalI; lanes 16 and 17 are XbaI / HindIII.

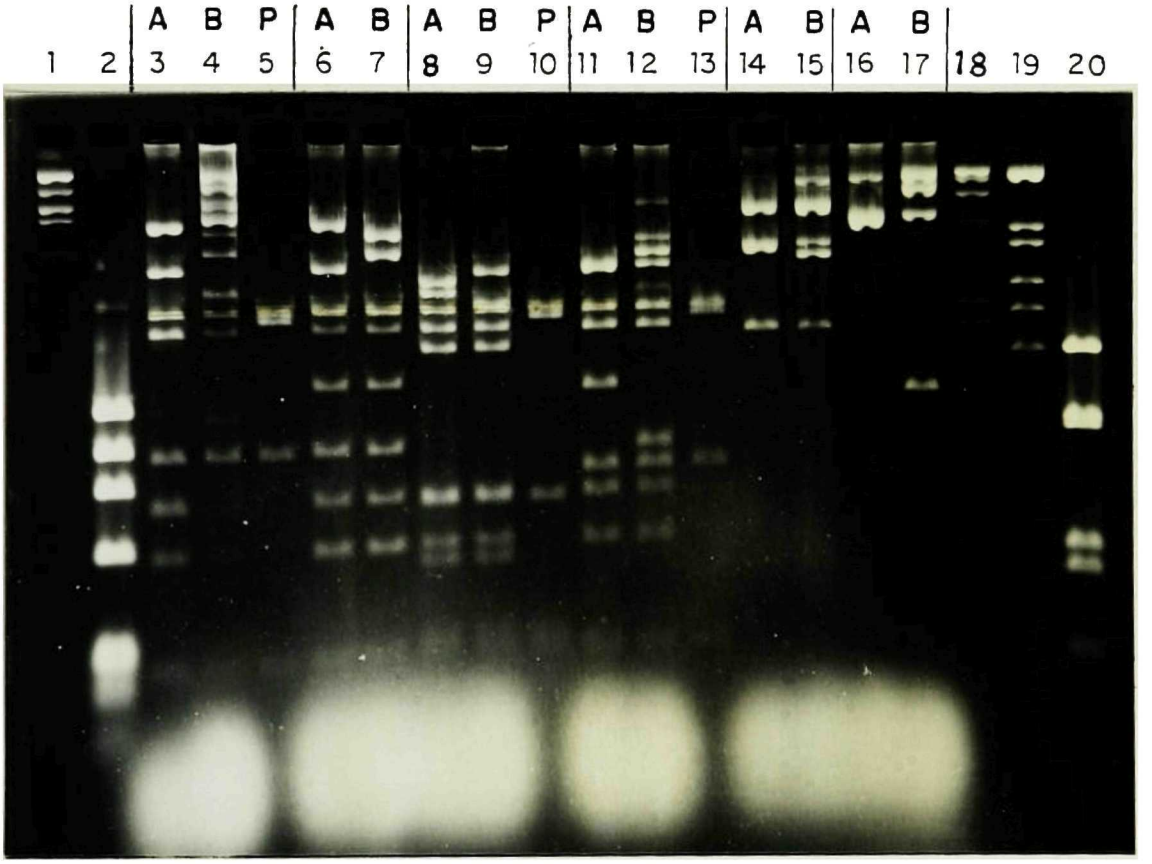

A B P A B A B P A B P A B A B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

FIGURE 28

Restriction endonuclease map for VZV EcoRI G. Location of the cleavage sites for the restriction endonucleases EcoRI, SalI, BglI, XbaI, BamHI and HindIII in the VZV EcoRI G fragment. This map is based on data present in figures 23 to 27 as described in the text. The sizes shown are for Ellen EcoRI G. The map position of the variable region is marked (BamHI / Sal I).

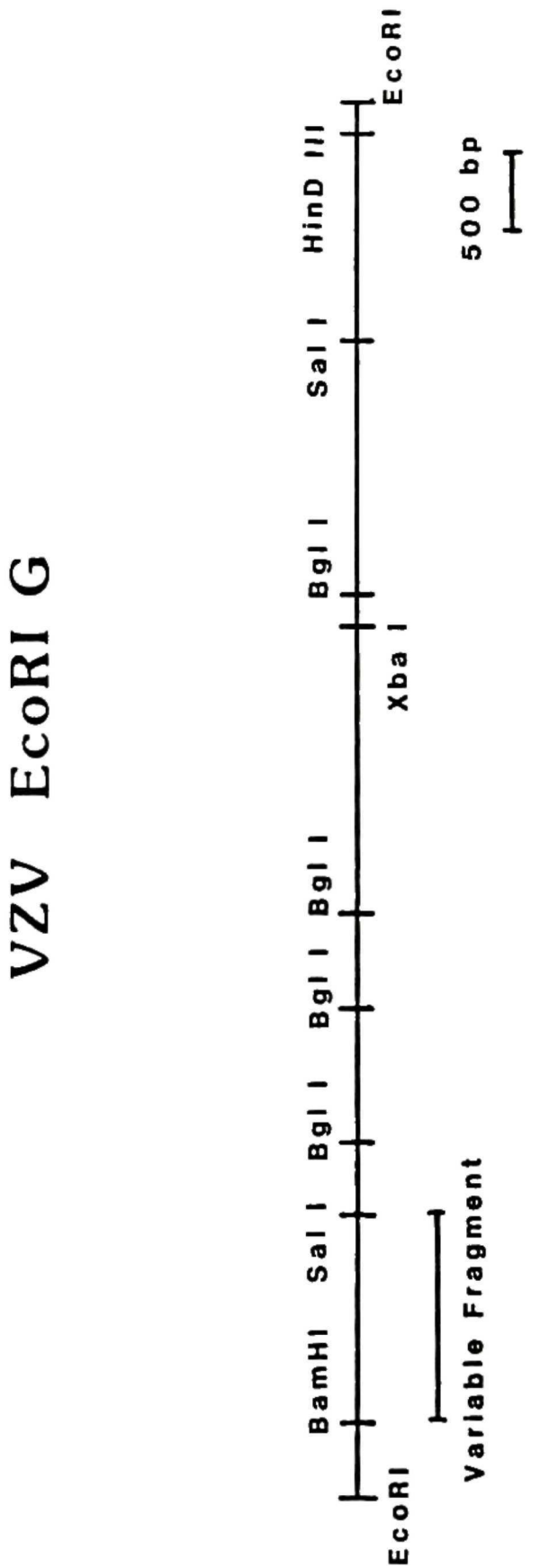
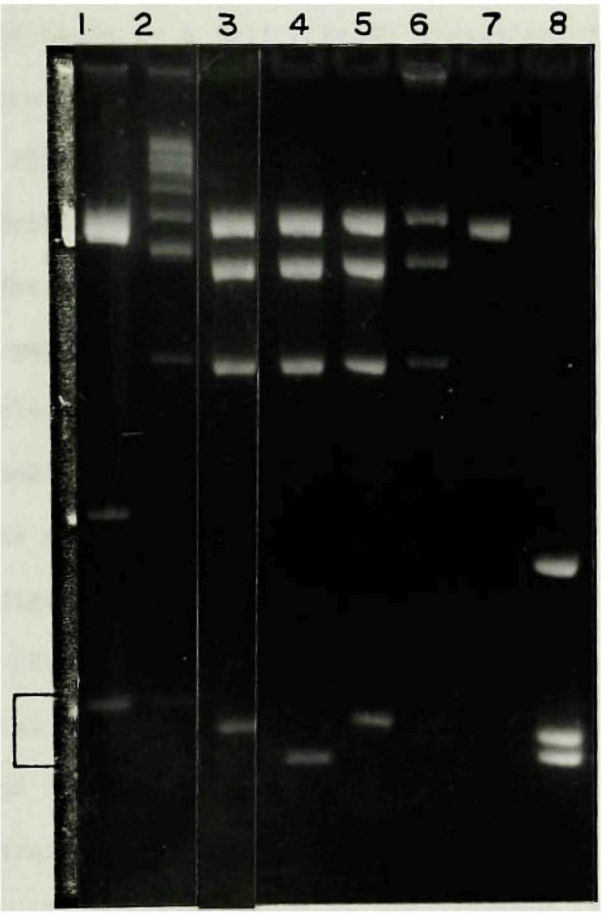

VZV EcoRI G
EcoRI
BamHI
Sal I
Bgl I
Bgl I
Bgl I
Bgl I
Xba I
Bgl I
Sal I
HinD III
EcoRI
Variable Fragment
500 bp

FIGURE 29

Digestion of the EcoRI G fragment from various strains with BamHI / SalI. Recombinant plasmid DNA containing the EcoRI G fragment from VZV strains Ellen (lanes 1 and 2), Oka (lane 3), Webster (lanes 4 and 5) and Scott (lanes 6 and 7), were digested with BglI / SalI and the bands were separated by electrophoresis in a 1.5% agarose gel at 50 V for 18 hours. The gel was stained with ethidium bromide and photographed with ultraviolet light. Lane 8 has HaeIII SV40 DNA fragments as molecular weight markers. The position of a variable BamHI / SalI fragment is marked by a bracket.


1
2
3
4
5
6
7
8

(Figure 27, lanes 6 and 7). The XbaI site must be toward the right end of the EcoRI G fragment to fit these double digestion data as seen in Figure 28.

BglI / EcoRI double digestion of VZV EcoRI G results in 5 bands; additional bands result from sites within the plasmid. The terminal EcoRI / BglI fragments were identified by comparison with BglI digestion and the plasmid bands in a BglI / EcoRI digestion of pBR325. The 2.2 Kb junction fragment is located at the left end because it is cleaved by BamHI (Figure 23) and the other junction fragment (3.1 Kb) is at the right end of EcoRI G because it is cleaved by HindIII (Figure 27, lanes 11 and 12). The internal BglI fragments, 800, 500 and 2000 bp, were positioned by partial digestion products and because the 2000 bp BglI fragment was cleaved by XbaI (Figure 27, lanes 6 and 7).

The BamHI / SalI fragment marked on the map in Figure 28 as the location of the strain variation is consistent with all this restriction endonuclease digestion data described above. It is within a terminal BglI fragment (2.2 Kb) and a terminal SalI fragment (1.8 Kb). These two fragments map at the left end of the EcoRI G fragment based on orientation and double digestions with BamHI. Figure 29 confirms that the EcoRI G strain variation is between the BamHI and SalI sites at the left end of G. In this gel there is one fragment (marked with a bracket), which is the correct size predicted by the mapping data, and which varies among different strains.

The EcoRI G strain difference has therefore been located to a 1300 to 1500 bp BamHI / SalI fragment which is 500 bp (the predicted size of the BamHI / EcoRI terminal fragment) from the left end of the EcoRI G fragment.

VZV EcoRI P fragment strain variation

The variable region in the U_L region of VZV DNA, which is positioned toward the left end of the restriction maps in Figure 19, was examined by detailed restriction mapping and electron microscopic heteroduplex analysis to determine the position and nature of this variation. The VZV EcoRI P fragment, which contains this variable region, was cloned from whole VZV DNA for strains Scott, Oka and Webster as described in Materials and Methods. A lambda clone containing the EcoRI H, P and Q fragments (Straus et al., 1982) was used as a source of the Ellen P fragment. An agarose gel of an EcoRI digestion of these EcoRI P fragment clones is shown in Figure 30. Independent clones from the same strain appear to be identical (for example, lanes 9 to 12 are all from the Oka strain). In addition, different preparations of the same recombinant plasmid appear identical (data not shown). Thus, unlike the EcoRI G fragment, the EcoRI P fragment strain difference appears to be stable in E. coli. Size estimates for the EcoRI P fragment from different strains are Scott, 1850; Webster, 1800; Ellen, 1700; and Oka, 1600 bp. The EcoRI P fragments from Scott and Oka were chosen for detailed comparison because the greatest size difference was seen between these two.

Electron Microscopic Analysis. Heteroduplex analysis was performed between the EcoRI P fragments from Scott and Oka to determine of both the number of variable sequences and their (its) location in the fragment. Equal amounts of gel-purified EcoRI P fragments from these two strains were mixed, denatured and allowed to renature before examination in the electron microscope. Two representative heteroduplex molecules are shown in Figure 31. These electron micrographs show that

FIGURE 30

Identification of recombinant plasmids which contain the VZV EcoRI P fragment. Plasmid DNA was isolated from colonies which contained the VZV EcoRI P fragment by colony hybridization. The DNA was digested with EcoRI and the fragments were separated in a 1% agarose gel at 50 V for 18 hours. The gel was stained with ethidium bromide and photographed with ultraviolet light. Lanes 1, 6 and 7 contain as molecular weight markers lambda cut with HindIII, SV40 cut with HindIII and Ad2 cut with EcoRI respectively. Lanes 2 and 3 contain EcoRI P from strain Scott; lanes 4 and 5 are P from Webster; lane 8 is a lambda clone from Ellen which contains the EcoRI H, P and Q fragments; lanes 9, 10, 11 and 12 are EcoRI P from Oka. The positions of the variable P fragments are marked with a bracket.

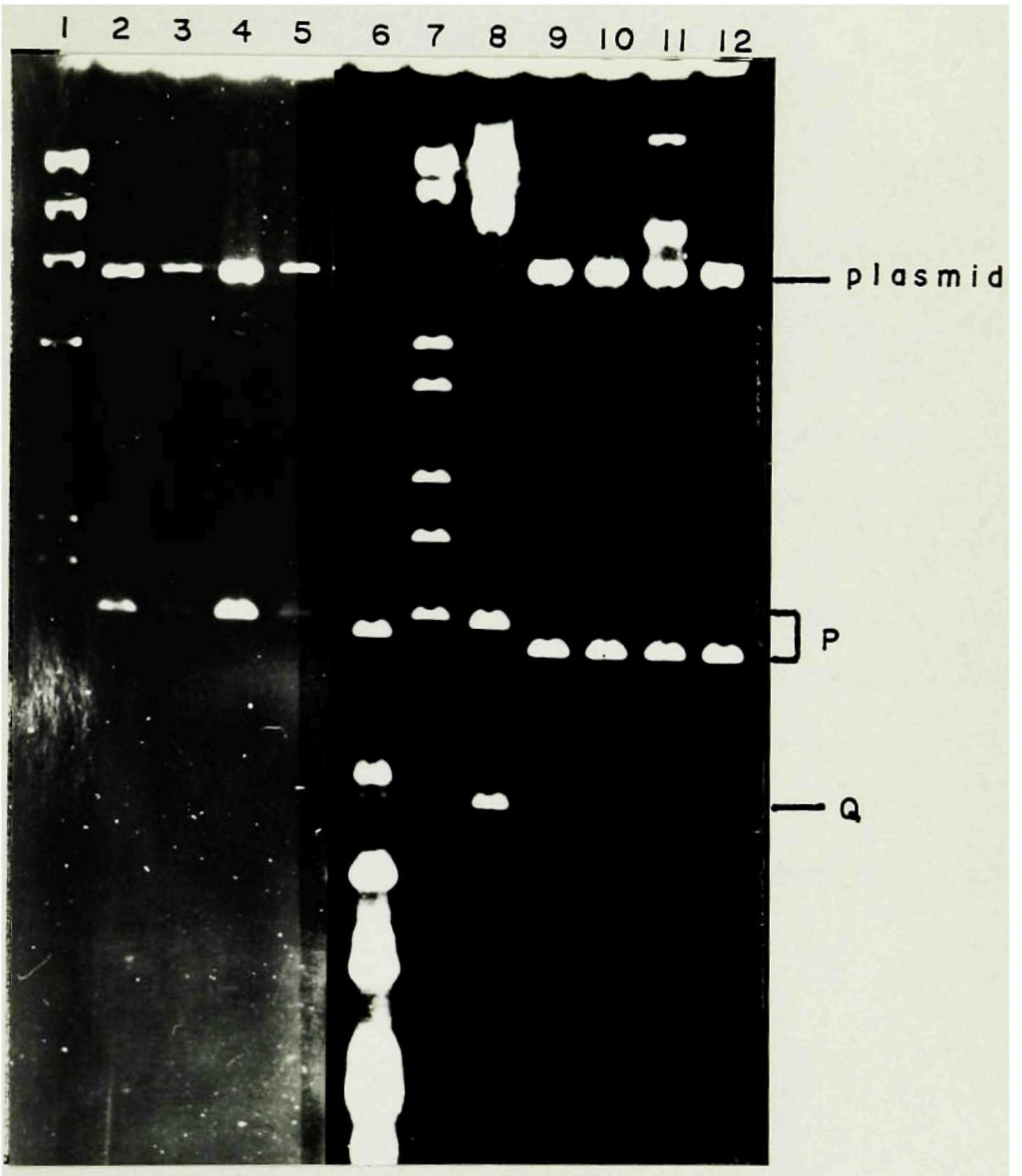

1 2 3 4 5 6 7 8 9 10 11 12
plasmid
P
Q

FIGURE 31

Electron micrographs of heteroduplex DNA molecules between the VZV EcoRI P fragments from strains Oka and Scott. Plasmids containing the EcoRI P fragment from Ellen and Scott were digested with EcoRI and the bands were separated in 5% polyacrylamide gels. The EcoRI P bands were cut out and extracted from the gel. Equal amounts of each DNA were mixed, denatured and allowed to reanneal before being spread and mounted for examination in the electron microscope as described in the Materials and Methods. These are two representative heteroduplex molecules. There is a single stranded insertion or deletion loop near the middle of both molecules which is the strain difference. The magnification is approximately 112,000X.

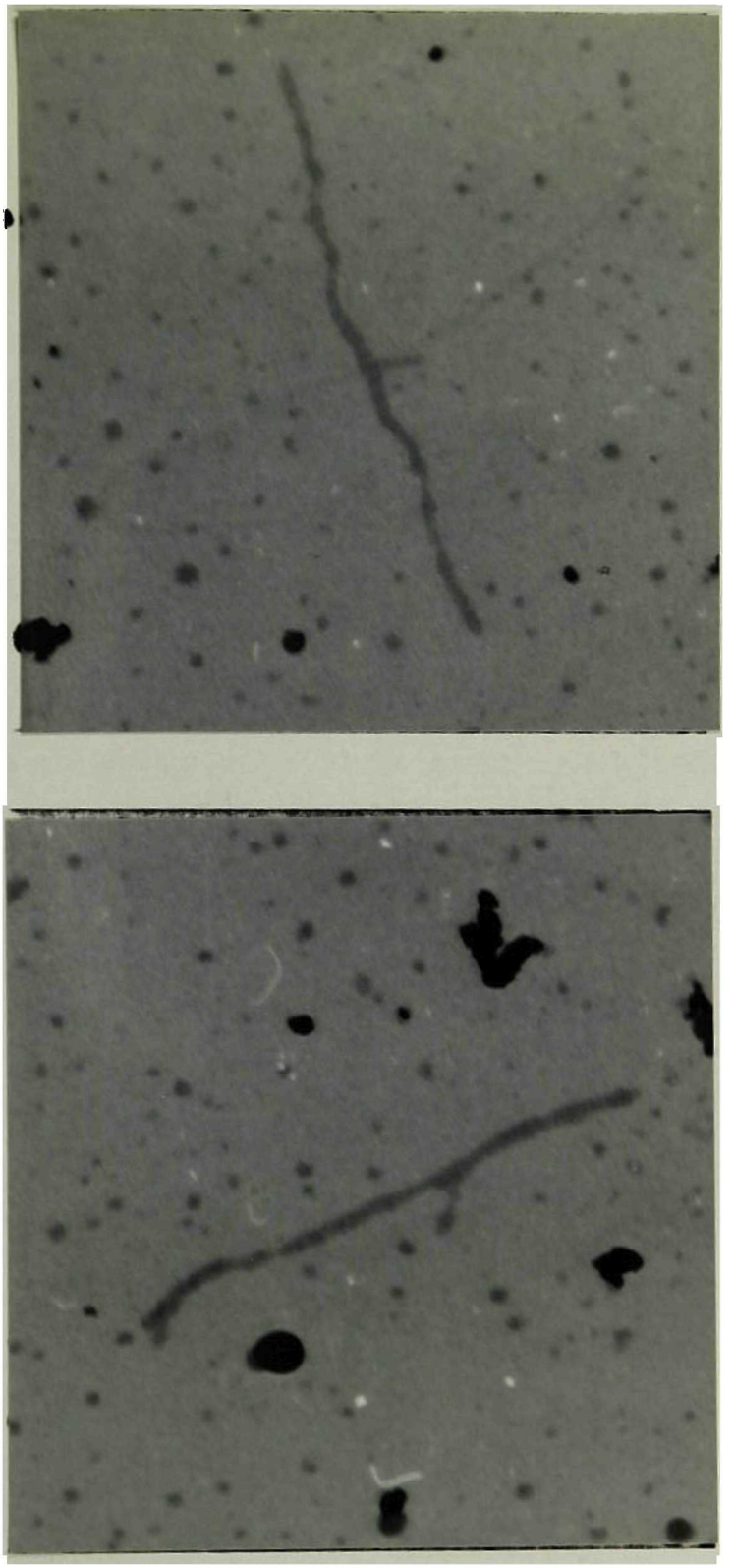
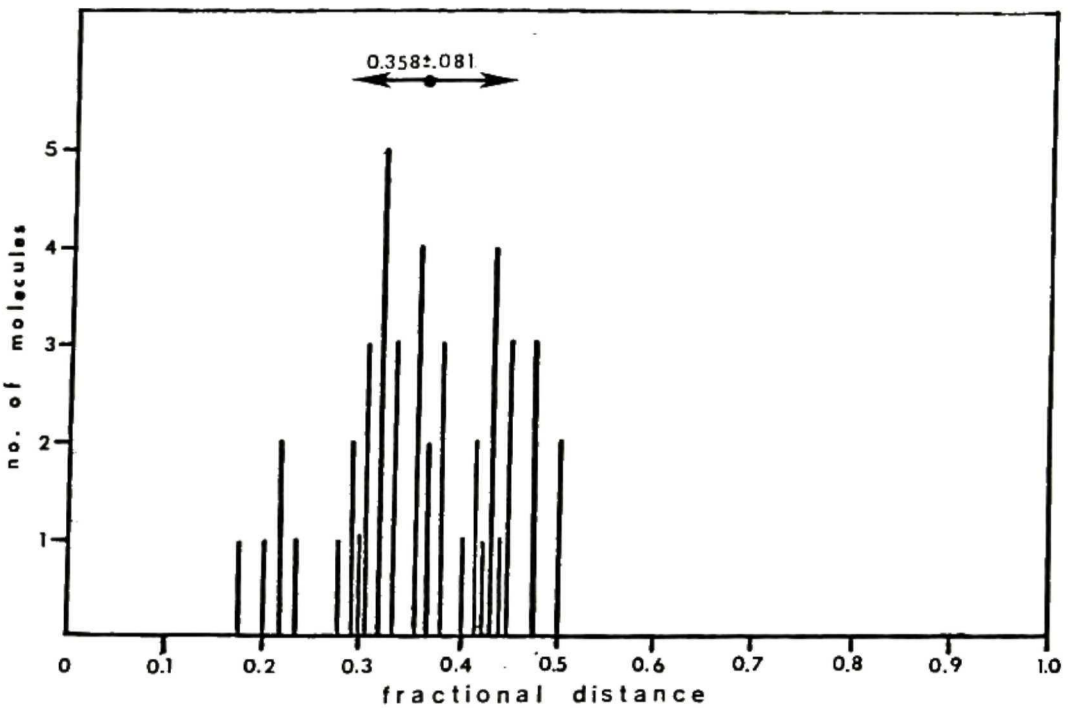


FIGURE 32

Summary of the heteroduplex analyses of VZV EcoRI P from strains Oka and Scott. The position of the insertion / deletion loop is plotted as the fractional distance from one end of the molecule vs. the number of molecules with a loop. A total of 46 heteroduplexes are shown on this Figure. The mean fractional position of the difference is also shown.


0.358±.081
no. of molecules
1
2
3
4
5
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
fractional distance

most of the molecule is double-stranded with a single-stranded insertion-deletion loop near the middle of the molecule. This appears to be the only difference, although additional very small differences might not be detected by this procedure. The size of this insertion or deletion is difficult to determine but appears to be close to the size of the difference between the EcoRI P fragments from the Scott and Oka strains (200-300 bp) estimated from agarose gels (Figure 30). A histogram summary of 46 heterduplex molecules between Scott and Oka EcoRI P is shown in Figure 32. These data suggest that the difference between the strains in the EcoRI P fragment is due to insertion or deletion of DNA at a defined location, approximately 40% from one end of the fragment.

Restriction Endonuclease Mapping. The difference in the EcoRI P fragment was also characterized by restriction endonuclease mapping. These studies were done to confirm the position of variation determined by heteroduplex analysis and to produce a physical map. The physical map of EcoRI P will be useful for determining the nucleotide sequence of the strain variation and for mapping of viral transcripts from this region in ongoing studies. The method used for mapping involved isolation of the cloned EcoRI P fragments from 5% polyacrylamide gels and comparison, by gel electrophoresis, of different single and double digestions of purified P fragment DNA which had been radiolabeled by either nick-translation or end-labeled with polynucleotide kinase (see Materials and Methods). Autoradiograms which support the restriction map in Figure 38 are shown in Figures 33 though 37. The sizes shown on the map in Figure 38 are for Scott EcoRI P but the variable area indicated was the only region which was found to vary in the strains

examined.

The restriction map of EcoRI P could not be aligned with the published maps for VZV DNA because it is not cleaved by any of the enzymes which have been used for mapping. The map is therefore oriented arbitrarily with the single HincII site toward the right side. Restriction sites for HinfI, DdeI, TaqI and HaeIII are positioned in relation to this HincII site. Lanes 8 and 9 in Figure 33 show the two bands which result from cleavage at the single HincII site for the P fragment from strains Scott and Oka, respectively. It is the largest HincII sub-fragment which varies (either 1400 or 1200 bp in Scott and Oka, respectively) with a smaller fragment (420 bp) which is the same size in both strains. The sum of these two fragment is very close to the size of uncut EcoRI P for these two strains. Small terminal fragments probably do not exist, because these same two fragments are end-labeled by polynucleotide kinase as seen in Figure 35 lane 5. Smaller internal HincII fragments were not seen and, if present, must be smaller than 25 bp.

HinfI Digests. Digestion of the EcoRI P fragment with HinfI results in 3 bands seen in Figure 33 lanes 6 and 7 (the largest two bands in lane 7 comigrate). The terminal HinfI fragments, identified by end-labeling, are 670 bp and 240 bp (lane 6, Figure 34). The variable fragment (860 or 670 bp) must therefore be internal. The terminal HinfI fragments are not cut by HincII (lane 2 Figure 34) and therefore the 240 bp fragment must be at the left end of the fragment. This is confirmed in lanes 3 and 4, Figure 35, where there is a new band of 180 bp which results from the cleavage of the 420 bp HincII band by HinfI (240+180=420 bp).

TaqI Digests. TaqI digestion of EcoRI P is shown in lanes 10 and 11 in Figure 33. The fragment sizes are 1100 or 850 bp (variable fragment), 400, 120 and 50 bp (common fragments). Partial digestion by TaqI (not shown) suggest that two bands co-migrate at both the 120 and 50 bp position. The TaqI sites were mapped in relation to the HincII and HinfI sites by the double digestions shown in Figure 36. The terminal TaqI fragments are the variable one, 1100 or 900 bp, and the one at 400 bp in size (lane 8 in Figure 34 and lane 5 in Figure 36). The variable fragment was placed on the right because it is cleaved into 240 and 650 or 850 bp fragments by HinfI (lanes 7 and 8, Figure 36) and the 400 bp fragment on the left because it is not cut with this enzyme. The variable TaqI sub-fragments could also be positioned at the left end of the EcoRI P fragment because they are cut by HincII into variable fragments (500 or 650 bp) and a 400 bp fragment in the TaqI / HincII double digestion in Figure 36, lanes 9 and 10. The remaining 120 and 50 bp fragments could not be definitively placed. Partial digestion (not shown) suggests that they map toward the left end of EcoRI P to the right of the terminal 400 bp TaqI fragment with the arrangement of 50 + 120 + 50 + 120 bp; this is the way these fragments are shown on Figure 38.

DdeI Digests. DdeI digestion of EcoRI P results in four fragments. The largest fragment (1200 or 1000 bp) is variable and the common fragments are 340, 220 and 110 bp. Partial digestion (lane 2, Figure 37) suggests there is an additional small fragment (not resolved on the gels but approximately 20 bp in size) which has not been placed on the map in figure 38. Figure 37 shows the double digests, with HincII and HinfI, and the end-labeled digests used to map the DdeI

sites. The DdeI terminal fragments are 340 and 220 bp (lane 1, Figure 37). Neither of these terminal fragments is cut by either HincII or HinfI (Figure 37, lanes 3 through 8). The 220 bp DdeI fragment must be at the right and the 340 bp fragment at the left of EcoRI P. If the postions of these two fragments were reversed, the 340 bp fragment would have been cut by HinfI (see map in Figure 38). The variable DdeI fragments are cut by HincII into 1200 or 840 bp and 80 bp fragments (lanes 5 and 6, Figure 37). For this to occur, the variable fragments must map next to the 340 bp terminal fragment at the left end of EcoRI P. This places the 110 bp fragment between the variable fragment and the 220 bp fragment at the right end. The 110 DdeI fragment in this position should be cut by HincII and HinfI. Lanes 5 and 6 in Figure 37 show that the 110 bp DdeI fragment is cut by HincII and lanes 4 and 6 in this figure show that it is cut by HinfI into sizes predicted by the map. HinfI also cuts the variable DdeI fragment into 820 or 580 bp variable sub-fragments and a common 360 bp fragment (lanes 3 and 4, Figure 37). The sizes of these fragments are consistent with the positioning of the DdeI sites as described above and shown in the map (Figure 38).

HaeIII Digests. HaeIII digestion of EcoRI P from strains Scott and Oka are shown in Figure 33, lanes 4 and 5 respectively. No variable fragment can be seen in the HaeIII digests. The intensity of the smallest band (approximately 35 bp) is greater than it should be for the size of this fragment and appears to be dependent on the size of the variation. That is, in the Scott digestion (lane 4, Figure 33), this fragment is more intense than the same fragment in the Oka digestion (lane 5, Figure 33). This suggests that the strain difference is due to

FIGURE 33

HaeIII, HinfI, HincII and TaqI sub-fragments of the EcoRI P fragments from Scott and Oka. Plasmids containing the EcoRI P fragments from Scott and Oka were digested with EcoRI and separated in 5% polyacrylamide gels. The P fragments were cut out and passively eluted as described in Materials and Methods. The gel-purified P fragments were nick-translated, digested with various restriction enzymes and the fragments separated in an 8% polyacrylamide gel at 100 V for 5 hours. The gel was dried and the bands visualized by autoradiography.
Lane 1 contains a 123 bp ladder (Bethesda Research Labs) as molecular weight markers. The other lanes contain either Scott (S) or Oka (O). Lane 2 and 3 are uncut; lanes 4 and 5, HaeIII; lanes 6 and 7, HinfI; lanes 8 and 9, HincII; lanes 10 and 11, TaqI.

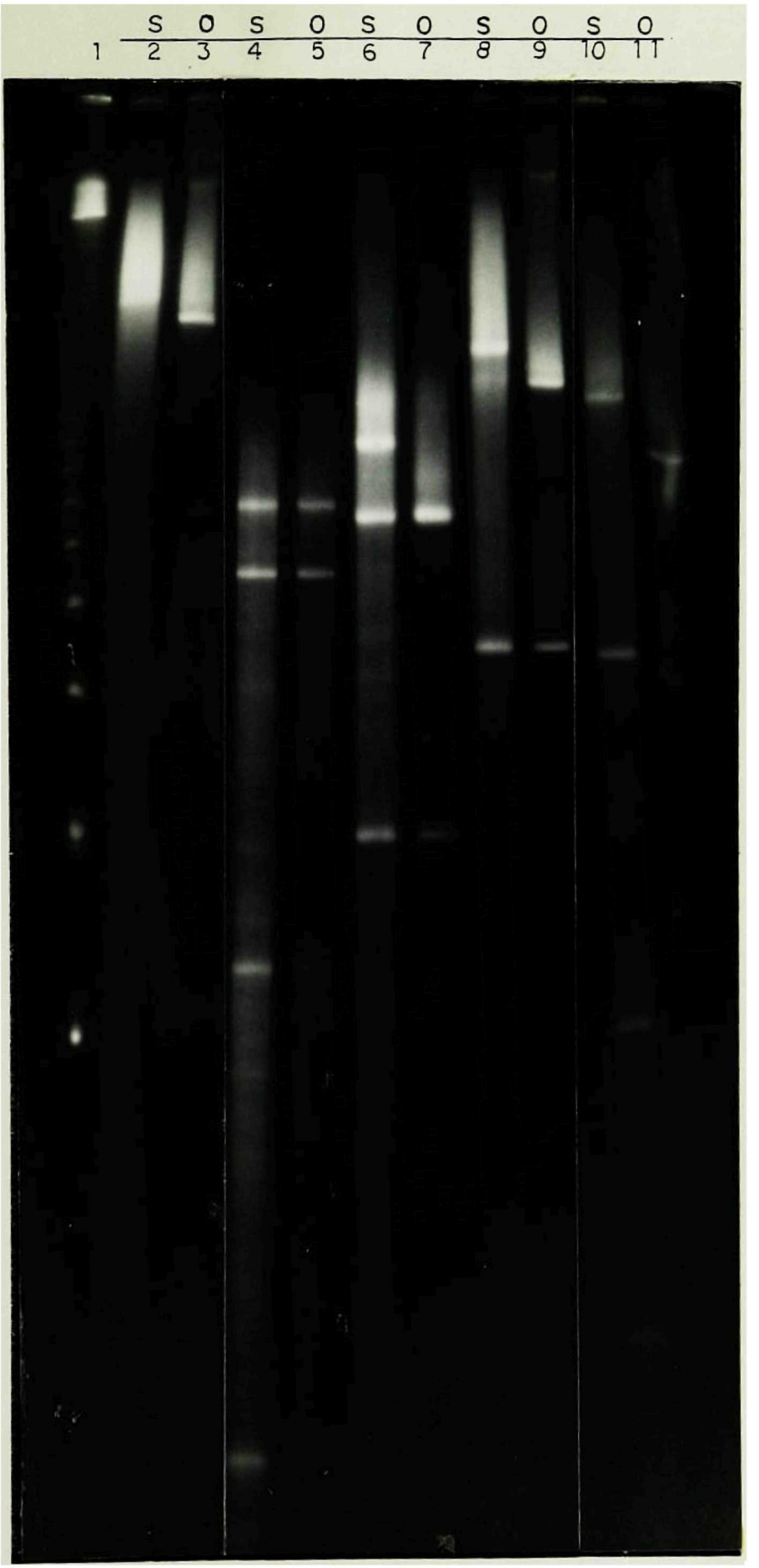

S O S O S O S O S O
1 2 3 4 5 6 7 8 9 10 11

FIGURE 34

Identification of the HincII, TaqI, HinfI and HaeIII terminal fragments of EcoRI P. A plasmid containing the EcoRI P fragment of VZV Oka was digested with EcoRI and separated in a 5% polyacrylamide gel. The P fragment was cut out and passively eluted as described in Materials and Methods. The gel-purified P fragment was either nick-translated (N) or kinase end-labeled (K), single or double-digested with various restriction enzymes and the fragments were separated in an 8% polyacrylamide gel at 100 V for 5 hours. The gel was dried and the bands visualized by autoradiography. Lanes 1 and 12 contain a 123 bp ladder (Bethesda Research Labs) as molecular weight markers. Lanes 2 and 3, HincII / HinfI; lane 4, HincII; lane 5 HinfI; lanes 6 and 7, HaeIII / HincII; lanes 8 and 9, TaqI.

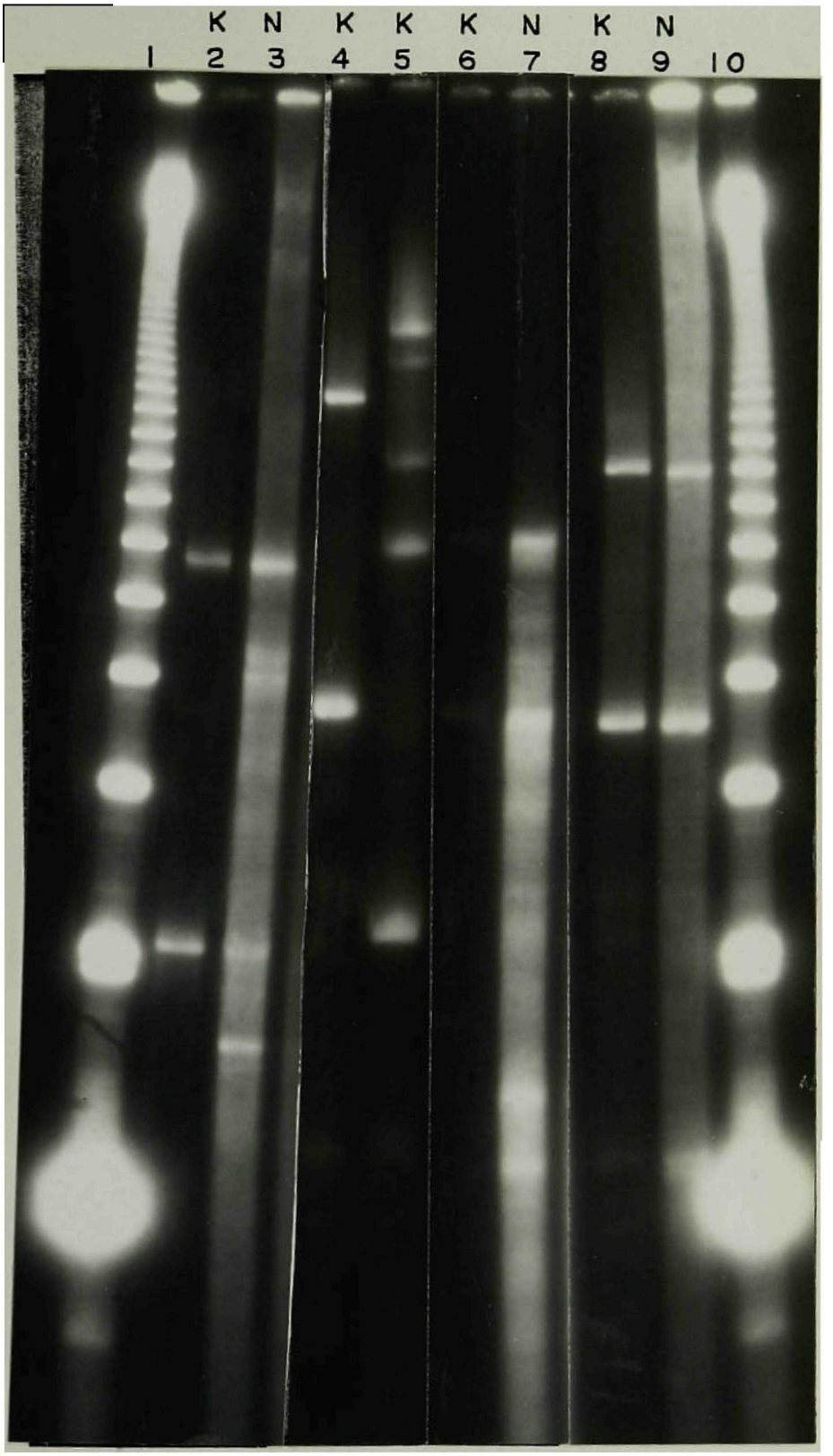

K
N
K
K
K
N
K
N
1
2
3
4
5
6
7
8
9
10

FIGURE 35

Mapping the HincII, HinfI and HaeIII sites in EcoRI P by double digestions. Plasmids containing the EcoRI P fragments from Scott and Oka were digested with EcoRI and separated in 5% polyacrylamide gels. The P fragments were cut out and passively eluted as described in Materials and Methods. The gel purified P fragments were nick-translated, single or double-digested with various restriction enzymes and the fragments were separated in an 8% polyacrlyamide gel at 100 V for 5 hours. The gel was dried and the bands visualized by autoradiography. Lanes 1 and 12 contain a 123 bp ladder (Bethesda Research Labs) as molecular weight markers. The other lanes contain either Scott (S) or Oka (O). Lane 2, HincII; lanes 3 and 4, HincII / HinfI; lane 5, HinfI; lanes 6 and 7, HaeIII / HincII; lanes 8 and 9, HaeIII / HinfI; lanes 10 and 11, DdeI.

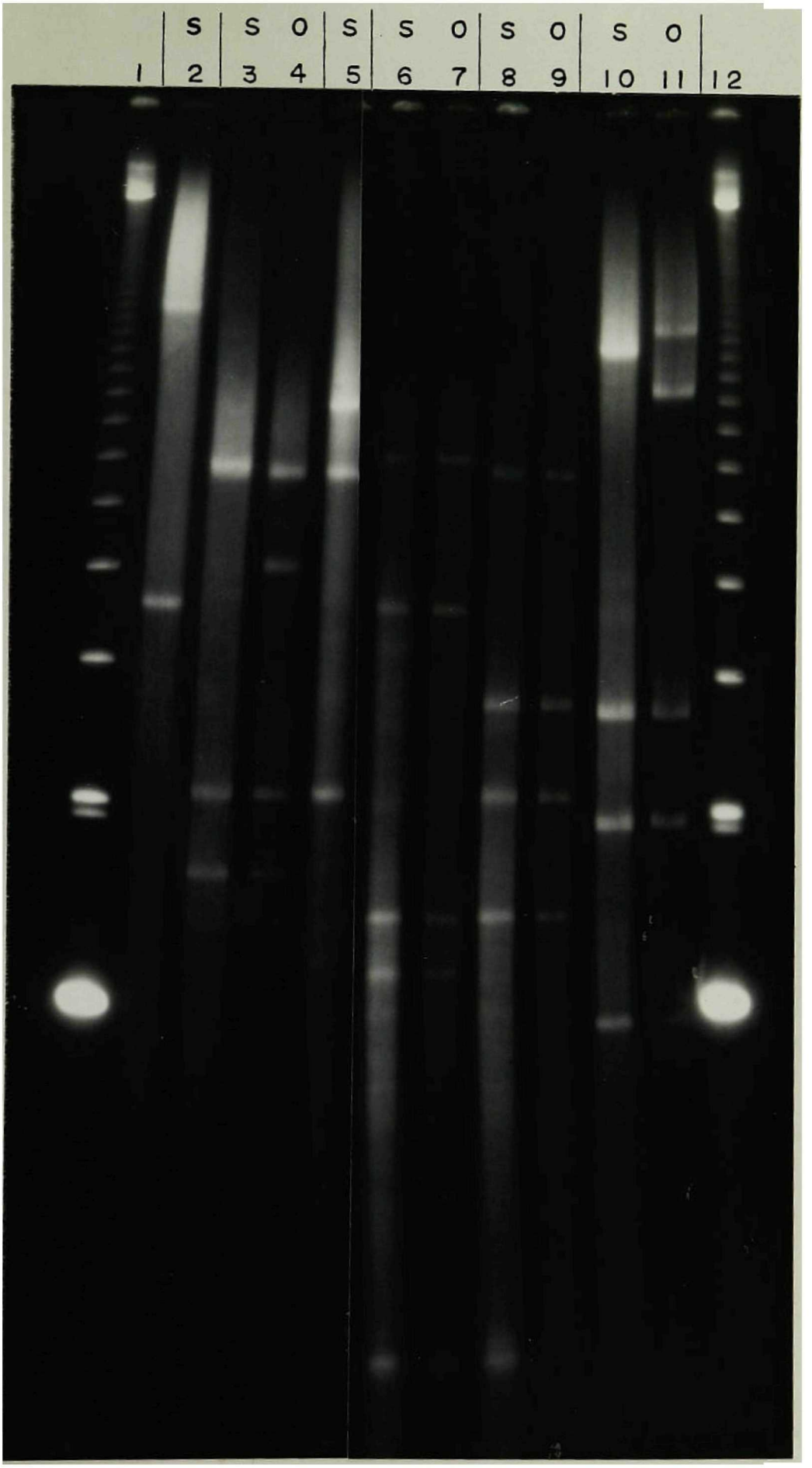

S S O S S O S O S O
1 2 3 4 5 6 7 8 9 10 11 12

FIGURE 36

Mapping the TaqI sites in EcoRI P. Plasmids containing the EcoRI P fragments from Scott and Oka were digested with EcoRI and separated in 5% polyacrylamide gels. The P fragments were cut out and passively eluted as described in Materials and Methods. The gel-purified P fragments were either nick-translated or kinase end-labeled, single or double-digested with various restriction enzymes and the fragments were separated in an 8% polyacrylamide gel at 100 V for 5 hours. The gel was dried and the bands were visualized by autoradiography. Lane 1 contains a 123 bp ladder (Bethesda Research Labs) as molecular weight markers. Lane 2, Oka P, kinase-labeled and HinfI-digested; lane 3, Oka P, nick-translated and HinfI-digested; lane 4, Oka P, kinase-labeled and double-digested with HinfI and HincII; lane 5, kinase-labeled Oka P digested with TaqI; lane 6, nick-translated Oka P digested with TaqI; lane 7, HinfI / TaqI-digested nick-translated Oka P; lane 8, HinfI / TaqI-digested nick-translated Scott P; lane 9, HincII / TaqI-digested nick-translated Oka P; lane 10, HincII / TaqI-digested nick-translated Scott P; lane 11, HincII nick-translated Oka P; lane 12, kinase-labeled Oka P double-digested with TaqI and HincII; lane 13, Oka P kinase-labeled and double-digested with TaqI and HinfI.

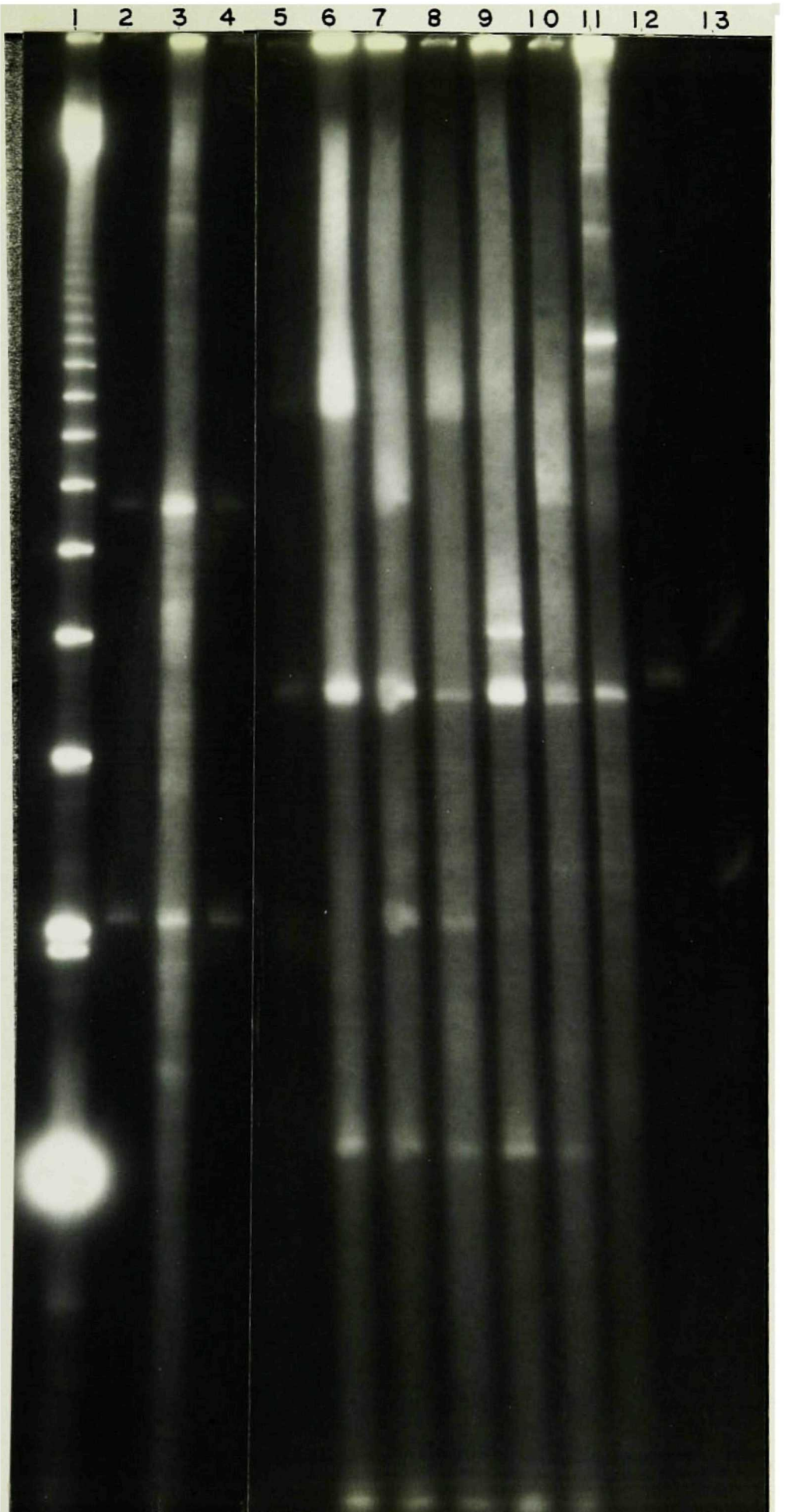

1
2
3
4
5
6
7
8
9
10
11
12
13

FIGURE 37

Mapping the DdeI sites in EcoRI P. Plasmids containing the EcoRI P fragments from Scott and Oka were digested with EcoRI and separated in 5% polyacrylamide gels. The P fragments were cut out and passively eluted as described in Materials and Methods. The gel-purified P fragments were nick-translated or kinase end-labeled, digested with various restriction enzymes and the fragments were separated in an 8% polyacrylamide gel at 100 V for 5 hours. The gel was dried and the bands were visualized by autoradiography. Lane 1, kinase-labeled Oka EcoRI P digested with DdeI; lane 2, nick-translated Oka digested with DdeI; lanes 3 and 4, nick-translated, DdeI / HinfI-cut Oka and Scott respectivly; lanes 5 and 6, nick-translated DdeI / HincII-cut Oka and Scott; lane 7 kinase-labeled Oka digested with DdeI / HinfI; and lane 8, kinase-labeled Oka digested with DdeI/ HincII.

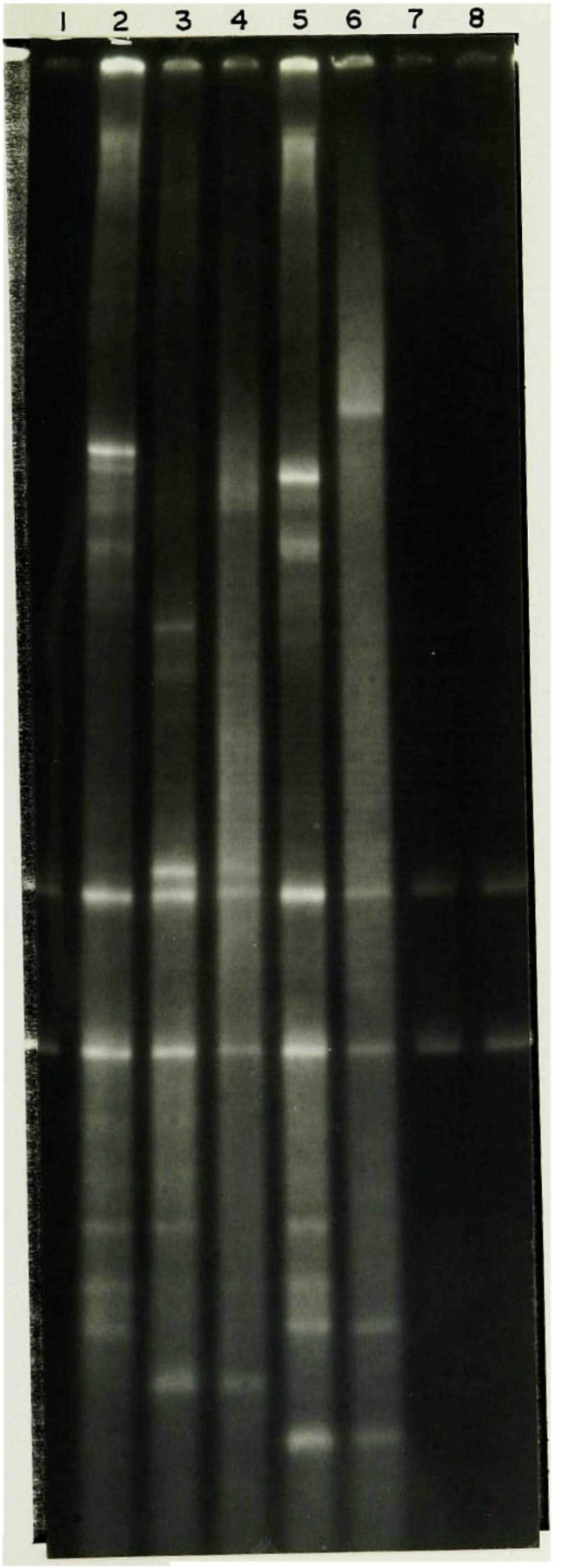
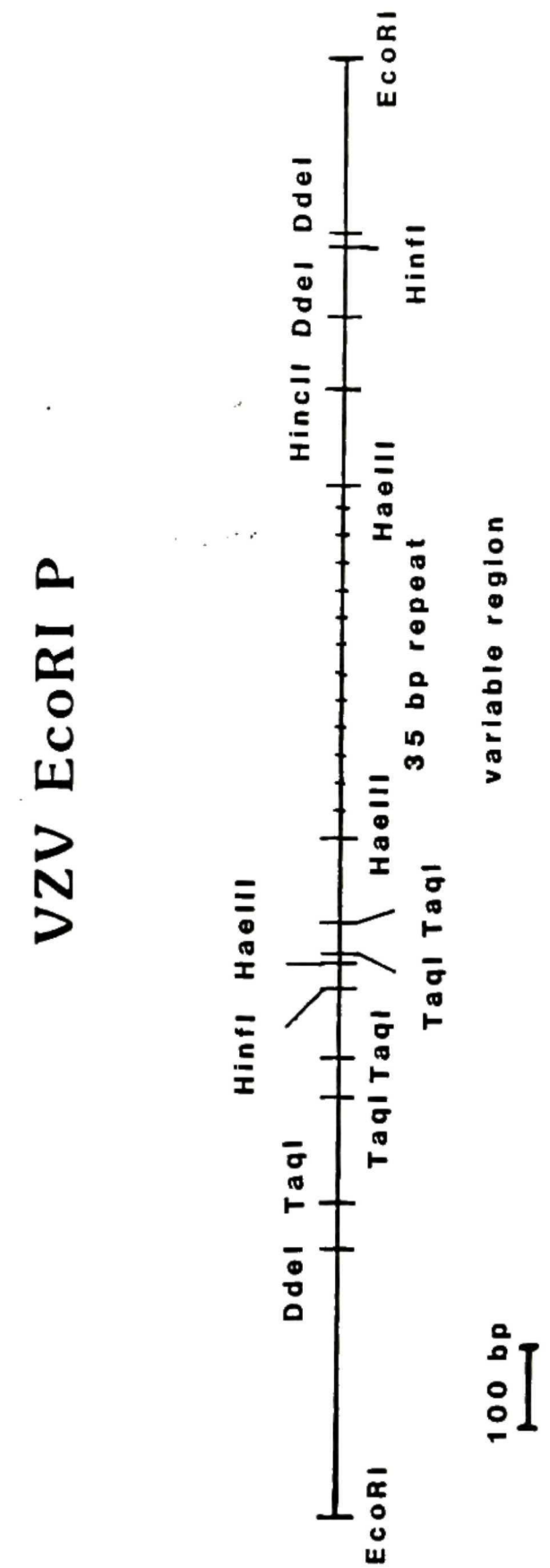

1
2
3
4
5
6
7
8

FIGURE 38

Restriction endonuclease map of VZV EcoRI P. Location of the cleavage sites for the restriction endonucleases EcoRI, Dde I, Taq I, HinfI, HaeIII and HincII in the VZV EcoRI P fragment. This map is based on data present in figures 33 to 37 as described in the text. The sizes shown are for Scott EcoRI P. The map position of the variable region is marked.


VZV EcoRI P
EcoRI
HincII DdeI DdeI
EcoRI
HinfI
HaeIII
35 bp repeat
variable region
HinfI HaeIII
HaeIII
TaqI TaqI
TaqI TaqI
DdeI TaqI
100 bp

a tandem DNA repeat sequence which is about 35 bp in size and that this sequence contains a HaeIII site. The terminal HaeIII fragments are 700 and 540 bp as determined by kinase end-labeling (lane 4, Figure 34). The positions of HaeIII sites were determined by double digestion with HincII and HinfI. The 540 bp terminal HaeIII fragment was placed at the right of EcoRI P because it is cut by HincII into 440 and 130 bp fragments (lanes 6 and 7, Figure 35). HinfI cuts the terminal 540 bp HaeIII fragment in half producing two fragments 240 bp in size. HinfI also cut the 700 bp terminal HaeIII fragment into 440 and 130 bp fragments (lanes 6 and 7, Figure 35). The 160 bp HaeIII fragment was placed next to the 700 bp terminal HaeIII fragment at the left of EcoRI P because if it was placed at the other end (adjacent to the 540 bp fragment), the tandem duplication would be cut by TaqI. The tandemly duplicated 35 bp HaeIII repeat fragments must therefore map between the 160 bp and the 540 bp terminal fragment.

The position for the strain variation shown in Figure 38 is consistent with the heterduplex mapping studies shown above (Figures 31 and 32). The results suggest that the EcoRI P difference between VZV DNA from different strains is located at a single site near the middle of the fragment and that it appears to result from insertions or deletions of 35 bp units. Thus, different strains of VZV seem to have different numbers of this 35 bp sequence which is tandemly duplicated.

VZV terminal and internal repeat strain variation

The third variable region in VZV DNA was mapped to within the inverted repeat sequences which bound the invertible sequences (see Figure 19). Strain variation of the type described here have frequently

been shown to occur in the repeat regions of other herpesviruses (see Introduction). A detailed examination of this inverted repeat sequence variation in VZV and a comparison with similar variable sequences in other herpesviruses would be an important step in understanding the significance of these sequences for herpesvirus replication

The VZV IR_S/TR_S variation was characterized by examination of the EcoRI E fragments from the four different VZV strains. The EcoRI A fragments from several different strains were also examined. Both fragments (E and A) have been shown to contain the repeat sequences (Straus et al., 1982) but because of the large size of these fragments, and the very small variation, it was difficult to see the difference in EcoRI digestion of whole VZV DNA (see Figures 12 and 15). It was easier to see the strain difference in the other two VZV EcoRI fragments which contain the repeat sequence, F and J. These fragments, however, could not be cloned directly because they are terminal (see Figure 8).

The EcoRI E and A fragments were cloned into pBR325 as described above, and transformants containing either EcoRI A or E were identified by colony hybridization and confirmed by molecular weight and Southern blot hybridization. An agarose gel of EcoRI-digested recombinant plasmid DNAs which contain EcoRI E fragments is shown in Figure 39, panel A, for two representative cloned isolates from each strain. A Southern blot of this gel is shown in panel B. Each recombinant (lanes 2 to 9) on this gel contains an EcoRI E fragment from one of the four strains identified by comigration with authentic EcoRI E fragment in a lambda clone from strain Ellen (in lanes 1 and 10) and by hybridization with this lambda clone in the blot. Figure 40 is an agarose gel of plasmid clones which contain the EcoRI A fragment, and, in one case,

FIGURE 39

Identification of recombinant plasmids which contain the VZV EcoRI E fragment. Plasmid DNA was extracted from transformants which, on colony blots, were positive for VZV EcoRI E. The DNA from two representative isolates for each of the four VZV strains was digested with EcoRI and the fragments were seperated by electrophoresis in a 0.7% agarose gel at 20 V for 18 hours. The gel was stained with ethidium bromide and photographed with ultraviolet light. The resulting gel is shown in panel A. Lanes 1 and 10 contain a lambda clone of VZV Ellen EcoRI E (Straus et al., 1982); lanes 2 to 9 are pBR325 clones of VZV EcoRI E from strain Ellen (lane 2 and 3); Scott (lane 4 and 5); Webster (lanes 6 and 7); and Oka (lanes 8 and 9). The DNA in this gel was transferred to nitrocellulose (Southern, 1975) and hybridized with ^{32}P-labeled lambda VZV Ellen EcoRI E. The autoradiogram is shown in panel B. The positions of the plasmid, EcoRI E and lambda bands on this gel and blot are marked.

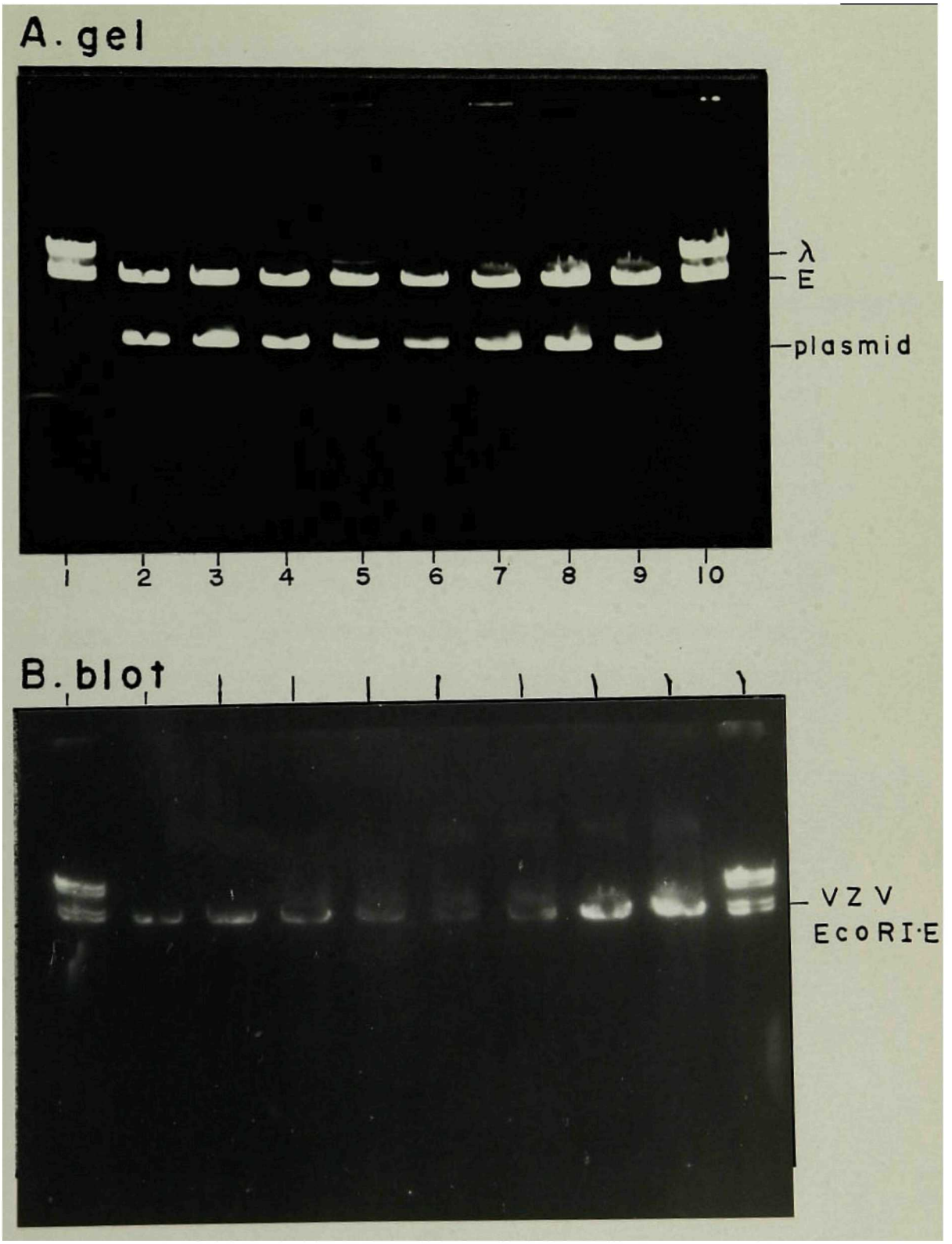

A. gel
λ
E
plasmid
1
2
3
4
5
6
7
8
9
10
B. blot
VZV
EcoRI-E

FIGURE 40

Identification of reconbinant plasmids which contain the EcoRI A fragment. Plasmid DNA was extracted from transformants which, on colony blots, were positive for VZV EcoRI E. Some of these clones contained a fragment which was larger than EcoRI E. Molecular weight determinations for this fragment indicated that it was VZV EcoRI A. The DNA from several representative isolates for three VZV strains was digested with EcoRI and the fragments were seperated by electrophoresis in a 0.7% agarose gel at 20 V for 18 hours. The gel was stained with ethidium bromide and photographed with ultraviolet light. The resulting gel is shown in panel A. Lane 1 contains a lambda clone of VZV Ellen EcoRI E (Straus et al., 1982); lanes 2 to 9 are pBR325 clones of VZV EcoRI A from strain Scott (lane 2 and 3); Webster (lanes 5, 6 and 7); Oka (lanes 8, 9 and 10); and lane 4 is both EcoRI E and A from strain Scott. The DNA in this gel was transferred to nitrocellulose (Southern, 1975) and hybridized with ^{32}P-labeled lambda VZV Ellen EcoRI E. The autoradiogram is shown in panel B. The positions of the plasmid, VZV EcoRI E, A and lambda bands on this gel and blot are marked.

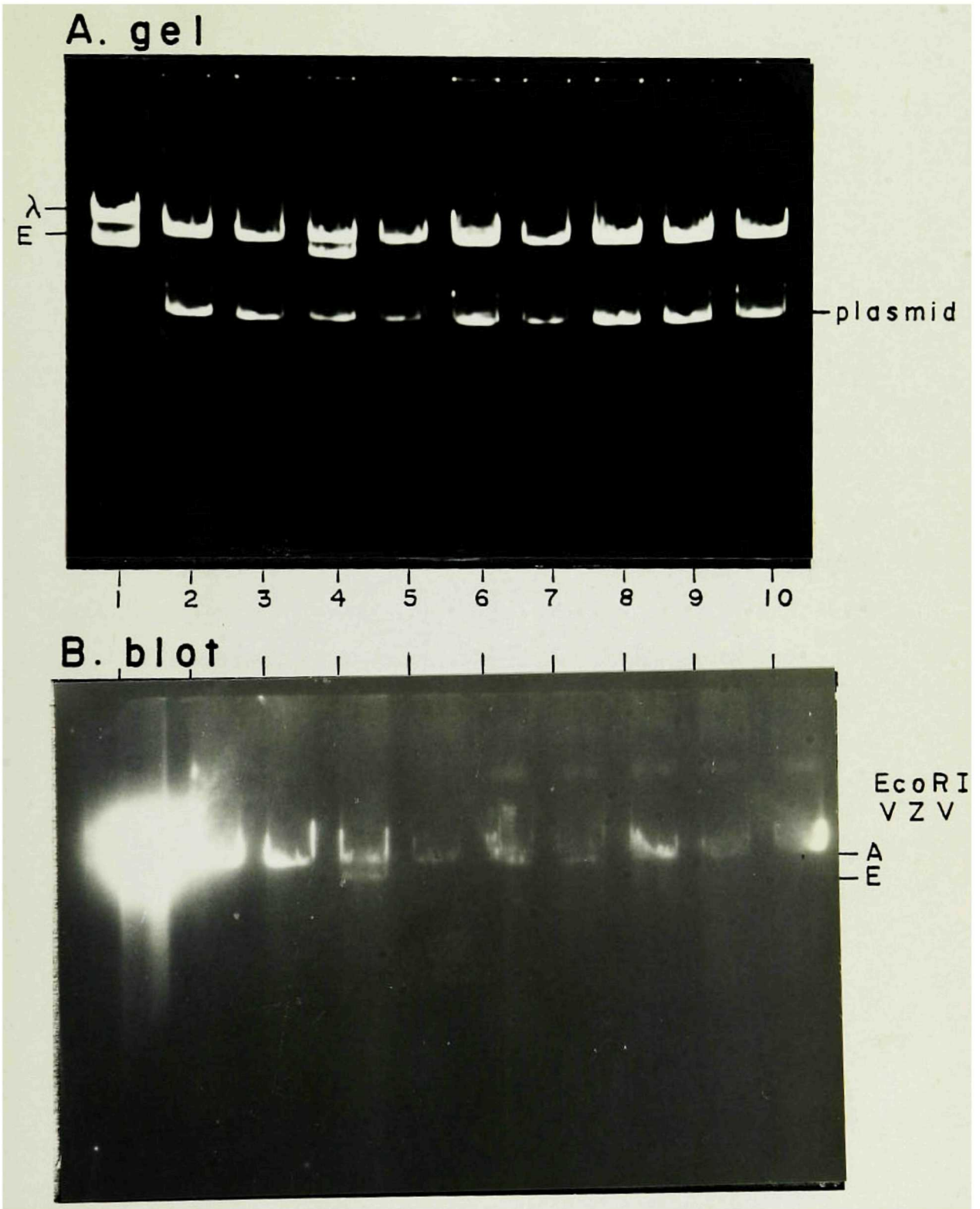

A. gel
λ
E
plasmid
1
2
3
4
5
6
7
8
9
10
B. blot
EcoRI
V Z V
A
E

FIGURE 41

BglI and BglI / EcoRI digestion of the EcoRI E fragment from four VZV strains. Recombinant plasmid DNA containing the EcoRI E fragment from VZV strains Ellen (E), Scott (S), Webster (W) and Oka (O) were digested with BglI or BglI / EcoRI and the bands were separated by electrophoresis in a 1.5% agarose gel at 50 V for 18 hours. The gel was stained with ethidium bromide and photographed with ultraviolet light. Bgl I / EcoRI digested pBR325 DNA (P) was included as a control. Lanes 1 and 2 contain Ad2 DNA digested with EcoRI and HindIII as molecular weight markers.

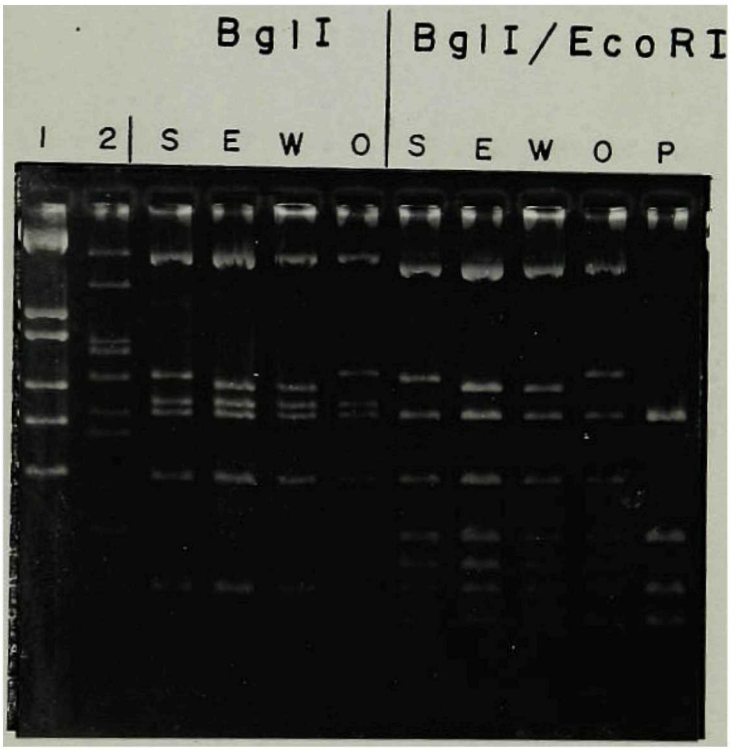

BglI
BglI/EcoRI
1
2
S
E
W
O
S
E
W
O
P

FIGURE 42

SalI, SalI / EcoRI and SmaI, SmaI / EcoRI digestion of the EcoRI fragment from four VZV strains. Recombinant plasmid DNA containing the EcoRI E fragment from VZV strains Ellen (E), Scott (S), Webster (W) and Oka (O) were digested with SalI, SalI / EcoRI, SmaI or SmaI / EcoRI and the bands were separated by electrophoresis in a 1.5% agarose gel at 50 V for 18 hours. The gel was stained with ethidium bromide and photographed with ultraviolet light. SalI / EcoRI and SmaI / EcoRI-digested pBR325 DNA (P) was included as a control. Lanes 1 and 2 contain Ad2 digested with EcoRI and SV40 digested with HindIII as molecular weight markers.

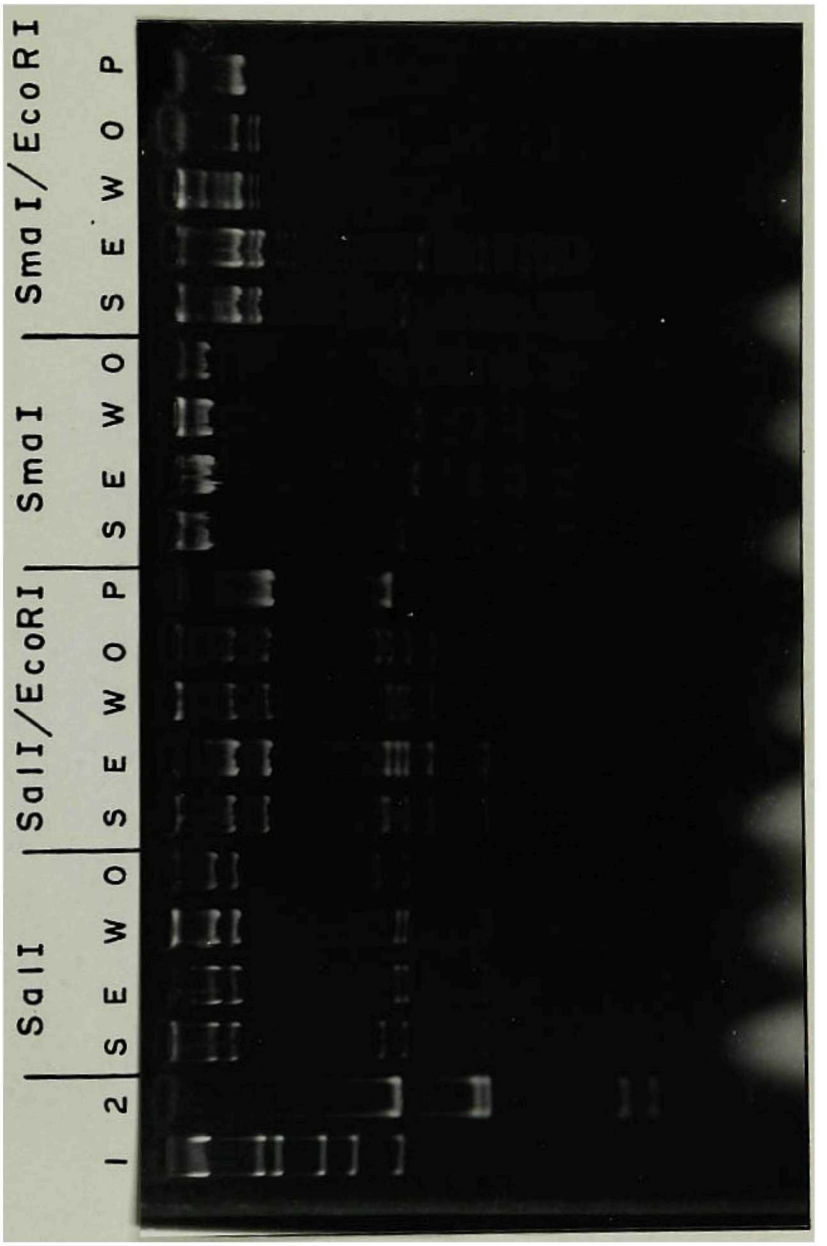
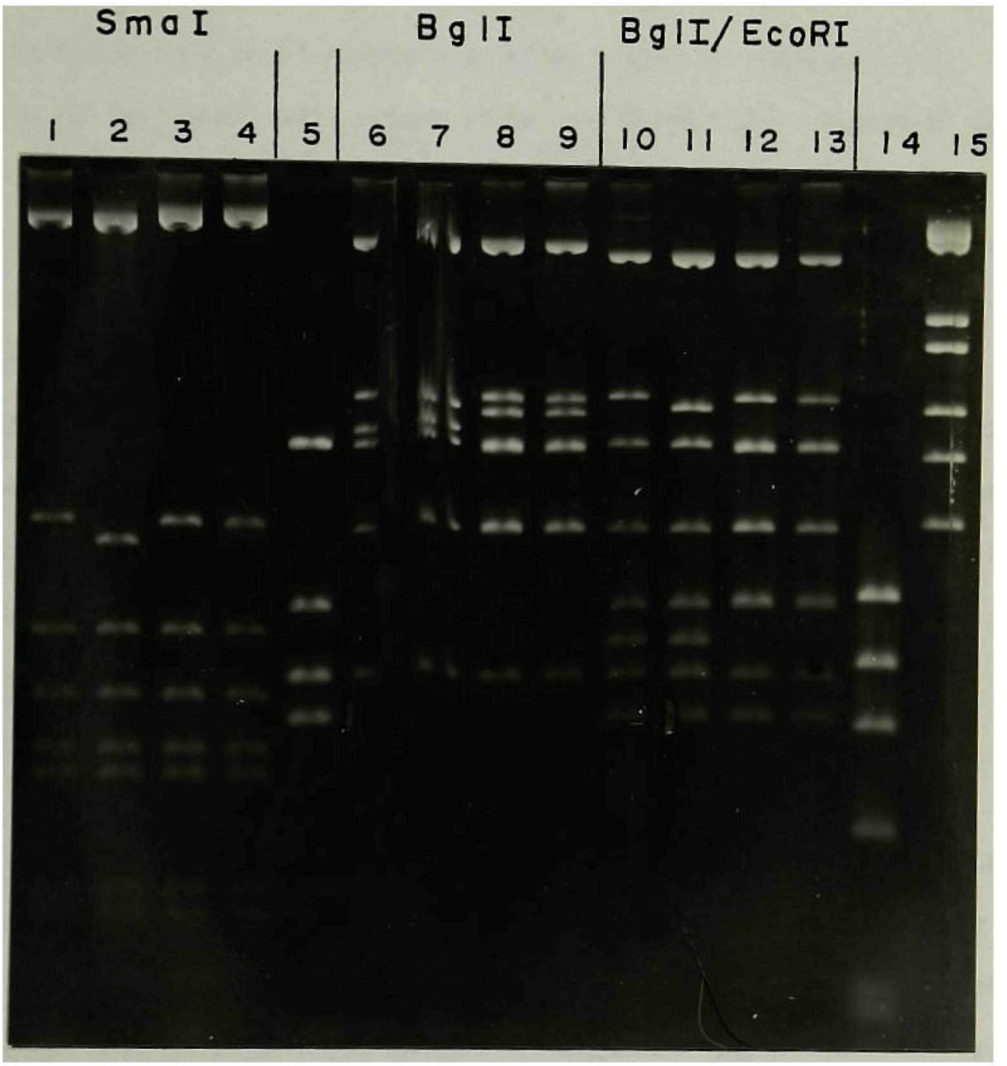

1 2
SalI
SEWO
SalI/EcoRI
SEWOP
SmaI
SEWO
SmaI/EcoRI
SEWOP

FIGURE 43

Comparison of EcoRI E and EcoRI A from different VZV strains.

Recombinant plasmid DNA containing the EcoRI E fragment from VZV strains Ellen and Scott are compared here with the EcoRI A fragment from Scott. These DNAs were digested with SmaI, BglI or BglI / EcoRI and the bands were separated by electrophoresis in a 1.5% agarose gel at 50 V for 18 hours. The gel was stained with ethidium bromide and photographed with ultraviolet light. BglI-digested pBR325 DNA (lane 5) was included as a control. Lanes 14 and 15 contain ΦX174 digested with HaeIII and Ad2 digested with EcoRI as molecular weight markers. Lanes 1, 6 and 10 are the EcoRI E fragment from strain Scott; lanes 2, 7 and 11 are Ellen EcoRI E; lanes 3, 8, 12 and 4, 9, 13, are two independent isolates of the EcoRI A fragment from Scott.


SmaI
BglI
BglI/EcoRI
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

both the EcoRI E and A fragments (lane 4). These fragments were identified as EcoRI A by their molecular weight and by blot hybridization with the lambda EcoRI E probe (Figure 40, panel B).

The strain differences among the EcoRI E or A fragments cannot be seen in these gels (Figures 39 and 40) because of their large size and the relatively small difference between them in different VZV strains as mentioned earlier (see above and Figure 12). Figure 41 is a gel of BglI and BglI / EcoRI digestions of plasmid clones containing the EcoRI E fragment from the four VZV strains. Figure 42 shows the same DNAs cut with SalI, SalI / EcoRI, SmaI, and SmaI / EcoRI. Now the strain difference is evident, in that with these single and double digestions, a single fragment migrates at a slightly different position for each strain. The size of the variable fragment in each single or double digestion is characteristic for that strain, does not change in different recombinants from the same VZV strain and does not change during amplification in E. coli, in contrast to the behavior of the EcoRI G fragment (see Figure 24, above).

The difference between strains in the EcoRI A fragment is identical to the difference in the EcoRI E fragments (Figure 43). Lanes 3 and 4 are two clones of the EcoRI A fragment from VZV strain Scott digested with SmaI. These are compared with SmaI digests of the EcoRI E fragments from strains Scott and Ellen in lanes 1 and 2 respectively. A single SmaI band, which is the fragment that varies between Ellen and Scott in the E fragment, varies between Ellen EcoRI E and Scott EcoRI A and is identical to the SmaI variable band in Scott E. Similar results are found with the BglI and BglI / EcoRI double digestions of Scott E and A compared with Ellen E, also shown on this figure.

Restriction mapping of EcoRI E. A restriction map of the EcoRI E fragment was then constructed using the enzymes PstI, BglII, BamHI, HindIII, BglI and SalI, by comparison of different combinations of single and double digestions with these enzymes. Supporting data for the map of EcoRI E from strain Ellen shown in Figure 51 are given in Figures 44 to 50. Several SmaI sites were also located (data not shown), but a complete map of the SmaI sites in EcoRI E could not easily be constructed because of the large number of sites (greater than 12). In addition, most of the SmaI fragments were small (less than 500 bp) and many were not cleaved by the other enzymes used in the mapping.

The orientation of the EcoRI E insert in each plasmid clone was determined by digestion with either PstI or BamHI. As mentioned previously, the orientation of plasmid clones can be used to map one or two restriction sites in the inserted fragment by comparison with the known sites in the vector (such as PstI and BamHI sites in pBR325). It is also important to know the orientation of these clones to prevent confusion when mapping by double digestion with enzymes other then the one used in the cloning. Examples of these digestions to determine orientation are shown in Figure 44. The position of the bands produced by digestion with these enzymes for the two different orientations are marked (either 1 or 2). The orientations of all EcoRI E clones were determined by these digestions (data not shown). Comparisons among the different EcoRI E clones for identification of the variable sub-fragment were carried out with clones in the same orientation (Figures 41, 42 and 43). Most of the mapping procedures are a comparison of different double digestions of the two different orientations of the EcoRI E fragment from strain Ellen which are arbitrarily labeled A or B in the

figures.

BglII digests. There is a single BglII site in VZV EcoRI E and no site in the plasmid vector. Digestion with BglII results in a single band the size of the vector plus the insert (approximately 18 Kb; Figure 45, lanes 5 and 6). BglII / EcoRI double digestion results in two fragments from EcoRI E, 8.5 and 3.5 Kb (Figure 45, lane 7). This BglII site was positioned toward the left end of EcoRI E (in U_L) to conform with the BglII map of whole VZV DNA (Dumas et al., 1981). This site was then used to map other restriction sites in double digestions with BglII and other enzymes (see below).

PstI digests. Digestion of cloned EcoRI E with PstI results in two fragments. The size of these fragments depends on the orientation of the inserted EcoRI E fragments (see Figure 44 and above). These two fragments are due to a single PstI site in both the plasmid and the EcoRI E insert. The single PstI site in the insert can be positioned by comparison of PstI (Figure 46, lanes 15 and 16) and PstI / EcoRI digestions (Figure 46, lanes 17 and 18) of EcoRI E clones in both orientations. The two fragments from EcoRI E digested with EcoRI / PstI are 1.1 and 11 Kb. The smaller PstI / EcoRI fragment from EcoRI E comigrates with the EcoRI / PstI fragment from the vector. This was deduced because this band stains more brightly than it should for its size in this gel (compare the plasmid control digested with EcoRI / PstI in lane 19 with the same digestion of EcoRI E fragments in lanes 17 and 18 in Figure 46). The size of the PstI / EcoRI fragment from EcoRI E can also be inferred from the difference between the known size of the EcoRI / PstI fragment from pBR325 (1.1 Kb) and the size of the small fragment in the PstI digestion of one of the orientations of EcoRI E

(2.2 Kb, lane 16, Figure 46). The PstI site is therefore approximately 1.1 Kb from the end of EcoRI E and is positioned at the left end on the map in Figure 51, to align this map with the PstI map for whole VZV DNA (Dumas et al., 1981). The PstI site, like the single BglII site, was used to position the recognition sites for other restriction endonucleases in the EcoRI E fragment.

HindIII digests. There are three HindIII sites in the EcoRI E fragment. Lanes 3 and 4 in Figure 46 are the two orientations of Ellen EcoRI E cut with HindIII and lane 5 is a HindIII / EcoRI double digestion. These digestions identified the terminal HindIII fragments as 4 Kb and 1.4 Kb. The 1.4 Kb fragment was positioned at the left end of EcoRI E because it was cleaved by PstI in Figure 46, lane 6 and 8. The other terminal fragment (4.0 Kb) must therefore be at the right end of EcoRI E. This position was confirmed by BamHI / HindIII double digestions (Figure 46, lanes 12 and 14). The position of these terminal fragments is consistent with the position of the plasmid HindIII site and the orientation of these two recombinants determined with other enzymes. The remaining 4.6 and 2.0 Kb fragments are internal because they are present in the HindIII digestion of both orientations of the EcoRI E clones (Figure 46, lanes 3 and 4). The 4.6 Kb HindIII fragment is cleaved by BglII (compare the HindIII digested clones in lanes 9 and 10 with the BglII / HindIII digestions in lane 12 and 13, Figure 45). The remaining 2.0 Kb internal HindIII fragment must therefore be between the 4.6 Kb internal fragment and the 4.0 Kb right terminal HindIII / EcoRI fragment.

BamHI digests. A BamHI digestion of either orientation of the EcoRI E plasmids results in three bands, one of which is common to both

orientations (Figure 44 and Figure 46, lanes 9 and 10). This means that there are two BamHI site in EcoRI E. This common band (4.6 Kb) must be internal with respect to the EcoRI sites and is positioned on the map between the two terminal BamHI / EcoRI fragments. The size of the two BamHI / EcoRI fragments are 6.4 and 1.0 Kb. The 6.4 Kb BamHI / EcoRI is located at the left terminus of EcoRI E because it is cleaved by BglII (Figure 48, lane 8, panel A is one orientation, panel B is the other). The 1.0 Kb BamHI / EcoRI fragment must be at the right terminus of EcoRI E.

BglI digests. Digestion of EcoRI E clones with BglI / EcoRI results in 4 fragments (Figure 48, lane 9). The other bands seen in these lanes are due to sites within the plasmid (Figure 48, lane 10). The terminal BglI fragments (1.1 and 6.3 Kb) were identifed by comparison of BglI digestions of either orientation and BglI / EcoRI digestion (lane 4, Figure 47 and lane 9 in Figure 48). The 6.3 Kb BglI / EcoRI fragment was placed at the left end of the EcoRI E map because it is cut by BglII (Figure 47, lane 5). The 1.1 Kb EcoRI / BglI fragment is therefore at the right terminus of EcoRI E. The 2.7 Kb internal variable BglI fragment was not cut by BamHI (Figure 48, lane 7, the second largest fragment) or HindIII (not shown). This fragment was therefore placed in the only remaining position which could produce these results, that is, next to the 1.1 Kb terminal BglI fragment toward the right terminus of the EcoRI E map. The remaining internal 1.9 Kb BglI fragment is between the the 2.7 Kb variable fragment and the 6.3 Kb terminal BglI fragment at the right terminus.

SalI digests. Digestion of VZV EcoRI E with SalI results in 6 fragments. The two terminal fragments were identified by comparison of

SalI digestion of either orientation (Figure 47, lane 3, in panels A and B) with SalI / EcoRI double digestion (in Figure 47, lanes 5, A and B). The 5.7 Kb terminal fragment was placed at the left, because only the large plasmid / EcoRI E junction fragments were cleaved by BglII (Figure 48, lanes 11 A and B). If the smaller 1.5 Kb EcoRI / SalI fragment was at the left terminus, an internal SalI fragment would be cut by BglII and it is not (see map in Figure 51). The 1.5 Kb SalI terminal fragment was therefore placed at the right of the EcoRI E map. The remaining internal SalI fragments were placed in the locations shown on the map in Figure 51 by double digestions with HindIII, BamHI and BglI. The 1.7 Kb fragment, (the third largest fragment in the SalI digest in lane 3 A and B, Figure 47) which was found to be the variable SalI fragment (see Figure 42), was not cleaved by any of these other enzymes and can only be next to the 1.5 Kb terminal fragment to meet these requirments. The 1.6 Kb fragment (the fourth largest fragment in lanes 6 A and B, Figure 47) was cleaved by BglI (Figure 47, lanes 9 A and B) and HindIII (lanes 6 A and B, Figure 47) but not BamHI (compare in Figure 48 SalI alone, in lanes 6 A and B, with HindIII / SalI in lanes 4 A and B). Based on these digestions the SalI 1.6 Kb fragment must be next to the 1.7 Kb variable fragment. The 1.1 Kb SalI fragment (fifth largest fragment in the SalI digestion in lanes 3 A and B, Figure 47 and Figure 48, lanes 6 A and B) must be to the left of the 1.7 Kb fragment because it is cut by BamHI (Figure 48, lanes 3 A and B) and BglI (Figure 47 lanes, 9 A and B). The 400 bp fragment must therefore be between the 1.1 Kb fragment and the 5.7 Kb left terminal fragment.

The SalI fragments of EcoRI E were the most difficult to map and the mapping is hardest to explain. To confirm the positions determined

FIGURE 44

Orientation of the EcoRI E clones by PstI and BamHI digestion.

Representive plasmid isolates which contained the VZV EcoRI E fragment were digested with PstI or BamHI and the fragments were separated in a 1.0% agarose gel at 50 V for 18 hours. The gel was stained with ethidium bromide and photographed with ultraviolet light. The positions of the two bands which identify inserts in different orientations are shown at each side. Lanes 1, 2 and 3 are Scott EcoRI E clones; lanes 4-9 are Ellen EcoRI E. All plasmid recombinants which contained the EcoRI E fragment were digested in a similar manner to determine insert orientation (data not shown). There is a common band in the BamHI digestions which must be internal and is seen to vary between the strains (compare lanes 3 and 4).

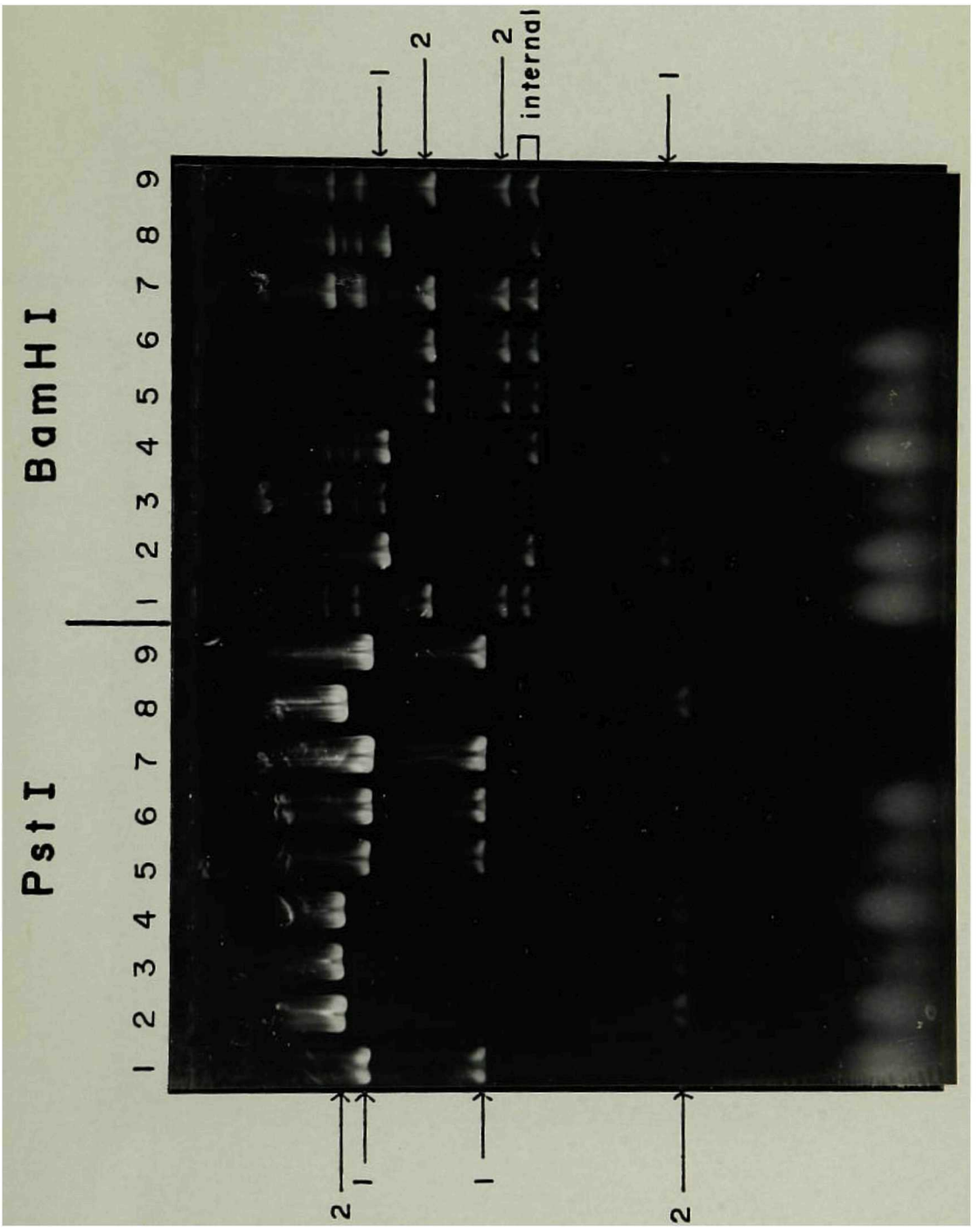

PstI
BamHI
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9
1
2
2
internal
1
2
1
1
2

FIGURE 45

Mapping the BglII and HindIII sites in the EcoRI E fragment. Plasmids containing either orientation of the Ellen EcoRI E fragment (A or B) were single or double-digested and the fragments were separated in a 1.5% agarose gel at 50 V for 18 hours. The gel was stained with ethidium bromide and photographed with ultraviolet light. The molecular weight markers (M) in lanes 3 and 4 are Ad2 cut with EcoRI and lambda cut with EcoRI respectively. A pBR325 digestion (P) is also included as a control. Lanes 1 and 2 are EcoRI; lanes 5 and 6 are BglII; lanes 7 and 8 are BglII / EcoRI double digestions; lanes 9 and 10 are HindIII; lane 11 is HindIII / EcoRI; and lanes 12 and 13 are BglII / HindIII.

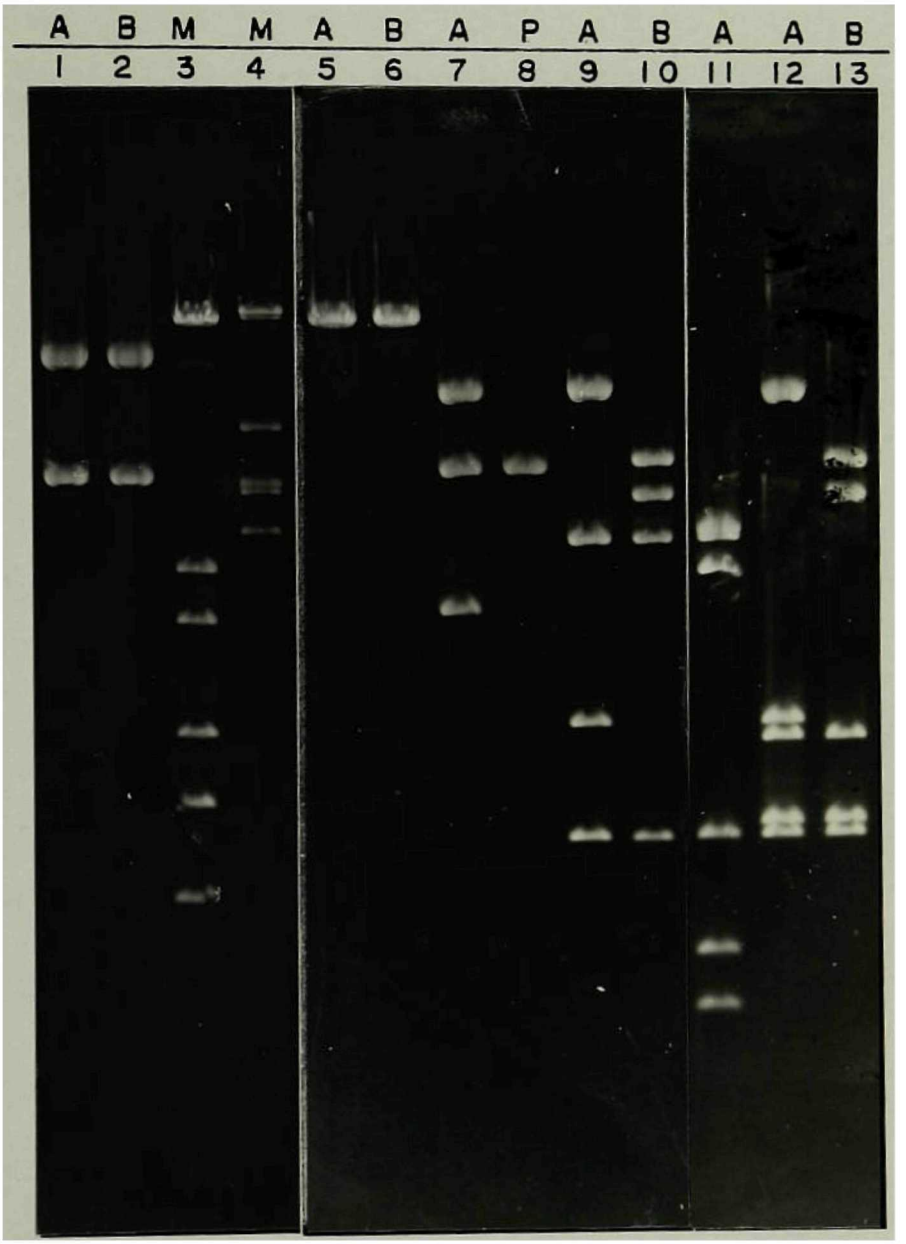

A B M M A B A P A B A A B
1 2 3 4 5 6 7 8 9 10 11 12 13

FIGURE 46

Mapping of EcoRI E with HindIII, PstI and BamHI. Plasmids containing either orientation of the Ellen EcoRI E fragment (A or B) were single or double-digested and the fragments were separated in a 1.5% agarose gel at 50 V for 18 hours. The gel was stained with ethidium bromide and photographed with ultraviolet light. Molecular weight markers (M) in lanes 1 and 2 are lambda cut with EcoRI and Ad2 cut with EcoRI respectively. Plasmid pBR325 digestions (P) are also included as controls. Lanes 3 and 4 are HindIII; lane 5 is HindIII / EcoRI; lanes 6, 7, and 8 are PstI / HindIII; lanes 9 and 10 are BamHI; lane 11 is BamHI / EcoRI; lanes 12, 13, and 14 are BamHI / HindIII; lanes 15 and 16 are PstI; and lanes 17, 18 and 19 are PstI / EcoRI.

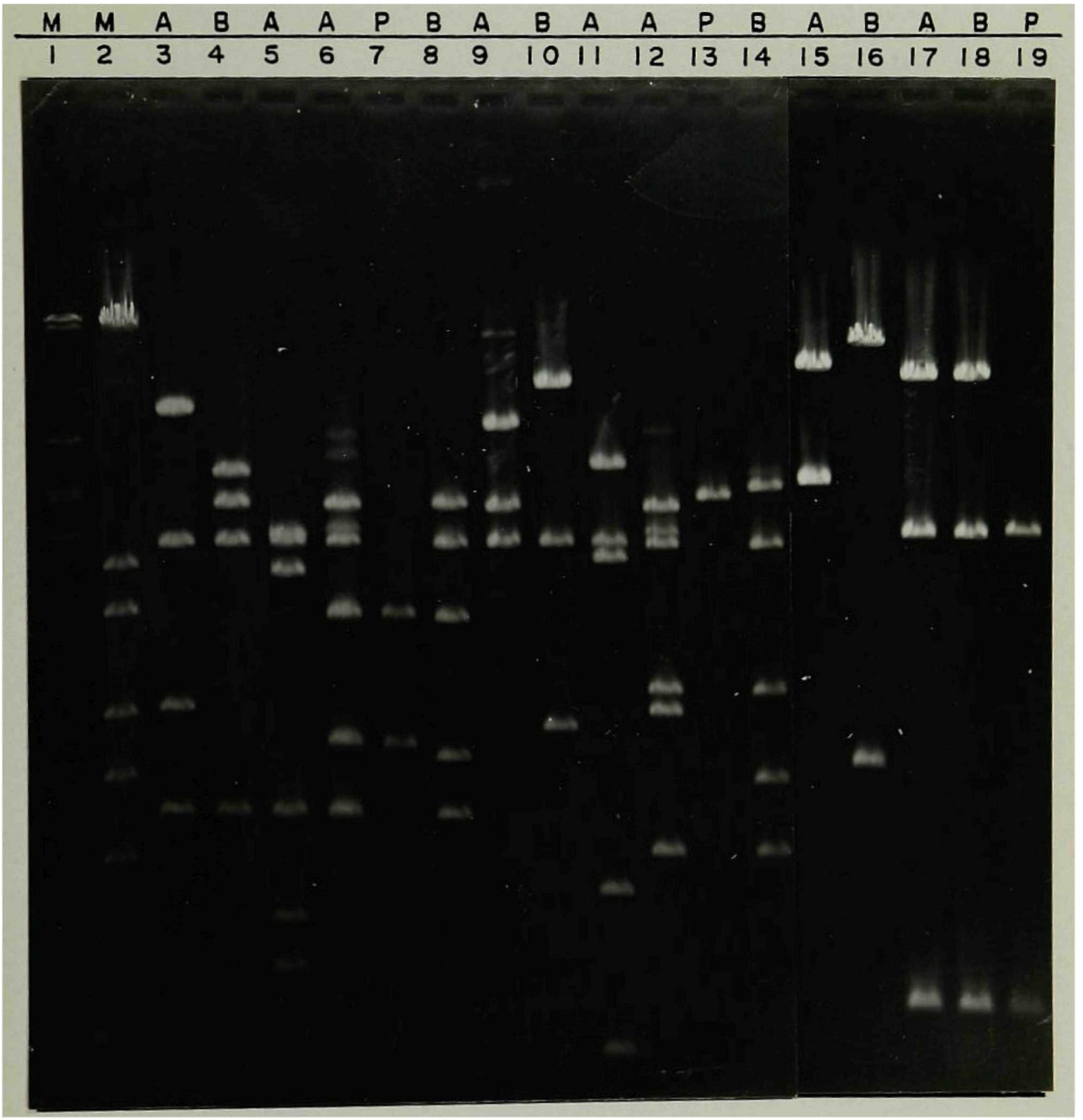

M M A B A A P B A B A A A P B A B A B P
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

FIGURE 47

Mapping of EcoRI E with HindIII, SalI, BglI and BglII. Plasmids containing either orientation of the Ellen EcoRI E fragment (panel A or B) were single or double-digested and the fragments were separated in a 1.5% agarose gel at 50 V for 18 hours. The gel was stained with ethidium bromide and photographed with ultraviolet light. Lanes with the same number in both panels were digested with the same enzyme(s). Lane 1 is HindIII; lane 2 is HindIII / EcoRI; lane 3 is SalI; lane 4 is BglI; lane 5 is BglI / BglII; lane 6 is HindIII / SalI; lane 7 and 8 are pBR325 digested with BglI / SalI and BglI respectively; and lane 9 is BglI / SalI.

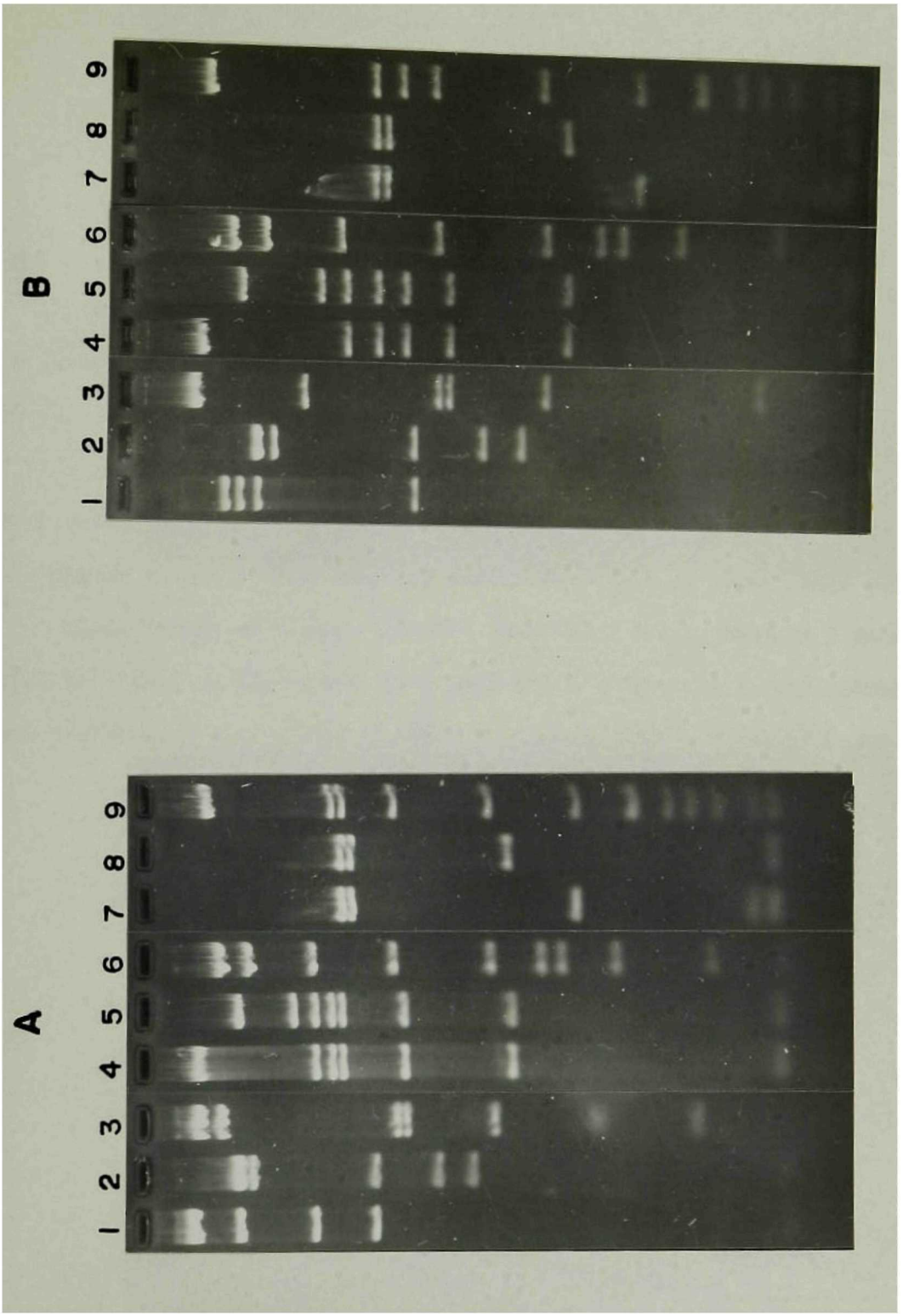

A
1 2 3 4 5 6 7 8 9
B
1 2 3 4 5 6 7 8 9

FIGURE 48

Mapping of EcoRI E with BamHI, SalI, BglI and BglII. Plasmids containing either orientation of the Ellen EcoRI E fragment (panel A or B) were single or double-digested and the fragments were separated in a 1.5% agarose gel at 50 V for 18 hours. The gel was stained with ethidium bromide and photographed with ultraviolet light. Lanes with the same number in both panels were digested with the same enzyme(s). Lane 1 is BamHI; lane 2 is BamHI / EcoRI; lane 3 is pBR325 BamHI / EcoRI; lane 4 is BamHI / SalI; lane 5 is EcoRI / SalI; lane 6 is SalI; lane 7 is BamHI / BglI; lane 8 is BamHI / BglII; lane 9 is BglI / EcoRI; lane 10 is pBR325 BglI / EcoRI; and lane 11 is BglII / SalI.

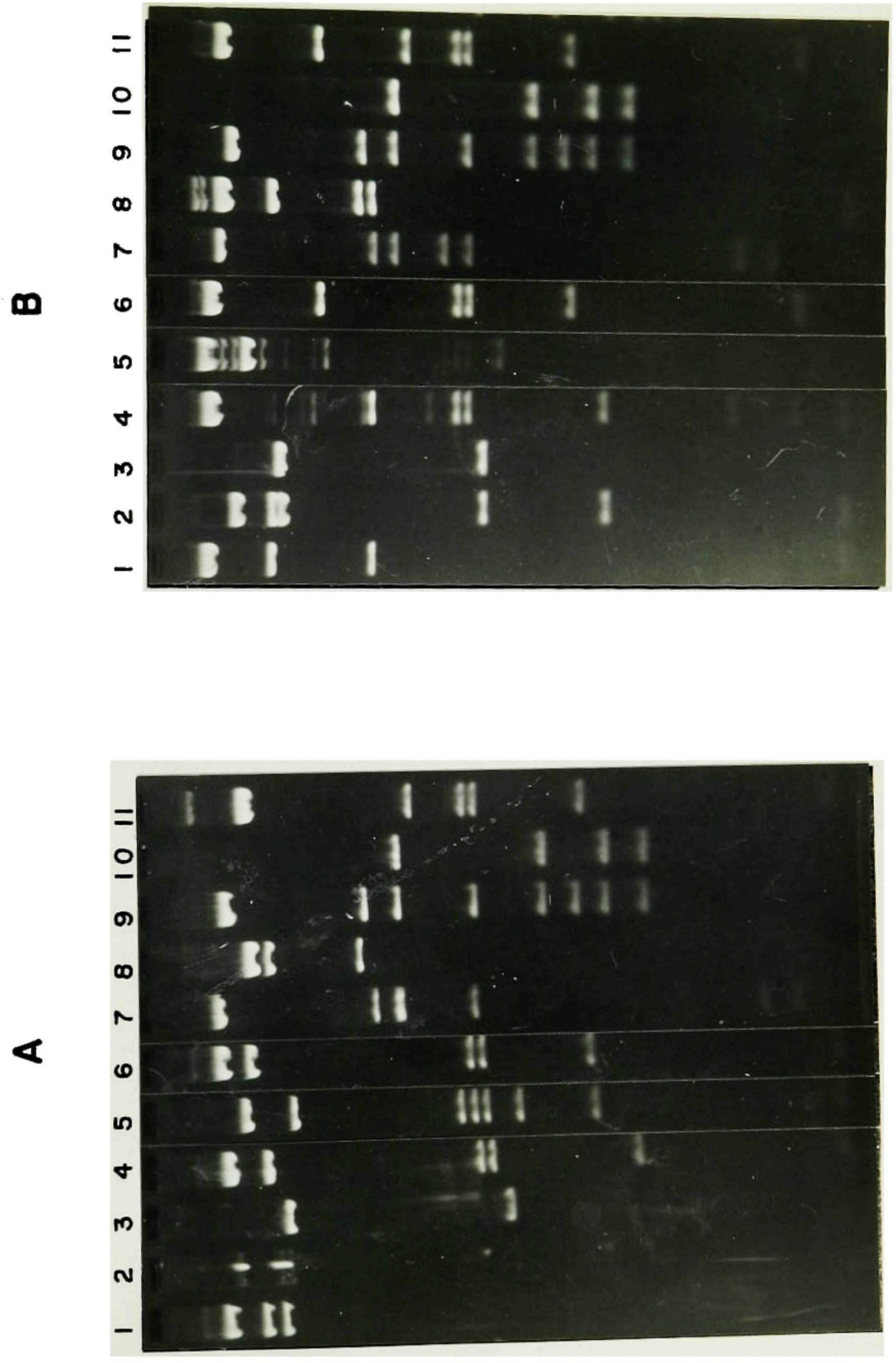

A
B
1 2 3 4 5 6 7 8 9 10 11

FIGURE 49

Double digestions of SalI sub-clones of Ellen EcoRI E. Plasmids containing one of the SalI fragments of the Ellen EcoRI E fragment were double-digested SalI and one other enzyme and the fragments were separated in a 1.5% agarose gel at 50 V for 18 hours. The gel was stained with ethidium bromide and photographed with ultraviolet light. Molecular weight markers in lane 1 is ΦX174 cut with HaeIII. Lanes 2-6 contain the 400 bp SalI fragment clone. Lane 2, SalI alone; lane 3, SalI / BglI; lane 4, SalI / HindIII; lane 5, SalI / BamHI; lane 6, SalI / SmaI. Lanes 7-10 contain plasmid clones of the 5.7 Kb EcoRI / SalI junction fragment from Ellen EcoRI. Lane 7 is SalI / BglI; lane 8 is SalI / HindIII; lane 9, SalI / BamHI; lane 10, SalI / SmaI. Lanes 11-15 are plasmid clones which contain the 1.1 Kb SalI fragment from Ellen EcoRI E. Lane 11 is SalI alone; lane 12, SalI / BglI; lane 13, SalI / HindIII; lane 14, SalI / BamHI; lane 15, SalI / SmaI. Lanes 16-19 contain the Ellen EcoRI E plasmid clone from which these sub-clones were constructed. Lane 16, SalI / BglI; lane 17, SalI / HindIII; lane 18, SalI / BamHI; and lane 19 is SalI / SmaI.

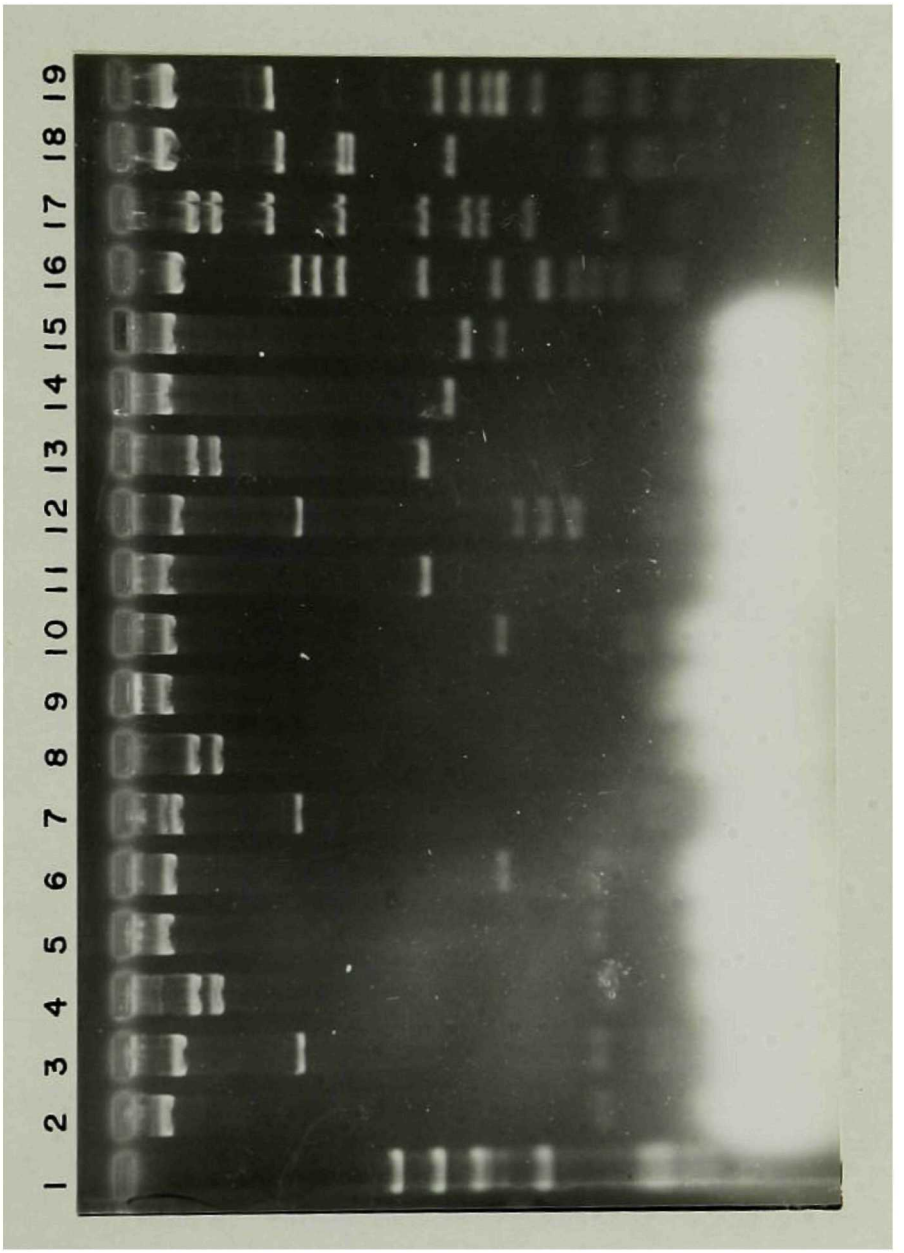

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

FIGURE 50

Double digestions of SalI sub-clones of Ellen EcoRI E. Plasmids containing one of the SalI fragments of the Ellen EcoRI E fragment were double digested SalI and one other enzyme and the fragments were separated in a 1.5% agarose gel at 50 V for 18 hours. The gel was stained with ethidium bromide and photographed with ultraviolet light. Molecular weight markers in lane 1 are ΦX174 HaeIII fragments. Lanes 2-6 contain the 1.7 Kb SalI variable fragment clone. Lane 2, SalI alone; lane 3, SalI / BglI; lane 4, SalI / HindIII; lane 5, SalI / BamHI; lane 6, SalI / SmaI; Lanes 7-10 contain plasmid clones of the 5.7 Kb EcoRI / SalI junction fragment from Ellen EcoRI. Lane 8 is SalI / BglI; lane 9 is SalI / HindIII; lane 10, SalI / BamHI; lane 11, SalI / SmaI. Lanes 11-15 are plasmid clones which contain the 1.6 Kb SalI fragment from Ellen EcoRI E. Lane 11 is SalI alone; lane 12, SalI / BglI; lane 13, SalI / HindIII; lane 14, SalI / BamHI; lane 15, SalI / SmaI. Lanes 16-19 contain the Ellen EcoRI E plasmid clone from which these sub-clones were constructed. Lane 16, SalI / BglI; lane 17, SalI / HindIII; lane 18, SalI / BamHI; and lane 19 is SalI / SmaI.

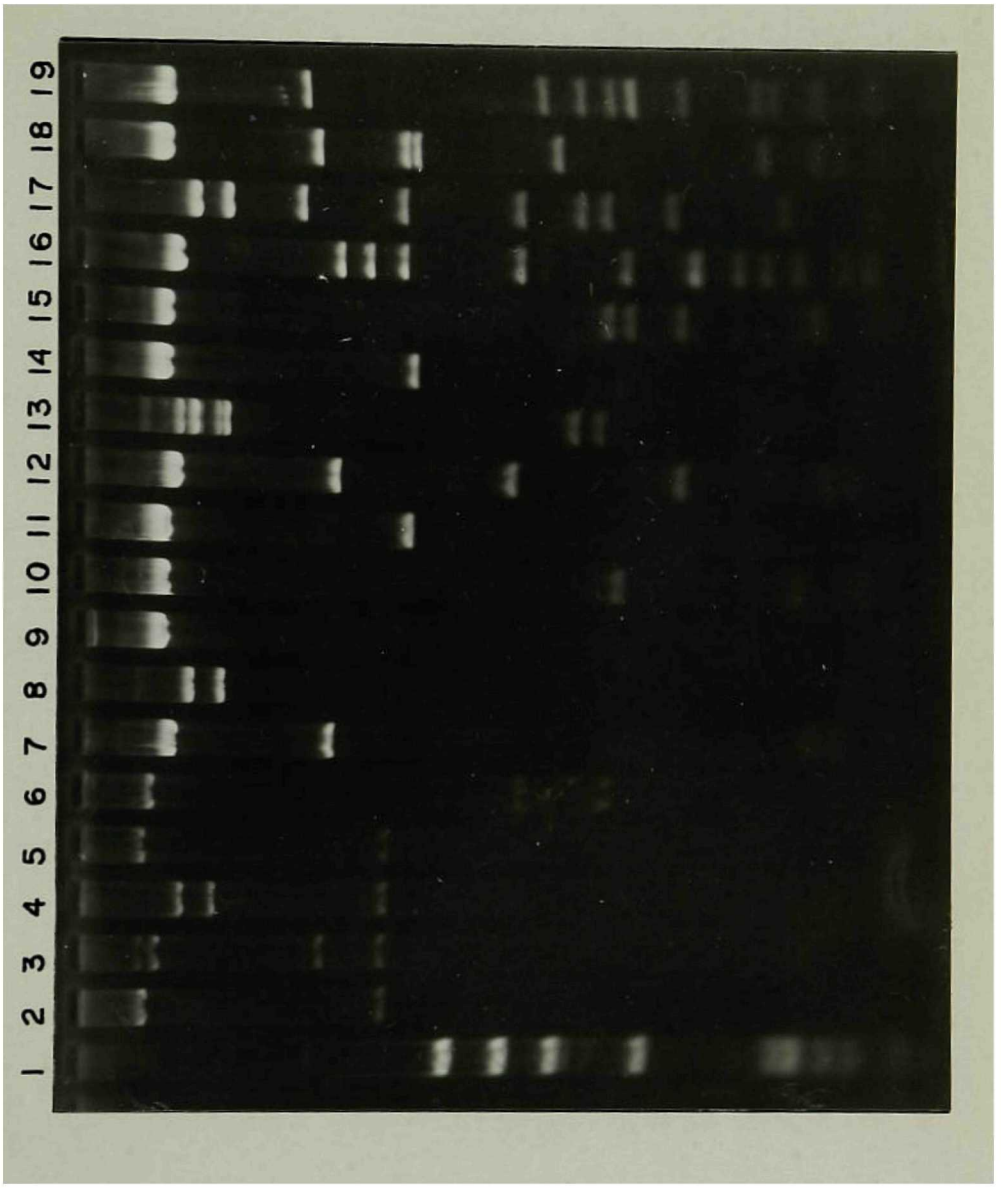
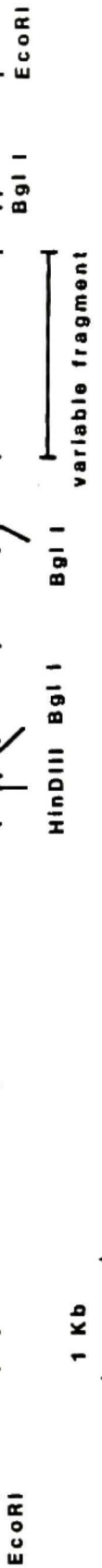

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

FIGURE 51

Restriction endonuclease map of VZV EcoRI E. Location of the cleavage sites for the restriction endonucleases EcoRI, Sal I, Bgl I, Pst I, BamHI and HindIII in the VZV EcoRI E fragment. This map is based on data present in figures 44 to 50 as described in the text. The sizes shown are for Ellen EcoRI E. The map position of the variable region is marked.

VZV EcoRI E


EcoRI
Pst I
HinDIII
Bgl II
Sal I
Sal I
BamHI
HinDIII
Sal I
HinDIII
Bgl I
Bgl I
Sal I
Sal I
BamHI
Bgl I
EcoRI
variable fragment
1 Kb

above for the SalI sites in EcoRI E, these SalI fragments were subcloned. Plasmids containing each of the SalI fragments were double-digested with SalI and BglI, HindIII and BamHI. These results are shown in Figures 49 and 50, and indicate that the 400 bp SalI fragment (seen in Figure 49, lane 2) is not cut by BglI (Figure 49, lane 3), is cut by HindIII (lane 4), and is not cut by BamHI (lane 5). The SalI 1.1 Kb fragment (Figure 49, lane 11) is cut by BglI (Figure 49, lane 12) is not cut by HindIII (Figure 49, lane 13) and is cut by BamHI (Figure 49, lane 14). The SalI 1.7 Kb variable fragment is not cut by any of these enzymes (Figure 50, lanes 2 to 5). The 1.6 Kb SalI fragment (Figure 50, lane 11) is cut by BglI (Figure 50, lane 12), is cut by HindIII (Figure 50, lane 13) and is not cut by BamHI (Figure 50, lane 14). These results are consistent with the positions of SalI sites determined by digestion of whole EcoRI E and are shown on the map in Figure 51.

Heteroduplex analysis. The EcoRI E strain difference was examined by heteroduplex analysis to confirm both the position determined by restriction mapping and if, like the EcoRI P strain difference (see above), the EcoRI E difference resulted from insertion or deletion of sequences at one specific location. EcoRI E-containing plasmids were digested with EcoRI, and the fragments were separated by gel electrophoresis and purified from the gel as described in the Materials and Methods. Equal amounts of the E fragment from Scott and Ellen were mixed, denatured and allowed to reanneal with each other before spreading and examination in the electron microscope. Three representative heteroduplex molecules between the EcoRI E fragments from the VZV Ellen and Scott strains are shown in the electron micrographs in Figure 52. In each of these heteroduplex molecules there is a single-

FIGURE 52

Electron micrographs of heteroduplex molecules between the EcoRI E fragments from VZV Scott and Ellen. Plasmids containing the EcoRI E fragment from Ellen and Scott were digested with EcoRI and the bands were separated in 1% low melting temperature agarose gels. The EcoRI E bands were cut out and extracted from the gel (see Materials and Methods). Equal amounts of each DNA were mixed, denatured and allowed to reanneal before being spread and mounted for examination in the electron microscope as discribed in the Materials and Methods. These are three representative heteroduplex molecules. There is a single stranded insertion or deletion loop near one end of each molecule which is the strain difference. The remainder of the molecules is double stranded. The magnification is approximately 112,000X.

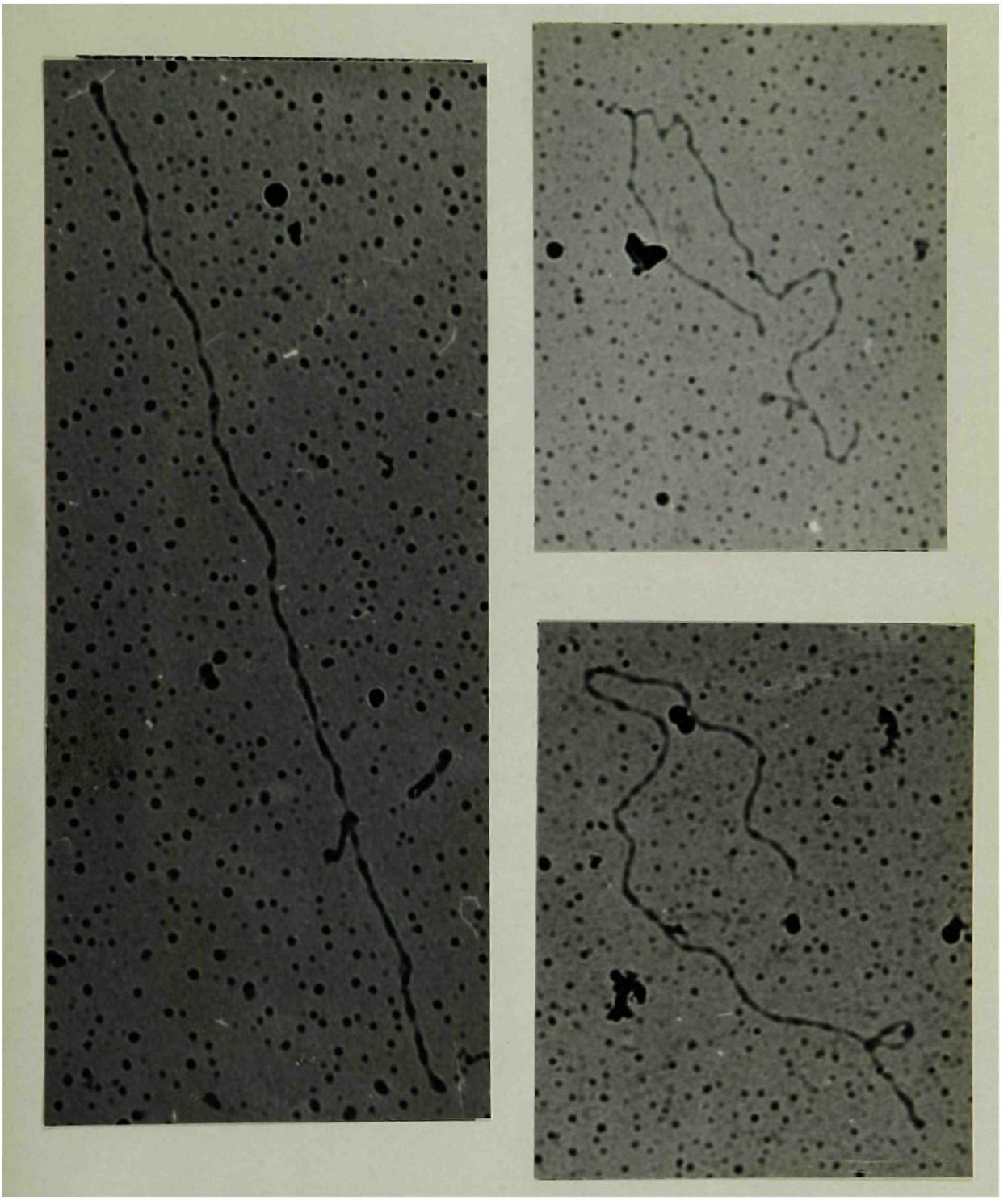
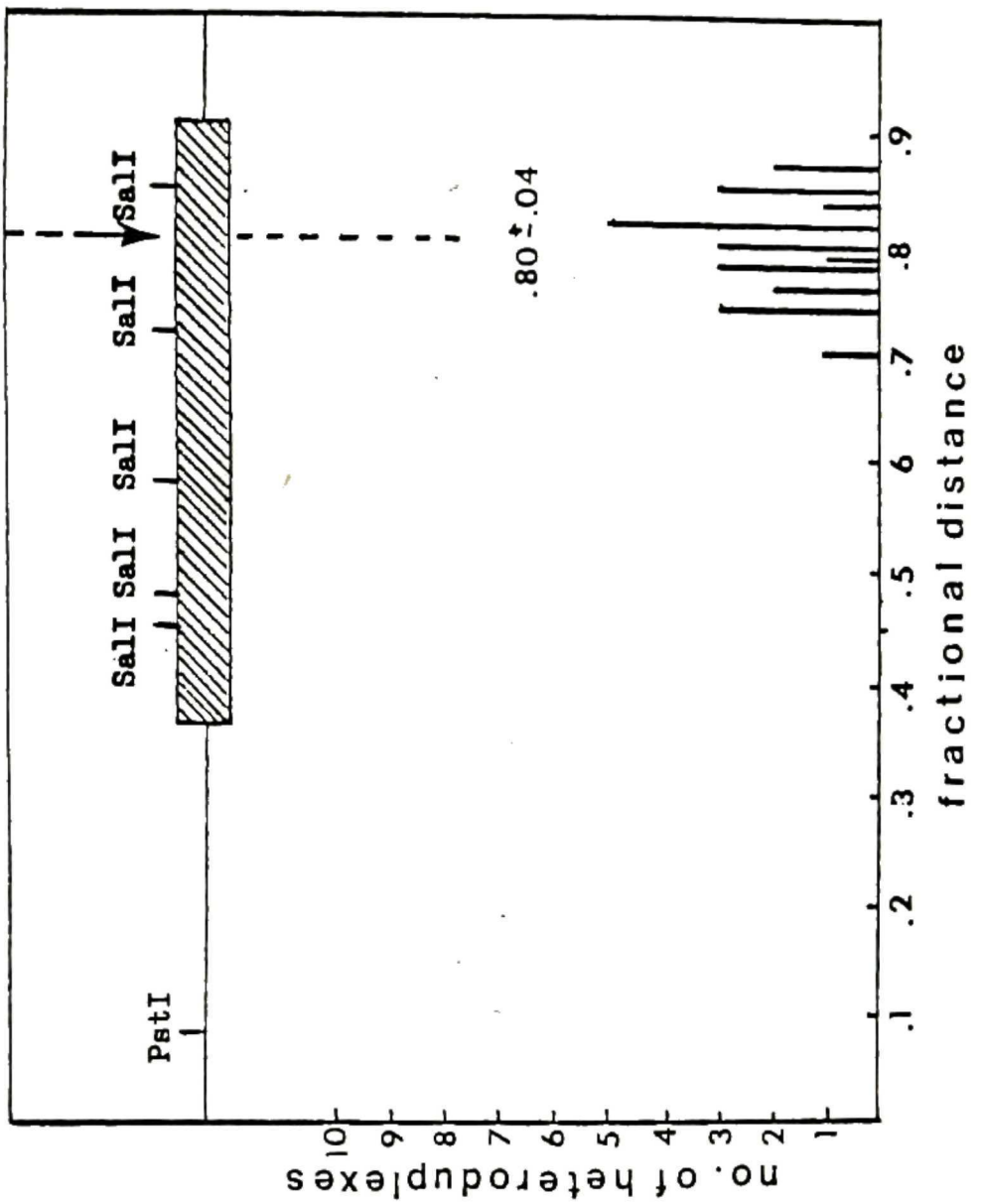


FIGURE 53

Summary of heteroduplex analyses of VZV Scott and Ellen EcoRI E. The position of the insertion / deletion loop (Figure 52) is plotted as the fractional distance from one end of the molecule vs. the number of molecules with a loop. A total of 21 heteroduplexes are shown on this figure. The mean fractional position of the difference is also shown. At the top of this figure is a simplified map of the EcoRI E fragment which shows the positions of the PstI site and the SalI sites.


PstI
SalI
SalI
SalI
SalI
SalI
.80 ± .04
no. of heteroduplexes
fractional distance
1
2
3
4
5
6
7
8
9
10
.1
.2
.3
.4
.5
.6
.7
.8
.9

stranded insertion or deletion loop near one end, with the rest of the molecule being double-stranded. A summary of the position of this insertion / deletion loop in 21 heteroduplex molecules between the EcoRI E fragments from VZV strain Ellen and Scott is shown in Figure 53. Included in this figure is a simplified map of EcoRI E with the position of the PstI site included for orientation and the positions of the SalI sites identified as described above. There is excellent correlation between the map position of the SalI fragment that varies and the insertion / deletion loop seen by heteroduplex analysis.

Nucleotide sequencing. The restriction mapping and heteroduplex data indicated that the strain difference in the VZV IR_S/TR_S sequences resulted from insertion or deletion of nucleotides in a defined region. The determination of the precise sequences involved is an important step in determining the function of this variable region, such as a element for replication or transcription, or perhaps its expression in a VZV protein. The EcoRI E fragment is too large to directly determine its nucleotide sequence by the Maxam and Gilbert procedure (1977; 1980). Since the smallest variable sub-fragment which could be mapped was a SalI fragment, the EcoRI E clones from all four strains were digested with SalI and the SalI variable fragments were cloned into pBR325. Clones containing the SalI variable fragment were identified by colony hybridization and confirmed by co-migration with this SalI fragment from each strain. An example of the subclones identified by colony hybridization as containing either the 1.7 Kb variable fragment or the 1.6 Kb fragment from strain Ellen is shown in Figure 54.

The SalI variable fragment was found to be cut once by the enzyme HincII and the larger of the two resulting fragments was variable

FIGURE 54

Identification of SalI sub-clones of Ellen EcoRI E which contain the variable SalI fragment. Plasmid sub-clones containing either the SalI 1.7 Kb variable fragment or the 1.6 Kb fragment from Ellen EcoRI E were digested with SalI and the fragments were separated in a 1.5% agarose gel at 50 V for 18 hours. The gel was stained with ethidium bromide and photographed with ultraviolet light. SalI-digested Ellen EcoRI fragments are in lane 1 for comparison. Similar digestions were done to identify all the SalI sub-clones from Ellen EcoRI E and the variable SalI fragments from the EcoRI E fragments of three other VZV strains (data not shown).

Sal I

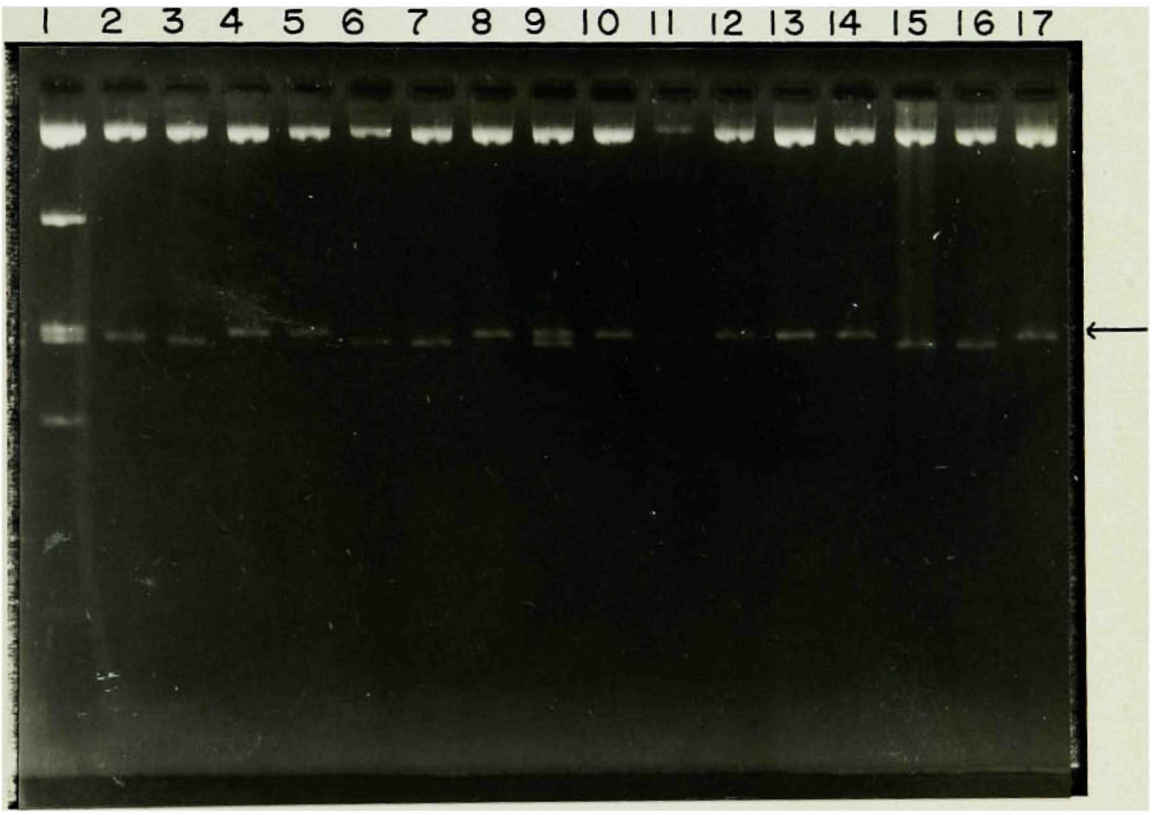
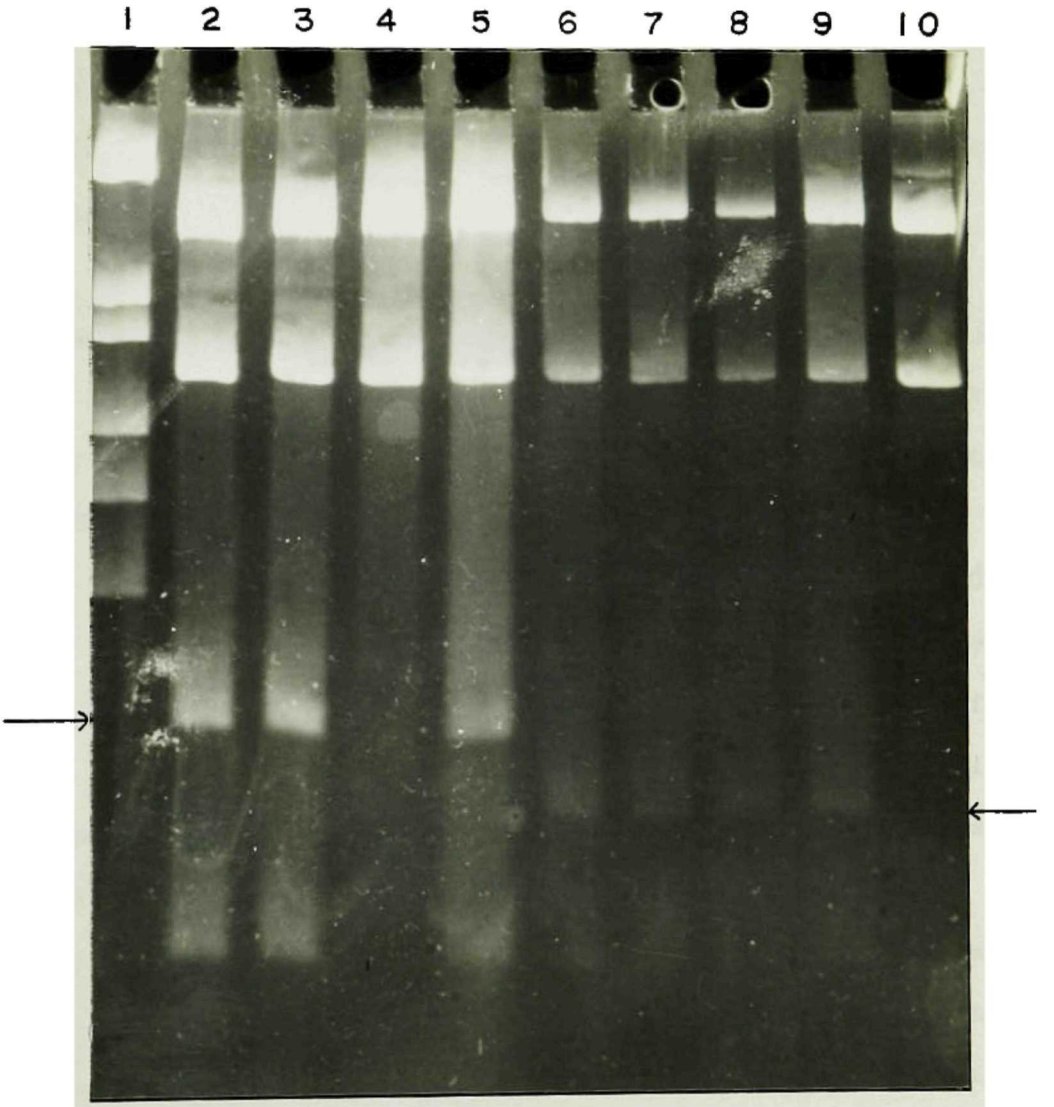


FIGURE 55

Comparison of the SalI variable fragments from different strains by SalI / HincII double digestion. Several independent plasmid sub-clones containing the SalI variable fragments from Oka (lanes 2-5) and Ellen (lanes 6-10) EcoRI E were double-digested with SalI / HincII and the fragments were separated in a 5% polyacrylamide gel at 50 V for 18 hours. The gel was stained with ethidium bromide and photographed with ultraviolet light. Molecular weight markers in lane 1 are Ad2 EcoRI fragments. The positions of the variable fragments are marked with arrows.


1
2
3
4
5
6
7
8
9
10

(Figure 55). This SalI / HincII fragment was sequenced using the Maxam-Gilbert method. The plasmids were digested with SalI followed by 5' end labeling with polynucleotide kinase and segregation of the labeled ends by digestion with HincII. A single AccI site was also found near the SalI site in the variable fragment. This site was also used for 5' end labeling and sequencing. The sequence was confirmed by sequencing the other strand using 3' labeling with Klenow polymerase at both the SalI and AccI sites (not shown). The results of sequence analysis of this region are shown in Figures 56 and 57.

A 27 bp tandemly duplicated sequence was found starting approximately 150 bases from the labeled end. The nucleotide sequence of this tandem repeat is shown in Figure 56. Figure 57 shows that this tandemly repeated DNA sequence is repeated a different number of times in the SalI / HincII variable fragments from VZV strains Oka and Ellen. The 27 bp sequence is repeated approximately 14 times in Oka and 6 times in Ellen. This difference in copy number of a repeated sequence was the only difference seen in the limited sequence analysis performed in this study but is sufficient in magnitude to account for the size difference between these fragments (approximately 200 bp). The exact copy number of tandem repeats could not be determined because the sequence was determined from only one direction and the precise ends of the repeats could not be resolved. It seems, however, that the number of times this sequence is repeated is not an integer. Examination of the banding pattern of the tandemly repeated sequence in Figure 57 suggests that the final partial copy of the repeats contains at least 10 bases of the repeated sequence.

FIGURE 56

DNA sequence of a 27 bp tandem repeat in the SalI variable fragment from EcoRI E. A sub-clone containing the SalI variable fragment from VZV strain Oka was digested with SalI, radiolabeled at the 5' termini with polynucleotide kinase and then digested with HincII. The fragments were separated in a 5% polyacrylamide gel. The variable SalI / HincII sub-fragment was located by ethidium bromide staining and ultraviolet light, cut from the gel and eluted (see Materials and Methods). This fragment was sequenced using the Maxam and Gilbert procedure in four separate reactions specific for the bases listed at the top of the figure, and analyzed in an 8% polyacrylamide sequencing gel (see Materials and Methods). A 27 bp tandemly-duplicated sequence was found in this fragment. The sequence for the first repeating unit is shown at the bottom. The bars show the beginning and end of each repeat unit. All repeats can not be seen in this figure (indicated by the dots at the top) because of over-modification and cleavage of bases in this gel.

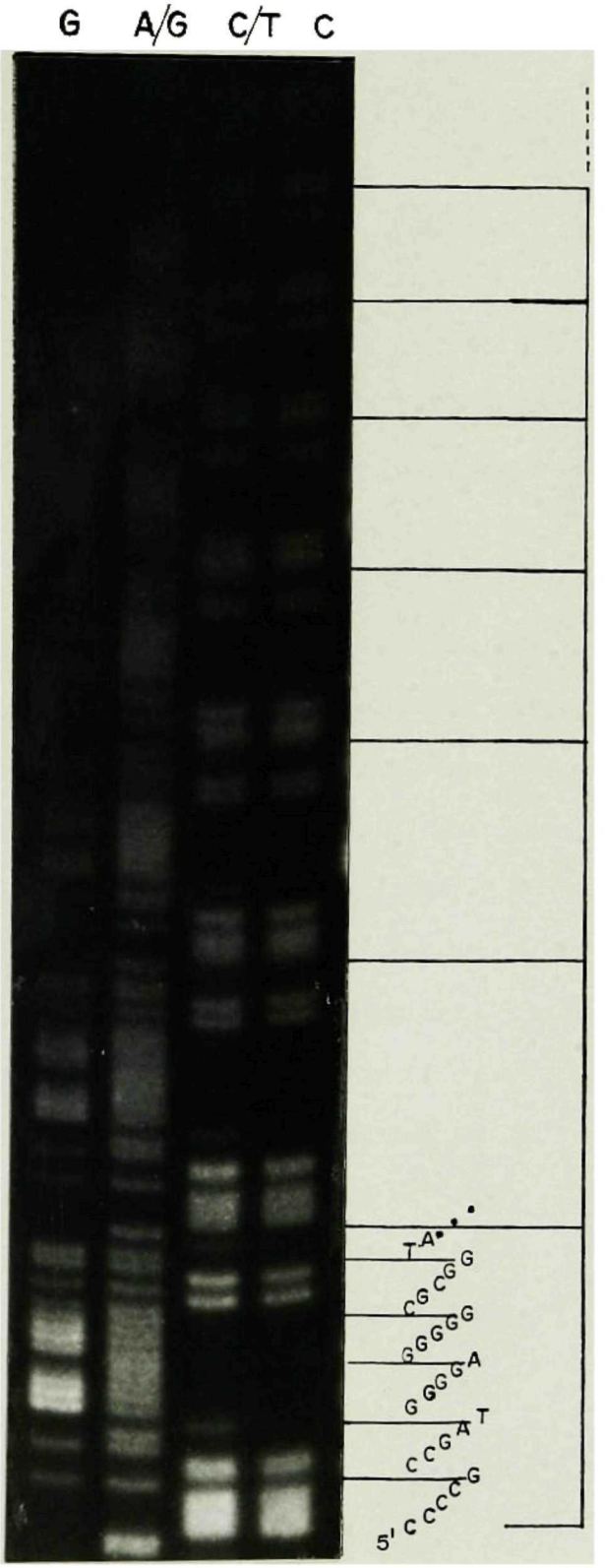
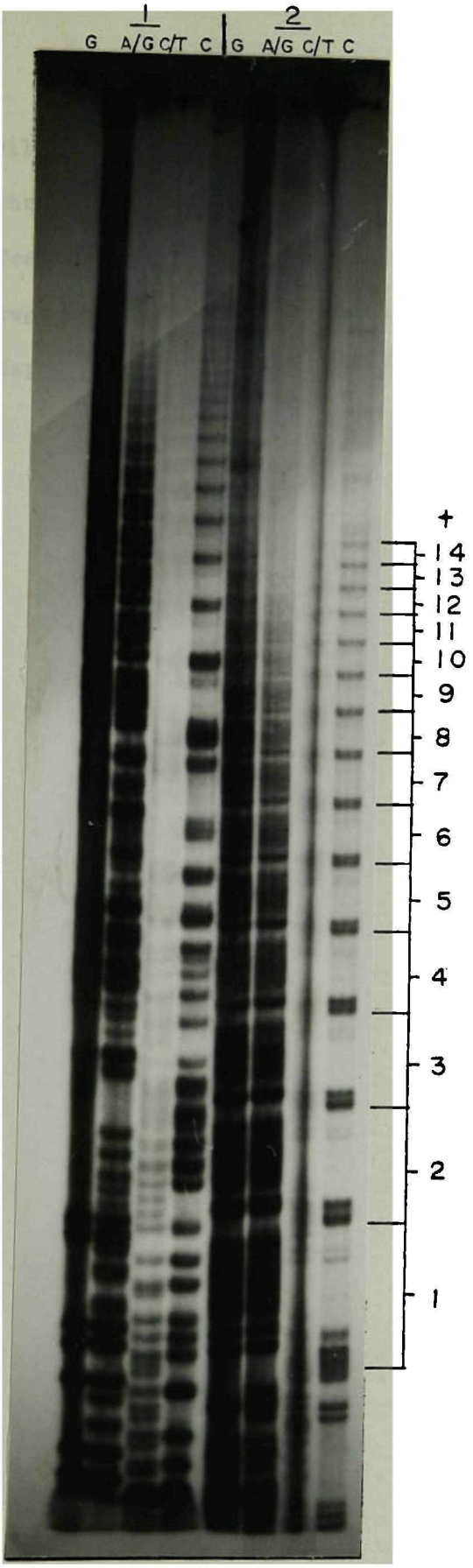

G
A/G
C/T
C
5'

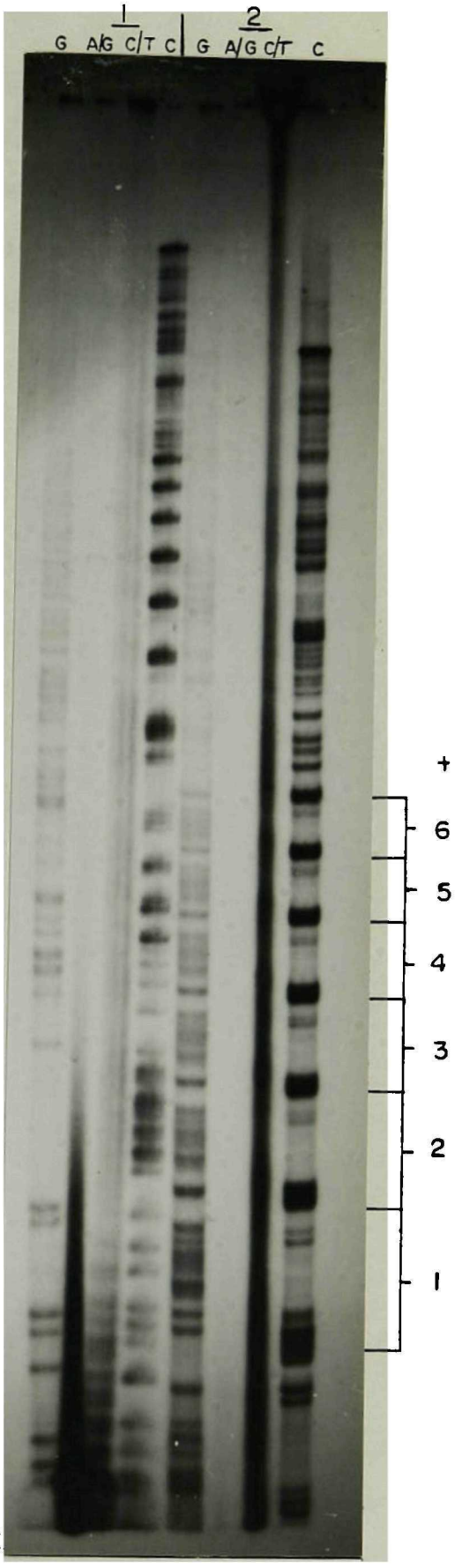
FIGURE 57

Comparison of the DNA sequence of SalI variable fragments from different strains. Sub-clones containing the SalI variable fragment from VZV strains Oka and Ellen were digested with SalI, radiolabeled at the 5' termini with polynucleotide kinase and then digested with HincII. The fragments were separated in a 5% polyacrylamide gel. The variable SalI / HincII sub-fragments were located by ethidium bromide staining and ultraviolet light, cut from the gel and eluted (see Materials and Methods). These fragments were sequenced using the Maxam and Gilbert procedure in four seperate reactions specific for the bases listed at the top of the figure, and analyzed in an 8% polyacrylamide sequencing gel with two loadings (labeled 1 and 2, see Materials and Methods). The 27 bp tandemly-duplicated sequence found in these fragments is present in different copy numbers for each strain and is the only difference seen. The bars show the beginning and end of each repeat unit. Exact numbers of repeating units could not be determined because sequencing was carried out from only one end of each the fragment. Thus, the numbers of repeat units listed at the sides of these gels are approximate (±1 unit).

OKA


1
2
G A/G C/T C G A/G C/T C
+
14
13
12
11
10
9
8
7
6
5
4
3
2
1

ELLEN


1
2
G A/G C/T C G A/G C/T C
+
6
5
4
3
2
1

Future studies. The data presented here are being utilised, and will be utilised in studies to determine the transcript maps of the three variable regions and the sequences of the EcoRI G and P fragments. In addition, the function of the repeat sequence variable region will be tested using a chloramphenicol acetyltransferase expression vector (see page 231).

DISCUSSION

Poor growth characteristics and cell-association in tissue culture have prevented detailed molecular examination of VZV. Basic features of viral replication, known in other viral systems, have not yet been characterized for VZV. Prior to the beginning of this work (1980), very little was known about VZV DNA, viral-coded proteins, VZV genetics or gene expression. The purpose of this study was to begin an examination of the molecular biology of VZV.

The experiments presented in this dissertation were designed to determine the basic structure of VZV DNA. This was accomplished using procedures which have been successfully employed in the examination of other herpesvirus DNAs. These studies identified a region of VZV DNA which is located at one terminus and inverts in orientation. Additional experiments identified three regions in the genome which vary in size in different VZV strains; the variation appears to be due to short tandemly-duplicated DNA sequences, in at least two of these regions, which are present in a variable number of copies in different strains.

In addition, the libraries of cloned EcoRI fragments from different VZV strains which were produced in the course of this work will be of use in future studies of VZV gene expression.

VZV invertible DNA region

Restriction endonuclease analysis of VZV DNA, with EcoRI and BglII, identified four fragments for each enzyme which are under-represented in relation to the other restriction fragments in these digests. These under-represented fragments could not be caused simply

by partial digestion of the DNA because reactions were carried out with greater than 10 fold excess restriction enzyme and for incubation times longer than are recommended by the enzyme manufacturer. The molarities of the under-represented fragments were determined by measurement of the areas under the peaks in densitometer tracings of these fragments separated by agarose gel electrophroesis. These determinations suggest that the under-represented fragments from certain digests (e.g. EcoRI and BglII) are present on the average in only half of the molecules in a preparation of VZV DNA, and are 0.5 M in relation to the remaining fragments in these digests, which are 1 M. Digestion with other enzymes (e.g. BamHI), however, produces no 0.5 M fragments but does result in a 2 M fragment.

The presence of under-represented fragments from restriction digests of other herpesvirus DNAs (e.g. HSV 1 and 2; Hayward et al., 1975b; Clements et al., 1976) has been used as evidence for the presence of invertible regions. Thus, the presence of 0.5 M fragments in restriction digests of VZV DNA suggests that a region of VZV DNA can invert in orientation relative to the remaining DNA. A consequence of the presence of this invertible region is that the sum of two of the 0.5 M DNA fragments in a given digest should equal the sum of the remaining two 0.5 M fragments. The reason for this is that two of the 0.5 M fragments share sequences with the other two 0.5 M fragments and are different only because of a change in position of a restriction site due to inversion. Thus, the sum of the EcoRI fragment A and F equals the sum of the remaining EcoRI fragments, E and J (Table 1). Similarly the sum of two of the BglII 0.5 M fragments (C,J) equals the sum of the other two 0.5 M BglII fragments (D,G) (Table 1). The 2 M fragment

produced by BamHI (J) suggests that this fragment is wholly located within repeat sequences (Figure 11).

The possibility of inverted repeat sequences (IR_S/TR_S) in VZV DNA was confirmed by electron microscopic examination of denatured and self-reannealed whole VZV DNA. Similar studies with other herpesviruses have identified inverted repeat sequences in herpesvirus DNA bracketing DNA regions which can invert (Sheldrick and Berthelot, 1974; Stevely, 1977). Our studies with denatured and self-reannealed VZV DNA identified a stem and loop structure, which is characteristic of inverted repeat sequences, at one end of the DNA molecule (Figure 7). The stem and loop structure is interpreted as indicating that one of the repeat sequences in VZV DNA is terminal and has self-hybridized (stem) to the internal repeat sequence which must be in an inverted orientation. These inverted repeats (IR_S/TR_S) are approximately 7Kb and are separated by about 6.5 Kb of unique sequence DNA (loop). Thus, the total molecular weight of the invertible region is estimated to be 20.5 Kb.

A maximum size for the invertible region can also be estimated from the size of a set of two of the 0.5 M fragments. These fragments must together contain the entire invertible region or they would be present in both isomers (see Introduction). Thus, the sum of two BglII 0.5 M fragments (eg. C + J, Table 1), which is smaller than the sum of two EcoRI 0.5 M fragments (eg. A + J, Table 1), results in a maximium of 20.8 Kb for the VZV invertible region. Thus, both methods result in similar sizes for the VZV invertible region.

The presence of inverted repeat sequences and of 0.5 M fragments in certain restriction digests which were related by inversion was

confirmed in further investigations designed to identify the terminal restriction endonuclease fragments in VZV DNA. Exonuclease III digestion of VZV prior to digestion with EcoRI places two 0.5 M fragments (EcoRI F and J) at one terminus of VZV and EcoRI C, a 1 M fragment, at the other (Figure 8). The remaining 0.5 M fragments (EcoRI A and E) must map across the internal (IR_S) as opposed to the terminal (TR_S) repeated sequence. Identification of the terminal EcoRI fragments was the first step in a major collaborative effort between our laboratory and that of S. E. Straus, which led to the construction of the first EcoRI map of VZV DNA (Straus et al., 1982).

Similar exonuclease treatment prior to BglII digestion, indentified the 0.5 M fragments D and J as terminal, and places BglII C and G internally. The 1 M BglII fragment at the unique terminus identified by exonuclease treatment is BglII L (Figure 9). This result differs slightly from those published by Dumas et al. (1981). Although they found the same 0.5 M fragments at the invertible terminus (D, J), they positioned the BglII Q fragment at the unique terminus; whereas this fragment is clearly present in the BglII / exonuclease III digestion shown in Figure 9. The reason for this difference is not clear, but exonuclease determinations for terminal fragments can be difficult to interpret, since the extent of digestion is not easy to control. It is conceivable that over-digestion with exonuclease by this group has led to a false result.

We can conclude, therefore, that VZV DNA is divided into to a long and short segment (U_L and U_S). The U_S segment is located near one terminus and can invert in orientation relative to U_L owing to the presence of flanking inverted repeat sequences (IR_S/TR_S, Figure 11).

These results have been confirmed by others (Dumas et al., 1981; Ecker and Hyman, 1982; Gilden et al., 1982; Davison and Scott, 1983; Mishra et al., 1984) based on similar experimental procedures.

A variety of herpesvirus DNAs have been found which invert parts of their DNA. These include HSV 1 and 2, CMV, PRV, BMV and EHVI, as described in the Introduction. In addition, other eukaryotic DNAs have been found to have invertible regions, such as the 2u circle in yeast and the mitochondrial DNA of Achlya ambisexualis (Guerineau et al., 1976; Hudspeth et al., 1983). However, the reason for DNA inversions in these eukaryotic DNAs is not understood. Hayward et al. (1975b) suggested, by analogy to bacteriophage mu, that inversions may be involved in the host range of HSV 1. In phage mu there is a segment of DNA, called the G loop, which inverts and changes the direction of transcription. In one orientation a phage tail is made which is specific for E. coli K12 and in the other orientation a tail for E. coli C is produced. In a lytic phage infection of either E. coli K12 or C, the G loop orientation which produces a tail for the infected strain predominates with a small proportion in the orientation specific for the other strain. Similar inversions have been shown to change transcription in bacteriophage P1 and Salmonella (van de Putte et al., 1980; Yun and Vapnek, 1977; Zieg et al., 1977).

However, since this suggestion was made, data have emerged which suggest that herpesvirus DNA inversions probably do not have such a role. For example, in herpesviruses both orientations appear to be present in approximately equal amounts, and the proportion of one orientation to the other does not seem to change by infection of a different cell line or type. Also, it seems that virions which contain

different isomers are equally infectious at least in pseudorabies virus (Ben-Porat et al., 1980) and, perhaps, also in HSV. Third, mutants of HSV have been generated which cannot invert or invert at a low frequency; these appear to be just as infectious on a variety of cell lines as wild type HSV (Davison and Wilkie, 1984; B. Roizman, personal communication). Finally, transcription across the L/S junction does not seem to occur in HSV, and such transcription would be required if different genomic isomers were to produce attachment proteins specific for different hosts (Clements et al., 1979; Watson et al., 1981).

Another reason for the presence of invertible regions could be that they are simply a consequence of the necessity for sets of inverted repeats. If the genes transcribed from the repeated sequences in herpesviruses are required to be diploid then the only arrangement which would not result in deletions would produce inversions. That is, the repeated sequences, which must be diploid, can only be in an inverted orientation, otherwise DNA sequences between the repeats would be deleted by intramolecular homologous recombination. If this hypothesis is true the specific sequence of the inverted repeats is not important for inversion and any duplicated sequence could cause inversion of DNA sequences between the inverted copies.

Despite ten years having passed since the discovery of herpesvirus DNA inversions, the point during viral replication that inversion occurs and the mechanism of inversion are not really understood. However, several revealing experiments have been carried out. Mocarski et al. (1980) and Smiley et al. (1981) have engineered a new joint region in the middle of the long unique region of HSV DNA. Co-infection of this construct with the virus genome results in the

formation of new isomers of HSV in addition to the normal four. The additional isomers result from new inversions cause by the new joint. In other experiments, Mocarski and Roizman (1982a), inserted an HSV "a" sequence (see Figure 3), a component of the L/S joint, in inverted orientation on either side of the HSV thymidine kinase gene. Transfection of this construct into eukaryotic cells resulted in the inversion of the thymidine kinase gene upon infection by HSV. They conclude from this that the "a" sequence must be involved in and is required for the inversion event which occurs by site-specific recombination. However, VZV does not contain an "a" sequence and therefore, either VZV inversion has a different mechanism or the "a" sequence is irrelevant.

Another model of herpesvirus DNA inversions, originally suggested by Sheldrick and Berthelot (1974), is based solely on homologous recombination between the inverted repeat sequences. In prokaryotes, it has been found that DNA sequences between inverted repeats invert in orientation and DNA sequences between direct repeats delete (Adelberg and Bergquist, 1972). This is thought to occur by homologous recombination. In support of this model, Lominiczi et al.(1984) have reported the isolation of a PRV strain which has evolved into a double inverting DNA molecule from the normal single inverting DNA by addition of a new inverted repeat at the normally unique end.

Ben-Porat et al. (1982) suggest that recombination in PRV infection is a very early event and occurs between parental molecules. Therefore, if inversion results simply from homologous intramolecular recombination then it may also be an early event. Evidence suggests that, indeed, inversion occurs early in infection (Roizman 1979a, b).

On the other hand, Roizman (1979a) has proposed a very complex model for inversion. By a series of recombination and strand switching steps during replication, this model predicts that any single infecting isomer of HSV DNA can produce all isomers.

If Ben-Porat (the simple model) is correct, then replication is not needed for recombination and all isomers are produced from a single infecting one by recombination before they all enter the replicating pool of DNA molecules. This could be tested if sufficient amounts of viral DNA could be isolated from infected cells which were blocked for replication by an inhibitor or from cells infected with a DNA^- mutant at the non-permissive temperature. Unfortunately, these experiments are difficult to carry out and to interpret, as we do not yet know enough about either replication or recombination in herpesvirus-infected cells to be able to make intelligent guesses as to which process is responsible for DNA inversions.

Very recently, Davison (1984) and Ruyechan et al. (1984) have suggested that the long region (U_L) of VZV DNA may also, at a low frequency, invert in orientation. Davison (1984) has identified an 88.5 bp sequence (the 0.5 bp is a single overhanging base at one end) that is inverted at either end of U_L and it is this sequence which is probably involved in the inversion. Davison (1984) suggests that inversion of U_L in VZV may be a by-product of replication but, once more, no experimental proof is available. One reason for the low frequency of this inversion may be that the repeat region (88.5 bp) is a very small target for homologous intramolecular recombination which may require longer stretches of inverted repeats (e.g. the size of IR_S and TR_S) for high frequency inversions. This could be tested using different sizes

of inverted repeat sequences in transfection assays with a gene sequence which could be selected for (e.g. TK) and whose orientation following transfection could be determined.

At present, then, there is no agreement among herpes virologists on either the functions of or the mechanism which results in the inversion of DNA sequences. It may be, however, that VZV, with its high and low frequency inversions might be a good model system with which to study the process further.

VZV DNA strain variation

DNA sequence variations in different viral strains have been reported for many herpesviruses (Allen et al., 1983; Dambaugh et al., 1980; Desrosiers and Falk, 1982; Hayward et al., 1975; Heller et al., 1981; Harai et al., 1981; Locker and Frenkel, 1979; Pritchett, 1980; Raab-Traub et al., 1978 Wathen and Pirtle, 1984).

There are two types of strain variations which can generally be identified using restriction endonucleases. The first is the gain or loss of a restriction site. This results in the fusion of fragments (leading to a loss) or the production of new fragments, and can be due to a change in a single base pair. This type of strain variation has been identified in VZV DNA (see Figure 18) but seems to be very uncommon. Martin et al. (1982) have reported different BglI fragments produced by digestion of the Oka vaccine strain of VZV DNA when compared to other VZV strains, and this could be caused by a point mutation which forms a new recognition site for BglI. In this dissertation, new restriction fragments have been found in the BamHI digestions of VZV strains Oka and Scott, which are different and appear to be

characteristic for these strains, and could also be caused by point mutations. In several other studies, however, no variation of this type was observed. Straus et al. (1983) examined 17 different clinical isolates of VZV DNA with up to 7 different restriction endonucleases and did not find a single isolate that had new fragments. Similarly, Iltis et al. (1977), Richards et al. (1979), and Oakes et al. (1977) examined several different clinical isolates and found no variation in numbers of restriction fragments. Ecker and Hyman (1981) and Ecker et al., (1984) have claimed to find differences between the Oka vaccine strain DNA and DNA from the parent of this strain which might be predicted to be of the restriction site alteration variety. They do not, however, elaborate on the type of difference they have observed and, from the published figures, it is unfortunately not possible to tell if they have identified contaminating host sequences or genuine differences between viral genomes.

Although the gain or loss of restriction sites is very uncommon in VZV, this type of strain difference appears to be common in HSV. The reason for this is not known, but it could be caused by differences in the fidelity of DNA replication by the viral encoded DNA polymerases of these viruses or by greater tolerance of point mutations in HSV then in VZV.

New restriction fragments have been used as markers in molecular epidemiology of HSV infections (Buchman et al., 1978; Halperin et al., 1980; Linneman et al., 1978; Sakaoka et al., 1984). Analogous molecular epidemiology of VZV infections cannot use the gain or loss of restriction sites as a marker since it does not occur frequently enough to be useful in these studies.

The second type of DNA strain variation seen in VZV DNA is the change in mobilites of specific restriction endonuclease fragments. This type of strain variation does seem to meet the requirements for use in molecular epidemiological studies of VZV infections; this variation is quite stable and occurs relatively frequently. The studies reported here were performed to determine the nature of the strain variation in VZV DNA.

Restriction endonuclease digestion of VZV DNA from different viral strains shows small changes in the migration of a small number of fragments. Epidemiologically-distinct strains have characteristic differences and related strains appear identical (Straus et al. 1983; this dissertation). In addition, these strain differences appear to be stable in human cell culture (Straus et al., 1981; Zweerink et al., 1981; this dissertation). These factors allow a precise examination of the positions of these strain differences and the determination of the molecular basis of strain variation in VZV DNA.

In the EcoRI patterns of different VZV DNAs, a limited number of variable restriction fragments were found. The 0.5M EcoRI fragments, for example, which contain the inverted repeat sequence (EcoRI A, E, F and J) and two fragments which map to the long unique region (EcoRI G and P) had small differences in mobilities in different strains (Figures 12 and 16). The differences in the fragments which contain the inverted repeats appeared to be analogous. Although it was difficult to measure these small differences in the larger fragments (e.g. EcoRI A and E), it was found that if one of these 0.5 M fragments increased in size (e.g. EcoRI J) compared to another strain, then all four (A, E, F, J) always increased in size. The two other strain-variable fragments appeared to

vary independently of the difference in the inverted repeats and of each other (Figures 15 and 16). Examination of other restriction digests and comparison of the map locations of the strain-variable fragments confirmed that three distinct regions of VZV DNA varied. These regions are the inverted repeats and two regions of U_L (Figure 19).

Other herpesvirus have also been examined for this type of strain variation. Allen et al. (1983) identified regions in EHV 1 which varied, and their map locations are very similar to those found in VZV. The inverted repeats as well as two region of U_L were found to vary in EHV 1 DNA. However, in EHV 1 there appears to be an additional strain variable region in U_S. Although not examined in detail in VZV, SmaI digestion of VZV DNA suggests that U_S also varies in a few strains. The SmaI E fragment, which maps entirely in U_S appears larger in VZV strains Scott and Ellen (Figure 17). Interestingly, Allen et al. (1983) also found that the EHV-1 strain-variable fragments were stable in cultured equine cells but not in monkey cell lines. Similarly, Wathen and Pirtle (1984) examined strain variation in PRV. These authors mapped PRV strain variable fragments to three regions of the genome and found that, although the differences were stable in porcine cell culture, the viral genome began to vary after several passages in pigs, the natural host of PRV. Significantly, the three regions of PRV which were found to vary are also in the inverted repeats near the junction with U_S and two regions in U_L. Thus, the locations of strain variations in PRV, EHV 1 and VZV all appear to be very similar.

The three regions of VZV DNA which vary in different strains were molecularly cloned into the plasmid vector pBR325 as EcoRI fragments from four different strains. Each variable fragment could

then be characterized for the precise location of the strain difference.

The EcoRI G fragment, which is one of the strain-variable regions which maps in U_L (Figure 19) was difficult to analyze. Although this region of the genome is stable in cultured human cells it seems to be very unstable when cloned in E. coli in a plasmid or lambda vector. Frequently, different recombinant isolates from the same VZV strain were found to contain different sizes of EcoRI G fragments (Figure 24). Differences were even seen in different preparations of the same isolate. Sub-digestion of the EcoRI G fragment with other restriction endonucleases showed that the region which was changing in E. coli was also apparently the same region which in whole VZV DNA was variable. The reason for this instability in E. coli of the EcoRI G recombinants is not known. However, it has been observed that an HSV 1 fragment (BglII I; S. Weller, personal communication) is also very unstable when cloned into E. coli. Close examination of the HSV 1 BglII I fragment has revealed that it contains an origin of DNA replication and at that this is the point at which deletions occur after E. coli cloning. On this basis it is predictable that the EcoRI G fragment of VZV also contains an origin of DNA replication, and this can readily be tested.

Restriction endonuclease mapping localized the strain-variable region in EcoRI G to a 1.2 to 1.5 Kb BamHI / SalI fragment located at the left end of EcoRI G. This position for the variable region is consistent with digestions of whole VZV DNA, which show that BamHI A (Figure 18) and HindIII E (Figure 16) varied, and the map positions of these fragments overlap with EcoRI G (Figure 19). VZV DNA sequence analysis of this region (A. Davison, personal communication) has

identified a 9 bp sequence tandemly-duplicated in this area. It is likely by analogy with our work with EcoRI E (see Results), that a variable copy number of this 9 bp sequence is solely responsible for the strain variation in EcoRI G. The DNA sequence of this area is currently being determined for the four strains used in these mapping experiments to test this hypothesis and to look for a potential DNA synthesis origin.

The EcoRI P fragment strain variation also maps in the U_L region of VZV DNA. These P fragments, unlike the EcoRI G fragments, did not vary as plasmid or lambda recombinants in E. coli. Heteroduplex analysis of strains Scott and Ellen EcoRI P hybrids showed that there is a single insertion or deletion loop, near the middle of the duplex, which is large enough to be responisble for the difference between these two strains. Comparison of restriction endonuclease digestions between strains Scott and Oka with five different enzymes showed that a single sub-fragment in each digest varied. In the HaeIII digest however, there did not appear to be any difference in migration of the fragments from these strains. There is a 35 bp HaeIII fragment in digests of EcoRI P which differs in intensity between the different strains. This indicates that different molar amounts of this fragment exist in different strains, suggesting that the difference between strains of VZV EcoRI P is due to a variable number of tandemly-duplicated direct repeats of DNA which are 35 bp in size and contain a HaeIII site. Restriction endonuclease mapping of the EcoRI P fragment places the tandemly duplicated 35 bp HaeIII fragment near the middle of this fragment. Thus, by electron microscopic heteroduplex analysis and restriction endonuclease mapping, the difference between EcoRI P

fragments from different VZV strains is a single insertion or deletion of variable numbers of a 35 bp tandemly-repeated DNA sequence near the middle of the fragment.

The strain difference which is located in the inverted repeats (IR_S and TR_S) which bound U_S was examined by comparing cloned EcoRI E fragments (which contain IR_S) from four different VZV strains. The EcoRI A fragments from different strains which also contain IR_S (with U_S in the other orientation), were also examined and compared with EcoRI E fragments for the position of the variable region. The results show that the variable sub-fragments are the same in both EcoRI A and E and must therefore map in the same location in IR_S. Restriction endonuclease mapping of EcoRI E showed that the variable region is located approxiamtely 2 Kb from one end of the fragment. The positions of restriction endonuclease sites in this fragment then allowed the orientation of the fragment to be determined in relation to the restiction maps for whole VZV DNA. This alignment shows that the strain difference is located toward U_S on the inside of IR_S. Electron microscopic heteroduplex analysis of EcoRI E fragments from strains Ellen and Scott showed a single insertion or deletion loop in predominantly duplex molecules. The location of the insertion or deletion loop is approximately 80% from one end of EcoRI E and is similar to the map position of the strain difference determined by restriction endonuclease mapping. The smallest sub-fragment from EcoRI E which varied was a SalI fragment of between 1550 and 1800 bp in size, depending on the strain. This SalI variable fragment was then subcloned from the EcoRI E fragments of different VZV strains. The largest difference in size is between the variable fragments of VZV strains

Ellen and Oka. These fragments were end-labeled and sequenced using the Maxam and Gilbert method (Maxam and Gilbert, 1977; 1980). Approximately 150 bp from the fragment's end a 27 bp tandemly-duplicated sequence was found. This tandemly-duplicated sequence is repeated a different number of time in the different strains and was the only difference found between these fragments. The sequence of this 27 bp repeat is shown in Figure 58.

These results show that two regions of VZV DNA which were found to vary in different viral strains (EcoRI P and E) are due to variable copy numbers of a short tandemly-duplicated directly repeated DNA sequence. Sequence data from the third VZV DNA variable region (EcoRI G) suggests that this difference may also be due to different numbers of tandem repeats (A. Davison, personal communication). Although the nucleotide sequence was determined only in the IR_S/EcoRI E region, the evidence from restriction endonuclease digestions suggests that the sequence of these repeats in different variable regions is not the same. For example, the EcoRI P duplication is cleaved by HaeIII into monomer repeats and is approximately 35 bp in size, while the EcoRI E tandem repeat is 27 bp and is not cleaved by HaeIII. In addition, the EcoRI E duplication contains a KpnI site which cleaves the repeat region into 27 bp monomer while the EcoRI P fragment is not cut by this enzyme (data not shown). The variable regions in EcoRI P and EcoRI G from different strains are currently being sequenced in our laboratory to determine if there are any similarities in base sequence among the tandem repeats which might suggest a common function or the mechanism for the variability.

There are at least two possible reasons why strain variation

might occur in VZV DNA. The first is that insertions or deletions occur in these regions during viral DNA replication in response to the environment in a particular host cell. Wathen and Pirtle (1984) have shown that PRV DNA shows variations only during passage in whole pigs, and that these changes occur in regions analogous to the locations of strain variations in VZV. In similar experiments, Allen et al.(1983) showed that EHV 1 DNA was stable when grown in equine cell cultures but began to change, in regions similar to those in VZV, when grown in cultures of monkey cells. Ben-Porat et al. (1984) have shown that the repeat region of PRV can vary in response to mutagenesis of a distal area of the genome. Taken together, these data suggest that variations in herpesvirus DNA occur in response to perturbation of replication such as changes in growth conditions in different cell types or because of interference with replication by mutation of a gene or regulatory sequence. DNA sequence variation could be a viral response which allows the virus to replicate more efficiently or it may be an effect which slows viral replication and is advantageous to the host cell. Allen et al. (1983) found that yields of EHV-1 were not significantly different if grown in horse or in monkey cells, but this effect may be a subtle one which might not change infectious particle production dramatically in a single growth cycle.

An alternative possiblity is that these strain variable regions are "hot spots" for recombination. Changes in copy number of tandemly duplicated repeat sequences might occur by unequal crossing-over during recombination. Unfortunately, there is currently no evidence that the two variable regions which map in U_L (EcoRI E and P) are recombinationally hyperactive because no genetic study has so far been

possible in VZV. However, we suspect that the IR_S/TR_S regions are active in recombination and thus the IR_S/TR_S strain difference might result by unequal crossing over in the repeat regions during inversion of U_S.

Two recent publications have suggested that the recombination process which results in inversion of DNA sequences is related to changes in the mobility of restriction endonuclease fragments from the IR_S/TR_S regions of PRV (Ben-Porat et al., 1984) and HSV1 (Varmuza and Smiley, 1984). Both groups plaque-purified virus which contained DNA with inverted repeats which were not identical. The differences between the repeats in the same DNA molecule have locations similar to the difference in the VZV IR_S/TR_S strain difference described here. These heterologous repeat sequences equalized to one size or the other very rapidly after infection, presumably either by intermolecular or by intramolecular homologous recombination at very high frequencies. Both groups propose that the recombination event which results in the equalization of these repeats also causes inversion of U_S in normally infected cells.

The sequence of two variable regions in the IR_S/TR_S region of HSV1 DNA have recently been determined. The HSV "a" sequence contains three sets of tandemly-repeated DNA, and two sets of these tandem repeat sequences are found a variable number of times in the "a" sequence both in different strains and in the same strain (Davison and Wilkie, 1981; Mocarski and Roizman, 1981). The other HSV strain-variable region is also located in IR_S/TR_S, in the "c" sequence (see Figure 3), which is a location similar to the VZV strain difference discussed above, and is due to variable copy numbers of a 22 bp direct repeat which is tandemly

duplicated. A comparison of one of the "a" sequence repeats and the "c" repeat of HSV with the VZV IR_S/TR_S tandem repeat is shown in Figure 58. The sequences are aligned for maximum homology, and several interesting points emerge. First, all the reiterated sequences are very high in G+C; the HSV "a" sequence repeat is 83% G+C, the reiteration in the "c" sequence of IR_S/TR_S is 86% G+C and the VZV repeated sequence is 81% G+C. This high G+C content is not too surprising for HSV which has an overall G+C content of 67% (Plummer et al., 1969) but VZV has an average G+C content of 47% (Straus et al., 1981) which makes this sequence highly unusual and interesting. Second, the size of the tandem reiterations in "c" sequence of HSV and VZV IR_S/TR_S repeat are very similar (22 bp in HSV, 27 bp in VZV) and third, the right end of the HSV "c" and "a" sequences matches the VZV sequence at 9 of 10 bases (Figure 58) . It is not clear if these base matches alone are significant, because the sequences are so short and could conceivably occur in random DNA sequences with such G+C contents. It does seem unusual however, that these homologies are seen in similar-sized tandemly-repeated sequences which are located in similar regions of VZV and HSV.

Tandemly-reiterated sequences which vary in different strains have also been found in other viruses. The terminal inverted repeats of vaccinia virus DNA consists of tamdem repeats of 70 bp (Wittek and Moss, 1980). In non plaque-purified virus, the ends of vaccinia DNA are heterogenous due to a variable number of tandem repeats in different viruses in the population. Plaque-purification eliminates this heterogeneity by selecting a single population with the same size of inverted repeat, probably because all viruses in the population now have the same number of terminal tandem repeats (Wittek et al., 1978; Wittek

FIGURE 58

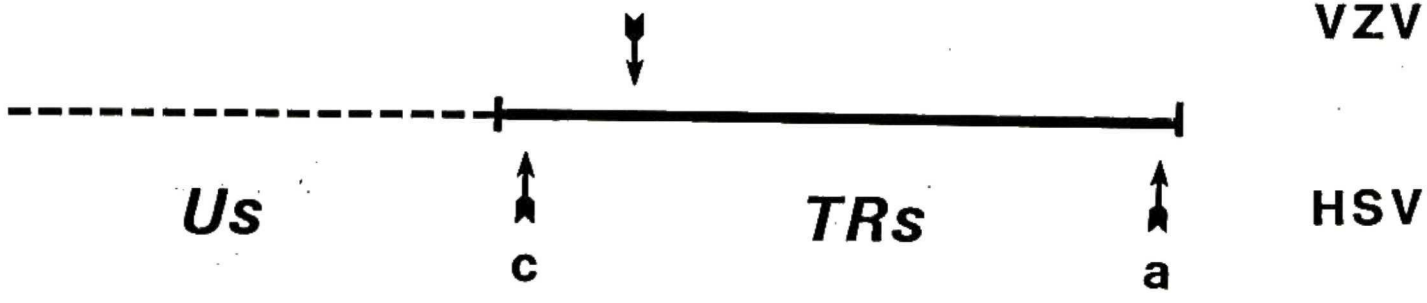
Comparison of the tandemly-repeated sequences in VZV and HSV. The tandemly-repeated DNA sequence which is responsible for variation in mobilities of restriction fragments that contain VZV IR_S or TR_S is compared to the sequences found in the "a" and "c" regions of HSV 1 DNA (Mocarski and Roizman, 1981; Davison and Wilkie, 1981; Murchie and McGeoch, 1982). The figure also shows the relative positions of these sequences in IR_S/TR_S of VZV and HSV.

REPEAT SEQUENCE HOMOLOGY

CCGCCGATGGGGAGGGGGCGCGGTACC VZV

GACGCGGGGGCGGAGGAGGGGG HSV 1 "c"

GCGAGGAGGGGG HSV 1 "a"



and Moss, 1980). Precisely how the terminal repeats increase or decrease in size, however, is not known. McFadden and Dales (1979) noted that a deletion in one of the vaccinia terminal repeats is also found in the other terminal repeat. This can be explained by high frequency recombination in the terminal repeats and, as suggested for HSV, the variability is possibly due to unequal crossing-over in the area of these tandem duplications.

Papovaviruses also contain tandem direct duplications which may vary in different isolates. In JC virus there are differences in mobilities of restriction fragments which map near the origin of replication in a non-coding region. These differences are seen in virus from cells in culture (Martin et al., 1983) or from virus isolated directly from patients (Grinnel et al., 1983; Martin and Foster, 1984). The DNAs of some polyoma virus strains contain duplications of different sizes (Ruley and Fried, 1983) and these differences are also located near the origin of replication. This area is known to be important in control of polyoma early gene expression (Ruley and Fried, 1983). SV40 and BK virus also seem to have tandem duplications in this area (Fiers et al., 1978; Yang and Wu, 1979; Seif et al., 1979). Although most of the DNA sequence organization of the papovaviruses is similar from virus to virus, the greatest differences among viruses occur in these tandemly-duplicated sequences of the early control region. It has been suggested that the differences are involved in host range phenomena and may contribute to the biological differences among these viruses.

The differences in this papovavirus control region are not exactly analogous to the strain variation seen in IS_S/TR_S of VZV and HSV

DNA. The base sequence, the size of the tandem duplication and perhaps the number of repeats all vary in different papovaviruses. There does appear, however, to be a consensus sequence, GGGXGGPuPu, which is present in most papovaviruses examined to date (Frisque et al., 1984). This is similar to the 'consensus sequence' GGAGGGG, which we have defined above, present in both HSV and VZV.

As we have noted above, the papovavirus tandem repeat sequence near the origin of replication has a positive effect on the level of transcription of the early genes caused via binding of a specific transcription factor, SP1 (Dynan and Tjian, 1983). The sequence important in this interaction is the consensus sequence. It seems possible that the tandem duplication which causes the strain variation in HSV and VZV also interacts with an analogous transcription factor, since the 'consensus sequences' for both virus groups are similar.

In this context, recent evidence from our laboratory and from others (J. Ostrove and A. Davison, personal communication) shows that the VZV IR_S/TR_S strain variation occurs in a non-coding region of the genome and is close to an origin of DNA replication. Experiments are planned to test the effect of the VZV tandem repeat element(s) after insertion into a plasmid containing a gene (chloramphenicol acetyltransferase) whose expression can readily be measured.

Finally, the inverted repeats in HSV and CMV share some homology with cellular sequences, which was measured by hybridization of HSV and CMV DNA to human cellular sequences (Ruger et al., 1984; Penden et al., 1982; Puga et al., 1982). The region in SV40 which contains the tandem duplication has also been shown to share homology with monkey cellular sequences (Mc Cutchan and Singer, 1981; Queen et al., 1981) and this is

further evidence for a common or similar function for the tandemly-repeated sequences among these viruses. Work on homology between VZV DNA and cellular DNA is underway.

REFERENCES

Abrahamsen, L.H. and M. Jerkofsky. 1981. Enhancement of varicella-zoster virus replication in cultured human embryonic lung cells treated with the pesticide carbaryl. Appl. Environ. Microbiol. 41: 652-656.

Achong B.G. and E.V. Meurisse. 1968. Observations on the fine structure and replication of varicella virus in cultivated human aminon cells. J. Gen. Virol. 3: 305-308.

Adelberg, E.A. and P. Bergquist. 1972. The stabilization of episomal integration by genetic inversion: A general hypothesis. Proc. Natl. Acad. Sci. U.S.A. 69: 2061-2065.

Allen, G.P., M.R. Yeargan and J.T. Bryans. 1983. Alterations in the equine herpesvirus-I genome after _in vitro_ and _in vivo_ passage. Infect. Immun. 40: 436-439.

Almeida, J.D., A.F. Howatson, M.G. Williams. 1962. Morphology of varicella (chickenpox) virus. Virol. 16: 353-355.

Asano, Y., and M. Takahashi. 1979. Studies on the polypeptides of varicella-zoster (V-Z) virus: Detection of varicella-zoster virus polypeptides in infected cells. Biken J. 22: 81-89.

Asano, Y., and M. Takahashi. 1980. Studies on the polypeptides of varicella-zoster (V-Z) virus: Synthesis of viral polypeptides in infected cells. Biken J. 23: 95-106.

Baer, R., A.T. Bankier, M.D. Biggin, P.L. Deininger, P.J. Farrell, T.J. Gibson, G. Hatfull, G.S. Hudson, S.C. Satchwell, C. Seguin, P.S. Tuffnell and B.G. Barrell. 1984. DNA sequence and expression of the B 95-8 Epstein-Barr virus genome. Nature 310: 207-311.

Bastian, F.O., A.S. Rabson, C.L. Yee and T.S. Tralka. 1972. Herpesvirus hominis: Isolation from human trigeminal ganglia. Science 178: 306-307.

Bastian, F.O., A.S. Rabson, C.L. Yee and T.S. Tralka. 1974. Herpesvirus varicella isolated from human dorsal root ganglia. Arch. Path. 97: 331-332.

Becker, P., J.L. Melnick and H.D. Mayor. 1965. A morphological comparison between the developmental stages of herpes zoster and human cytomegalovirus. Exp. Mol. Path. 4: 11-23.

Ben-Porat, T., F.J. Rixon and M.L. Blankenship. 1979. Analysis of the structure of pseudorabies virus. Virol. 95: 285-294.

Ben-Porat, T., R. Veach and B. Ladin. 1980. Replication of herpesvirus DNA: Virions containing either isomer of pseudorabies virus DNA are infectious. Virol. 102: 370-380.

Ben-Porat, T., L. Brown and R.A. Veach. 1982. Recombination occurs mainly between parental genomes and precedes DNA replication in pseudorabies virus-infected cells. J. Virol. 44: 1134-143.

Ben-Porat, T., A. Deatly, R.A. Veach and M.L. Blankenship. 1984. Equalization of the inverted repeat sequences of the pseudorabies virus genome by intermolecular recombination. Virol. 132: 304-314.

Birnboim, H.L. and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7: 1513-1523.

Bolivar, F. 1978. Construction and characterization of new cloning vectors: Derivatives of plasmid pBR322 carrying unique EcoRI sites for selection of EcoRI generated recombinant DNA molecules. Gene 4: 121-136.

Bornkamm, G.W., H. Delius, B. Fleckenstein, F. J. Werner and C. Mulder. 1976. The structure of Herpesvirus saimiri genomes: Arrangement of heavy and light sequences in the M genome. J. Virol. 19 154-161.

Bormkamm, G.W., H. Delius, U. Zimber, J. Hudewentz and M.A. Epstein. 1980. Comparison of Epstein-Barr virus strains of different origin by analysis of the viral DNAs. J. Virol. 35: 603-608.

Boyer, H.W. and D. Roulland-Dussoix. 1969. A complementation analysis of the restriction and modification of DNA in Escherichia coli. J. Mol. Biol. 41: 565-472.

Brunell, P.A. and H.L. Casey. 1964. Crude tissue culture antigen for determination of varicella-zoster complement fixing antibody. Pub. Hlt. Rept. 79: 839-842.

Brunell, P.A. 1979. Varicella-Zoster virus. Ch. 117, pp. 1295-1306. in Principals and practice of infectious diseases. G.I. Mandel, R.G. Douglas and J.E. Bennett ed., John Wiley and Sons, New York.

Brunell, P.A., A.A. Gershon, S.A. Uduman and S. Steinberg. 1975. Varicella-zoster immunoglobulins during varicella, latency and zoster. J. Infect. Dis. 132: 49-54.

Bruusgaard, E. 1932. The mutual relationship between zoster and varicella. Brit. J. Derm. 44: 1-24.

Buchman, T.G. and B. Roizman. 1978a. Anatomy of bovine mammillitis virus DNA: Restriction endonuclease maps of four populations of molecules that differ in the relative orientation of their long and short components. J. Virol. 25: 395-407.

Buchman, T.G., and B. Roizman. 1978b. Anatomy of bovine mammillitis DNA: Size and arrangements of deoxynucleotide sequences. J. Virol. 27: 239-254.

Buchman, T.G., B. Roizman, G. Adams and B. H. Stover. 1978. Restriction endonuclease fingerprinting of herpes simplex virus DNA: A novel epidemiological tool applied to a nosocomial outbreak. J. Infect. Dis. 138: 488-498.

Buchman, T.G., B. Roizman and A.J. Nahmias. 1979. Demonstration of exogenous genital reinfection with herpes simples virus type 2 by restriction endonuclease fingerprinting of viral DNA. J. Inf. Des. 140: 295-304.

Buchman, T.G., T. Simpson, C. Nosal, B. Roizman and A.J. Nahmias. 1980. The structure of herpes simplex virus DNA and its application to molecular epidemiology. Ann. N.Y. Acad. Sci. 354: 279-290.

Caunt, A.E. 1963. Growth of varicella-zoster virus in human thyroid tissue culture. Lancet ii: 982-983.

Cebrian, J., C. Kashka-Dierich, N. Berthelot and P. Sheldrick. 1982. Inverted repeat nucleotide sequences in the genome of Marek's disease virus and the herpesvirus of the turkey. Proc. Natl. Acad. Sci. U.S.A. 79: 555-558.

Cheng, Y.C., T.Y. Tsou, T. Hackstadt and L.P. Mallavia. 1979. Induction of thymidine kinase and DNase in varicella-zoster virus-infected cells and kinetic properties of the virus induced thymidine kinase. J. Virol. 31: 172-177.

Choppin, P.W., and A. Scheid. 1980. The role of viral glycoproteins in adsorption, penetration and pathogenicity of viruses. Rev. Infect. Dis. 2: 40-61.

Chousterman, S., M. Lacasa and P. Sheldrick. 1979. Physical map of the channel catfish virus genome: Location of the sites for restriction endonucleases EcoRI, HindIII, HpaI and XbaI. J. Virol. 31: 73-85.

Clements, J.B., R. Cortini and N.M. Wilkie. 1976. Analysis of HSV substructure by means of restriction endonucleases. J. Gen. Virol. 30: 243-256.

Clements, J.B., J. McLauchlan and D.J. McGeoch. 1979. Orientation of herpes simplex virus type 1 immediate early mRNAs. Nuc. Acid Res. 7: 77-91.

Clewell, D.B. and D.R. Helinski. 1969. Supercoiled circular DNA-protein complex in Escherichia coli: Purification and induced conversion to an open circular DNA form. Proc. Natl. Acad. Sci. U.S.A. 62: 1159-1166.

Clewell, D.B. and D.R. Helinski. 1972. Effect of growth conditions on the formation of the relaxation complex of supercoiled ColE1 deoxyribonucleic acid and protein in Escherichia coli. J. Bact. 110: 1135-1146.

Cohen, S.N., A.C.Y. Chang and L. Hsu. 1972. Nonchromosonal antibiotic resistance in bacteria : genetic transformation of Escherichia coli by R-factor DNA. Proc. Natl. Acad. Sci. U.S.A. 69: 2110-2114.

Cook, M.L. and J.G. Stevens. 1968. Labile coat: Reason for non-infectious cell-free VZV in culture. J. Virol. 2: 1458-1464.

Cook, M.L.and J.G. Stevens. 1970. Replication of varicella-zoster virus in cell culture: An ultrastructural study. J. Ultrastr. Res. 32: 334-350.

Cruickshank, J.G., H.S. Bedson and D.H. Watson. 1966. Electron microscopy in the rapid diagnosis of smallpox. Lancet 2: 527-530.

Dambaugh, T., N. Raab-Traub, M. Heller, C. Beisel, M. Hummel, A. Cheung, S. Fennewald, W. King and E. Kieff. 1980. Variations among isolated of Epstein-Barr virus. Ann. N.Y. Acad. Sci. 354: 309-325.

Davison, A.J. and N.M. Wilkie. 1981. Nucleotide sequences of the joint between the L and S segments of herpes simplex type I and II. J. Gen Virol. 55: 315-331.

Davison A.J. and N.M. Wilkie. 1983. Inversion of the two segments of the herpes simplex virus genome in intertypic recombinants. J. Gen. Virol. 64: 1-18.

Davison A.J. and J.E. Scott 1983. Molecular cloning of the varicella-zoster virus genome and derivation of six restriction endonuclease maps. J. Gen. Virol. 64: 1811-1814.

Davison, A.J. 1983. DNA sequence of the U_S component of the varicella-zoster virus genome. EMBO J. 2: 2203-2209.

Davison A.J. 1984. Structure of the genome termini of varicella-zoster virus. J. Gen Virol. in press.

Delius, H. and J.B. Clements. 1976. A partial denaturation map of herpes simplex virus type I DNA: evidence for inversion of the unique DNA regions. J. Gen. Virol. 33: 125-133.

Denniston, K.J. , M.J. Madden, L.W. Enquist and G. Vande Woude. 1981. Characterization of coliphage lambda hybrids carrying DNA fragments from herpes simplex virus type I defective interfering particles. Gene 15: 365-378.

Denhardt, D.T. 1966. A menbrane-filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Commun. 23: 641-646.

Desrosiers, R.C. and L.A. Falk. 1982. Herpesvirus saimiri strain variability. J. Virol. 43: 352-356.

Dobersen, M.J., M. Jerkofsky and S. Greer. 1976. Enzymatic basis for the selective inhibition of varicella-zoster by 5-halogenated analogues of deoxycytidine. J. Virol. 20: 478-486.

Dolin, D., R.C. Reichman, M.H. Mazur and R.J. Whitley. 1978. Herpes zoster-varicella infections in immunosuppressed patients. Ann. Int. Med. 89: 375-288.

Dugaiczyk, A., H.W. Boyer and H.M. Goodman. 1975. Ligation of EcoRI endonuclease-generated DNA fragments into linear and circular structures. J. Mol. Biol. 96: 171-184.

Dumas, A.M., J.L.M.C. Geelen, W. Maris and J. van der Noordaa. 1980. Infectivity and molecular weight of varicella-zoster virus DNA. J. Gen. Virol. 47: 233-235.

Dumas, A.M., J.L.M.C. Geelen, M.W. Weststrate, P. Wertheim and J. van der Noordaa. 1981. XbaI, PstI and BglII restriction enzyme maps of the two orientations of the varicella-zoster virus genome. J. Virol. 39: 390-400.

Dynan, W.S. and R. Tjian. 1983. The promoter-specific transcription factor SP1 binds to upstream sequences in the SV40 early promoter. Cell 35: 79-87.

Ecker, J.R. and R.W. Hyman. 1981. Varicella-zoster virus vaccine DNA differs from the parental virus DNA. J. Virol. 40: 314-318.

Ecker, J.R. and R.W. Hyman. 1982. Varicella zoster virus DNA exists as two isomers. Proc. Natl Acad. Sci. U.S.A. 79: 156-160.

Ecker, J.R., L. Kudler and R.W. Hyman. 1984. Variation in the structure of varicella-zoster viurs DNA. Intervirol. 21: 25-37.

Esiri, M.M. and A.H. Tomlinson. 1972. Herpes zoster: Demonstration of virus in trigeminal nerve and ganglion by immunofluorescence and electron microscopy. J. Neurol. Sci. 15: 35-48.

Feldman, S., W.T. Huges and C.B. Daniel. 1975. Varicella in children with cancer: seventy-seven cases. Pediatr. 56: 388-397.

Feldman, S. and E. Epp. 1976. Isolation of varicella-zoster virus from blood. J. Pediatr. 88: 265-267.

Fenner, F. 1948. The pathogenesis of the acute exanthems: An interpretation based on experimental investigations with mouse pox (infectious ectromelia of mice). Lancet 2: 915-920.

Fiers, W., R. Contreras, G. Haegerman, R. Rogiers, A. van der Voorde, H. van Herverswyn, J. van Herreweghe, G. Volckaert and M. Ysebaert. 1978. Complete nucleotide sequence of SV40 DNA. Nature 273: 113-120

Frenkel, N., R.J. Jacob, R.W. Honess, G.S. Hayward, H. Locker and B. Roizman. 1975. Anatomy of herpes simplex virus DNA: Characterization of defective DNA molecules and biological properties of virus populations containing them. J. Virol. 16: 153-167.

Frenkel, N., H.Locker, W. Batterson, G.S. Hayward and B. Roizman. 1976. Anatomy of herpes simplex virus DNA: Defective DNA originates from the S component. J. Virol. 20: 527-531.

Frisque, R.J., G.L. Bream and M. T. Cannella. 1984. Human polyomavirus JC virus genome. J. Virol. 51: 458-469.

Fukuchi, K., M. Sudo, Y.S. Lee, A. Tanaka and M. Nonoyama. 1984. Structure of Marek's disease virus DNA: Detailed restriction enzyme map. J. Virol. 51: 102-109.

Garland, J. 1943. Varicella following exposure to herpes zoster. N. Engl. J. Med. 228: 336-337.

Gershon, A.A., R. Raker, S. Steinberg. B. Topf-Olstein and L.M. Drusin. 1976. Antibody to varicella-zoster virus in parturinet women and their offspring during the first year of life.. Pediatr. 58: 692-696.

Gershon, A.A. 1980. Live attenuated varicella-zoster vaccine. Rev. Inf. Dis. 2: 393-407.

Gershon, A.A., S.P. Steinberg, L. Gelb and the National Institute of Allergy and Infectious Diseases Collaborative Varicella Vacine Study Group. 1984. Clinical reinfection with varicella-zoster virus. J. Infect. Dis. 149: 137-142.

Gilden, D.H., Z. Wroblewska, V. Kindt, K.G. Warren and J.S. Wolinski. 1978. Varicella-zoster virus infection of huamn brain cells and ganglion cells in tissue culture. Arch. Virol. 56: 105-117.

Gilden, D.H., Y. Shram, A. Friedmann, M. Wellish M. Devlin, N. Fraser and Y. Becker. 1982. The internal organization of the varicella-zoster virus genome. J. Gen. Virol. 60: 371-374.

Gilden, D.H., A. Vafai, Y. Shtram, Y. Becker, M. Devlin and M. Wellish. 1983. Varicella-zoster virus DNA in human sensory ganglia. Nature 306: 478-480.

Gold, E. 1966. Serologic and virus-isolation studies of patients with varicella or herpes-zoster infections. New Engl. J. Med. 274: 181-185.

Gordon, J.E. and F.M. Meader. 1929. The period of infectivity and serum prevention of chickenpox. JAMA 93: 2013-2015.

Gordon, J.E. 1962. Chickenpox: an epidemiological review. Amer. J. Med. Sci. 244: 262-389.

Grafstrom, R.H., J.C. Alwine, W.L. Steinhart and C.W. Hill. 1974. Terminal repetitions in herpes simplex virus type 1 DNA. Cold Spring Harbor Sym. Quant. Biol. 39: 679-681.

Grinnel, B.W., B.L. Padgett and D.L. Walker. 1983. Comparison of infectious JC virus DNA's cloned from human brain. J. Virol. 45: 299-308.

Grose, C., D. Perrotta, P.A. Brunell and G.C. Smith. 1979. Cell-free varicella-zoster virus in cultured human melanoma cells. J. Gen. Virol. 43: 15-27.

Grose, C. 1980. The synthesis of glycoproteins in human melanoma cells infected with varicella-zoster virus. Virol. 101: 1-9.

Grose, C., B.J. Edmond and W.E. Friedrichs. 1981. Immunogenic glycoproteins of laboratory and vaccine strains of varicella zoster virus. Infect. Immun. 31: 1044-1053.

Grose, C. and W.E. Friedrichs. 1982. Immunoprecipitable polypeptides specified by varicella-zoster virus. Virol. 118: 86-95.

Grose, C., D.P. Edwards, W.E. Friedrichs, K.A. Weigle and W.L. McGuire. 1983. Monoclonal antibodies against three major glycoproproteins of varicella-zoster virus. Infect. Immun. 40: 381-388.

Grunstein, M. and D. Hogness. 1975. Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. U.S.A. 72: 3961-3965.

Guerineau, M., C. Grandchamp and P.P. Slonimski. 1976. Circular DNA of a yeast episome with two inverted repeats: Structural analysis by a restriction enzyme and electron microscopy. Proc. Natl. Acad. Sci. U.S.A. 73: 3030-3034.

Hackstadt, T. and L.P. Mallavia. 1978. Deoxypyrimidine nucleoside metabolism in varicella zoster virus infected cells. J. Virol. 25: 510-517.

Halperin, S.A., J.O. Hendley, C. Nosal and B. Roizman. 1980. DNA fingerprinting in investigation of apparent nosocomial acquisition of neonatal herpes simplex. J. Pediatr. 97: 91-93.

Hayward, G.S., N. Frenkel and B. Roizman. 1975a. Anatomy of herpes simplex virus DNA: Strain differences and heterogeneity in the locations of restriction endonuclease cleavage sites. Proc. Natl. Acad. Sci. U.S.A. 72: 1768-1772.

Hayward, G.S., R.J. Jacob, S.C. Wadsworth and B. Roizman. 1975b. Anatomy of HSV DNA: evidence for the four populations of molecules that differ in relative orientation of their long and short components. Proc. Natl. Acad. Sci. U.S.A. 72: 4243-4247.

Head, H. and A.W. Campbell. 1900. The pathology of herpes zoster and its bearing on sensory localization. Brain 23: 353-519.

Heller, M., T. Dambaugh and E. Kieff. 1981. Epstein-Barr virus DNA: Variations among viral DNAs. J. Virol. 38: 632-648.

Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. 26: 365-369.

Hondo, R., H. Shibuta and M. Matumoto. 1976. A improved plaque assay for varicella virus. Arch Virol. 51: 355-359.

Honess, R.W. and D.H. Watson. 1977. Unity and diversity in the herpesviruses. J. Gen. Virol. 37: 15-37.

Hope-Simpson, R.E. 1954. Studies on shingles: Is the virus ordinary chickenpox virus? Lancet ii: 1299-1392.

Hope-Simpson, R.E. 1965. The nature of herpes zoster: A long-term study and a new hypothesis. Proc. Royal Soc. Med. 58: 9-20.

Huang, E.S. and S.M. Huong. 1980. Cytomegalovirus: Genetic variation of viral genomes. Ann. N.Y. Acad. Sci. 354: 332-346.

Hudspeth, M.E.S., D.S. Shumard, C.J.R. Brandford and L.I.Grossman. 1983. Organization of Achlya mtDNA: A population with two orientations of a large inverted repeat containing the rRNA genes. Proc. Natl. Acad. Sci. U.S.A. 80: 142-148.

Hyman, R.W., J.R. Ecker and R.B. Tenser. 1983. Varicella-zoster virus RNA in human trigeminal ganglia. Lancet ii: 814-816.

Iltis, J.P., J.E. Oakes, R.W. Hyman and F. Rapp. 1977. Compairison of the DNAs of varicella-zoster viruses isolated from clinical cases of varicella and herpes zoster. Virol. 82: 345-352.

Jezek, Z., W. Hardjotanojo and A.G. Rangaraj. 1981 Facial scarring after varicella: A comparison with variola major and variola minor. Am. J. Epidermiol. 114: 798-903.

Juel-Jensen, B.E. and F.O. MacCallum. 1972. Herpes simplex, varicella and zoster: Clinical manifestations and treatment. pg. 79-97 J.B. Lippincott Co. Philadelphia.

Kaschka-Dierich, C., F.J. Werner, I. Bauer and B. Fleckenstein. 1982. Structure of nonintegrated circular Herpesvirus saimiri and Herpesvirus ateles genomes in tumor cell lines and in vitro transformed cells. J. Virol. 44: 295-310.

Kilpatrick, B.A., E.S. Huang and J.S. Pagano. 1976. Analysis of cytomegalovirus genomes with restriction endonucleaes HindIII and EcoRI. J. Virol. 18: 1095-1105.

Kundratitz, K. 1925. Über die ätiologie des zoster und über seine beziehungen zu varizellen. Wien. Klin. Wschr. 38: 502-503.

Langridge, J., P. Langridge and P.L. Bergquist. 1980. Extraction of nucleic acids from agarose gels. Anal. Biochem. 103: 264-271.

Leclair, J.M., J.A. Zaia, J.M. Levin, R.G. Congdon and D.A. Coldman. 1980. Airborne transmission of chickenpox in a hospital. N. Engl. J. Med. 302: 450-453.

Leventon-Kriss, S., R. Yoffa, L. Ronnan and M. Madan 1978. Seroepidemologic aspects of varicella zoster virus infections in an Israeli Jewish population. Isr. J. Med. Sci. 14: 766-770.

Linneman, C.C. Jr., T.G. Buchman, I.J. Light J.L. Ballard and B. Roizman. 1978. Transmission of herpes simplex virus type I in a nursery for the newborn: Identification of viral isolated by DNA "fingerprinting". Lancet i 964-966.

Locker, H. and N. Frenkel. 1979. BamHI, KpnI and SalI restriction enzyme maps of the DNAs of herpes simplex virus strain Justin and F : Occurrence of heterogeneities in defined regions of the viral DNA. J. Virol. 32: 429-441.

Lominiczi, B., M.L. Blankenship and T. Ben-Porat. 1984. Deletions in the genome of pseudorabies virus vaccine strains and existence of four isomers of the genome. J. Virol. 49: 970-979.

Lonsdale, D.M., S.M. Brown, J.H. Subak-Sharpe, K.P. Warren and H. Koprowski. 1979. The polypeptide and DNA restriction enzyme profiles of spontaneous isolates of herpes simplex type I from explants of human trigeminal, superior, cervical and vagus ganglia. J. Gen. Virol. 43: 151-171.

Lonsdale, D.M., S.M. Brown, J. Lang and J.H. Subak-Sharp. 1980. Variations in herpes simplex isolated from human ganglia and a study of clonal variation in HSV 1. Ann. N.Y. Acad. Sci. 354: 291-308.

Ludwig, H., H.G. Haines, N. Biswal and M Benyesh-Melnick. 1972. The characterization of varicella-zoster virus DNA. J Gen. Virol. 14:111-114.

Maniatis, T., E.F. Fritsch and J. Sambrook. 1982. Molecular Cloning, A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Mar, E.C., Y.S. Huang and E.S. Huang. 1978. Purification and characterization of varicella-zoster virus induced DNA polymerase. J. Virol. 26: 249-256.

Martin, J.D., B.L. Padgett and D.L. Walker. 1983. Characterization of tissuce culture-induced heterogeneity in DNA's of independent isolates of JC virus. J. Gen. Virol. 64: 2271-2280.

Martin, J.D. and G.C. Foster. 1984. Multiple JC virus genomes from one patient. J. Gen. Virol. 65: 1405-1411.

Martin, J.H., D.E. Dohner, W.J. Wellinghoff and L.D. Gelb. 1982. Restriction endonuclease analysis of varicella-zoster vaccine virus and wild-type DNA. J. Med. Virol. 9: 69-76.

Maxam, A.M. and W. Gilbert. 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. U.S.A. 74: 560-564.

Maxam, A.M. and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Meth. Enzymol. 65: 499-560. L. Grossman and K. Moldave (ed).

McCutchan, T. and M.F. Singer. 1981. DNA sequences similar to those around the simian virus 40 origin of replication are present in the monkey genome. Proc. Natl. Acad. Sci. U.S.A. 78: 95-99.

McFadden, G. and S. Dales. 1979. Biogenesis of poxviruses: mirror-image deletions in vaccinia virus DNA. Cell 18: 101-108.

Meurisse, E.V. 1969. Laboratory studies on the varicella-zoster virus.. J. Med. Microbiol. 2: 317-325.

Meyers, M.G., H.L. Duer and C.K. Hausler. 1980. Experimental infection of guinea pigs with varicella-zoster virus. J. Infect. Dis. 142: 414-420.

Miller, L.H. and P.A. Brunell. 1970. Zoster, reinfection or activation of a latent virus? Am. J. Med. 49:480-483.

Mishra, L., D.E. Dohner, W.J. Wellinghoff and L.D. Gelb. 1984. Physical maps of varicella-zoster virus DNA derived with 11 restriction enzymes. J. Virol. 50: 615-618.

Mocarski, E.S., L.E. Post and B. Roizman. 1980. Molecular engineering of the herpes simplex virus genome: Insertion of a second L/S junction into the genome causes additional genome inversions. Cell 22: 243-255.

Mocarski, E.S. and B. Roizman. 1981. Site-specific inversion sequence of the herpes simplex virus genome: Domain and structural features. Proc. Natl. Acad. Sci. U.S.A. 78: 7047-7051.

Mocarski, E.S. and B. Roizman. 1982. Herpesvirus-dependent amplification and inversion of cell-associared viral thymidine kinase gene flanked by viral "a" sequences and linked to an origin of viral DNA replication. Proc. Natl. Acad. Sci. U.S.A. 79: 5626-5630.

Mulder, C., J.R. Arrand, H. Delius, W. Keiler, V. Pattersson, R.J. Roberts and P.A. Sharp. 1974. Cleavage maps of DNA from adenovirus types 2 and 5 by restriction endonucleases EcoRI and HpaI. Cold Spring Harbor Symp. Quant. Biol. 39: 397-401.

Murchie, M.J. and D.J. McGeoch. 1982. DNA sequence analysis of an immediate-early gene region of the herpes simplex virus type I genome (map coordinates 0.950 to 0.978) J. Gen. Virol. 62: 1-15.

Nelson, A.M. and J.W. St. Geme. 1966. On the respiratory spread of varicella-zoster virus. Pediatr. 37: 1007-1009.

Nevins, J.R. 1981. Mechanism of activation of early viral transcription by the adenovirus E1A gene product. Cell 26: 213.

Oakes, J.E., J.P. Iltis, R.W. Hyman and F. Rapp. 1977. Analysis by restriction enzyme cleavage of human varicella-zoster virus DNAs. Virol. 82: 353-361.

Ogino, T., T. Otsuka and M. Takahashi. 1977. Induction of deoxypyrimidine kinase activity in human embryonic lung cells infected with varicella-zoster virus. J. Virol. 21: 1232-1235.

Okuno, T., K. Yamanishi, K. Shiraki and M Takahashi. 1983. Synthesis and processing of glycoproteins of varicella-zoster virus (VZV) as studied with monoclonal antibodies to VZV antigens. Virol. 129: 357-368.

Overall, J.C. and L.A. Glasgow. 1970. Virus infections of the fetus and newborn infant. Fetal Neonatal Med. 77: 315-333.

Peden, K., P. Mounts and G.S. Hayward. 1982. Homology between mamalian cell DNA sequences and human herpesvirus genomes detected by a hybridization procedure with high-complexity probe. Cell 31: 71-80.

Plotkin, S.A., S. Stein, M. Snyder and P. Immesoete. 1977. Attempts to recover varicella virus from ganglia. Ann. Neurol. 2: 249.

Plummer, G., C.R. Goodheart, D. Henson and C.P. Bowling. 1969. A comparative study of the DNA density and behavior in tissue cultures of fourteen different herpesviruses. Virol. 39: 134-139.

Preblud, S. and L. D'Angelo. 1979. Chickenpox in the United States. 1972-1977. J. Infect. Dis. 140: 257-260.

Preblud, S.R. 1981. Age-specific risks of varicella complications. Pediatr. 68: 14-17.

Puga, A. E.M. Cantin and A.L. Notkins. 1982. Homology between murine and human cellular DNA sequences and the terminal repetitions of the S component of herpes simplex type 1 DNA. Cell 31: 81-87.

van de Putte, P., S. Cramer and M. Giphart-Gassler. 1980. Invertible DNA determines hose specificty of bacteriophage mu. Nature 286: 218-222.

Queen, C., S.T. Lord, T.F. McCutchan and M.F. Singer. 1981. Three segments from the monkey genome that hybridize to simian virus 40 have common structural elements. Mol. Cell. Biol. 1: 1061-1068.

Raab-Traub, N., R. Pritchett and E. Kieff. 1978. DNA of Epstein-Barr virus: Identification of restriction enzyme fragments which contain DNA sequences that differ among strains of Epstein-Barr virus. J. Virol. 27: 388-398.

Rapp, F., J.P. Iltis, J.E. Oakes and R.W. Hyman. 1977. A novel approach to study the DNA of herpes zoster virus. Intervirol. 8: 272-280.

Richards, J.C., R.W. Hyman and F. Rapp. 1979. Analysis of the DNAs from seven varicella-zoster isolates. J. Virol. 32: 812-822.

Rifkind, D. 1966. The activation of varicella-zoster virus infections by immunosuppressive therapy. J. Lab. Clin. Med. 68: 463-474.

Rigby, P.W.J., M. Dieckmann, C. Rhodes and P Berg. 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. 113: 237-251.

Roberts, C.R. 1984. Characterization of the proteins in varicella-zoster virus infected cells. Ph.D. dissertation.

Roizman, B. 1979a. The structure and isomerization of herpes simplex genomes. Cell 16: 481-494.

Roizman, B. 1979b. The organization of the herpes simplex virus genome. Ann. Rev. Genet. 13: 25-57.

Roizman, B. and M. Tognon. 1982. Restriction enzyme analysis of herpesvirus DNA: Stability of restriction endonuclease patterns. Lancet i: 677.

Ross, A.H. 1962. Modification of chickenpox in family contacts by administration of gamma globulin. N. Engl. J. Med. 267: 369-376.

Rüger, R., G.W. Bornkamm and B. Fleckenstein. 1984. Human cytomegalovirus DNA sequences with homologies to the cellular genome. J. Gen. Virol. 65: 1351-1364.

Ruley, H.E. and M. Fried. 1983. Sequence repeats in a polyoma virus DNA region important for gene expression. J. Virol. 47: 233-237.

Ruyechan, W.T., S.A. Dauenhauer, D.J. O'Callaghan. 1982. Electron microscopic study of equine herpesvirus type 1 DNA. J. Virol. 42: 297-300.

Ruyechan, W.T., T.A. Casey, W. Reinhold, A.C. Weir, M. Wellman, S.E. Straus and J. Hay. 1984. Distribution of G+C rich regions in varicella-zoster virus DNA. J. Gen. Virol. in press.

Sakaoka, H., T. Aomori, I. Ozaki, S. Ishida and K. Fujinaga. 1984. Restriction endonuclease cleavage analysis of herpes simplex virus isolates obtained from three pairs of siblings. Infect. Immun. 43: 771-774.

Schmidt, N.J. and E.H. Lennette. 1976. Improved yeilds of cell-free varicella-zoster virus. Infect. Immun. 14: 709-715.

Schydlower, M., R.M. Lampe and C. Gross. 1984. Varicella and herpes zoster. N. Engl. J. Med. 311: 329.

Scott-Wilson, J. J. 1978. Why "Chicken" pox? Lancet i: 1152.

Seif, I., G. Khoury and R. Dhar. 1979. The genome of human papovavirus BKV. Cell 18: 963-977.

Sheldrick, P. and N. Berthelot. 1974. Inverted repetitions in the chromosome of herpes simplex virus. Cold Spring Harbor Symp. Quant. Biol. 39: 667-678.

Shemer, Y., S. Leventon-Kriss and I. Sarov. 1980. Isolation and polypeptide charaterization of varicella-zoster virus. Virol. 106: 133-140.

Shibuta, H., T. Ishikawa, R. Hondo, Y. Aoyama, K. Kurata, and M. Matumoto. 1974. Varicella virus isolation from spinal ganglion: Brief report. Arch. ges. Virusforsch. 45: 382-385.

Shiraki, K., T. Okuno, K. Yamanishi and M Takahashi. 1982. Polypeptides of varicella-zoster virus (VZV) and immunological relationship of VZV and herpes simplex virus (HSV). J. Gen. Virol. 61: 255-269.

Shiraki, K. and M. Takahashi. 1982. Virus particles and glycoproteins excreted from cultured cells infecred with varicella-zoster virus (VZV). J. Gen. Virol. 61: 271-275.

Singha, D.P. 1976. Chickenpox: a disease predomenintly affecting adults in rural West Bengal, India. Int. J. Epidemiol. 5: 367-374.

Skare, J., W.P. Summers and W.C. Summers. 1975. Structure and function of herpesvirus genome: Comparison of five HSV I and two HSV 2 strains by cleavage of their DNA with EcoRI restriction endonuclease. J. Virol. 15: 726-732.

Smiley, J., B. Fong and W.C. Leung. 1981. Construction of a double jointed herpes simplex virus DNA molecule: Inverted repeats are required for segment inversion and direct repeats promote deletions. Virol. 113: 345-362.

Smith, I.W., N.J. Maitland, J.F. Peutherer and D.H.H. Robertson. 1981. Restriction enzyme analysis of herpesvirus-2 DNA. Lancet ii: 1424.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98: 503-517.

Steele, R.W. 1982. Immunology of varicella-zoster virus. pp. 73-88. in Immunology of Human Infections Part II. A.J. Nahmias and R.J. O'Reilly (ed.) Plemum Press, London.

Stevely, W.S. 1977. Inverted repetition in the chromosome of pseudorabies virus. J. Virol. 22: 232-234.

Stow, N.D., J.H. Subak-Sharpe and N.M. Wilkie. 1978. Physical mapping of herpes simplex virus type 1 mutation by marker rescue. J. Virol. 28:182-192.

Straus, S.E., H.S. Aulakh, W.T. Ruyechan, J. Hay, T.A. Casey, G.F. VandeWoude, J. Owens and H.A. Smith. 1981. Structure of varicella-zoster virus DNA. J. Virol. 40: 516-525.

Straus, S.E., J. Owens, W.T. Ruyechan, H.E. Takiff, T.A. Casey, G.F. Vande Woude and J. Hay. 1982. Molecular cloning and physical mapping of varicella-zoster virus DNA. Proc. Natl.Acad. Sci. U.S.A. 79: 993-997.

Straus, S.E., J. Hay, H. Smith and J. Owens. 1983. Genome differences among varicella-zoster virus isolates. J. Gen. Virol. 64: 1031-1041.

Straus, S.E., W. Reinhold, H.A. Smith, W.T. Ruyechan, D.K. Henderson, R.M. Blaese and J. Hay. 1984. Endonuclease analysis of viral DNA's from varicella and subsequent zoster infections in the same patient. New Engl. J. Med. in press.

Takahashi, M., Y. Okuno, T. Otsuka, J. Osame, A. Takamizawa, T. Sasada amd T. Kubo. 1975. Development of a live attenuated varicella vaccine. Biken J. 18: 25-33.

Takahashi, M. 1983. Chickenpox virus. Adv. Virus Res. 28: 285-356.

Taylor-Robinson, D. and A.E. Caunt. 1972. Varicella virus. Virol. Monogr. 12: 4-88. Springer-Verlag. New York.

Thomas, M. and R.W. Davis. 1975. Studies on the cleavage of bacteriophage lambda DNA with EcoRI restriction endonuclease. J. Mol. Biol. 91: 315-328.

Thomson, F.H. and C.M. Aberd. 1916. The aerial conveyance of infection, with a note on the contact-infection of chicken-pox. Lancet i: 341-344.

Tyzzer, E.E. 1909. The histology of the skin lesions in varicella. Philipp. J. Sci. 1: 349-372.

Ullrich, A., J. Shine, J. Chirgwin, R. Pictet, E. Tischer, W.J. Rutter and H.M. Goodman. 1977. Rat insulin genes: Construction of plasmids containing the coding sequences. Science 196: 1313-1319.

Vaczi, L., L. Geder, M. Koller and E. Jeney. 1963. Influence of temperature on the multiplication of varicella virus. Acta. Microb. Hung. 10: 109-115.

Varmuza, S.L. and J.R. Smiley. 1984. Unstable heterozygosity in a diploid region of herpes simplex virus DNA. J. Virol. 49: 356-362.

Von Bokey, J. 1909. Uber den atiologischen zusammenhang der varizellen mit gewissenfallen von herpes zoster. Wien klin Wchschr. 22: 1323-1327.

Wadsworth, S., R.J. Jacob and B. Roizman. 1975. Anatomy of herpes simplex virus DNA: Size, composition and arrangment of inverted terminal repetitions. J. Virol. 15: 1487-1497.

Wahl, G.M., M. Stern and G.R. Stark. 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization using dextran sulfate. Proc. Natl. Acad. Sci. U.S.A. 76: 3683-3687.

Wagner, M.J. and W.C. Summers. 1978. Structure of the joint regions and the termini of the DNA of herpes simplex virus type 1. J. Virol. 27: 374-387.

Wathen, M.W. and E.C. Pirtle. 1984. Stability of pseudorabies virus genome after in vivo serial passage. J. Gen. Virol. 65: 1401-1404.

Watson, R.J., M. Sullivan and G.F. Vande Woude. 1981. Structures of two spliced herpes simplex virus type 1 immediate-early mRNA's which map at the junctions of the unique and reiterated regions of the virus DNA S component. J. Virol. 37: 431-444.

Weibel, R.E., B.J. Neff, B.J. Kuter, H.A. Guess, C.A. Rothenberger, A.J. Fitzgerald, K.A. Connor, A.A. McLean, M.R. Hilleman, E.B. Buynak and E.M. Sclnick. 1984. Live attenuated varicella virus vaccine: Efficacy trial in healthy children. N. Engl. J. Med. 310: 1909-1915.

Weller, T.H. 1953. Serial propagation in vitro of agents producing inclusion bodies derived from varicella and herpes zoster. Proc Soc. Exp. Biol. Med. 83: 340-346.

Weller, T.H. and A.H. Coons. 1954. Florescent antibody studies with agents of varicella and herpes propagated in vitro. Proc. Soc. Exp. Biol. 86: 789-794.

Weller, T.H., H.M. Witton and E.J. Bell. 1958. The etiologic agents of varicella and herpes zoster: isolation, propagation, and cultural characteristics in vitro. J. Exp. Med. 108: 843-868.

Weller, T.H. and H.M. Witton. 1958. Etiological agents of varicella and herpes zoster: Serologic studies with the viruses as propagated in vitro. J. Exp. Med. 108: 869-889.

Weller, T.H. 1983a. Varicella and herpes zoster: Changing concepts of the natural history, control, and importance of a not-so-benign virus. N. Engl. J. Med. 309: 1362-1368.

Weller, T.H. 1983b. Varicella and herpes zoster: Changing concepts of the natural history, control and importance of a not-so-benign virus. N. Engl. J. Med. 309: 1434-1440.

Westrate, M.W., J.L.M.C. Geelen and J. van der Noorda. 1980. Human cytomegalovirus DNA: physical maps for the restriction endonucleases BglII, HindIII and XbaI. J. Gen. Virol. 49: 1-21.

Whalley, J.M., G.R. Robertson and A.J. Davison. 1981. Analysis of the genome of equine herpesvirus type 1: Arrangement of the cleavage sites for restriction endomucleases EcoRI, BglII and BamHI. J. Gen. Virol. 57: 307-323.

Wildy, P., W.C. Russell and R.W. Horne. 1960. The morphology of herpes virus. Virol. 12: 204-222.

Wittek, R., H.K. Muller, A. Menna and R. Wyler. 1978. Length heterogeneity in the DNA of vaccinia virus is eliminated on cloning the virus. FEBS Letters 90: 41-46.

Wittek, R. and B. Moss. 1980. Tandem repeats within the inverted terminal repetition of vaccinia virus DNA. Cell 21: 277-284.

Wolff, M.H. 1978. The proteins of varicella-zoster virus. Med. Microbiol. Immunol. 166: 21-28.

Yang, R.C.A. and R. Wu. 1979. BK virus DNA: Complete nucleotide sequence of a human tumor virus. Science 206: 456-462.

Yun, T. and D. Vapnek. 1977. Electron microscopic analysis of bacteriophage P1, P1Cm and P7: Determination of genome sizes, sequences homology and location of antibiotic resistance determinants. Virol. 77: 376-385.

Zieg, J., M. Silverman, H. Helmen and M. Simon. 1977. Recombinational switch for gene expression. Science 196: 170-172.

Zweerink, H.J. and B.J. Neff. 1981. Immune response after exposure to varicella zoster virus: Characterization of virus-specific antibodies and their corresponding antigens. Infect. Immun. 31: 436-444.

Zweerink, H.J., D.H. Morton, L.W. Stanton and B.J. Neff. 1981. Restriction endonuclease analysis of the DNA from varicella-zoster virus: Stability of the DNA after passage in vitro. J. Gen. Virol. 55: 207-211.