

UNCLASSIFIED

AD NUMBER
ADB241963
NEW LIMITATION CHANGE
TO Approved for public release, distribution unlimited
FROM Distribution authorized to U.S. Gov't. agencies only; Proprietary Info.; Sep 98. Other requests shall be referred to US Army Medical Research and Materiel Comd., Fort Detrick, MD 21702-5012.
AUTHORITY
U.S. Army Medical Research and Materiel Command ltr. dtd July 23, 2001.

THIS PAGE IS UNCLASSIFIED

AD _____

GRANT NUMBER DAMD17-97-1-7163

TITLE: Catalytic Antibodies and DNA Site-Specific Recombination

PRINCIPAL INVESTIGATOR: E. Aubrey Thompson, Ph.D.

CONTRACTING ORGANIZATION: Baylor College
Houston, Texas 77030

REPORT DATE: September 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commanding General
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Distribution authorized to U.S. Government agencies only (proprietary information, Sep 98). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

NOTICE

USING GOVERNMENT DRAWINGS, SPECIFICATIONS, OR OTHER DATA INCLUDED IN THIS DOCUMENT FOR ANY PURPOSE OTHER THAN GOVERNMENT PROCUREMENT DOES NOT IN ANY WAY OBLIGATE THE U.S. GOVERNMENT. THE FACT THAT THE GOVERNMENT FORMULATED OR SUPPLIED THE DRAWINGS, SPECIFICATIONS, OR OTHER DATA DOES NOT LICENSE THE HOLDER OR ANY OTHER PERSON OR CORPORATION; OR CONVEY ANY RIGHTS OR PERMISSION TO MANUFACTURE, USE, OR SELL ANY PATENTED INVENTION THAT MAY RELATE TO THEM.

LIMITED RIGHTS LEGEND

Award Number: DAMD17-97-1-7163

Contractor: Baylor College

Location of Limited Rights Data (Pages): 1-25

Those portions of the technical data contained in this report marked as limited rights data shall not, without the written permission of the above contractor, be (a) released or disclosed outside the government, (b) used by the Government for manufacture or, in the case of computer software documentation, for preparing the same or similar computer software, or (c) used by a party other than the Government, except that the Government may release or disclose technical data to persons outside the Government, or permit the use of technical data by such persons, if (i) such release, disclosure, or use is necessary for emergency repair or overhaul or (ii) is a release or disclosure of technical data (other than detailed manufacturing or process data) to, or use of such data by, a foreign government that is in the interest of the Government and is required for evaluational or informational purposes, provided in either case that such release, disclosure or use is made subject to a prohibition that the person to whom the data is released or disclosed may not further use, release or disclose such data, and the contractor or subcontractor or subcontractor asserting the restriction is notified of such release, disclosure or use. This legend, together with the indications of the portions of this data which are subject to such limitations, shall be included on any reproduction hereof which includes any part of the portions subject to such limitations.

THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.

Patricia Mlodron

2/16/99

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.				
1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE September 1998		3. REPORT TYPE AND DATES COVERED Annual (1 Sep 97 - 31 Aug 98)
4. TITLE AND SUBTITLE Catalytic Antibodies and DNA Site-Specific Recombination			5. FUNDING NUMBERS DAMD17-97-1-7163	
6. AUTHOR(S) E. Aubrey Thompson, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Baylor College Houston, Texas 77030			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES			19990225188	
12a. DISTRIBUTION / AVAILABILITY STATEMENT Distribution authorized to U.S. Government agencies only (proprietary information, Sep 98). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) This is a report for the period from November, 1997 to September, 1998. The goal of this project is to develop catalytic antibodies that will allow efficient DNA transfer by promoting site-specific recombination. Such catalytic antibodies may provide a very powerful means to manipulate DNA transfer at defined sequences with obvious implications for gene therapy of breast cancer. In order to engineer enzymes that catalyze site-specific recombination, we have immunized mice and are continuing immunize mice with a synthetic Holliday structure. There are no positive clones identified so far, though we have screened over 1000 hybridoma clones. We have worked out the conditions for RT-PCR amplifying 6 pairs of light chains (out of 7) and all 9 pairs for heavy chains of antibody IgG ₁ Fab variable region. Soon we will be able to construct a Fab expression library to screen for the Fabs that resolve the Holliday structure. Successful completion of this project will provide a new technology of DNA transfer for both biomedical research and therapeutic purposes.				
14. SUBJECT TERMS Breast Cancer			15. NUMBER OF PAGES 26	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Limited	

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

N/A Where copyrighted material is quoted, permission has been obtained to use such material.

✓ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

✓ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

✓ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

✓ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

✓ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

✓ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Aubrey Thompson 24 Sept 98
PI - Signature Date

Table of Contents

	pages
Front Cover	1
Report Documentation Page	2
Foreword	3
Table of Contents	4
Introduction	5-6
Body	6-10
Conclusion	10
References	11-12
Appendices	13-25

INTRODUCTION

Gene therapy is an emerging chemotherapeutic approach to cancers, and breast cancer is a particularly attractive target in this regard (reviewed by Avalosse *et al.*, 1995, Mastrangelo *et al.*, 1996). Most gene transfer techniques result in low transfection efficiency and random insertion of the transgene into host genome. High efficiency gene transfer into defined genomic sites is critical for therapeutic purposes (see review by Yanez and Porter, 1998).

Ideal genetic therapy would involve the transfer of a functioning gene into a defined site on the genome in target cells. DNA site-specific recombination would allow insertion of a functional gene into a specified genomic locus, thereby placing the transgene under normal control. Conversely, sequence-specific deletion of a functional gene would provide a powerful way of studying its effect on cell processes. Site-specific recombination allows integration into and/or excision of a DNA fragment from specific sites (Gu *et al.*, 1994). Unfortunately, the application of this process is limited because we lack enzymes that catalyze site-specific recombination. The goal of this project is to generate antibodies that will catalyze site-specific recombination.

Antibodies have inherent structural specificity, which is advantageous for generation of nucleotide sequence-specific reagents. More recently, it has been recognized that the inherent variability in the secondary and tertiary structure of antigen-binding sites results in the generation of rare antibodies that catalyze bond rearrangement of the antigen. Catalytic antibodies were first described in 1986 (Tramontano *et al.*, 1986), and this new class of enzymes promises to be of considerable value to chemistry, biology and medicine (reviewed by Shokat and Schultz, 1990; Benkovic, 1992; Lerner, 1993; Tramontano, 1994; Schultz and Lerner, 1995). We propose to generate sequence specific antibodies that possess integrase activity and are able to catalyze recombination at defined nucleotide sequences. This pilot project aims to demonstrate the feasibility of creating such catalytic antibodies.

We have determined that the Holliday structure (Figure 1*) is antigenic by using a synthetic DNA Holliday structure to elicit immune response in mice. DNA Holliday structure is the intermediate of integrase-mediated site-specific recombination. Formation of the Holliday structure and its subsequent resolution into two strand-exchanged products is mediated by an integrase, which is a single polypeptide enzyme with no known cofactors. All integrases contain a highly conserved amino acid tetrad, Arg-His-Arg-Tyr (Abremski and Hoess, 1992). A typical integrase is the 50 kDa FLP protein, which catalyzes site-specific recombination of 2 μ m plasmid in *Saccharomyces cerevisiae* (Gates and Cox, 1988). A single protein mediates the resolution and formation of the Holliday structure. It is reasonable that an antibody that resolves the Holliday structure will also catalyze its formation, since the chemistry of formation is identical to that of resolution. Antibodies that catalyze the formation and resolution of the Holliday structure will thus be engineered integrases. These integrases will be able to catalyze site-specific recombination and provide very important new reagents for DNA transfer. The target sequence specificity will be derived from the antigen used to elicit the immune response.

We have designed a set of 4 oligonucleotides (Oligo1, 60 nucleotides (nt), Oligo2, 42nt, Oligo3, 53nt, and Oligo4, 71nt) (Figure 2). A Holliday structure (hereinafter called HS) can be annealed from these four oligonucleotides as shown in Figure 3. HS has four arms with different lengths (Figure 3). Five hybrids (HS, R, S, Y, and Z) are predicted from annealing of the 4

* All figures are attached to Appendices.

oligonucleotides (Figure 3). However, we routinely detected HS, Y and Z in the mixture of these oligonucleotides as shown in Figure 4. The resolution of HS will generate two alternative pairs of double-stranded DNAs (Figure 5), which is the basis of our integrase assay. HS can elicit immune response in mice as shown in Figure 6. The anti-serum contained antibodies that specifically bind to the cruciform center structure of HS as shown in Figure 7.

BODY

Our short-term goal is to test the hypothesis that catalytic antibodies can recombine DNA fragments *in vitro*, and to develop catalytic antibodies that can do so. Three technical objectives were proposed: 1) Produce hybridomas that secrete antibodies against the Holliday structure and screen for monoclonal antibodies that resolve the structure; 2) Construct an Fab expression library and screen for Fabs that resolve the Holliday structure; 3) Determine if these catalytic antibodies (Fabs) recombine DNAs of defined sequences *in vitro*. We are now pursuing the first two technical objectives as planned. We have not obtained a positive clone so far. As soon as we obtained a positive clone we will test it as stated in technical objective 3.

The following are the methods originally proposed with an additional section **Results and Discussion** describing what has been accomplished and the reason for changing the original plan.

Technical Objective 1: Production of Hybridomas and Screening of Monoclonal Antibodies That Resolve the Holliday Structure:

Serum from HS-immunized mice contains a mixed population of antibodies. Our initial goal is the preparation of specific monoclonal antibodies with the desired specificity. We will isolate and screen hybridomas to this end. We have designed a functional assay to screen for antibodies that resolve HS, as illustrated in Figure 5. The decision not to use ELISA is based on the use of HS both as the antigen and as the substrate for the screening assay. An antibody with enzymatic activity will rapidly dissociate from its product after resolution of HS. ELISA detects stable antibody-antigen complexes. Since we expect the antibody-product complex to be unstable, a catalytic antibody cannot be detected by ELISA. The catalytic activities of HS-specific antibodies will be determined by a two-step screening method as described below. The substrate used for the first step of screening is HS labeled with ^{32}P on the 5'-ends of the three shorter arms. Biotin will be affixed to the 5'-end of the longest arm, to allow flexibility to the substrate after it is bound to a solid surface. The biotin will be used as an anchor to immobilize the substrate onto a microtest plate covered with streptavidin. Supernatants from individual hybridomas will be incubated with the immobilized substrate. After a period of incubation, part of the reaction mixture from the individual supernatants will be counted with a liquid scintillation counter to detect the release of ^{32}P from the bound substrate. The release of ^{32}P indicates that the culture supernatant contains one of the following activities: 1) phosphatase; 2) exonuclease; 3) endonuclease; or 4) integrase. The reaction mixtures that contain released ^{32}P will be adjusted to 10% (v/v) of cold trichloroacetic acid (TCA) to precipitate macromolecule-bound ^{32}P (i.e. labeled DNA fragments). TCA does not precipitate ortho-phosphate or nucleotide monophosphates that would be released by phosphatases or exonucleases. The TCA-precipitated DNA will be filtered onto Whatman GF/C glass-fiber membranes. The membranes will be exposed to X-ray film. Release of TCA-precipitable ^{32}P from immobilized HS will indicate that the supernatant contains an endonuclease or an integrase. The supernatants from such

hybridomas will be subjected to a second round of screening.

The second step will be to determine whether the ^{32}P from TCA-precipitable DNA is due to resolution of HS. This substrate HS will be labeled by ^{32}P at the 5'-ends of all four arms and incubated with these selected hybridoma supernatants. The reaction products will be resolved by electrophoresis on native 7% polyacrylamide gels before autoradiography. If the products of the reaction are one of the two pairs of DNA fragments, 60 & 53bp, or 71 & 42bp, as illustrated in Figure 5, we will conclude that the supernatant contains an integrase-like enzyme.

Hybridoma supernatants contain antibodies as well as other secretable proteins. In order to determine whether the resolution of HS is due to the antibodies in the selected supernatants, we will deplete the antibodies using Protein G Sepharose beads. If depletion of antibodies from these supernatants diminishes the resolution of HS, we will conclude that the antibody resolves HS and is a potential integrase. Its ability to catalyze DNA recombination *in vitro* will be examined, as described in the objective 3. The detailed methodology is given below.

a. Hybridoma Production: Custom immunization of mice and generation of hybridomas will be contracted to ImmunoTech Laboratories, CTTC, Houston, Texas. Mice will be immunized and anti-serum will be tested as described in Figure 6 legend. After mice show a positive ELISA, their spleens will be removed to isolate splenic cells for production of hybridomas. The fusion experiments will be performed following their routine protocol.

Results and Discussion: Due to US Army Medical research and Material Command Animal Use Regulations, we do not immunize mice and generate hybridomas commercially. we perform these tasks by ourselves on the campus of Baylor College of Medicine. We have successfully made 2 fusions (Balb/c) mice) and screened over 1000 hybridomas. Two more fusions (Balb/c mice) are underway, and two more mice (MRL/lpr) are in the process of immunization. We also will use (NZW x BXSB) F1 mice, which spontaneously develop autoimmune diseases, for immunization.

b. Substrates: All oligonucleotides will be synthesized and purified as described in Figure 2 legend. HS will be annealed and purified as well. Biotin will be added at the 5'-end of Oligo1 during oligomer synthesis (Genosys Biotechnologies, The Woodlands, Texas). 5'-labeling of ^{32}P of oligonucleotides will be carried out as described in Figure 4 legend.

Results and Discussion: We used two sources of oligo providers. One is Genosys Biotechnologies, The Woodlands, Texas. The other is Gibco BRL Life technologies. The former does not have consistent qualities for the oligos requested for purification. So we also used the latter.

c. Primary Screening: The primary screening substrate, HS, is formed by annealing biotinylated Oligo1 and ^{32}P -labeled Oligo2, Oligo3 and Oligo4, as described in Figure 4 legend. The purification of this substrate is not necessary, because we measure the release of ^{32}P from bound substrate. Two structures, HS and Y (Figure 3), will be immobilized onto Streptavidin-coated plate because they have biotin affixed. Streptavidin will be coated on microtest plates by incubating with 5mg/ml streptavidin, 200 μl /well at 4°C overnight. The plates will be washed with PBST (PBS plus 0.05% Tween-20, v/v). Biotinylated ^{32}P -labeled HS (100 μl , 5×10^4 cpm/well) will be added for one hour at room temperature to immobilize the substrate. The wells will be washed twice with PBST to remove unbound substrate. Hybridoma supernatants (5 μl) will be mixed with 95 μl TSEP buffer (50mM Tris-HCl, pH7.4, 7mM spermidine, 5mM Na_2EDTA and 75mM KCl, Lange-Gustafson and Nash, 1984) and incubated with immobilized substrate in the wells at 25°C for one hour. After incubation, 5 μl of the reaction mixtures will be

counted in a liquid scintillation counter. The remaining reaction mixtures will be mixed with ice-cold TCA to a concentration of 10% (v/v) to precipitate released DNA. The TCA-precipitated DNA will be filtered through Whatman GF/C glass-fiber membrane, and these will be exposed to X-ray film for autoradiography. The control will be the substrate incubated with the supernatant of an irrelevant hybridoma.

Results and Discussion: The primary screening method was not reliable due to high fluctuation of background release of bound substrate. The release was in the range of 10-30% of total bound substrate. The high fluctuation made it very difficult to interpret the data. Therefore, we skip the first step and screen every clone by electrophoresis as described in *d. Determination of Catalytic Activity* without removal of proteins in the samples. In order to reduce the amount of work and still obtain reliable results, we adapted a screening method described by Israel (1993). The hybridoma supernatants from 4 wells across a row or 4 wells down a column in a 96-well dish are pooled. The matrix of 16 is reduced to 8 reactions. A positive pool of an across row and a positive pool of a down column identifies a positive hybridoma clone as diagramed in Figure 8. We have not obtained a positive clone so far after screening over 1000 hybridoma from the two mice. Figure 9 shows one example of such screening.

d. Determination of Catalytic Activity: Supernatants (1-2 μ l) which release macromolecule-bound 32 P will be incubated with gel-purified substrate HS (5×10^3 cpm), labeled with 32 P at the 5'-ends of all four arms, in TSEP buffer at 25°C for various time periods in a final volume of 30 μ l. As a control, we will use an irrelevant hybridoma supernatant. After incubation, sodium dodecyl sulfate (SDS, 10%, w/v) and proteinase K (10mg/ml) will be added to final concentrations of 0.8% SDS and 500 μ g/ml of proteinase K. This reaction will be carried out at 37°C for an additional 20 minutes before extraction with phenol/chloroform (1:1, v/v, phenol: TE-saturated phenol). The supernatants are then mixed with one fifth volume of loading buffer (Figure 4 legend) and resolved by electrophoresis on 7% native polyacrylamide gels (1 X TBE). The gels will be dried and exposed to X-ray film. If the supernatant contains an integrase, there should be two distinct bands in addition to the substrate HS, as illustrated in Figure 5. These bands will be purified, and their sizes confirmed, on a DNA sequencing gel (Ausubel *et al.* 1987b). The size of each product will be compared with 32 P-labeled standard molecular size markers. If the bands have the defined sizes of 60 & 53bp, or 71 & 42bp, the supernatants contain integrase-like activities.

Results and Discussion: Since no positive clones have been identified at this time, we have not performed this experiment.

e. Depletion of Antibodies from Supernatants: These selected supernatants will be incubated individually with Protein G Sepharose beads at room temperature for one hour to deplete antibodies. After incubation, the beads will be sedimented. The resulting supernatants will be tested for their ability to resolve HS as described in *c*.

Results and Discussion: Since no positive clones have been identified at this time, we have not performed this experiment.

Technical Objective 2: Construction of an Fab Expression Library and Screening of Monoclonal Fabs That Resolve the Holliday Structure:

Most of the reported catalytic antibodies are monoclonal antibodies. In practice, however, isolating hybridomas that secrete antibodies with the desired catalytic activities is often a difficult and laborious job. If the efficiency of fusion of splenic cells and myeloma cells is low,

one may fail to recover the cells that secrete the desired catalytic antibodies. Beyond that, the labor-intensive screening process may take many months. Recent advances in molecular biology permits the construction of Fab expression libraries, which provide an alternative approach to isolation of mono-specific antibodies.

Theoretically, an entire antibody repertoire can be expressed by an Fab expression library. This approach allows Fab cDNAs to be cloned and expressed in bacteria. The process is rapid and may be completed in a relatively short period of weeks to months. IgG Fab cDNAs are DNA copies from entire light-chain (κ) and one-third of the N-terminal heavy-chain (Fd). The composition of the Fab expression library may not necessarily represent the antibody repertoire that was secreted by the cell population from which the genes were obtained. Rather, the library is randomly assembled from a pool of IgG Fd and κ cDNAs. We will construct Fab expression libraries from spleen cells of HS-immunized mice. The libraries will express secreted Fabs using a bacteriophage T3 system (Stewart *et al.* 1995), and the culture supernatants from individual clones will be screened for the ability to resolve HS as described in the objective 1. We will work on both hybridoma production and Fab expression library at the same time. This will increase both the probability of isolating catalytic antibodies and the diversities of catalytic antibodies and/or Fabs that can be isolated. Following are the detailed methodology:

a. Construction of Combinatorial Repertoire: Fab expression libraries derived from the spleens of HS immunized mice will be constructed essentially following the methods described by Stewart *et al.* (1995). The construction is illustrated in Figure 10.

Results and Discussion: We are in the process of defining conditions for RT-PCR amplification of antibody variable regions. We have successfully amplified 6 pairs of light chains (out of 7) and all 9 pairs for heavy chains of Fab fragments (Figure 11). Soon we will be able to construct a Fab expression library to screen for the Fabs that resolve the Holliday structure.

b. Screening: The individual cloned bacterial culture medium will be screened for their activities of resolving HS as described in the objective 1. Bacterial culture supernatants usually contain detectable DNases. However, the assay buffer can eliminate DNase activity because the buffer contains high concentration of EDTA and is Mg^{+2} -free. Known integrases do not require Mg^{+2} as a cofactor, so presumably catalytic antibodies/Fabs with integrase activity will also not require Mg^{+2} .

Results and Discussion: Since no Fab library has been made at this time, we have not performed this experiment.

Technical Objective 3: Site-Specific Recombination *in vitro*:

The goal of this project is to test the hypothesis that catalytic antibodies can recombine DNA fragments *in vitro* and to develop catalytic antibodies that can do so. The ability of selected catalytic antibodies (and Fabs) to catalyze recombination of two DNA duplexes of defined sequences will be tested *in vitro*. If the selected catalytic antibodies (or Fabs) are integrases, these antibodies (or Fabs) should be able to catalyze a full circle of integrase-mediated DNA site-specific recombination.

The catalytic antibodies and/or Fabs will be purified first. The selected catalytic antibodies will be purified by protein G chromatography. Fabs will be purified by polyaspartic acid cation-exchange chromatography.

Two groups of substrates will be used to test this hypothesis. One group is the pair of 60 & 53bp (Oligo5, 60nt, Oligo6, 60nt, Oligo7, 53nt, and Oligo8, 53nt, Figure 12); the other is 71 & 42bp

pair (Oligo9, 42nt, Oligo10, 42nt, Oligo11, 71nt, and Oligo12, 71nt, Figure 13). These DNAs are the products of two alternative HS resolutions. One pair of the substrates (i.e. 60 & 53bp) can undergo reciprocal strand exchange to form HS, which can then be resolved into the other pair (i.e. 71 & 42bp), or *vice versa* (Figure 14). (Note: Self-recombined products will not be detected by gel electrophoresis because they will be resolved into the same products.) The completion of this objective will demonstrate the feasibility of our long-term goals and provide the basis for a more ambitious future program to exploit catalytic antibodies for gene therapy.

a. Purification of Catalytic Antibodies: Catalytic antibodies will be purified from hybridoma supernatants by protein G Sepharose chromatography (Dean, 1992). The purity of isolated antibodies will be determined by SDS polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with silver or Coomassie brilliant blue. The antibody concentration will be determined by measuring A_{280} ($1 \text{ OD}_{280} = 0.8 \text{ mg/ml}$, Harlow and Lane, 1988b) or by the Bradford method in reference to BSA as described (Ausubel *et al.* 1987c).

Results and Discussion: Since no positive clones have been identified at this time, we have not performed this experiment.

b. Purification of Fabs: Catalytic Fabs will be purified from large *E.coli* cultures as described by Stewart *et al.* (1995). Briefly, culture supernatants will be collected by centrifugation and concentrated by ultrafiltration through a tangential flow apparatus. The concentrated material will be dialyzed against 20mM sodium MOPS, 0.4mM Ca acetate, pH6.3. Then the sample will be loaded onto a polyaspartic acid cation-exchange column; Fab fragments will be eluted with a linear gradient of Ca acetate from 1mM to 25mM and pH from 6.3 to 7.5, buffered with 40mM MOPS. Fab-containing fractions will be identified by SDS-PAGE, combined and concentrated by ultrafiltration. The final sample will be dialyzed against 100mM sodium HEPES, 50mM NaCl. The Fab concentration will be measured by the Bradford method.

Results and Discussion: Since no positive clones have been identified at this time, we have not performed this experiment.

c. Recombination in vitro: The substrate duplex DNAs (60bp and 53bp, or 71bp and 42bp) will be annealed from appropriate oligonucleotides and labeled with ^{32}P as described in Figure 4 legend. Recombination reactions will be performed as described in the objective 1 and analyzed by their sizes in reference to molecular size standards.

Results and Discussion: Since no positive clones have been identified at this time, we have not performed this experiment.

CONCLUSION

This is a pilot project to test the feasibility of generating antibodies that can recombine DNA fragments *in vitro*. Successful completion of these objectives will confirm the working hypothesis, and provide the basis for a more ambitious program to exploit catalytic antibodies for gene therapy. Our primary goal in this project is to create such antibodies, though we have not obtained such antibodies so far. We are now immunizing MRL/*lpr* mice and future (NZW x BXSB) F1 mice to obtain such antibodies. We will also pursue the objective 2.

REFERENCES

- Abremski, K.E. and R.H. Hoess (1992) Evidence for a Second Conserved Arginine Residue in the Integrase Family of Recombination Proteins. *Protein Eng.* 5:87-91;
- Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl (1987a) Phosphatases and Kinases. *in* *Current Protocols in Molecular Biology*, edited by Wiley Interscience, pp3.10.3-3.10.5;
- Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl (1987b) Denaturing Gel Electrophoresis for Sequencing. *in* *Current Protocols in Molecular Biology*, edited by Wiley Interscience, pp7.6.1-7.6.11;
- Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl (1987c) Quantitation of Proteins. *in* *Current Protocols in Molecular Biology*, edited by Wiley Interscience, pp10.1.1-10.1.3;
- Avalosse, B., F. Dupont and A. Burny (1995) Gene Therapy for Cancer. *Curr. Opinion in Oncol.* 7:94-100;
- Benkovic, S.J. (1992) Catalytic Antibodies. *Annu. Rev. Biochem.* 61:29-54;
- Bernard, O, S. Cory, S. Gerondakis, E. Webb and J.M. Adams (1983) Sequence of the Murine and Human Cellular *myc* Oncogenes and Two Modes of *myc* Transcription Resulting from Chromosome Translocation in B Lymphoid Tumors. *The EMBO J.* 2:2375-2383;
- Dean, J.C. (1992) *in* *Methods in Molecular Biology*, Vol. 10, Immunological Protocols, edited by M. Manson, The Humana Press, Inc., pp43-63;
- Duckett, D.R., A.I.H. Murchie, S. Diekmann, E. von Kitzig, B. Kemper and D.M.J. Lilley (1988) The Structure of the Holliday Junction, and Its Resolution. *Cell* 55:79-89;
- Gates, C.A. and M.M. Cox (1988) FLP Recombinase Is an Enzyme. *Proc. Natl. Acad. Sci. USA* 85:4628-4632;
- Gu, H., J.D. Marth, P.C. Orban, H. Mossmann and K. Rajewsky (1994) Deletion of a DNA Polymerase β Gene Segment in T Cells Using Cell Type-Specific Gene Targeting. *Science* 265:103-106;
- Harlow, E. and D. Lane (1988) Chapter 8: Storing and Purifying Antibodies. *In* *Antibodies: A Laboratory Manual*. Cold Spring Harbor Lab. Press, Plainview, NY. P311;
- Holliday, R. (1964) A Mechanism for Gene Conversion in Fungi. *Genet. Res.* 5:282-304;
- Huse, W.D., L. Sastry, S.A. Iverson, A.S. Kang, M. Altling-Mees, D.R. Burton, S.J. Benkovic and R.A. Lerner (1989) Generation of a Large Combinatorial Library of the Immunoglobulin Repertoire in Phage Lambda. *Science* 246:1275-1281;
- Isreal, D.I. (1993) A PCR-Based Method for High Stringency Screening of DNA Libraries. *Nucleic Acids Res.* 21:2627-2631;
- von Kitzing, E., D.M.J. Lilley, and S. Diekmann (1990) The Stereochemistry of a Four-Way DNA Junction: A Theoretical Study. *Nucleic Acid Res.* 18:2671-2683;
- Lerner, R.A. (1993) Catalytic Antibodies: The Concept and the Promise. *Hospital Practice* 28:53-59;
- Mastrangelo, M. J., D. Berd, F. E. Nathan and E. C. Lattime (1996) Gene Therapy for Human Cancer: An Essay for Clinicians. *Seminars in Oncol.* 23:4-21;
- Sambrook, J., E.F. Fritsch and T. Maniatis (1989) Recovery of Synthetic Oligonucleotides by Electrophoresis Through a Denaturing Polyacrylamide Gel. *in* *Molecular Cloning, a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring

Harbor, pp11.23-11.28;

Schultz, P.G. and R.A. Lerner (1995) From Molecular Diversity to Catalysis: Lessons from the Immune System. *Science* 269:1835-1842;

Shokat, K.M. and P.G. Schultz (1990) Catalytic Antibodies. *Annu. Rev. Immunol.* 8:335-363;

Stewart, J.D., I. Lee, B.A. Posner and S.J. Benkovic (1995) Expression of Properly Folded Catalytic Antibodies in *Escherichia coli*. in *Methods in Enzymology*. by Academic Press, Inc. Vol. 249, pp507-519;

Tramontano, A., K.D. Janda and R.A. Lerner (1986) Catalytic Antibodies. *Science* 234:1566-1570;

Tramontano, A (1994) Immune recognition, Antigen Design, and Catalytic Antibody Production. *Applied Biochem. Biotech.* 47:257-275;

Yanez, R.J. and A.C. Porter (1998) Therapeutic Gene Targeting. *Gene Therapy* 5:149-159;

APPENDICES

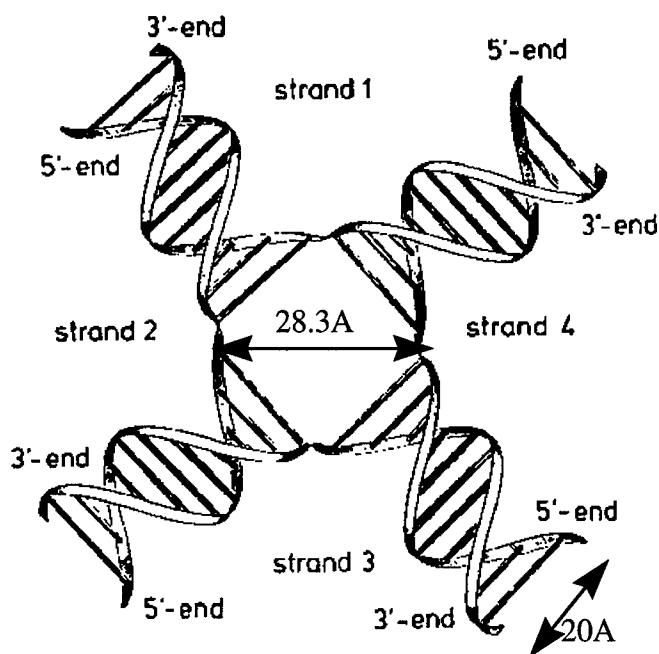


Figure 1. A ribbon model for the extended Holliday structure (von Kitzing *et al.*, 1990). The central base pairs at the junction do not stack. In each case the major groove of the base pair at the junction is pointing towards the viewer. The structure has a central size of 28.3 X. This is an X-shaped molecule that is formed from four DNA strands derived from two double-stranded DNAs. The structure is connected by a covalently closed joint (Holliday, 1964). The Holliday structure is formed by nicking and religation with the reciprocal exchange of single strands between two DNA duplexes. The structure is resolved by a second cycle of nicking and religation to form two new DNA duplexes. The formation and resolution of the Holliday structure are the processes required for integrase-catalyzed site-specific recombination.

A.

Oligo1 (60nt): 5'-CGCTACAGGAGTTACAGCCTAGTAATCACACGTCCTGCCTCGAGAAGGG
CGTACATGCTC-3'

Oligo2 (42nt): 5'-GAGCATGTACGCCCTTCTCGAGGCAGGACATCGACTAGTAGC-3'

Oligo3 (53nt): 5'-GCTACTAGTCGATGTCCTGCCTCGAGAAGGGCCATTGAACAGTCATGCT
GTCC-3'

Oligo4 (71nt): 5'-GGACAGCATGACTGTTCAATGGCCCTTCTCGAGGCAGGACGTGTGATTA
CTAGGCTGTAACCTCTGTAGCG-3'

B.

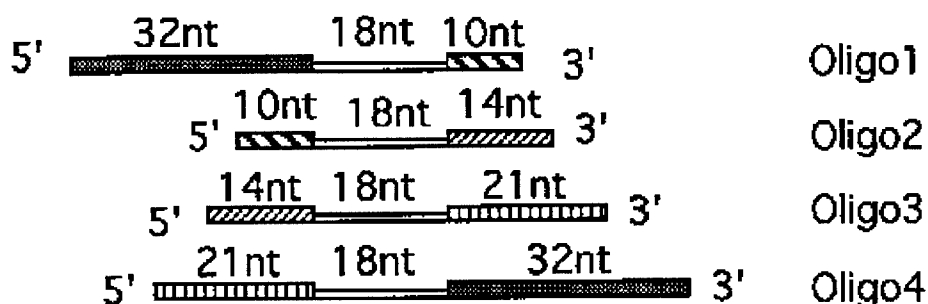


Figure 2. Oligonucleotide sequences of HS (A) and schematic diagrams of the oligonucleotides (B). All four oligonucleotide sequences have three important regions, an 18nt sequence at the center and two different lengths of flanking sequences. Bold face letters in A and open boxes in B show the 18nt central sequences. Filled boxes or cross-hatched represent the flanking sequences. The 18nt 5'-TCCTGCCTCGAGAAGGGC-3' sequences at the center of Oligo1 and Oligo3 are complementary to the central sequence 5'-GCCCTTCTCGAGGCAGGA-3' of Oligo2 and Oligo4. This sequence has a core palindromic sequence 5'-CTCGAG-3' (an *Xho*I site, underlined) and is from human *c-myc* (-102 to -84 base pairs (bp), relative to P2 promoter; Bernard *et al.*, 1983). The flanking sequences are designed to allow HS formation. The 32nt sequence at the 5'-end of Oligo1 is uniquely complementary to a 32nt sequence at the 3'-end of Oligo4. The 10nt at the 3'-end of Oligo1 are complementary to the 10nt at the 5'-end of Oligo2. The 14nt at the 3'-end of Oligo2 are complementary to the 14nt at the 5'-end of Oligo3. The 3'-end of Oligo3 (21nt) is complementary to the 21nt at the 5'-end of Oligo4. These oligonucleotides were synthesized by solid phase automated synthesis and purified from 8% polyacrylamide-8M urea gels by standard procedures (Sambrook *et al.* 1989).

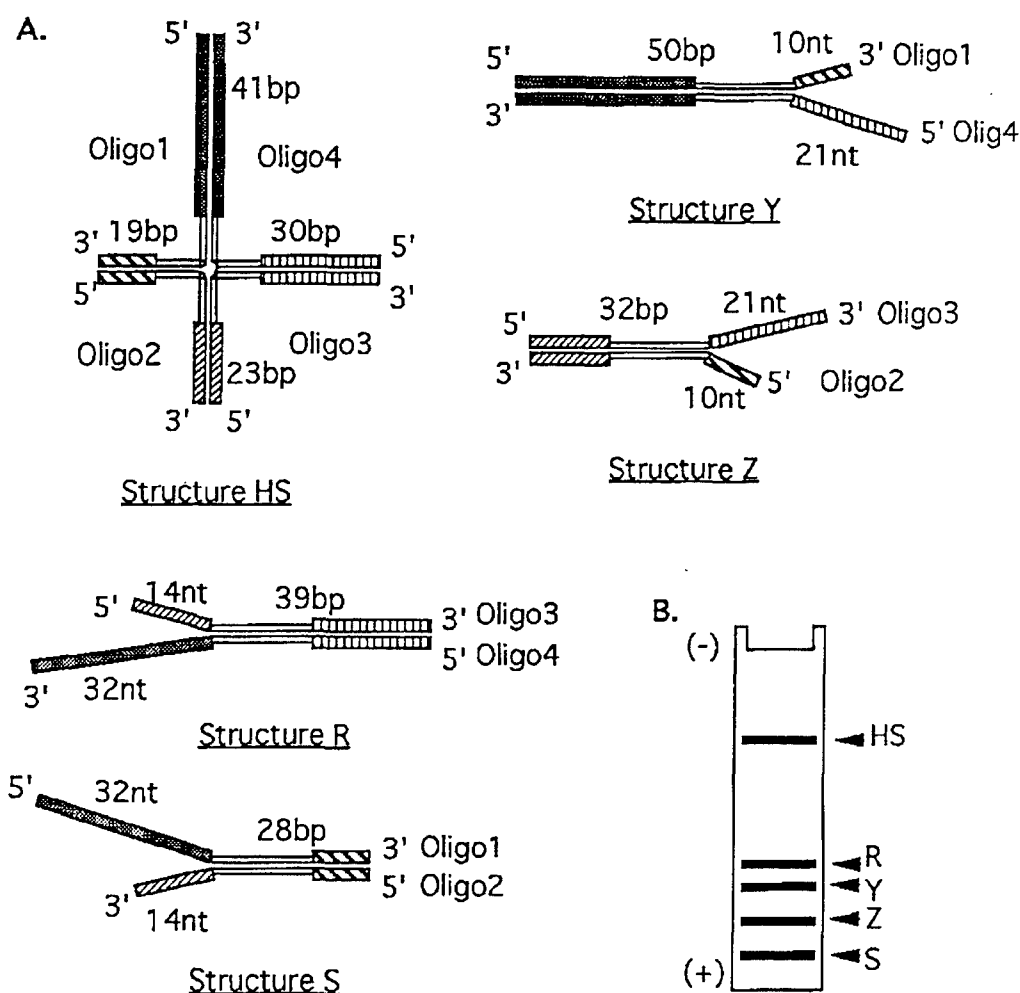


Figure 3. Schematic diagrams of predicted hybrids (A) and their relative mobilities (B). Five possible hybrids can form from a mixture of the four oligonucleotides. One will be HS. HS has four different lengths of arms (41, 30, 23 and 19bp). Other hybrids will be partial duplex DNAs annealed from the complementary sequences of Oligo3 and Oligo4, Oligo1 and Oligo2, Oligo1 and Oligo4, and Oligo2 and Oligo3. These structures are identified as R, S, Y and Z. R, S, Y and Z have different double-stranded portions of 39, 28, 50 and 32bp with two single-stranded tails, respectively. All of these hybrids have different sizes and degrees of double-stranded character, and all can be separated by their electrophoretic mobilities. HS is the largest molecule and will have the slowest mobility, as illustrated in the panel B on a native polyacrylamide gel. S is the smallest and will have the fastest mobility. Filled boxes or cross-hatched represent the flanking sequences. Open boxes represent the 18nt central sequences. The relative proportion of the hybrids differs in the hybridization mixture, because the hybrids have different T_m s. We routinely detect only three hybrids, HS, Y and Z, as will be shown in Figure 4.

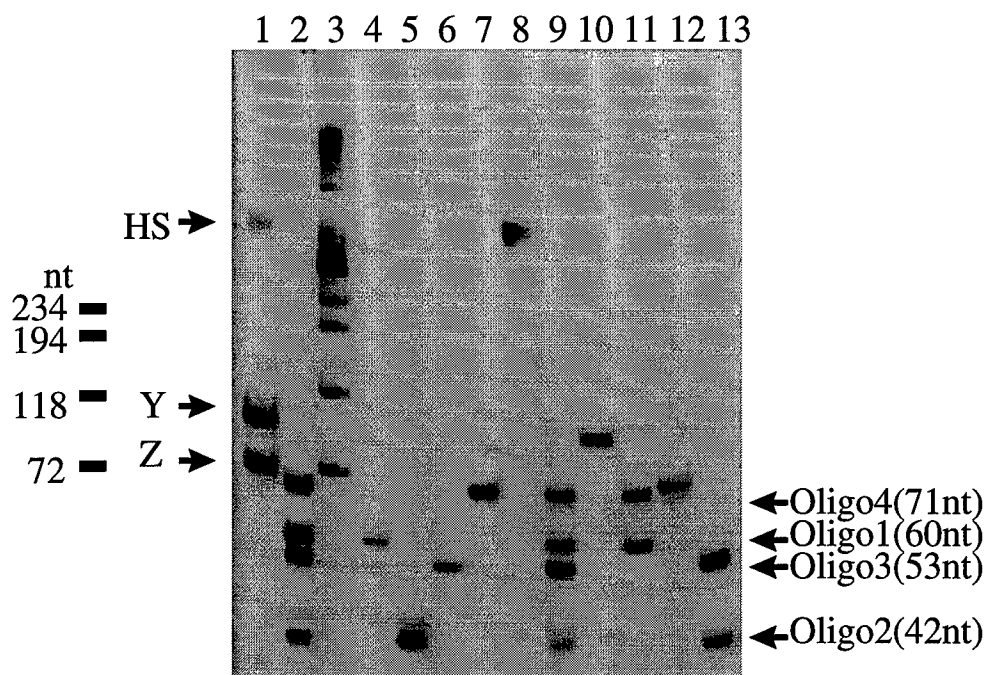


Figure 4. Formation and characterization of HS. Lane 3 is a set of ^{32}P -labeled single-stranded molecular size markers derived by denaturing *Hae*III digested bacteriophage phiX174 DNA. Lanes 4 through 7 contain ^{32}P -labeled oligonucleotides Oligo1 (60nt), Oligo2 (42nt), Oligo3 (53nt), and Oligo4 (71nt), respectively. The four ^{32}P -labeled oligonucleotides have different sizes and can be distinguished by their electrophoretic mobilities on the gel. The smallest one, Oligo2 (42nt), has the fastest mobility (lane 5); and the biggest one, Oligo4 (71nt) has the slowest (lane 7). Stoichiometric amounts of the four oligonucleotides were mixed in TE buffer (10mM Tris-HCl, pH8.0, 1mM Na_2EDTA) (Duckett *et al.*, 1988), denatured at 100°C for 5 minutes, incubated at 65°C for 2 hours and allowed to cool slowly to room temperature. The mixture is resolved into three major entities, HS, Y, and Z (lane 1). This mixture of HS, Y, and Z was heat-denatured in urea to show that the mixture contained the four individual oligonucleotides (lane 2). The hybrids were purified by eluting the corresponding bands from a native 5% preparative polyacrylamide gel, with recovery by ethanol precipitation. Lanes 8, 10 and 12 are purified hybrids HS, Y and Z, respectively. Lanes 9, 11 and 13 are the denatured hybrids, respectively. Since HS has the slowest electrophoretic mobility and dissociates into a stoichiometric mixture of all four labeled component oligonucleotides, Oligo1, Oligo2, Oligo3 and Oligo4, as shown in lane 9, we assumed that HS is a Holliday structure. The mixture, or the oligonucleotides or the marker were labeled by $\gamma\text{-}^{32}\text{P}$ -ATP at their 5'-ends by T4 polynucleotide kinase according to Ausubel *et al.* (1987a). The denaturation was carried out by heating the samples in 5M urea at 95°C for 3 minutes. Non-denatured samples were mixed with one fifth volume of loading buffer (50% [v/v] glycerol, 0.025% [w/v] xylene cyanol, and 0.025% [w/v] bromphenol blue). All the samples were then loaded on a 7% native polyacrylamide gel electrophoresed in 1 X TBE buffer (100mM Tris, 2mM Na_2EDTA , 100mM boric acid). After electrophoresis the gel was dried and exposed to X-ray film.

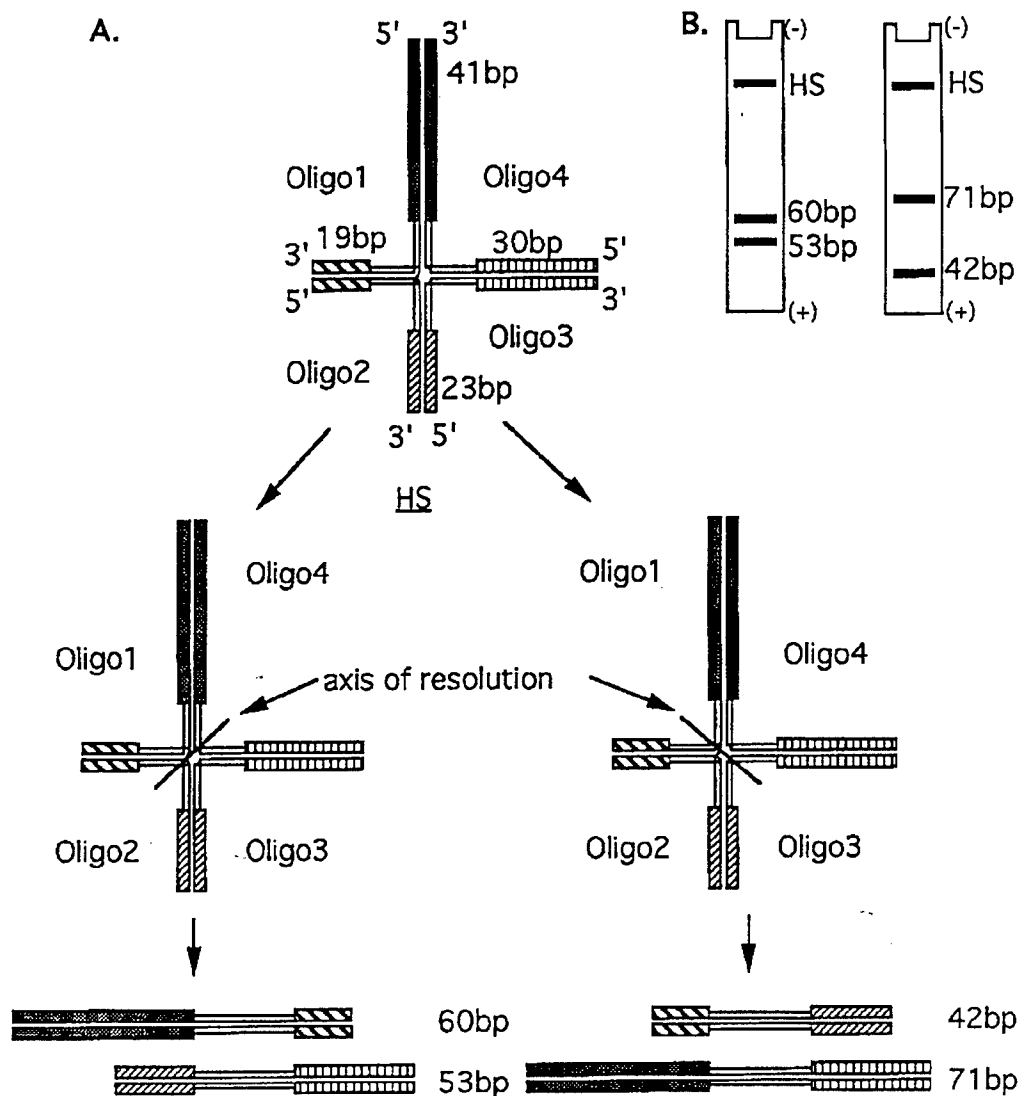


Figure 5. Schematic diagrams of HS resolution (A) and assay by gel electrophoresis (B). The resolution of HS will give rise to alternative pairs of fully double-stranded DNA products. One pair will be 60 & 53bp, the other 71 & 42bp. All these double-stranded DNA products and the hybrid HS differ in their electrophoretic mobilities under non-denaturing conditions. These mobility differences form the basis of our assay for resolution of HS. Filled or cross-hatched boxes represent the flanking sequences. Open boxes represent the 18nt central sequences (see Figure 2).

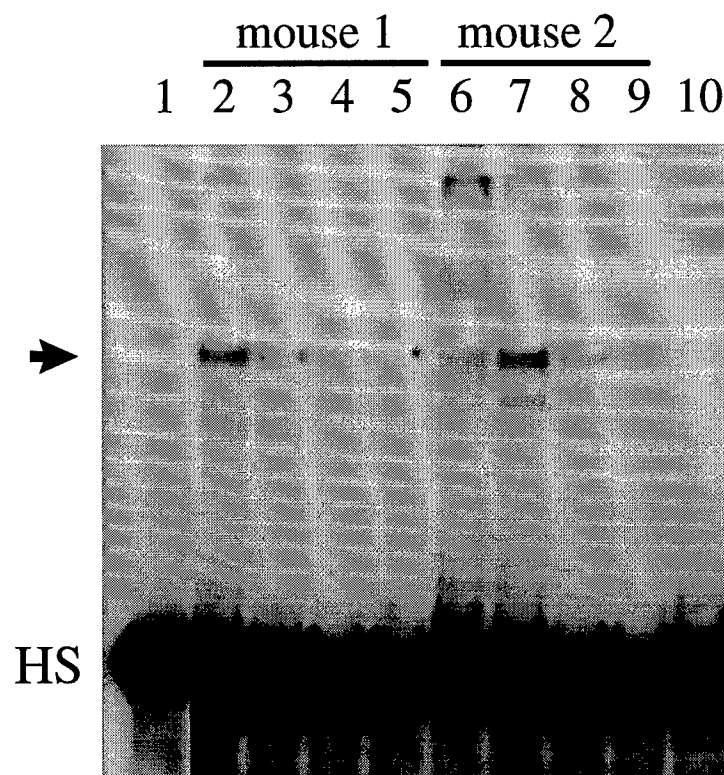


Figure 6. Formation of protein-HS complexes. The immunogen was a mixture (7.5 μ g) of HS, Y and Z (Figure 3) plus methylated thyroglobulin (5 μ g) in 10mM Tris-HCl (pH7.4), 1mM Na₂EDTA. Two female Balb/c mice were initially immunized with complete Freund's adjuvant, and incomplete Freund's adjuvant was used for subsequent boosts. The immunization was repeated on days 14, 28 and 42. Blood was collected on days 35 and 46, and the serum was tested for the presence of anti-HS antibodies by ELISA (Enzyme-linked Immunosorbent assay). After the mice showed positive ELISA results, an electrophoretic mobility shift assay was performed to characterize interaction between anti-HS antibodies and purified ³²P-labeled HS. All lanes contain an equal amount of ³²P-labeled HS and were incubated with: lane 1, buffer alone; lane 10, normal mouse serum (1:20); lanes 2 to 5, and 6 to 9 with serial dilutions (1:20, 1:40, 1:80 and 1:160) of anti-sera from mouse 1 and 2, respectively. Sera from immunized mice contain proteins that bind stably to HS (lanes 2 and 9). Normal mouse serum does not contain HS-binding protein, as shown in lane 10. Serum or anti-serum or PBS (phosphate buffered saline: 137mM NaCl, 2.7mM KCl, 4.3mM Na₂HPO₄, 1.4mM KH₂PO₄, pH7.2) diluted serum (0.5 μ l) was incubated with purified ³²P-labeled HS (2 X 10⁴ cpm, 10fmol) in the presence of 1 μ g polydI.dC in 10 μ l. Other components in this binding system were 66.6mM KCl, 13.3mM HEPES, pH7.9, 6.6mM MgCl₂, 13.3% glycerol (v/v), 0.13mM Na₂EDTA, 0.13mM Na₂EGTA, pH8.0, 3mM DTT (dithiothreitol), 2 μ g BSA (bovine serum albumin). The binding reaction was carried out for one hour at room temperature, and the samples then were electrophoresed in a low ionic strength (0.5 X TBE) 5% polyacrylamide. The gel was dried and exposed to X-ray film. The arrow indicates the protein-HS complexes.

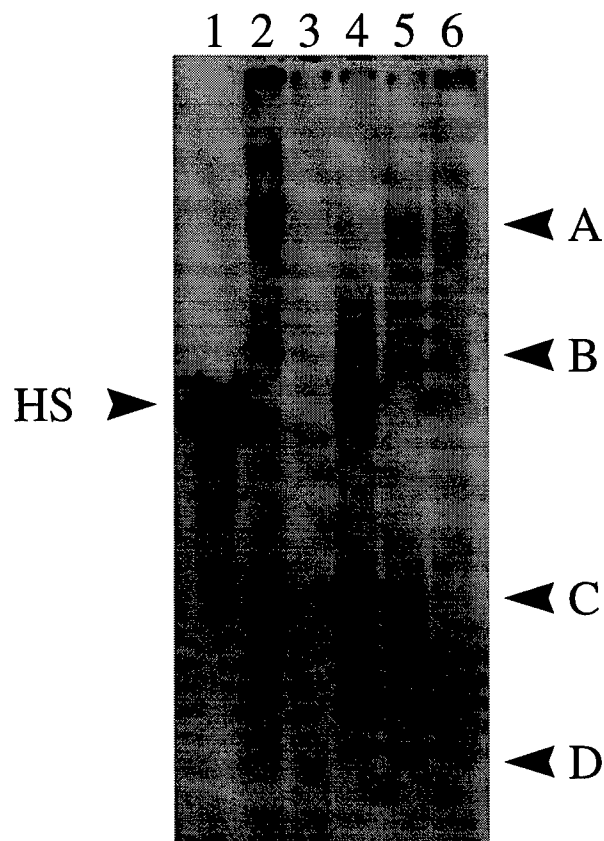


Figure 7. Binding specificity of antibodies to HS. The binding specificity of these proteins was tested, using serum from mouse 1. (**Note:** This serum was taken after one more boost to the mouse and so was different from the mouse 1 serum used in Figure 6, lane 2.). All lanes contain equal amount of purified ^{32}P -labeled HS and were incubated with: lane 1, buffer alone; lanes 2, mouse 1 anti-serum (1:20) only; lanes 3-6, both mouse 1 serum (1:20) and goat anti-mouse IgG, purified non-labeled HS (30x in molar excess), Y (100x) and Z (100x), respectively. In the presence of the anti-serum, ^{32}P -labeled HS was distributed into four major entities, designated A, B, C, and D (lane 2). Addition of goat anti-mouse IgG blocked the formation of A and B (lane 3). This blockage indicates that A and B are HS-antibody complexes. A could be displaced by addition of a 30-fold molar excess of non-labeled HS (lane 4). However, addition of a 100-fold molar excess of Y or Z had no effect (lane 5 and 6, respectively). The results indicate that the antibodies are saturable and bind specifically to the central structure of HS. The electrophoretic mobilities of C and D were unaltered by addition of any competitor. Since both C and D have greater electrophoretic mobilities than the probe (HS), we doubt that these are DNA-protein complexes. Finally, the electrophoretic mobilities of C and D are different from those of the dimeric hybrids that we have called Y and Z (Figure 4). Although the identity of C and D remains to be established, the electrophoretic mobility of these two entities is consistent with the hypothesis that they represent a pair of resolved, double-stranded products of an antibody-catalyzed integrase reaction. We therefore conclude that some of the antibodies in the serum may possess integrase activity. The assay condition is as described in Figure 6 legend. Double-stranded DNA was annealed by mixing stoichiometric amount of the appropriate oligonucleotides in TE buffer.

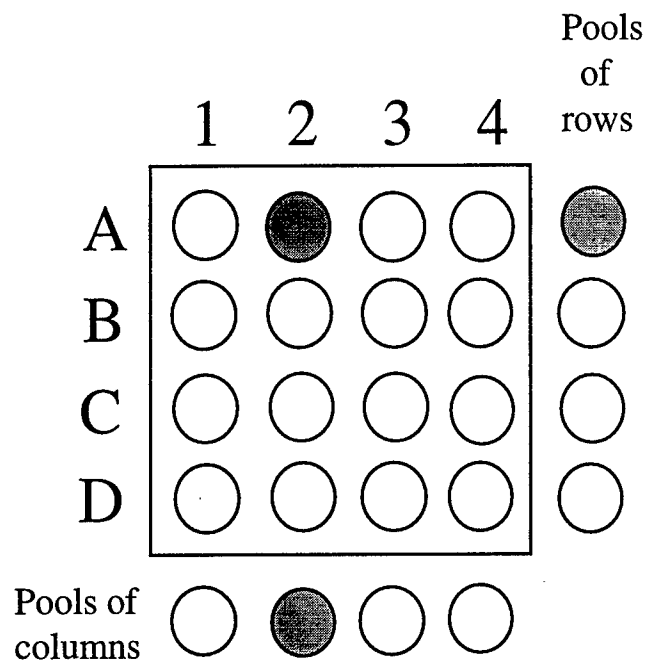


Figure 8. Schematic diagram of the screening procedure (adapted from Isreal, 1993). The hybridoma supernatants from 4 wells across a row or down a column are pooled. The matrix of 16 is reduced to 8 reactions. If both of the pools of row A and column 2 are tested positive (hatched circles). The clone at A2 position is a positive one (shaded circle).

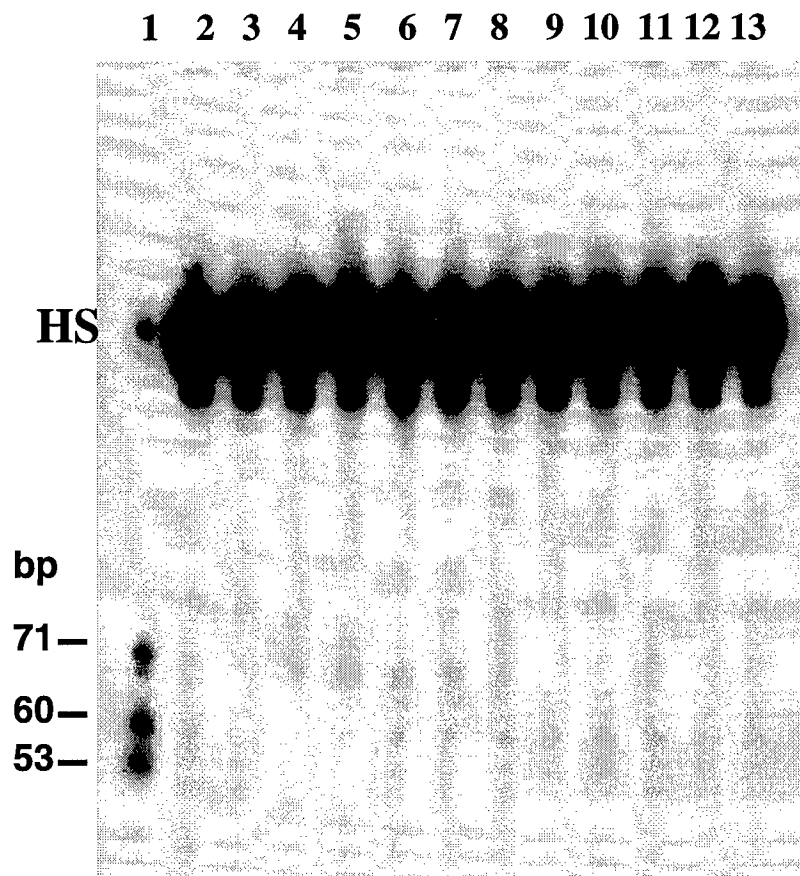


Figure 9. Examples of screening by electrophoresis. Lane 1 is the marker containing expected products resolved from HS. All other lanes contained equal amount of purified, ^{32}P -labeled HS and were incubated with hybridoma culture medium (lane 2) and 11 pools of hybridoma supernatants (lanes 3-13). The reactions were carried out at room temperature for 1.5-2 hours, and the samples were then separated on 8% polyacrylamide gel by electrophoresis in 1xTBE buffer. The gel was dried and exposed onto x-ray film.

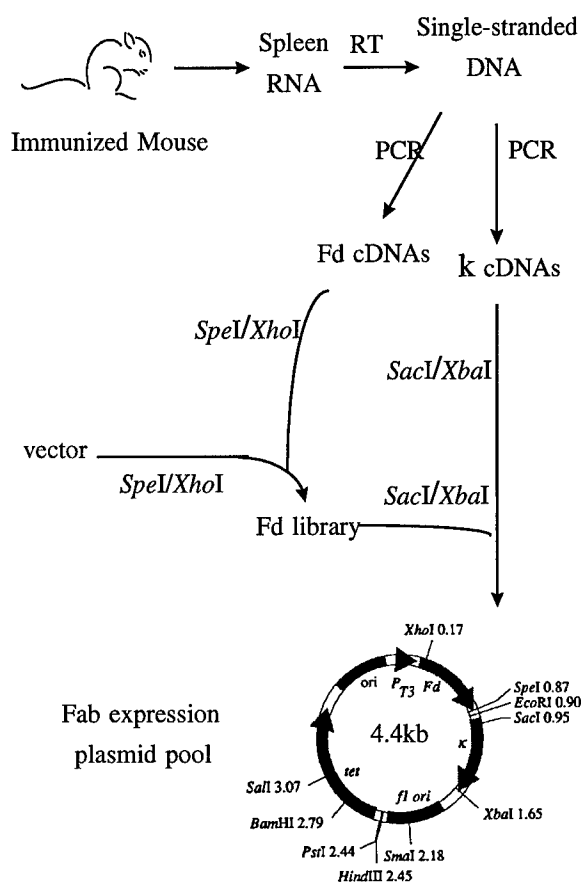


Figure 10. Construction of Fab expression library. The Fab expression library is derived from splenic RNAs. Briefly, total RNA will be isolated from spleens of HS-immunized mice. The RNAs will then be reverse transcribed into single-stranded DNA with oligo(dT) priming. The single-stranded DNA will be used as templates for generation of IgG Fd and κ cDNA by PCR. The Fd and κ genes will be amplified separately with murine IgG primers, including one Fd 3' primer, nine Fd 5' primers, one κ 3' primer and five κ 5' primers (Huse *et al.*, 1989). Each pair of primers will give rise to a product of about 700bp. All the primers contain a restriction enzyme site: the Fd 3' primer has a *SpeI* site, the Fd 5' primer an *XhoI* site, the κ 3' primer an *XbaI* site, and the κ 5' primer a *SacI* site. These enzyme sites allow the double-stranded PCR products to be cloned into an Fab overexpression plasmid vector. This vector features a single bacteriophage T3 promoter in front of the Fd and κ cDNAs. The PCR products will be digested by corresponding restriction enzymes and the DNA will be gel-purified. *SpeI/XhoI*-digested Fd cDNA will be ligated into the *SpeI/XhoI* sites of the vector and amplified in bacterial host. This pool of Fd-containing plasmids will then be purified, digested

with *SacI* and *XbaI*, and ligated with *SacI/XbaI*-digested κ cDNA to form a second pool of Fab expression plasmids. Each plasmid in this pool will contain a randomly assembled pair of Fd and κ cDNAs, and each plasmid will express a unique Fab. For protein overexpression, the *E. coli* host will be BL21 (*F'*, *ompT*, *hsdS_B*). Since the Fab cDNAs are under control of T3 promoter, pTG119 will be cotransformed into *E. coli* BL21 with the Fab expression plasmids. pTG119 supplies the T3 RNA polymerase under control of the inducible *lacUV5* promoter. After transformation, the *E. coli* culture will be cloned by limiting dilution. RT: reverse transcription. P_{T3}: T3 promoter (Stewart *et al.*, 1995).

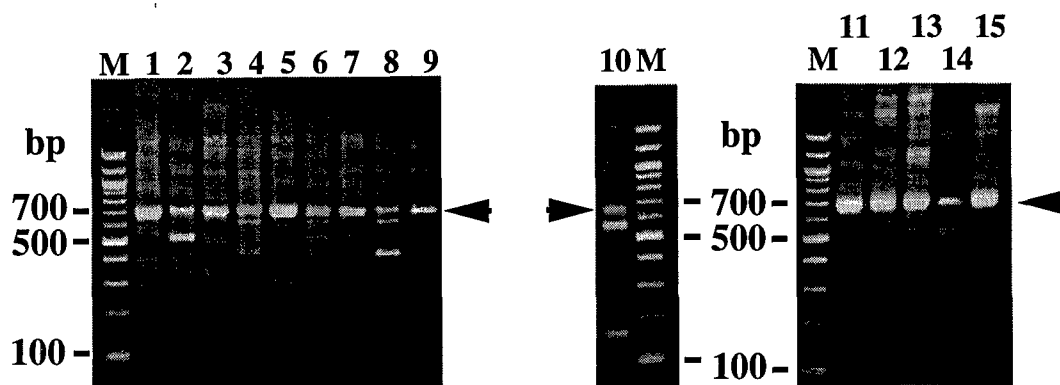


Figure 11. RT-PCR amplification of spleen IgG₁ Fab variable region. Lanes M are DNA size marker (100bp DNA marker, New England BioLabs). Lanes 1-9 are IgG₁ Fab heavy chains Hc1, Hc2, Hc3, Hc4, Hc5, Hc6, Hc7, Hc8, and Hc9, respectively. Lanes 10-15 are IgG₁ Fab light chains κ Lc2, Lc3, Lc4, Lc5, Lc6, and Lc7, respectively. The samples were separated on 0.8% agarose gel by electrophoresis in 1x TBE. The gel was stained by ethidium bromide and visualized by UV emission.

Oligo5 (60nt): 5'-CGCTACAGGAGTTACAGCCTAGTAATCACACGTCCTGCCTCGAGAAGGG
CGTACATGCTC-3'

Oligo6 (60nt): 5'-GAGCATGTACGCCCTTCTCGAGGCAGGACGTGTGATTACTAGGCTGTAA
CTCCTGTAGCG-3'

Oligo7 (53nt): 5'-GCTACTAGTCGATGTCCTGCCTCGAGAAGGGCCATTGAACAGTCATGCT
GTCC-3'

Oligo8 (53nt): 5'-GGACAGCATGACTGTTCAATGGCCCTTCTCGAGGCAGGACATCGACTAG
TAGC-3'

Figure 12. Oligonucleotide sequences of duplex DNAs used in *in vitro* recombination assays (A). Bold face letters show the central sequences as indicated in Figure 2. Underlined letters are the *Xho*I sites.

Oligo9 (42nt): 5'-GCTACTAGTCGATGTCCTGCCTCGAGAAGGGCGTACATGCTC-3'

Oligo10 (42nt): 5'-GAGCATGTACGCCCTTCTCGAGGCAGGACATCGACTAGTAGC-3'

Oligo11 (71nt): 5'-CGCTACAGGAGTTACAGCCTAGTAATCACACGTCCTGCCTCGAGAAGG
GCCATTGAACAGTCATGCTGTCC-3'

Oligo12 (71nt): 5'-GGACAGCATGACTGTTCAATGGCCCTTCTCGAGGCAGGACGTGTGATTA
CTAGGCTGTAACTCCTGTAGCG-3'

Figure 13. Oligonucleotide sequences of duplex DNAs used in *in vitro* recombination assays (B). Bold face letters show the central sequences as indicated in Figure 2. Underlined letters are the *Xho*I sites.

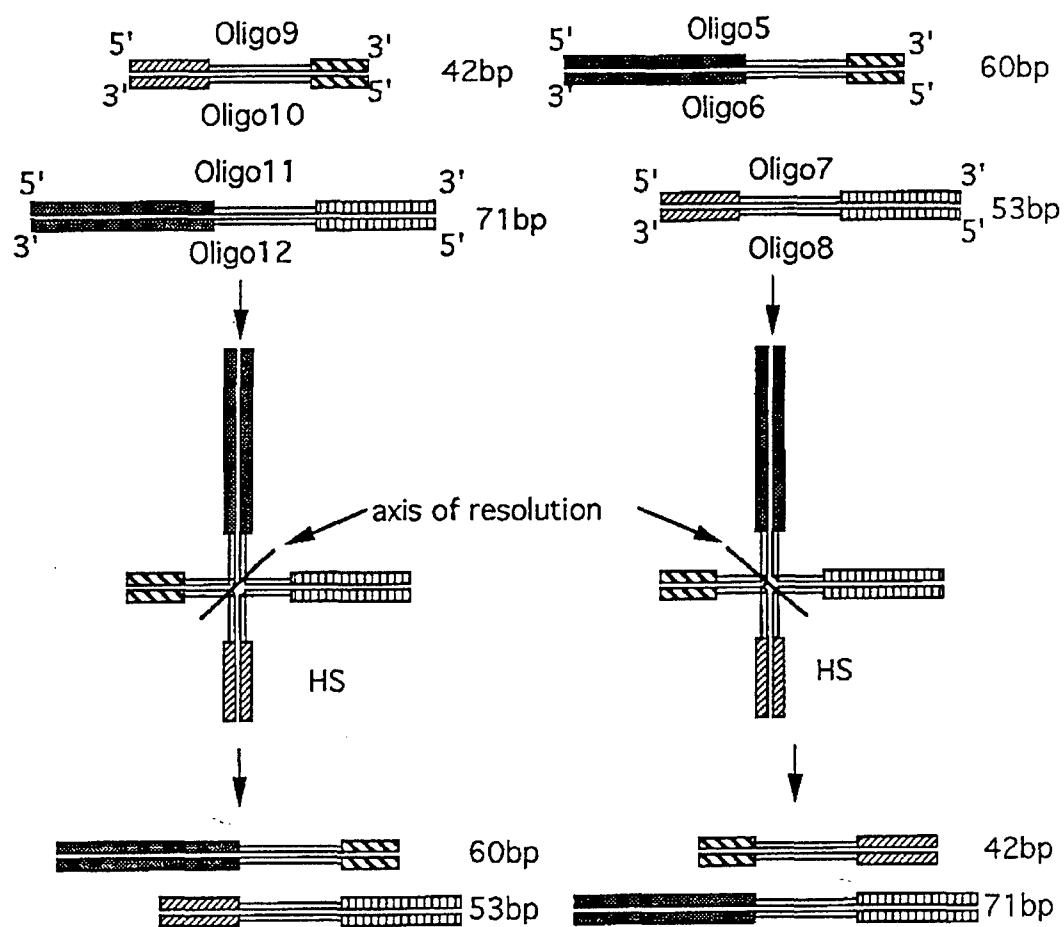


Figure 14. Schematic diagram of DNA recombination *in vitro*.



DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

Rec'd
7/23/2001

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management