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19. ABSTRACT (Continue on reverse if necessary and identify by block number) The goal of this project was to elucidate at the molecular level some of the features that make archaeobacteria unique and distinguish them from eubacteria and eucaryotes. Three types of genes, encoding rRNAs, ribosomal proteins and superoxide dismutase are phylogenetically conserved in all three kingdoms. The structure, organization, regulation and expression of these genes and their gene products has been examined in several archaeobacterial species and compared to their eubacterial and eucaryotic counterparts. The major findings from this work are:					
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- i) The promoter recognition signals in halophilic archaeobacteria consist of two sequence elements centered about -30 and -40 nucleotides upstream of the transcription initiation site. The consensus for these sequences are TTAA (-30) and TTCGA (-40). Transcription termination signals are tracts of T residues in the (+) strand of the template DNA and are often preceded by a G + C rich sequence, sometimes possessing inverted repeat symmetry. The transcripts of protein encoding genes are generally monocistronic, initiated close to the ATG translation initiation codon, and noticeably lack the Shine-Dalgarno ribosome binding sequence.
- ii) Ribosomal RNA operons are transcribed from one up to nine tandem promoters in the 5' flanking region. The initial step in processing precursor 16S and 23S from the primary transcript involves cleavage by an endonuclease that is also used to excise the intron from certain tRNA gene transcripts. The primary sequence and secondary structure of the cleavage site is highly conserved among archaeobacteria.
- iii) Ribosomal protein genes are organized into eubacterial-like operons. The L1e-L10e-L12e halophilic operon contains a putative regulatory sequence in the 5' transcribed leader that could function in autogenous translational control.
- iv) Archaeobacterial ribosomal proteins exhibit substantial structural and sequence similarity to eucaryotic ribosomal proteins and less similarity to the eubacterial equivalent ribosomal proteins.
- v) Halophilic archaeobacteria contain a Mn containing superoxide dismutase that is homologous to the eubacterial Mn or Fe SOD and unrelated to the eucaryotic CuZn enzyme. The sod gene and a related gene, slg, have been cloned and sequenced from *H. cutirubrum* and their regulation and expression has been characterized. These two sequences represent a unique example of divergent evolution driven by selection at the molecular level of a duplicated sequence within a genome.

Abbreviations:

Hcu	-	Halobacterium cutirubrum
Hha	-	Halobacterium halobium (same species as Hcu)
Hvo	-	Halobacterium volcanii
Hma	-	Halobacterium marismortui
Sso	-	Sulfolobus solfataricus
Eco	-	Escherichia coli
Sce	-	Saccharomyces cerevisiae

Research Objectives:

- i) To characterize the principles of gene organization and regulation of gene expression in archaeobacteria.
- 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 archaeobacteria to inhabit extreme environments.

Accomplishments:

Ribosomal protein genes: A 5.2 Kbp *Cla*I-*Bam*HI fragment of *Hcu* genomic DNA was cloned using synthetic oligonucleotides complementary to the coding regions of partially sequenced *Hcu* 50S subunit ribosomal proteins. The fragment was completely sequenced and found to contain the genes encoding the proteins equivalent to the *Eco* L11, L1, L10 and L12 ribosomal proteins. In addition, two open reading frames designated ORF and NAB were also detected. The transcripts from this region were extensively characterized by Northern hybridization, S1 nuclease protection and primer extension analysis. Four promoters were located in front of ORF, NAB, L11e and L1e genes, respectively. The first three initiate transcription at or adjacent to the respective ATG translation initiation codons whereas the fourth initiates to produce a transcript with a 75 nucleotide long untranslated leader sequence. Preceding each transcription initiation site are two motifs that appear to be conserved; they are the general archaeobacterial motif TTAA centered at about position -30 and a halophile specific motif TTCGA centered at about position -40. Transcription termination occurs at poly T tracts in the DNA (+) strand that are often preceded by GC rich sequences sometimes containing inverted repeat symmetry. Within the coding regions, poly T sequences are noticeably under represented and the TTT Phe codon is not used.

The tricistronic mRNA encodes the L1e, L10e and L12e ribosomal proteins that are produced in a 1:1:4 stoichiometry. Each of these cistrons is preceded by what appears to be the equivalent of the eubacterial Shine-Dalgarno ribosome recognition sequence. Monocistronic mRNAs lack these sequences and may initiate translation by a eucaryotic thread on type mechanism. The L1e binding sequence on 23S rRNA has been defined previously. We identified a region in the 5' untranslated leader to the L1e-L10e-L12e mRNA that resembles in primary sequence and secondary structure this rRNA binding site. We propose that this mRNA leader sequence is used to autogenously regulate translation of the mRNA by a mechanism similar to that employed in eubacteria. Thus halophilic archaeobacteria retain the same gene order and possibly also the same regulatory mechanism for controlling ribosomal protein synthesis that is found in eubacteria.

Ribosomal protein structure: The complete amino acid sequences of the L11e, L1e, L10e and L12e ribosomal proteins from *Hcu* and *Sso* have been deduced from the respective gene sequences and compared to the amino acid sequences of the respective eucaryotic and eubacterial equivalent ribosomal proteins. On the large ribosome subunit, the 1:4 complex of L10e and L12e in loose association with L11e binds to 23S rRNA at about nucleotides 1050-1120 to form a prominent stalk structure. This structure functions as a factor binding domain with associated GTPase activities during the protein synthesis cycle. The L1e protein binds to 23S rRNA near nucleotide 2150 and forms a prominent ridge on the large subunit opposite the stalk structure. The L1e protein stabilizes peptidyl tRNA binding to the P site and stimulates GTPase activity at the factor binding domain. Alignments of amino acid sequences of these four proteins from eubacteria, eucaryotes and archaeobacteria indicate that (i) archaeobacteria represent a distinct phylogenetic group and (ii) in general, the archaeobacterial proteins more closely resemble their eucaryotic rather than the eubacterial equivalent proteins. Similarities between the L11e, L1e, L10e and L12e proteins of *Eco*, *Hcu*, *Sso* and *Sce* are presented in the accompanying table.

Our work has provided some valuable insights into the structure and evolution of the L10e-L12e complex and has the important potential to help us identify functional domains within the respective proteins. Eucaryotes including *Sce* possess two different L12e genes

designated type I and II; in *Sce*, each of these is again duplicated to give four L12e genes designated IA, IB, IIA and IIB genes. Within the universally shared factor binding domain of all L12e proteins (from eubacteria, archaeobacteria and eucaryotes), the type I versus type II split appears to be the most ancient. This implies that the common ancestor contained two L12e genes and that one of these (probably the type II gene) was lost from the eubacterial and archaeobacterial lines. Although the factor binding domain remains a constant feature of all L12e proteins, the eubacterial protein clearly differs in its overall structure from the archaeobacterial and eucaryotic equivalents. In the *Eco* L12, the factor binding domain is shifted to the carboxy terminus of the protein and the amino terminus appears to have originated by a partial duplication event. The archaeobacterial-eucaryotic L12e proteins have the factor binding domain at the amino terminus and a unique charged region at the carboxy terminus that is not present in the eubacterial protein. Furthermore, a unique gene fusion placing the distal 75% of the L12e gene at the 3' end of the L10e gene is evident in both archaeobacteria and eucaryotes. This domain in the two proteins is highly conserved, implying a strong constraint on sequence divergence. In eubacteria, most of this fusion sequence has been removed from the contemporary L10 gene by subsequent deletion and there is virtually no homology left from the ancient fusion events. These and other detailed analyses of the structure, function and evolution of the domains of the L11e, L1e, L10e and L12e proteins is continuing.

Superoxide dismutase in the archaeobacteria: When life originated about 3.5×10^9 years ago, the earth's atmosphere was anaerobic. Oxygen began to accumulate in the atmosphere about 2×10^9 years ago as a by-product of the photosynthetic process. Molecular oxygen is toxic to living organisms because of the reactivity of the superoxide anion. The enzyme superoxide dismutase carries out the dismutation of superoxide to molecular oxygen and peroxide. Two separate SOD activities have evolved independently during evolution, the CuZn enzyme of eucaryotes and the Mn or Fe enzyme of eubacteria (and eucaryotic organelles). We have shown that the halophilic SOD is homologous to the eubacterial enzyme. The enzyme activity has been purified to near homogeneity from Hcu and the gene encoding the activity has been cloned, sequenced and its activity has been characterized.

TABLE 2. Similarities between the L11e, L1e, L10e, and L12e ribosomal proteins of *E. coli*, *H. cuirubrum*, *S. solfataricus*, and *S. cerevisiae*

	Length	Identities	Gaps
L11e			
Hcu/Sso	164	65 (40%)	1
Hcu/Eco	138	46 (33%)	1
Sso/Eco	139	45 (32%)	2
L1e			
Hcu/Sso	214	66 (31%)	4
Hcu/Eco	211	58 (27%)	12
Sso/Eco	220	49 (22%)	10
L10e			
Hcu/Sso	343	90 (26%)	1
Hcu/Eco	169	40 (24%)	6
Sso/Eco	169	35 (21%)	6
Hcu/Sce	329	76 (23%)	7
Sso/Sce	322	72 (23%)	7
Eco/Sce	163	27 (17%)	6
L12e			
Hcu/Sso	110	46 (42%)	1
L12e Globular Domain			
Hcu/Sso	56	28 (50%)	0
Hcu/Eco	44	13 (30%)	7
Sso/Eco	44	12 (27%)	7
Hcu/Sce IA	57	16 (28%)	2
/Sce IB	57	18 (32%)	2
/Sce IIA	54	9 (17%)	6
/Sce IIB	54	16 (30%)	6
Sso/Sce IA	57	18 (32%)	2
/Sce IB	57	17 (30%)	2
/Sce IIA	54	12 (22%)	6
/Sce IIB	54	17 (31%)	6
Sce IA/Sce IB	58	32 (55%)	2
/Sce IIA	55	8 (15%)	6
/Sce IIB	55	14 (25%)	6
Sce IB/Sce IIA	54	9 (17%)	6
/Sce IIB	54	11 (20%)	6
Sce IIA/Sce IIB	52	27 (52%)	0

NOTE: The average length in amino acids over the region of comparison of the two sequences is determined as the length of the comparison region minus half of the total gaps. Identities are given as the number (and percentage) of perfect matches over the region of comparison. Gaps are given as the number of deletions (or insertions) required to achieve alignment of the two sequences within the region of comparison. The L12e globular domain is the region indicated in Fig. 4B. In Hcu and Sso L12e this region extends from position 1 to 56 of Fig. 4A. Comparisons with *Eco* L12 are with the C domain and not the N-terminus in Fig. 4B.

In addition to the sod gene which encodes the authentic SOD activity that has been purified, we cloned a second gene designated slg from the Hcu genome which is highly similar to sod. This slg gene is actively transcribed; no sod-like activity corresponding to the product of the slg gene has been detected; the transcriptional regulation of slg is different from sod and the 5' and 3' flanking regions are unrelated. Within the coding region, the four codons used to specify the amino acid residues used to bind Mn in the protein are conserved in both genes. The genes have 87% nucleotide sequence identity whereas the proteins they encode have only 83% amino acid sequence identity. Mutations occur randomly at first, second and third codon positions and transversions outnumber transitions. Most mutational differences between slg and sod are confined to two limited regions; other regions totally lack differences. These two gene sequences are apparently in the initial stage of an unusual mode of divergent evolution. Presumably, this divergence is being driven by strong selection at the molecular level for either acquisition of new or partition and refinement of ancestral functions in one or both of the respective gene products.

An attempt to clarify the intriguing sod-slg relationship in Hcu was made by examining the homologous sequences from the related halophile Hvo. This organism also has two copies of a sod related sequence; amazingly both copies are nearly identical, differing only at codons two and three as follows:

codon		1	2	3	4		199	200	
amino acid		M	S	D	Y		F	E	TER
<u>sod</u> 1	...	AACACCTTAC	ATG	TCA	GAC	TAC	...	TTC	GAC TAA CGCGTACGC...
		...		...	...			...	...
<u>sod</u> 2	...	GTTACACATT	ATG	AGC	---	TAC	...	TTC	GAG TAA CCGGATCAT...
			M	S	-	Y		F	E TER

The remaining 197 codons are identical and homology abruptly ends at the cistron boundaries. We suspect that the duplication of the primordial sod gene was an ancient event and that in Hcu selection at the molecular level is operating to produce two proteins with different (although probably related) function whereas in Hvo selection is operating to conserve structure and function. We are beginning to exploit the Hvo DNA transformation system to address questions relating to the function, regulation and evolution of the sod genes in halophilic archaeobacteria halophilic rRNA operons.

Ribosomal RNA genes: In halophilic archaeobacteria, rRNA operons have the following gene order: 16S, 23S and 5S. An ala tRNA gene is located in the 16S-23S intergenic space and a cys tRNA is sometimes positioned distal to the 5S gene. The 5' flanking region contains from one up to nine tandemly repeated promoter sequences each containing the TTAA (-30) and TTCGA (-40) conserved sequences. The 16S and 23S genes are flanked by inverted repeat processing sequences that are highly conserved in both primary sequence and secondary structure. Excision at these sites liberates precursor 16S and 23S rRNAs from the primary transcript; it appears likely that the endonuclease responsible is identical to enzymes used to excise introns from the 23S rRNA genes.

During a comparison of leader sequences from Hcu, Hvo and Hha, we have observed an 80 nucleotide long region that immediately precedes the 16S processing site that is more highly conserved than is the 16S gene sequence between these organisms. Only the last few nucleotides of this sequence are required for recognition by the endonuclease. The function of the remainder of this conserved sequence is being investigated.

The species Hma has two rRNA operons, designated HC8 and HH10. The first is preceded by four tandem promoters whereas the second is preceded by only a single promoter. In addition, the HH10 operon appears to lack the normal 16S rRNA processing sequence. We have begun a complete sequence comparison of these two operons. Remarkably, our results indicate extensive differences between the two 16S coding regions. These include base substitutions as well as insertion-deletion. In virtually all organisms, the sequence of multiple rRNA genes are virtually identical. The functional significance of this sequence variation in the two Hha operons is currently under investigation.

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