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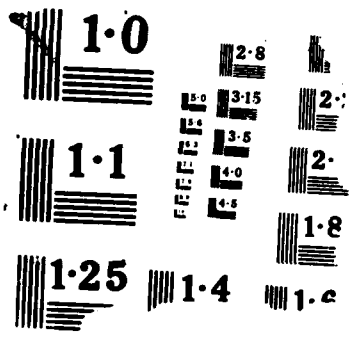
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Genomic restriction fragments from Halobacterium cutirubrum (Hcu) and Sulfolobus acidocaldorius (Sac) encoding ribosomal protein L11e, L1e, L10e and L12e were cloned and sequenced. The sequences were analyzed and the reading frames identified. The deduced L10e and L12e protein sequences were aligned with eubacterial and eucaryotic proteins and their structure-function and evolutionary relationships were defined. The N-terminal amino acid sequence of the purified superoxide dismutase enzymes from Hcu was determined, an oligonucleotide was prepared and the gene was cloned and sequenced. The sequence has been aligned to other SOD sequences. The gene transcripts have been characterized and the regulation of transcription (induction) by oxygen radicals has been demonstrated and characterized.

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Abbreviations: Hcu - Halobacterium cutirubrum
Hha - Halobacterium holobium (same species as Hcu)
Hvo - Halobacterium volcanii
Sac - Sulfolobus acidocaldorius
Eco - Escherichia coli

Research Objectives

(i) to characterize the principles of gene organization and regulation of gene expression in archaebacteria; (ii) to elucidate the evolutionary relationship between these novel organisms and the traditional eubacterial and eucaryotic organisms; (iii) to understand in biophysical and molecular terms some of the mechanisms that allow archaebacteria to inhabit extreme environments.

Progress - Year 1

Ribosomal protein genes: The ribosoml A protein complex forms the stalk structure on the large ribosome subunit and is comprised of four copies (two dimers) of L12e and one copy of L10e ribosomal protein. The L10e protein either binds to ribosomal RNA either directly or through the L11e protein which forms a bulge at the base of the stalk. This domain on the large subunit is the site of factor binding and associated GTPase activities during the protein synthesis cycle and is a conserved and defined feature on the ribosome from all organisms (1). In Eco the genes encoding the L11, L1, L10 and L12 ribosomal proteins are located within a 3.0 Kb region of genomic DNA. This region has been cloned, sequenced and extensively characterized (2,3).

Partial amino acid sequences of many archaebacterial ribosomal proteins have been determined (4). We used the sequences from the Hcu L11e and L12e proteins to deduce and synthesize oligonucleotide sequences complementary to the corresponding genes. We cloned initially a 1.2 Kb Pst-Bam fragment encoding L12e and subsequently a 5.2 Kb Cla-Bam fragment containing both L11e and L12e. The sequence of the entire 5.2 Kb fragment was determined and it was found to contain several long open reading frames. Four of these open reading frames were shown to encode the L11e, L1e, L10e and L12e ribosomal proteins. The gene order as present in Eco is conserved in Hcu although the transcriptional organization will certainly be much different (work in progress).

Using a similar strategy the corresponding genomic region of Sac has been cloned and sequenced. It contains the same four genes in the same order. From the Hcu and Sac nucleic acid sequence we have deduced the amino acid sequences for the four ribosoml proteins and have begun to align the protein to their eubacterial and eucaryotic equivalent and deduce their evolutionary relationships. This analysis has been completed for the L10e and L12e protein (5).

There are presently four complete sequences of L10e proteins (one eubacterial, two archaebacterial and one eucaryotic) and 16 complete sequences of L12e (eight eubacterial, three archaebacterial and five eucaryotic). Using these sequences we have carried out inter-kingdom L10e and L12e alignments. The L10e protein from the three kingdoms were found to be co-linear. (A cDNA sequence published by Rich and Steitz was shown by us to encode the human L10e

protein (6)). The eubacterial protein is much shorter than the archaebacterial and eucaryotic proteins because of two large deletions, one internal and one at the carboxy terminus. The two archaebacterial L10e proteins were most similar; they exhibit 27% amino acid identity with only a single deletion-insertion. Inter-kingdom comparisons exhibit 15-25% amino acid identity with 5-7 deletion insertions.

The L12e protein from archaebacteria and eucaryotes can also be aligned and are co-linear. The eubacterial protein could not be made to fit this pattern although two regions appear to have homologous domain in the archaebacterial and eucaryotic proteins. The C-terminal domain of the Eco L12e protein has been shown to contain (i) a conserved face for interaction with extrinsic translation factors; (ii) an anion (potential GTP) binding site; and (iii) a putative L12e-L12e dimerization site (1). This domain is separated from the N-terminus by an unstructured ala-pro-rich hinge sequence and aligns to a region near the amino terminus of the archaebacterial and eucaryotic proteins. Inter-kingdom comparisons range from 18-36 percent amino acid sequence identity over their domain. The second region conserved in all proteins is the ala-pro-rich sequence. In eubacteria this sequence precedes the facter interaction domain; in archaebacteria and eucaryotic it follows the facter interaction domain. The N-terminus of the eubacterial L12e protein exhibits more similarity to its own carboxy end than to any region within the archaebacterial-eucaryotic protein. The carboxy termini of the archaebacterial and eucaryotic protein are highly similar to each other and apparently not represented in the eubacterial L12e protein.

Intraspecies comparisons between L10e and L12e proteins indicate that the L10e protein of archaebacteria and eucaryotes contains a partial copy of the L12e protein fused to its carboxy terminus. (In eubacteria most of this fusion has been removed from L10e by the carboxy terminal deletion and the L12e protein has been restructured.) For the two Sac proteins the 31 carboxy teminal residues of L10e and L12e are identical; conservation at the nucleotide level is also perfect. This homology extends further into the central regions of the L10e and L12e proteins and includes a 26 amino acid long module, reiterated thrice in archaebacterial L10e, twice in eucaryotic L10e and once in the corresponding L12e protein. Careful examination indicates that a single copy of the modular sequence is also present in the L12e and L10e protein of eubacteria. This modular sequence may play a role in L12e dimerization, L10e-L12e complex formation and the function of the L10e-L2e complex in translation.

Based upon these alignments and shared homologies a model has been constructed to depict the evolution of the primordial L10e and L12e genes and proteins in the three phylogenetic groups (5).

Superoxide dismutase: Previously we had purified and characterized the SOD enzyme activity from Hcu (7). An N-terminal amino acid sequence was determined, an oligonucleotide was synthesized and the gene encoding the SOD enzyme was cloned as a 1.1 Kb Sau3A fragment and sequenced. The fragment contain a single long open reading frame encoding a protein of 200 amino acids. The first 56 codons of the ORF correspond to the amino acid sequence determined from the purified protein. The gene encodes a typical Mn/Fe ion type enzyme as evidenced by the fact that (i) it has an acidic pI; (ii) it

contains no cys residues; (iii) it is relatively abundant in tyr and trp residues; (iv) it exhibits 41% amino acid identity with the Mn SOD of Bacillus stearothermophilus and (v) it conserves the four residues, his 29, his 76, asp 158 and his 162 that in the Bacillus enzyme (position 26, 81, 163 and 167 respectively) are utilized for Mn binding.

By S1 nuclease protection and primer extension analysis we have shown that the SOD mRNA is about 242 nucleotides in length. It begins two nucleotides in front of the ATG translation initiation codon and terminates about 40 nucleotides downstream of the TAA translation termination codon, in a T_5 sequence that is preceded by a GC-rich sequence. This terminator sequence appears similar to other Hcu terminators. Analysis of this and other 5' flanking sequences have yet to reveal the concensus sequence involved in promoter recognition by RNA polymerase in Hcu (that is, there is little conservation between the bacterio-opsin, SOD, rRNA and r-protein 5' flanking sequences).

Like bacterio-opsin, the mRNA encoding SOD starts only two nucleotides in front of the ATG translation initiation codon. There is no homolog of the Shine Dalgarno purine-rich sequence of eubacteria that is complementary to the 3' end of 16S rRNA on the SOD mRNA. In contrast some r-protein genes of Hcu retain this sequence. Thus it seems that Hcu ribosomes can initiate translation by two separate mechanisms: at the first AUG on the transcript (SOD and bacterio-opsin) or by a Shine Dalgarno interaction at internal AUG codons.

We have discovered that the SOD gene in Hcu is subject to regulation. Addition of paraquat, a generator of oxygen radicals, to an exponential phase culture results in the 5-10x induction of SOD enzyme activity. After several days of growth in the presence of paraquat the SOD activity returns to normal. The culture has become resistant to the inhibitor and the frequency suggests that resistance arises by transposition insertion (as in bacterio-opsin negative mutants of Hha; 8) into a gene specifying sensitivity (i.e. transport of paraquat across the cell envelope). This observation is being investigated.

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Objectives for Year 2

Gene encoding r-protein

1) Carry out inter-kingdom alignment and analysis of L11e and L1e protein;
2) Define the transcriptional organization of the L11e, L1e, L10e and L12e gene clusters in Hcu and Sac, map 5' and 3' transcript ends, cap 5' transcript ends and analyze for conserved promoter and terminator sequences.

Superoxide dismutase

3) Cap mRNA and characterize induction by paraquat at the level of transcription;
4) Look for DNA binding protein to the SOD promoter region by in vivo foot-printing;
5) Clone and characterize SOD gene from a genetically stable strain Hvo, isolate paraquat-sensitive (null) and resistant (constitutive) mutants;
6) Utilize the transformation system of Hvo to characterize the genetic regulation of the SOD gene.

Processing of rRNA

7) Characterize promoter and processing signals in the separate 16S-23S and 5S transcription units of Sac;
8) Utilize cloned copies of the 16S inverted repeat processing signal from Hcu to develop an assay for RNaseIII-like activity;
9) Attempt to purify Hcu RNaseIII-like activity, clone the gene and study substrate specificity by *in vitro* site-specific mutagenesis of cloned 16S inverted repeat sequence.

Publications

May, B. and P. Dennis (1987) Superoxide dismutase from the extremely halophilic archaebacterium *Halobacterium cutirubrum*. J. Bacteriol. 169, 1417-1422.

Shimmin, L., C. Ramirez, A. Matheson and P. Dennis (1988) Sequence alignment and evolutionary comparison of the L10e and L2e ribosomal proteins from archaebacteria, eubacteria and eucaryotics. Submitted for publication (copy enclosed to ONR).

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