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19. ABSTRACT (Continue on reverse if necessary and identify by block number) The objective of this research is to test the hypothesis that the complex proteins with novel functions arose in the course of evolution by combining structural domains having partial functions and to discover the rules that govern successful recombinations. The research focuses on the enzymes that catalyze <u>de novo</u> pyrimidine biosynthesis. While the reactions are the same in most organisms, there are striking differences in the structure and regulation of these enzymes. However we postulate that all of these proteins are composed of a small number of functional domains whose basic architecture is very ancient. Our goal is to determine the structural organization of the pyrimidine biosynthetic enzymes in several organisms that represent a broad cross section of the phylogenetic tree. Domain specific synthetic oligonucleotide probes are being used to analyze poly A+ RNA and to screen genomic or cDNA libraries to determine the domain structure of the pyrimidine biosynthetic proteins. The structure and mode of regulation of interesting structural variants will then be analyzed in detail.				
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ANNUAL REPORT ON CONTRACT N00014-87-K-0081

PRINCIPAL INVESTIGATOR: David R. Evans

CONTRACTOR: Wayne State University
Detroit, Michigan

CONTRACT TITLE: The Evolution and Analysis of the Functional
Domains of the Chimeric Proteins that Initiate
Pyrimidine Biosynthesis.

PROJECT PERIOD: December 1, 1986 - July 31, 1988

RESEARCH OBJECTIVE: To determine the structural organization and trace the evolutionary development of the complex multi-domain proteins involved in de novo pyrimidine biosynthesis.

PROGRESS:

In mammals and other higher eukaryotic organisms, glutamine dependent (GLN) carbamyl phosphate synthetase (CPS), aspartate transcarbamylase (ATC) and dihydroorotase (DHO), enzymes that catalyze the first three steps of the pathway, are consolidated into a single chimeric polypeptide called CAD.

We have completed sequencing the mammalian CAD cDNA and have begun to analyze the evolutionary relationships between proteins from various organisms. Phylogenetic trees have been constructed using programs developed in Dr. Morris Goodman's laboratory (Wayne State University). Our results thus far indicate that the gene duplication giving rise to separate pyrimidine and arginine specific carbamyl phosphate synthetases occurred prior to the divergence of fungi and higher eukaryotes and that the fusion of GLN and CPS domains may have occurred only once during the course of evolution. Also the CPS and DHO domains, as defined by comparison to the prokaryotic monofunctional proteins, overlap suggesting that the trifunctional mammalian protein was generated by insertion of most of the DHO gene into a long linker region that connects the CPS and ATC domains in lower eukaryotic species. A long linker of 132 amino acid residues connects the carboxyl end of the DHO and the amino end of the ATC domains. This inter-domain connecting chain segment has a very unusual structure; high in proline, glycine and charged residues and extremely hydrophilic.

Based on sequence comparisons, we have designed nine mixed, inosine containing DNA probes directed against each of the major functional domains (1 GLN, 2 CPS, 4 DHO and 2 ATC probes). The probes were synthesized, end labelled with [32-P] and are being used for colony and plaque hybridization, dot blots and Northern and Southern analysis. The probes were tested by hybridization with mRNA from hamster, shark, sponge and earthworm; all give strong signals. These will be used to screen the DNA libraries we have obtained thus far: i) the protozoan, *Entamoeba histolytica* (from Dr. Marion Huber and Dr. Shmuel Rozenblatt at the Weizman Institute of Science, Israel), ii) the sea urchin (Dr. Hiraku Shimada, University of Tokyo, Japan) and iii) *Pseudomonas aeruginosa* (from Dr. A.M. Chakrabarty, University of Illinois at Chicago).

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We have begun to look in detail at organization of the enzyme system in several different organisms:

1. *Pseudomonas fluorescens*: We have shown that the CPS, ATC and DHO activities are carried by separate proteins. ATC has been isolated and in contrast to previously published results, appears to be composed of two polypeptides with molecular weight 43 kDa and 35 kDa. Thus the structural organization appears to be quite different than other prokaryotic and eukaryotic ATCases, although we suspect that the 35 kDa species is the catalytic domain. We are now attempting to determine i) whether these are separate subunits or proteolytic fragments, ii) the location of the active site, iii) the function of the non-catalytic polypeptide and iv) the stoichiometry of the complex. These results will be confirmed by analyzing clones encoding these proteins in the *Pseudomonas* library using the domain specific probes.

2. *Thermoplasma acidophilum*: Cells (ATCC strain 25905) were grown at pH 1.7, 590 and the DNA isolated. A major problem encountered in working with this organism is that the acidic conditions causes extensive depurination, so that we are devising techniques that will ensure recovery of intact DNA. We have also obtained purified *Thermoplasma acidophilum* DNA from Dr. Dennis G. Searcy (University of Massachusetts). The genomic DNA was restricted by several enzymes and one conspicuous Kpn I fragment has been cloned into pBS to demonstrate the feasibility of the approach. We are now constructing a *Thermoplasma* library and hope to obtain genomic libraries of other archaeobacteria.

In the case of the eukaryotic organisms we are also examining the mRNA directly. Poly A⁺ RNA has been isolated from each of the following organisms and will be analysed by Northern blotting.

3. *Lumbricus terrestris* (earthworm)
4. *Axinella polycapella* (marine sponge)
5. *Squalus acanthias* (shark)

The shark is especially interesting since Dr. Paul Anderson (University of Minnesota) has evidence that unlike other higher eukaryotic organisms the CPS, ATC and DHO are separate polypeptides in this species. In collaboration with Dr. Anderson we are trying to confirm this surprising discovery. Liver, spleen and testes extracts, prepared under conditions which minimize proteolysis, have been analyzed by Western blotting using antibodies directed against mammalian CAD and its ATC and DHO domains. While we can readily detect the related protein carbamyl phosphate synthetase III in liver extracts, we have not been able to find the pyrimidine biosynthetic enzymes in any of these tissues either because the method is insufficiently sensitive or because of the failure of the antibodies to cross react with the shark enzymes. Northern blots of mRNA is a more promising approach. We have isolated RNA from each of these shark tissues and found by dot blot analysis that it specifically hybridizes with domain specific DNA probes. We are now doing Northern analysis to determine if the pyrimidine biosynthetic enzymes are encoded by separate mRNAs and the size of each of mRNA molecule.

WORK PLAN (Year 2-3): We anticipate that the structural organization of the *Pseudomonas* and shark enzymes will be deciphered using a combination of molecular biology and protein methods during the next six months. Several other

organisms will be examined, using cDNA libraries or by isolating and probing poly A+ RNA directly. To fill out the broad outline of the phylogenetic tree we will focus on archebacteria, protozoa, primitive invertebrates, algae and higher plants. At this stage we will have a clear idea as to which structural variants are good candidates for more detailed studies planned for the third year. These studies would include cloning and partial sequencing and characterization of the functional properties of the isolated proteins.

INVENTIONS: None

PUBLICATIONS AND REPORTS:

The Domain Structure of Aspartate Transcarbamylase From *Pseudomonas Fluorescens*. Sandra T. Bergh, Emily J. Zaragoza and David R. Evans (1988) FASEB Journal 2: A1548. Presented at the American Society for Biochemistry and Molecular Biology, May, 1988.

TRAINING ACTIVITIES:

One postdoctoral, one graduate student and one research assistant are supported by this contract.

Women or minorities - 3

Non-citizens - 1 (citizen of Ethiopian)

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