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UNIVERSITY OF ALBERTA

The ribosomal RNA genes of *Halobacterium cutirubrum*

BY

Wan L. Lam

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NAME OF AUTHOR: Wan Lam

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Wan L. Lam

(Student's signature)

3303 112B St.

Edmonton, Alberta

(Student's permanent address)

March 26, 1986

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND REASEARCH

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Reasearch for acceptance, a thesis entitled,

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Kenneth L. Roy
(Supervisor)

W. J. Rogers

Frank Nargang

J. Campbell

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ABSTRACT

The complete nucleotide sequence of the *Halobacterium cutirubrum* 23S ribosomal RNA gene and its flanking regions has been determined. The gene sequence shares 59% homology with its *Escherichia coli* counterpart and is 53% homologous to the *Methanococcus vannielli* 23S rRNA gene, suggesting divergence within the archaebacteria. The secondary structure of the RNA has been derived from the primary sequence according to the existing prokaryotic models. Certain regions on the hypothetical structure resemble the functional sites on the *E. coli* large subunit RNA.

The 16S/23S spacer showed no significant homology to its *M. vannielli* counterpart except for the occurrence of an alanine tRNA gene. A 5S rRNA gene, a cysteine tRNA gene, as well as an open reading frame have been identified downstream from the 23S rRNA coding sequence. No obvious promoter or Shine-Dalgarno sequence is located immediately upstream from this open reading frame. Two possible termination signals (dyad symmetries) were found. One is structurally similar to the rho-independent terminator and the other resembles the anti-termination structure downstream from *E. coli* rrn operons.

Expression of the rRNA genes and tRNA genes in *H. cutirubrum* has been demonstrated by RNA-DNA hybridization analysis. Transcription of the alanine tRNA gene was shown in a eukaryotic system (HeLa cell lysate). No expression of the two tRNA genes was detected in *E. coli*.

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INTRODUCTION

Archaebacteria

A group of organisms, formerly classified as eubacteria, demonstrated unusual features that do not fit the definition of eubacteria. These observations have led to the postulation of a third line of descent, the archaebacteria. The extreme halophiles, the methanogens, certain *Thermoproteales* and the *Sulfolobales* comprise the archaebacteria (Woese et al.,1978; Magrum et al.,1978; Woese, 1981; Garrett, 1985). Unlike the eubacteria, archaebacteria lack diaminopimelic and muramic acids in their cell wall; instead they usually have a simple proteinaceous covering (Kander and Konig, 1985). Their lipids are built from ether (rather than ester) linked phytanyl chains; straight carbon chains are replaced by polyisoprenoid (branched) chains (Langworthy, 1985). The genus *Halobacterium* possess a photosynthetic system unlike any other photosynthetic bacteria. The photosynthetic pigment, the bacterial rhodopsin, is similar to eukaryotic visual pigments (Magrum et al., 1978).

The translational apparatus of archaebacteria shows features different from that of the eubacteria. The characteristic modification pattern and unique sequences in the tRNAs (Gupta, 1984; Kuchino et al., 1982), the use of non-formylated methionine tRNA (Bayley and Morton, 1978), the unique structral features of their ribosomes (Lake, 1983; Lake et al., 1984), are some of the distinguishing features. As well, their DNA-dependent RNA polymerases are immunologically related to their eukaryotic counterparts (Zillig et al., 1982a; Zillig et al.; 1982b, Zillig et al., 1985; Huet et al., 1983). Like the eukaryotes, their elongation factors are sensitive to diphtheria toxin (Kessel and Klink, 1980; Kessel and Klink, 1981; Klink, 1985).

Archaebacteria became widely recognized as a separate phylogenetic branch independent of the eubacteria when Woese and Fox (1977) presented evidence for their

evolutionary divergence using RNase T1 oligonucleotide pattern comparisons of 16S ribosomal RNAs (Woese and Fox, 1977). Detailed comparative analysis of the sequences of ribosomal RNAs (as well as the organization of their genes) has become a powerful tool for examining phylogenetic relationships.

Organization of ribosomal RNA genes

The conservation in rRNA gene organization within phylogenetically related groups of organisms has been studied (Takahashi et al.,1976; Neumann et al., 1983; Hofman et al, 1979; Doolittle and Pace, 1971; Ellwood and Nomura, 1982; Henckes et al., 1982; Bollen, 1982).

In prokaryotes, rRNA genes exist as a co-transcriptional unit of 16S-23S-5S rRNA genes (Brosius et al. 1981; Smith and Dubnau, 1968). The rRNA molecules are about 1540, 2900 and 120 nucleotides in length, respectively. Chloroplast rRNAs are similar (Grey and Doolittle, 1982; Orozco et al., 1980). Mitochondrial rRNAs show a wide range of variability and some mitochondria appear to lack 5S rRNA (Brimacombe and Stiege 1985). The rRNA operon may exist in mutiple copies. There are at least seven copies in *E. coli* (Ellwood and Nomura, 1982) and at least ten in *Bacillus subtilis* (Stewart et al., 1982). Eukaryotic nuclear rRNA genes are typically tandemly repeated and transcribed in the order 5'-18S-5.8S-28S-3' by RNA polymerase I (Rosenthal and Doering, 1983; Long and Dawid, 1980). The 5S rRNA genes are also usually tandemly repeated and separately transcribed by RNA polymerase III (Walker and Pace, 1983; Fedoroff, 1979; Reeder, 1984).

The organization of the rRNA genes has been determined by Neumann et. al. (1983) in a small number of archaebacteria. A common organizational pattern was not found. Members from the same subgroup tend to share a similar pattern. In the sulfur-dependent and some thermophilic archaebacteria (for example, *Desulfurococcus mobilis*, *Thermoplasma acidophilum* , and *Thermoproteus tenax*) rRNA genes are widely

separated (Tu and Zillig, 1982; Neumann et al., 1983). Multiple rRNA operons were identified in some archaebacteria (Jarsch et al., 1983a; Neumahn et al., 1983). Four sets of rRNA genes are found in *Methanococcus vannielii*. Additional 5S rRNA genes separated from the rRNA operon are observed in *Sulfolobus acidocaldarius*,*Thermococcus celer* and *Thermofilum pendens* (Neumann et al.,1983). Wich et. al. (1984) demonstrated an apparent operon coding for a 5S rRNA and seven tRNAs in *Methanococcus vannielii*. The linkage between rRNA genes in the halophiles (and the methanogens to a lesser extent) closely follows that of the eubacteria. Both *Halobacterium volcanii* and *Halobacterium marismortui* have two separate rRNA operons containing the typical eubacterial transcriptional unit of 5'-16S-23S-5S-3', whereas *Halobacterium halobium* and *Halobacterium cutirubrum* have only a single copy (Neumann et al., 1983; Daniels et al., 1985b; Mevarech, personal communication; Hofman et al., 1979; Lam, unpublished data; Hui and Dennis, 1985).

Transfer RNA genes

Transfer RNA genes are often found in the inter-cistronic spacer as well as the flanking regions of eubacterial rRNA operons (Loughney et al., 1982; Wawrousek and Hansen, 1983; Wawrousek et al., 1984; Campen et al.,1980; Green and Vold, 1983; Nakajima et al., 1981; Vold, 1985; Morgan et al., 1977). This observation also holds true for the cyanobacteria (Williamson and Doolittle, 1983; Tomioka and Sugiura, 1984) and some archaebacteria (Jarsch and Bock, 1983b; Leffers and Garrett, 1984; Hui and Dennis, 1985; Mankin et al., 1985) as well. So far, no tRNA genes are found associated with the nuclear rRNA cistrons of eukaryotes (Grey and Doolittle, 1982; Reeder, 1984).

A large number of eukaryotic and eubacterial tRNAs and tRNA genes have been sequenced (Sprinzl et al., 1985a, Sprinzl et al., 1985b). Sequences of the majority of archaebacterial tRNAs avaliable came from *Halobacterium volcanii* (Gu et al., 1983; Gupta, 1984). Characteristic modification patterns and unique sequences found in

archaebacterial tRNAs are described by Gupta (1984; 1985).

Interverning sequences

It was a striking discovery to find interverning sequences (IVS) in archae- bacterial tRNAs; IVS have been considered exclusive to eukaryotes. Two tRNA genes, for serine and leucine, containing IVS in the anticodon region were identified in the archaebacterium, *Sulfolobus solfataricus* (Kaine et al., 1983). Daniels et al. (1985a) demonstrated a 105 bp long interverning sequence in *Halobacterium halobium*. The size of this IVS is larger than any archaebacterial or eukaryotic nuclear tRNA intron discovered so far.

Interverning sequences in ribosomal RNA genes, have also been found in some plastid, mitochondrial and lower eukaryotic rRNA genes as well (Heckman and RajBhandary, 1979; Tabak et al., 1984; Burke and RajBhandary, 1982; Cech et al., 1983; Michel and Dujon, 1983; Allet and Rochaix, 1979). Recently, an intervening sequence, 620 nucleotides long, has been located in the 23S rRNA gene of the archaebacterium, *Desulfurococcus mobilis* (Kjems and Garrett, 1985).

Ribosomal RNA sequence homology as a measure of genetic relatedness

The concept of using the degree of homology in ribosomal RNA sequences for taxanomic classification is well accepted (Woese et al., 1975; Fox et al., 1977a; Fox et al.,1977b; MacKay et al., 1983; Stackebrandt and Woese, 1984; Raue et al., 1985). A large number of 5S and 5.8S rRNA sequences are avaliable (Erdmann et al., 1985). Phylogenetic trees for eubacteria, fungi and green plants have been derived through sequence comparison (Hori and Osawa, 1979; Dekio et al., 1984; Hori et al., 1985; Walker, 1985).

The use of larger rRNAs (rather than the 5S rRNA) for such comparative analysis is likely to be more informative and more reliable. Woese and Fox (Fox et al., 1977b;

Woese and Fox,1977) examined a number of RNase T1 digestion patterns obtained from the 16S or 18S rRNA of prokaryotes, eukaryotes and organelles. RNase T1 digestion products were subjected to two dimensional electrophoresis to produce an oligonucleotide fingerprint. The individual oligonucleotides were then sequenced. A set of oligonucleotide catalogs of these organisms was constructed. The association coefficient (S_{AB}) was derived by comparisons of all possible pairs of such catalogs, using the following formula, $S_{AB} = 2N_{AB}/ N_A+N_B$, in which N_A, N_B and N_{AB} are the total numbers of nucleotides in sequences of hexamers (or larger) in the catalogs for organisms A and B. This coefficient is a measure of the evolutionary relatedness of organisms.

Divisions within the archaebacteria

Based on 16S rRNA sequence comparisons (and oligonucleotide cataloging), the prokaryotes are subdivided (Stackebrandt and Woese 1984). The eubacteria comprise ten major subgroups. The archaebacteria form two major subdivisions, (i) the methanogens and the extreme halophiles and (ii) the sulfur metabolizers. The genus *Thermoplasma* does not totally conform to either of these major groupings. Such a branching pattern is consistent with the subdivision suggested by the rRNA gene organization mentioned above.

The halophilic, methanogenic branch is considered to be more closely related to the eubacteria. The sulfur metabolizers and the thermophiles are more related to the eukaryotes (Neumann et al, 1983; Matheson, 1985). In addition to the rRNA gene organizations and sequence homologies mentioned above, a number of major differences between these two branches have been observed (Lake et al., 1984; Garrett, 1985). For example, the subunit molecular weights and the immunological properties of DNA-dependent RNA polymerases of the eukaryotic polymerase I (I) are more similar to those of the *Sulfolobus* than those of the halophiles and the methanogens (Schnabel et

al., 1983; Zillig et al., 1985). The secondary structures of 5S rRNAs from *Sulfolobus acidocaldarius* and *Thermoplasma acidophilum* resemble the eukaryotic 5S pattern more closely than the 5S rRNA from the halophiles and the methanogens (Stahl et al., 1981; Fox et al., 1982; DeWachter et al., 1982). The sequence of the *Halococcus* initiator tRNA resembles that of eubacteria while the *Sulfolobus* initiator tRNA is closer to the eukaryotes, and that of *Thermoplasma* is intermediate (Kuchino et al., 1982; Lake et al., 1984). Significant amounts of polyadenylated mRNA are founded in *Sulfolobus* (Lake et al., 1984). The mechanism of cell division is another indication: the halophiles and methanogens undergo equal cell division by septum formation as eubacteria do. The sulfur metabolizers divide unequally by budding, constriction, branching, and other mechanisms (Stetter and Zillig, 1985). Archaebacteria utilize glycolipids with ether linkages. The membrane lipids of *Sulfolobus* contain cyclopentanol C40-biphytanyl chains; also their neutral lipids contain branched alkyl benzenes. The membrane lipids of the halophilic and methanogenic archaebacteria contain neither (Lake et al., 1984; Langworthy, 1985).

Electron microscopy has shown that ribosomes from the eubacteria, the eukaryotes and the two subdivisions of archaebacteria can be distinguished (Lake, 1983; Lake et. al.,1984). For example, the archaebacterial bill on the head of the small ribosome subunit is common to both archaebacteria and eukaryotes but is absent in eubacteria. The [redacted]karyotic lobes are not seen in eubacteria and most archaebacteria. The sulfur-dependent archaebacteria share common structral domains with eukaryotes that are absent in the halophiles and the methanogens.

These differences mentioned above, though based on very limited comparisons, have raised suspicions concerning the validity of the currently accepted classification scheme in the archaebacteria. Do they represent a common line of descent?

Primary structures

Nucleotide sequence determination of a large rRNA molecule is technically difficult. Advances in recombinant DNA methodology and DNA sequencing technologies have allowed the determination of the complete nucleotide sequence of the genes for larger ribosomal RNAs. Sequence determination of both the coding and the non-coding strands provides independent verification of the sequence. The flanking sequences of rRNA genes provide information on the signals for initiation and termination of transcription as well as for the processing of transcriptional products (Brimacombe and Stiege, 1985; Liebke and Hatfull, 1985).

The majority of the eubacterial and archaebacterial 16S and 23S rRNA gene sequences available have been published within the past two years. Table 1 shows a listing of most known prokaryotic 16S and 23S rRNA gene sequences.

Table1. Known sequences of eubacterial and archaebacterial 16S and 23S ribosomal RNA genes.

Eubacteria				
Anacystis nidulans	16S	Tomioka and Sugiura, 1983	23S	Kumano et.al.,1983 Douglas and Doolittle, 1984
Bacillus brevis	16S	Kop et.al., 1984 a	---	----------
Bacillus stearothermophilus	---	----------	23S	Kop et.al.,1984 b
Bacillus subtilus	16S	Green et.al., 1985	23S	Green et.al., 1985
Escherichia coli	16S	Brosius et.al., 1978	23S	Brosius et.al.,1980 Branlant et. al., 1981
Mycoplasma capricolum	16S	Iwami et.al., 1984	---	----------
Proteus vulgaris	16S	Carbon et.al., 1981	---	----------
Archaebacteria				
Halobacterium volcanii	16S	Gupta et.al., 1983	---	----------
Halobacterium halobium	16S	Mankin et.al., 1985	---	----------
Halobacterium cutirubrum	16S	Hui and Dennis, 1985	23S	this work
Halococcus morrhuae	16S	Leffers and Garrett, 1984	---	----------
Methanococcus vannielii	16S	Jarsch and Bock, 1985 b	23S	Jarsch and Bock, 1985 a
Methanobacterium formicicum	16S	Lechner et al., 1985	---	----------
Thermoproteus tenax	16S	Leinfelder et al., 1985	---	----------

Comparative analysis of rRNA sequences has revealed intriguing homo- logies that suggest a shared ancestry for the three major kingdoms. For example, the eukaryotic 5.8S and the chloroplast 4.5S rRNAs correspond to the 5'- and 3'- terminal sequences of bacterial 23S rRNAs respectively (Otsuka et. al.,1983; Mackett et al., 1981; Edwards and Kossel, 1981). Drosophila melanogaster has a 2S rRNA in its large ribosomal subunit corresponding approximately to the 3'- 25 nucleotides of the 5.8S rRNA (Pavlakis et al., 1979; Jordan et. al., 1976).

Post-transcriptional modifications

An obvious disadvantage to sequencing the genes rather than the rRNAs is the lack of informations on post-transcriptional modifications. Processes such as splicing of interverning sequences and modifications of bases have to be determined separately. These post-transcriptional modifications may be essential to the production of a functional ribosome.

In eubacteria, the majority of modifications are base methylations. *E. coli* 16S rRNA is the best studied model. In eukaryotes, both base modifications and ribose methylations have been identified. *Xenopus laevis* 18S rRNA is a good example. Modified bases tend to occur in positions in the sequences which are analogous to those in eubacteria, while the ribose methylations are more widely distributed (Brimacombe and Stiege, 1985; Noller, 1984; Starzky, 1984).

In most cases, the post-transcriptional modifications in *E. coli* 16S rRNAs occur in regions that are accessible to chemical probes in the intact 30S rRNA subunits (Noller, 1974). These regions furthermore tend to be highly conserved in primary and secondary structures.

Secondary structures

A number of rRNA gene sequences and their hypothetical secondary structures are

now avaliable for direct comparison. This allows reliable measurements of phylogenetic relatedness. The fine structural differences imply their evolutionary divergence. Furthermore, the identification of highly conserved regions suggests important sequences responsible for the maintainance of a functional structure (Thompson and Hearst, 1983b).

Secondary structure models, though mostly hypothetical at this point, reveal a common core structure (Luehrsen et al., 1981; Fox et al., 1982; Woese 1983; Fox, 1984; Kuntzel et al., 1983; Noller and Woese, 1981; Noller, 1984, Brimacombe and Stiege, 1985; Raue et al., 1985). Using this core structure as a reference, evolutionary divergence between organisms can be measured. Detailed structural differences in large rRNAs, such as the presence of extra sequences or the absence of a hairpin, are therefore extremely informative in the phylogeny of an organism. Fox et al. (1982; 1985) predicted subdivisions of archaebacteria based on their proposed 5S rRNA secondary structures.

Larger rRNAs contain more structral domains and therefore are more informative for detailed comparison. Deduced structures can be subsequently confirmed experimentally. Current techniques for secondary structure determination involve base-specific chemical and enzymatic probing for single strands (which correspond to open loop regions) and double strands (which correspond to stem structures). Treatment with various specific cross-linking agents allows probing for interactions within and between substructures (for review see, Woese et al., 1980; Branlant et al., 1981; Qu et al. 1983; Expert-Benzacon and Hayes, 1985b; Hui and Cantor, 1985; Inoue and Cech, 1985; Maly and Brimacombe, 1983; Noller and Woese, 1981; Thompson and Hearst, 1983a; Peattie, 1979; Peattie and Gilbert, 1980; Vary and Vournakis, 1984). Although experimental evidence is somewhat limited it supports the predicted structures.

Secondary structure comparison yields structral homology and suggests functional domains. The 9S and 12S kinetoplast rRNAs from the kinetoplastid protozoan, *Leishmania tarentolae* show limited sequence homology to *E. coli* 16S and 23S rRNAs respectively. However, the secondary structure of the 9S rRNA conforms remarkably to

portions of the *E. coli* 16S rRNA. It looks like a 16S rRNA with regions missing (De La Cruz et. al., 1985a). Similarly, the 12S RNA can be superimposed on the *E. coli* 23S rRNA secondary structure model (De La Cruz et al., 1985b). The domains for tRNA-binding and peptide transfer are preserved. The 3'-terminal region of bacterial 23S rRNA has structural homology with the 3'-terminal region of eukaryotic 28S and the chloroplast 4.5S rRNA (Mackett et al. 1981). Compensating base changes maintain structurally important stems. Non-Watson-Crick base pairing is occasionally found in corresponding stems in rRNAs from different organisms. In addition to the the well known G-U pairing, G-A pairing, pyrimidine-pyrimidine mismatches as well as A-C pairing are sometimes found. Some significant regions, such as ribosomal protein binding sites, the peptide transfer center and tRNA interaction sites, have been identified on the *E. coli* 16S and 23S rRNA through RNA-RNA and RNA-protein crosslinking experiments (Woese et al., 1983; Barta et al., 1984; Raue et al., 1985). Homologous sites on the structural models of other organisms can be inferred by structral comparison (for example, De La Cruz et al., 1985b; Spencer et al., 1984; Nazar, 1985).

Methods for tertiary and quaternary structure determinations are extremely limited (Pieler and Erdmann, 1982). X-ray crystallography has been useful in elucidating the tertiary structure of tRNAs (Robertus et al., 1974; Schevitz et al. 1979). Crystals of larger RNA molecules are difficult to obtain. Three-dimensional models may be derived from unfolding experiments using specific crosslinking reagents (Atmadja et al., 1985; Expert-Benzacon, 1985a; Klein et al. 1985; Inoue and Cech, 1985).

The processing and folding of the rRNA transcripts, the binding of ribosomal proteins, and the formation of the ribosomal subunits are all mysteries.

This work

We attempt to show the evolutionary relationship of the archae- bacterium *Halobacterium cutirubrum* to other archaebacteria and to eubacteria, through analysis of

its ribosomal RNA genes. Our approach is (i) to examine the organization of its rRNA genes, (ii) to determine their primary sequences and (iii) to deduce their secondary structures. Detailed comparative analysis of the gene sequences and secondary structures may contribute to an understanding of the phylogenetic lineage of the extreme halophilic archaebacteria.

We present here, the rRNA gene organization in *Halobacterium cutirubrum* as well as the DNA sequences and secondary structures of (i) the 3'-half of the 16S rRNA gene, (ii) the inter-cistronic spacer region, which includes a $tRNA^{Ala}$ gene, (iii) the 23S rRNA gene, (iv) the 5S rRNA gene and (v) a $tRNA^{Cys}$ gene, which is located in the 3' flanking region of this gene cluster.

MATERIALS AND METHODS

Bacterial Strain and Media

Halobacterium cutirubrum (NRC-34001) was obtained from the NRC culture collection, Ottawa, with the help of Dr. D. Kushner. Cultures were maintained in NRC-Halobacterium medium 2 at 37°C with vigorous shaking.

Escherichia coli strains HB101, JM83 and JM103 (carrying recombinant plasmid or phage) were grown in L-medium (1% tryptone, O.5% yeast extract, 0.5% sodium chloride and 0.1% glucose, pH 7.5) or yeast tryptone medium (0.8% tryptone, 0.5% yeast extract and 0.5% sodium chloride) with the appropiate antibiotics added (see Table 2). JM103, which harbours the F factor necessary for M13 bacteriophage infection, was maintained on minimal medium plus glucose.

DNA and RNA Isolations

Halobacterium DNA was isolated from exponentially growing cells lysed in one tenth growth volume of 0.5% sodium dodecyl sulfate, 10mM tris-HCl (pH 8) and 1mM EDTA. Proteins were removed by repeated phenol extractions followed by chloroform extractions. DNA was spooled out of the aqueous phase, which contains all nucleic acids, into an overlaying ethanol phase (Marmur 1961). DNA was dissolved to a concentration of approximately 1 mg/ml in 10 mM Tris-HCl, 1 mM EDTA, pH 7.8 and stored over chloroform at 4°C.

Plasmid DNA (pAT153 and pHcr1) was isolated by the cleared lysate procedure (Clewell and Helinski 1969) from *E. coli* HB101, and further purified on a CsCl gradient (1.1 g CsCl and 250 µg ethidium bromide per ml of cleared lysate, centrifuged at 40 K rpm for at least 36 hours). Ethidium bromide was extracted from the plasmid DNA with butanol. The DNA was precipitated from the aqueous phase at -20°C after the addition of 2.5 volumes of 70% ethanol.

For rapid, analytical scale plasmid isolations, the quick plasmid preparation procedures of (i) McCormick et al. (1981) and (ii) Birnboim and Doly (1979) were employed.

Restriction fragments were isolated from gel slices by standard methods described in the literature (Smith, 1980; Lizardi et al., 1984).

Various species of RNA were isolated from a preparation of total nucleic acids by a combination of electrophoretic and chromatographic methods. The 16S and the 23S rRNA were separated from the DNA and low molecular weight RNAs by precipitation from 2M NaCl at 4°C. The two RNA species were then separated on a preparative 1.5% agarose gel. A load of 100 µg per 6 cm^2 was electrophoresed in 40 mM tris-acetate, 2 mM EDTA, pH 7.8, (TEA buffer) at 11 volts/cm until the orange G tracking dye was at least 12 cm from the origin. RNA bands, visualized under UV light (300 nm) after ethidium bromide staining, were excised and recovered by the electroelution method (Smith, 1980).

Transfer RNA and crude 5S rRNA were isolated from the soluble fraction of the 2 M NaCl by ethanol precipitation. To free these low molecular weight RNAs from contaminating large RNAs and DNA, a redissolved sample (approximately 200-500 µg) was passed through a 60 cm long Sephedex G-100 superfine column (1 cm in diameter), equlibrated and eluted in 1 M NaCl, 1 mM EDTA, 10 mM tris-HCl, pH 7.5. Transfer RNA used for 3' end labelling in the Southern cross experiment was isolated from the total nucleic acids using a small Whatman DEAE cellulose (DE-52) column, equlibrated in 0.1 M NaCl, 0.1 M tris-HCl, pH 8.0 and eluted with 1 M NaCl, 0.5 M tris-HCl, pH 8.0. Fractions were analysed by polyacrylamide gel electrophoresis. 2.5 volumes of 95% ethanol were added to the combined desired fractions to precipitate the RNA at -20°C.

In vitro ^{32}P-labelled intact 5S rRNA was isolated by the elution method of Smith (1980) from a preparative 12% polyacrylamide gel (acryl:bis, 38:2) cast in 8 M urea,

0.1 M TEB buffer (100 mM tris-borate, 2 mM EDTA, pH 8.3).

Plasmids and phage strains

Plasmids and phages used in this project are summarized in Table 2.

Table 2. Plasmids and phages used in this project are listed.

DNA	Marker	*E. coli* Host strain	Use	Reference
Plasmids				
pAT 153	Amp^r, Tc^r	HB101	construction of pHcr1	Twigg and Sherratt, 1980
pBR 322	Amp^r, Tc^r	HB101	size markers	Bolivar and Backman, 1979
pHcr1	Amp^r	HB101	carrying *H.c.* rRNA genes	this work
pUC 8	Amp^r, $LacZ^+$	JM83	subcloning pHcr1	Vieira and Messing, 1982
pUC 13	Amp^r, $LacZ^+$	JM83	subcloning pHcr1	Vieira and Messing, 1982
pHt4-11	Amp^r	HB101	transcriptional studies	Roy et al., 1982
Phages				
M13mp8	$LacZ^+$	JM103	subcloning pHcr1	Vieira and Messing, 1982
M13mp9	$LacZ^+$	JM103	subcloning pHcr1	Vieira and Messing, 1982
M13mp18	$LacZ^+$	JM103	subcloning pHcr1	Yanisch-Perron et al., 1985
M13mp19	$LacZ^+$	JM103	subcloning pHcr1	Yanisch-Perron et al., 1985
Lamda CI857		-----	size markers	

Enzymes and Reagents

Restriction endonucleases were purchased from Bethesda Research Laboratory, New

England Biolabs, P-L Biochemicals (Pharmacia), and Boehringer-Mannheim. Restriction endonuclease digestions were carried out under the conditions recommended by the manufacturers. Recognition sequences for these enzymes are summarized by Roberts (1985).

Radioisotopes ([a^{32}P]dATP, [a^{32}P]dCTP, [a^{32}P]GTP, [V^{32}P]ATP, [$5'^{32}$P]pCp all at >3000 Ci/mmol and [a^{35}S]dATP at >1000 Ci/mmol) were purchased from New England Nuclear (NEN) and Amersham. Non-radioactive reagents and enzymes used in DNA and RNA labelling reactions were obtained from PL (with the execption of DNase I for nick translation, which was purchased from Sigma). The ribonuclease T_1 used in two dimensional tRNA finger printing experiments was also obtained from Sigma.

Deoxy and dideoxy nucleotides, forward and reverse DNA primers, and the Klenow fragment of DNA polymerase for dideoxy-DNA sequencing were purchased from PL and BRL.

Nitrocellulose for Southern transfers, colony hybridizations and plaque lifts was supplied by Millipore and Schliecher and Schuell. The DBM paper for northern transfers was made according to the method of Alwine et al. (1979). NA-45 DEAE membrane for the isolation of restriction fragments from agarose gels was purchased from Schliecher and Schuell.

New England Nuclear supplied the HeLa cell in vitro transcription system. Transcription reactions were carried out under standard conditions (Manley et al., 1980; 1982).

Cloning of restriction fragments

Fifty µg of genomic DNA was digested with Bgl II and Hind III. Digested DNA was size fractionated on a 10 to 40% sucrose gradient (in 1 M NaCl, 20 mM tris-HCl, pH 7.5) centrifuged at 36000 rpm for 14 hours in a SW 40 rotor. 400 µl fractions were

collected. 40 µl aliquots of alternate fractions were electrophoresed on a 0.5% agarose gel. A Southern transfer of this gel was hybridized to ^{32}P-16S rRNA (labelled by the polynucleotide kinase method). Radioautography revealed the desired fractions which contained DNA fragments of approximately 11 kb in size. Pooled fractions were precipitated with 3 volumes of 95% ethanol.

The plasmid vector, pAT 153 was digested with Bam HI, Eco RV and Hind III. Eco RV cleaves the 346 bp excised Hind III-Bam HI fragment to minimize recircularization.

The ligation reaction was carried out at a total DNA concentration of 60 ng/µl at a molar ratio of 5:1, passenger to vector at 11°C for 12 hours (also see Weiss and Richardson, 1967). The ligation mixture was used to transform competent *E. coli* HB101 (Morrison, 1979). Insertion at the Bam HI and Hind III sites of pAT 153 inactivates the tetracycline resistance gene. Transformants were initially screened on L agar with ampicillin (50µg/ml). The ampicillin resistant colonies were then tested for tetracycline sensitivity. Amp^r Tet^s colonies were spotted on a sterile nitrocellulose disc (pore size .22 µm) and placed on L-agar with ampicillin. A replica of this filter was treated with 0.5 M NaOH, 1.5 M NaCl to lyse cells and to facilitate DNA binding to nitrocellulose. After neutralization (in 3 M NaOAc, pH 5), the filter was prepared for hybridization in the same manner as a Southern transfer.

Hundreds of ampicillin resistant, tetracycline sensitive transformants were screened by the colony hybridization technique (Hanahan and Meselson, 1980; Grunstein and Hogness, 1975) using ^{32}P-labelled 16S and 23S rRNAs as a probe.

Plasmid DNA was isolated from the hybridization positive colonies by the method of Birnboim and Doly (1979). Plasmid DNA samples were then electrophoresed on a 0.75% agarose gel for size determination. Purified recombinant plasmids of 14 to 15 kb were subjected to Southern hybridization analysis for confirmation using ^{32}P-16S rRNA as the

probe. Only one out of the fifty hybridization positive colonies screened actually contained the *H. cutirubrum* ribosomal rRNA genes. This plasmid is named pHcr1.

Certain regions of interest on pHcr1 were subcloned into the pUC and M13 vectors for DNA sequencing analysis. The following fragments were purified from agarose gels: a 2.7 kb Kpn I/Bam HI fragment, a 1.2 kb BamHI/Sal I fragment, a 4.5 kb Sma I fragment, a 1.2 kb and a 1.0 kb Pst I fragment. These fragments were separately mapped in detail and were further digested with selected four base specific restriction enzymes to generate suitable sized products for subcloning. Standard procedures for subcloning and screening were described by Messing (1983).

^{32}P-labelling of RNA

Labelling of RNA was achieved by two methods.

(a) 3'-OH end labelling (England et al., 1980)

About 2.5 times molar excess of [5'^{32}P]pCp to RNA was used. T_4 RNA ligase catalyzes the reaction in 50 mM Hepes (pH 7.5)- 20 mM $MgCl_2$- 4 mM dithiothreitol- 4µg/ml BSA, at 4 °C. The efficiency of the labelling was checked by PAGE, followed by radioautography (Laskey, 1980).

(b) 5' end labelling (Chaconas and van de Sande, 1980)

Heat degradation at 90°C in alkaline hydrolysis buffer (5 minutes for HMW RNA and 10 minutes for tRNA in 0.5 M Na_2CO_3 / $NaHCO_2$, pH 9) causes random chain breakage thus generating a variety of 5'-OH terminii for the T_4 polynucleotide kinase catalyzed reaction. It is important to know that *H. cutirubrum* 5S rRNA does not have a 5' terminal phosphate (Nazar and Matheson, 1978), therefore intact 5S rRNA can be labelled directly without alkali-heat degradation. A typical reaction involves a 20 minute incubation at 37°C of 12 pmoles of heat degraded RNA, 12 pmoles of [V^{32}P]ATP, in 10 mM

$MgCl_2$- 5 mM dithiothreitol- 75 mM tris-HCl, pH 7.2. The reaction is stopped by adding excess KH_2PO_4 followed by heat inactivation at 70°C for 3 minutes.

Labelling of DNA fragments

DNA fragments were labelled with ^{32}P by three methods.

(a) Nick translation (Kelly et al., 1970; Rigby et al., 1977)

The nick translation method is used to generate radioactive DNA probes. Random nicking of the double stranded DNA template by DNase I (Sigma) provides 3'-OH ends for DNA synthesis, and thus the incorporation of [$a^{32}P$]dNTP into the newly synthesized strand by the *E. coli* DNA polymerase I. Nick translated DNA was separated from dNTPs using a small Sephadex G50 column.

(b) Fill in (Klenow and Henningsin, 1970)

DNA restriction fragments having 3'-recessed ends (such as are generated by Bam HI, Hind III, Eco RI) can be labelled using the Klenow fragment of the *E. coli* DNA polymerase I. This enzyme adds nucleotides to the recessed 3'-OH terminus using the 5'-overhang of the opposite strand as a template. Incorporation of a radioactive substrate (eg. [$a^{32}P$]dATP) allows labelling of the DNA fragment at the desired position at each end. 3'-exonuclease activity allows flush ends to be labelled, as well as 3'-overhanging ends.

(c) Kinase labelling (Maxam and Gilbert, 1980)

Polynucleotide kinase transfers the V-phosphate of [$V^{32}P$]ATP to the 5'-OH terminus of the DNA fragment. 5'-OH termini must be generated by alkaline phosphatase treatment.

Mapping of DNA fragments

Mapping of a DNA fragment involved single and combined digests using a variety of restriction endonucleases. The digestion products were separated by agarose or

polyacrylamide gel electrophoresis and sized according to their mobility compared with known molecular weight markers. These digestion products were then ordered according to the size of the fragments generated by partial restriction digestions of the full length DNA fragment labelled at one end. A radioautograph of the gel separating these partially digested products reveals the distance of the cut sites from the labelled end, since only those products extending from the labelled end would be detected by radioautography. The locations of the restriction sites were then confirmed by repeating the partial digestion experiment with the full length DNA fragment labelled at both ends.

Electrophoresis and immobolization of DNA and RNA

Electrophoretic techniques were modified from those described by Edwin Southern (1979). Agarose gel electrophoresis of DNA samples was carried out in 40 mM tris-acetate, 2 mM EDTA, pH 7.8 (TEA buffer), at 5 volts per cm. Orange G was used as the tracking dye. The agarose concentration of the gels ranged from 0.35% to 1.5%. Gels used for 16S and 23S rRNA isolations were run at 11 volts per cm. Gel apparatus were constructed by Dr. K. L. Roy following the design by E. Southern (1979).

Polyacrylamide gels (PAG) of various concentrations and degree of cross-linkage were employed for the separation of fragments ranging from 50 to 1500 nucleotides in size. 5% PAG cross-linked at acryl:bis, 29:1, electrophoresed at 15 volts per cm in TEA buffer were commonly used to fractionate DNA samples. Strand separation gels consisted of 5% polyacrylamide cross-linked at acryl:bis, 30:0.6. They are cast and run at < 8 volts per cm in 0.1 M tris-borate and 2 mM EDTA, at pH 8.3 (0.1 M TEB buffer) as described by Maxam and Gilbert (1980).

Transfer RNA and 5S rRNA samples were separated on a 10 % PAG (acryl:bis, 38:2) at 15 volts per cm in 0.1 M TEB buffer.

5%, 6%, 8% and 20% polyacrylamide gels (cross-linked at acryl:bis, 38:2) were cast in 8.3 M urea and TEB at the concentration of the running buffer for DNA sequencing (Maxam and Gilbert, 1980; Sanger et al.,1978; Smith and Calvo, 1980). Gels (19 cm

wide x 85 cm tall x 0.25 mm thick) were pre-run at 70 Watts then electrophoresed at 75 Watts in 50 or 60 mM TEB buffer. Shorter gels (34 cm x 40 cm x 0.4 mm) were pre-run at 45 Watts and electrophoresed at 50 Watts in 0.1 M TEB buffer. Meter long gels (115 cm x 24 cm x 0.25 mm) were usually run at only 3000 to 5000 Volts due to the limited voltage output of the power pack. A 1/4 inch thick aluminum plate was placed over the front glass plate to provide even heat distribution.

Transfer and immobilization of denatured DNA and glyoxalated RNA (MacMaster and Carmichael, 1980) on to nitrocellulose membrane were described by Southern (1975). Electroblotting of RNA onto DBM paper (northern transfers) was performed as described by Wahl et. al. (1979).

Hybridization analysis

The conditions for both DNA-DNA and RNA-DNA hybridization were described by E. Southern (1975). Techniques used in the Southern cross experiments are described in the figure legend.

DNA sequencing

Both the chemical sequencing method (Maxam and Gilbert, 1977; 1980) and the enzymatic sequencing method (Sanger et al., 1977) were employed.

The chemical sequencing method, which requires DNA fragments labelled at one terminus, involves the random chemical modification of the bases in base specific reactions. The DNA strand is then cleaved at the modified base by a piperidine catalyzed beta-elimination reaction. The sequencing reactions were carried out as described by Maxam and Gilbert (1980) with some of the modifications described by Smith and Calvo (1980) and Rubin and Schmid (1980).

The enzymatic sequencing method, which utilizes DNA fragments subcloned into M13 or pUC vectors, involves chain extension from a 17 base DNA primer annealed to the vector sequence just upstream from the subcloned fragment. The chain extension is

terminated by the incoporation of a dideoxynucleotide in four independent base-specific reactions (Sanger, Nicklen and Coulson, 1977). In certain reactions deoxyguanosinetriphosphate was replaced by deoxyinosinetriphosphate to reduce interference with size fractionation on polyacrylamide gel due to the formation of very stable secondary structures. In some reactions, the lower energy [$a^{35}S$]dATP was employed (instead of [$a^{32}P$]dATP) to give better resolved images on radioautograms.

Computer programs for compiling sequencing data

Programs written by Staden (1978; 1979; 1980a; 1980b) and Delany (1982) were obtained. Unfortunately, the adaptation of these programs to the MTS system was only partially completed and therefore not yet completely functional. Staden's *Transrp* program for the translation of open reading frames (ORF) was operational. It was used to translate the ORF downstream from the 5S rRNA gene. The Bionet system was essential in the allignment of the 23S rRNA sequences from *E. coli* and *H. cutirubrum* .

In vitro transcription

In vitro transcription experiments using a HeLa cell lysate were carried out under conditions recommended by the manufacturer (NEN), adapted from a procedure described by Manley et al. (1980; 1982).

Radioautography

Kodak X-OMAT AR X-ray film was used for radioautography. Exposures were enhanced fluorographically by Dupont Cronex Lightning Plus and Par Speed intensifying screens at -90°C (Laskey,1980). Gels containing ^{35}S-labelled DNA fragments were first fixed in 10% acetic acid followed by 15% ethanol then dried onto Whatman 3MM paper before radioautography.

RESULTS

Gene organization

Southern transfers of electrophoretically separated genomic DNA digests were separately hybridized to radioactively labelled rRNAs and mixed tRNAs. The hybridization patterns revealed a single copy of 16S, 23S and 5S rRNA genes clustered and arranged in that order. DNA sequence analysis of the rRNA gene cluster revealed two associated transfer RNA genes. The rRNA genes on the cloned DNA fragment (see below) were first located by RNA-DNA hybridizations before beginning DNA sequence analysis.

Cloning and mapping of rRNA genes

An 11 kilobase Bgl II/ Hind III fragment containing all the rRNA genes was defined by rRNA-DNA hybridization analysis. This fragment was cloned into the Bam HI and Hind III sites of the plasmid vector pAT 153 as described in Material and Methods (also see Figure 1). This plasmid is named pHcr1 (for plasmid *Halobacterium cutirubrum* rRNA, the first).

A restriction map of pHcr1 was constructed (see Figure 2). The method for mapping is explained in Material and Methods. Restriction sites were located according to their distance from a number of fixed points (generated by labelling of the Bam HI, Eco RI, Eco RV and Hind III sites in independent experiments). To confirm the locations of various restriction sites, the sizes of various single and combined digestion products were compared to their sizes predicted by the map. Detailed physical maps of regions used for DNA sequencing analysis are given along with the sequencing strategies below.

Primary sequence determination

The nucleotide sequence of the *H. cutirubrum* 23S rRNA gene, including the 5'- and

Figure 1. A schematic diagram to summarize the cloning of the 11kb Hind III/ Bgl II fragment containing the *H. cutirubrum* rRNA gene cluster. Experimental conditions are described in Materials and Methods.

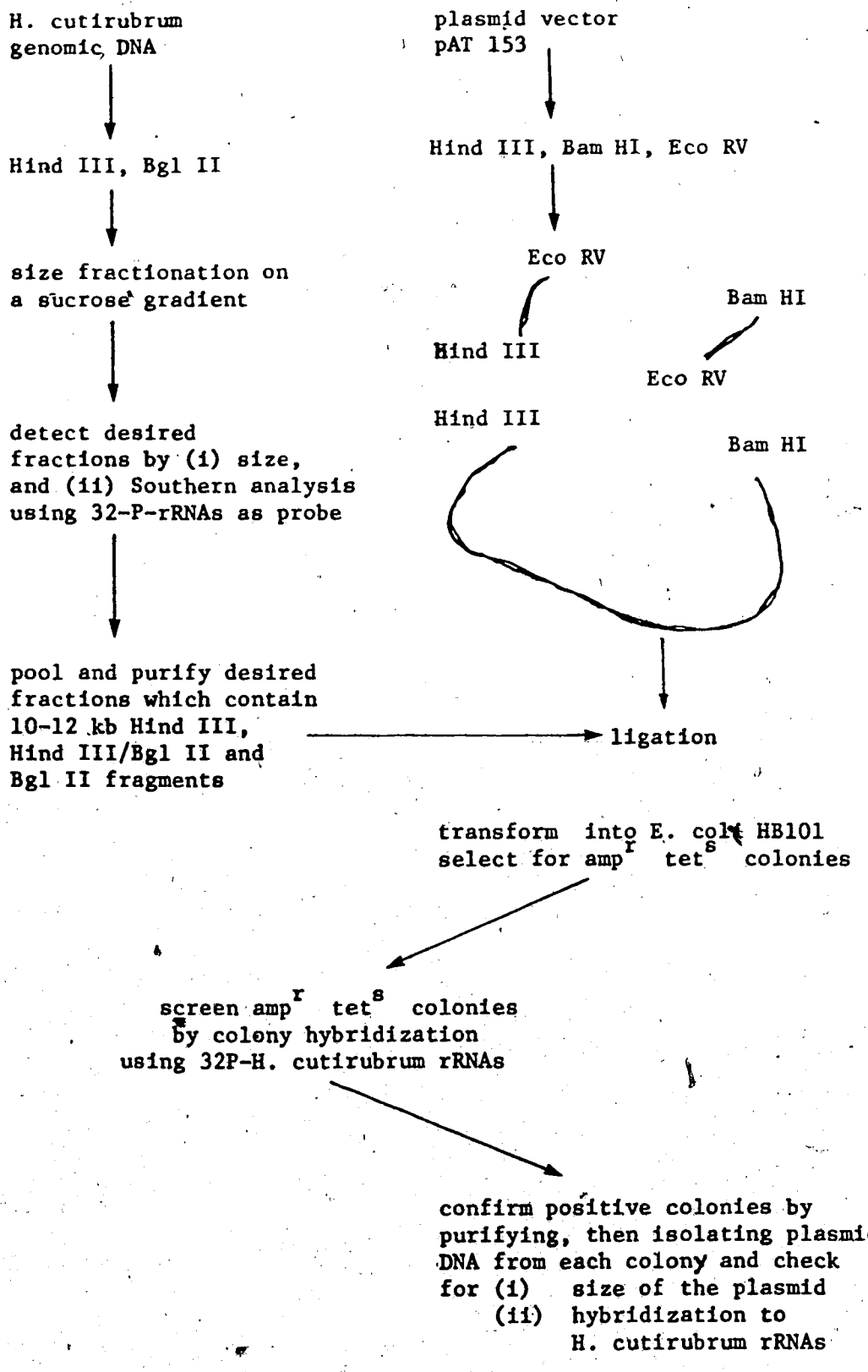
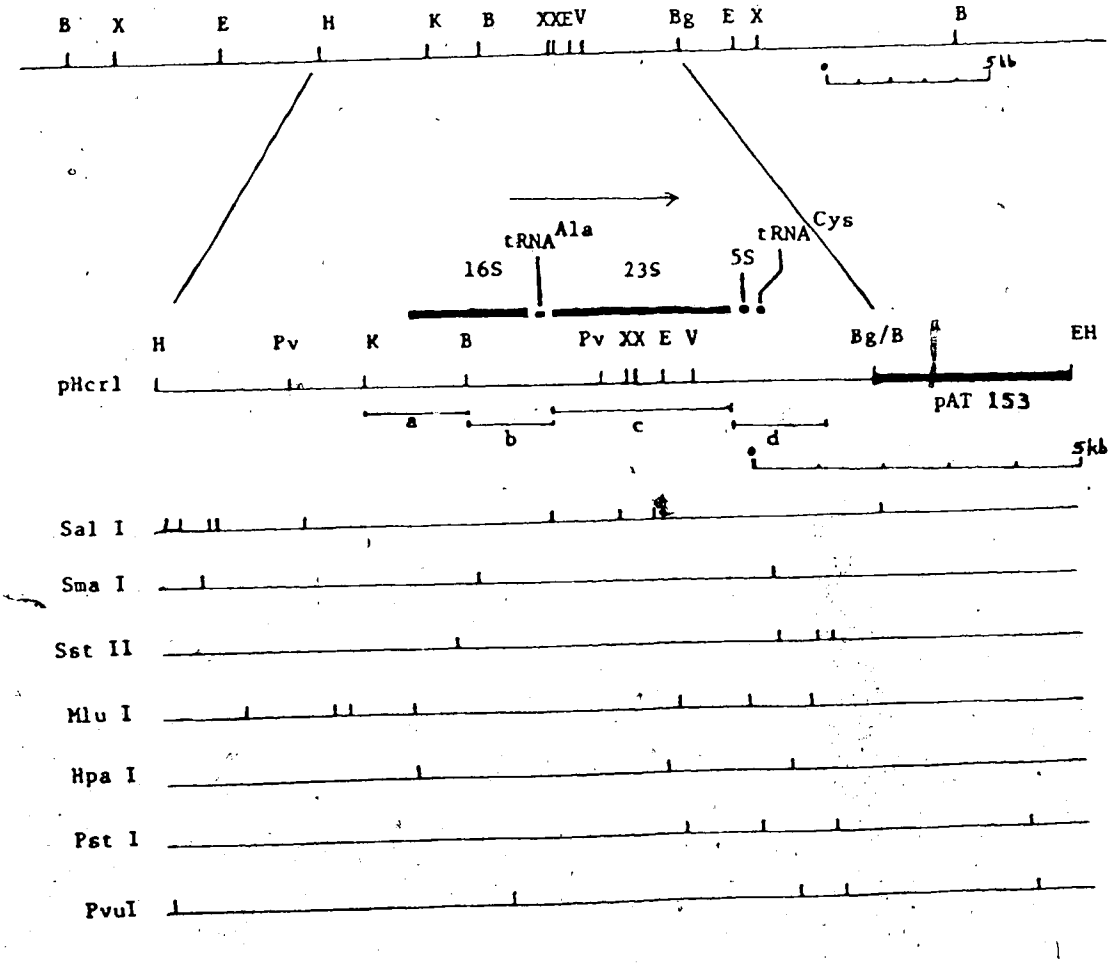

H. cutirubrum genomic DNA
Hind III, Bgl II
size fractionation on a sucrose gradient
detect desired fractions by (i) size, and (ii) Southern analysis using 32-P-rRNAs as probe
pool and purify desired fractions which contain 10-12 kb Hind III, Hind III/Bgl II and Bgl II fragments
plasmid vector pAT 153
Hind III, Bam HI, Eco RV
Eco RV
Hind III
Bam HI
Eco RV
Hind III
Bam HI
ligation
transform into E. coli HB101
select for amp^r tet^s colonies
screen amp^r tet^s colonies by colony hybridization using 32P-H. cutirubrum rRNAs
confirm positive colonies by purifying, then isolating plasmid DNA from each colony and check for (i) size of the plasmid
(ii) hybridization to H. cutirubrum rRNAs

Figure 2. A physical map of the recognition sites of hexanucleotide specific restriction endonucleases. The locations of some restriction sites near the rRNA cluster on the *H. cutirubrum* chromosome are indicated on the top line. The 11 kb Hind III/ Bgl II fragment is expanded from the top line. The regions sequenced are indicated by a, b, c, and d. The location of the rRNA genes, as well as the two tRNA genes found associated with this gene cluster, are indicated on the expanded line. Some of the restriction endonuclease cleavage sites on the Hind III/ Bgl II fragment are alligned below the expanded line. The direction of transcription is indicated by an arrow. The abbrevations for restriction sites are as follows: B= Bam HI, Bg= Bgl II, E= Eco RI, H= Hind III, K= Kpn I, Pv= Pvu II, V= Eco RV and X= Xho I.


B X E H K B XXEV Bg E X B
5kb
tRNA Ala
16S
23S
5S
tRNA Cys
H Pv K B Pv XX E V Bg/B EH
pHcrl
pAT 153
a
b
c
d
5kb
Sal I
Sma I
Sst II
Mlu I
Hpa I
Pst I
PvuI

the 3'- flanking regions has been determined (covering most of regions b, c and d in figure 2). The strategy for DNA sequence determination of the 16S-23S spacer region is summarized in figure 3. Figure 3 also shows the sequencing strategy for the 23S rRNA gene and its 3' flanking region. The restriction fragments used in DNA sequence analysis are cataloged in Table 3.

Table 3. Properties of the restriction fragments used in the determination of the 5 kb of DNA sequence downstream from the Bam HI site are summarized below. The fragment numbers correspond to those indicated on figure 3. The following abbrevations are used: CH= chemical chain cleavage method, D= dideoxy chain termination method, CS= coding strand, NCS= non-coding strand.

fragment number	description	size (in kb)	method of sequencing	M13 vector	strand sequenced
1	BamSal2.7	2.7	CH	------	NCS
2	BamSal1.2	1.2	CH	------	CS
3	SauBamSal8	0.52	D	mp8	CS
4	SauKpnBam6	0.9	D	mp8	NCS
5	SauKpnBam7	0.9	D	mp9	CS
6	SauBamSal13	0.52	D	mp8	NCS
7	SauBamSal3F	0.22	CH	------	NCS
8	SauBamSal3S	0.22	CH	------	CS
9	SauBamSal7	0.20	D	mp8	NCS
10	SauBamSal2	0.20	D	mp8	NCS
11	SauBamSal3	0.20	D	mp8	NCS
12	SauBamSalHinf	0.19	CH	------	CS
13	SauBamSalHaeL	0.12	CH	------	CS
14	SauBamSalHaeS	0.10	CH	------	NCS
15	SauBamSal11	0.20	D	mp8	CS
16	SauBamSal12	0.31	D	mp8	NCS
17	SalBamSau	0.31	CH	------	NCS
18	Sal8-4	1.08	D	mp8	NCS
19	SauSma2-85b	0.66	D	mp18	NCS
20	SauSal5-1,5,10	0.66	D	------	NCS
21	SauSma2-18	0.66	D	mp18	NCS
22	PvuBam	2.2	CH	------	NCS
23	SauSal5Taq	0.34	CH	------	NCS
24	SauSal5-9	0.66	D	------	CS
25	Sal8-8	1.04	D	mp8	CS
26	SauSma2-44	0.45	D	mp18	NCS
27	PvuEcoRI8-2	0.84	D	mp8	NCS
28	SauSma2B-37	0.17	D	mp8	NCS
29	Sal8-5	0.49	D	mp8	NCS
30	Xho8-1	0.19	D	mp8	NCS
31	SauSma2B-23	0.17	D	mp18	NCS
32	Xho8-6	0.19	D	mp8	CS

Table 3 continues.

fragment number	description	size (in kb)	method of sequencing	M13 vector	strand sequenced
33	Sal8-17	0.49	D	mp8	CS
34	SauSma2-51,54,67	0.17	D	mp18	NCS
35	SauSma2-19	0.23	D	mp18	NCS
36	EcoRIPvu9-3	0.84	D	mp9	CS
37	EcoRIXho19-1	0.84	CH	------	NCS
39	SauSma2B-79b	0.05	D	mp18	NCS
40	EcoRIEcoRV	0.55	CH	------	CS
41	EcoRIEcoRV9-4	0.55	D	mp9	NCS
42	SauSma2-69	0.33	D	mp18	NCS
43	EcoRIEcoRV8-6	0.55	D	mp8	CS
44	SauSma2-79a	0.33	D	mp18	CS
45	Pst3-8-1	1.17	D	mp8	NCS
46	HinP9-17	0.80	D	mp9	NCS
47	EcoRVSma9-23	1.11	D	mp9	NCS
48	MluPst3CfoL	0.55	CH	------	NCS
49	HinP9-5	0.80	D	mp9	CS
51	EcoRVSma9-24	1.11	D	mp9	CS
50	MluPst3CfoS	0.25	CH	------	CS
52	Pst3-8-5	1.17	D	mp8	CS
53	SmaSal9-4	1.88	D	mp9	CS
54	Pst4-9-5	1.08	D	mp9	NCS
55	Pst4-9-1	1.08	D	mp9	CS
56	HaePst3-102	0.25	D	mp9	NCS

The DNA sequence of the spacer region (including the $tRNA^{Ala}$ gene) is given in figure 4. Figure 5 shows the complete nucleotide sequence of the 23S rRNA gene. The sequence downstream from the 23S rRNA gene, including the 5S rRNA gene and the $tRNA^{Cys}$ gene, as well as an open reading frame, is presented in figure 6. All DNA sequences shown are given as the non-coding (or the RNA transcript-like) strand.

The nucleotide sequence determination of the 16S rRNA gene and its promoter region (denoted as region a in figure 2) is partially completed. The abortion of this sequencing project was due to the publication of the sequence of interest by Hui and Dennis (1985). The partial sequence data obtained is not presented.

Secondary structure derivation

Figure 3. The strategy to sequence the *H. cutirubrum* 23S rRNA gene and its flanking regions. This figure deals with regions b, c, and d in figure 2. Arrows indicate direction and extent of the sequences of each DNA fragment analysed. Descriptions of the analysed fragments and the method of analysis are given in Table 3. A physical map is included to define the location of the arrows, in relation to the genes. Abbrevations for the restriction endonuclease recognition sites are: B= Bam HI, E= Eco RI, H= Hpa I, M= Mlu I, P= Pst I, Pv= Pvu II, V= Eco RV, S= Sau 3AI, Sa= Sal I, Sm= Sma I, Ss= Sst II and X= Xho I. Only two of many Hin PI sites (denoted as Hp) in this region are indicated.

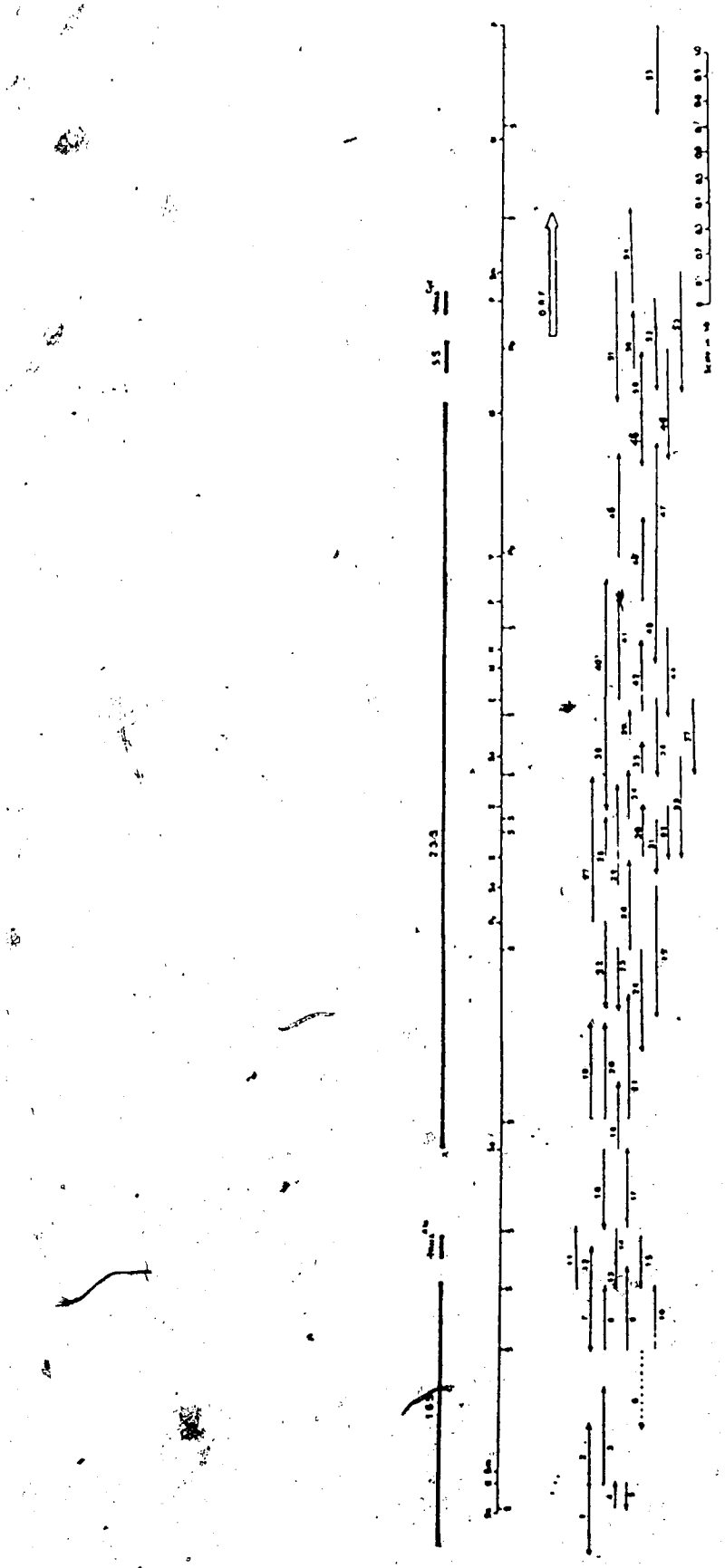
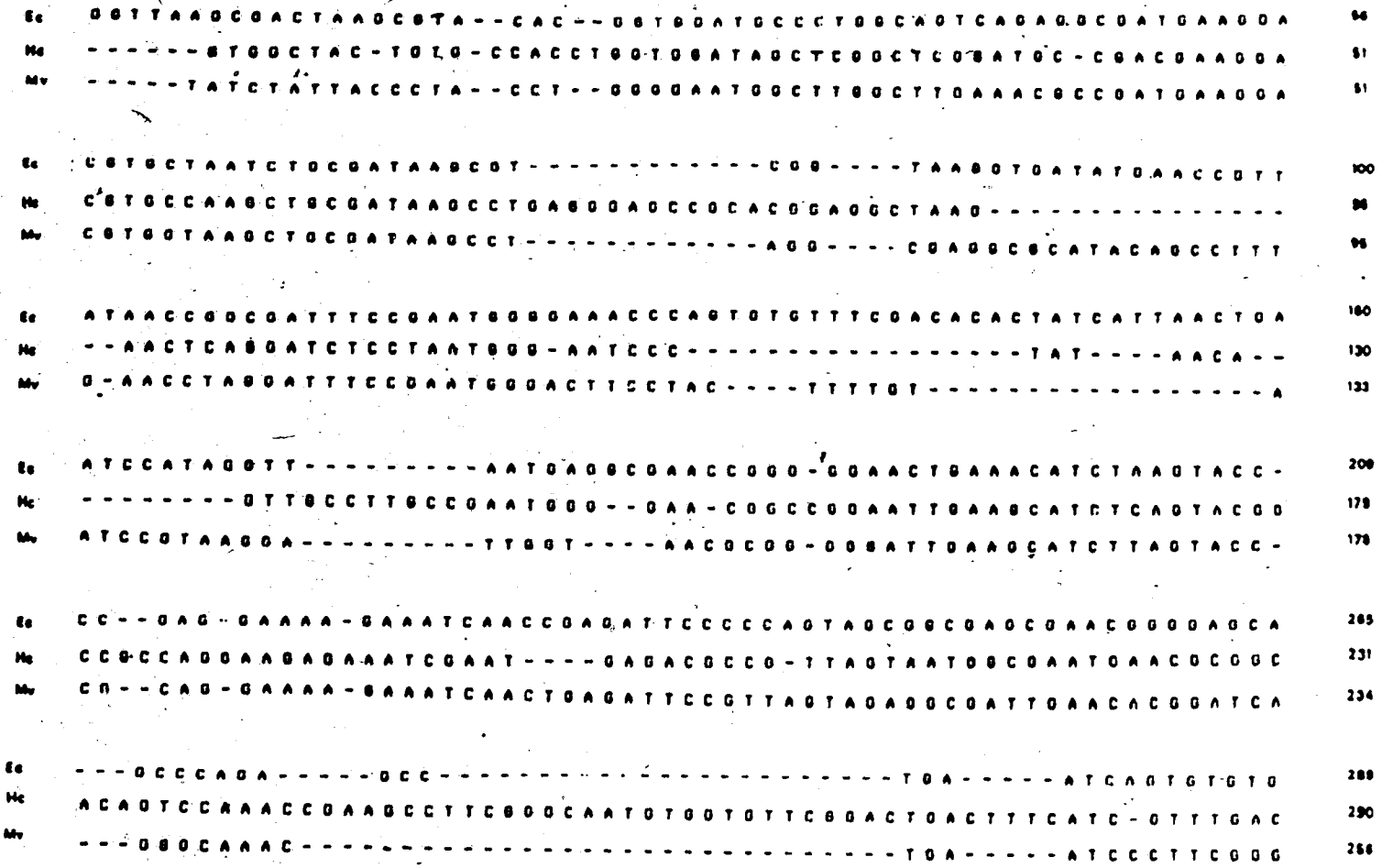
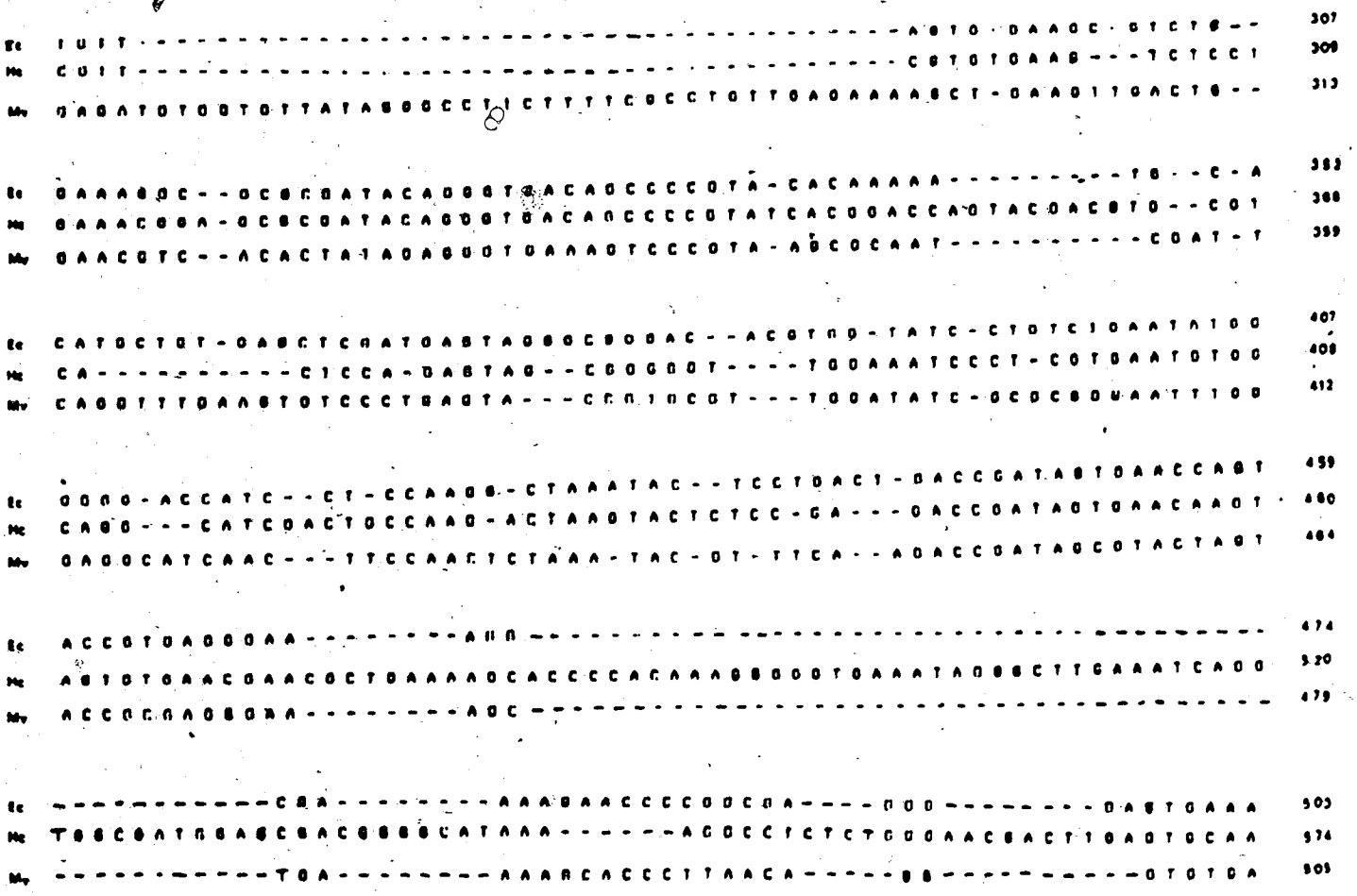


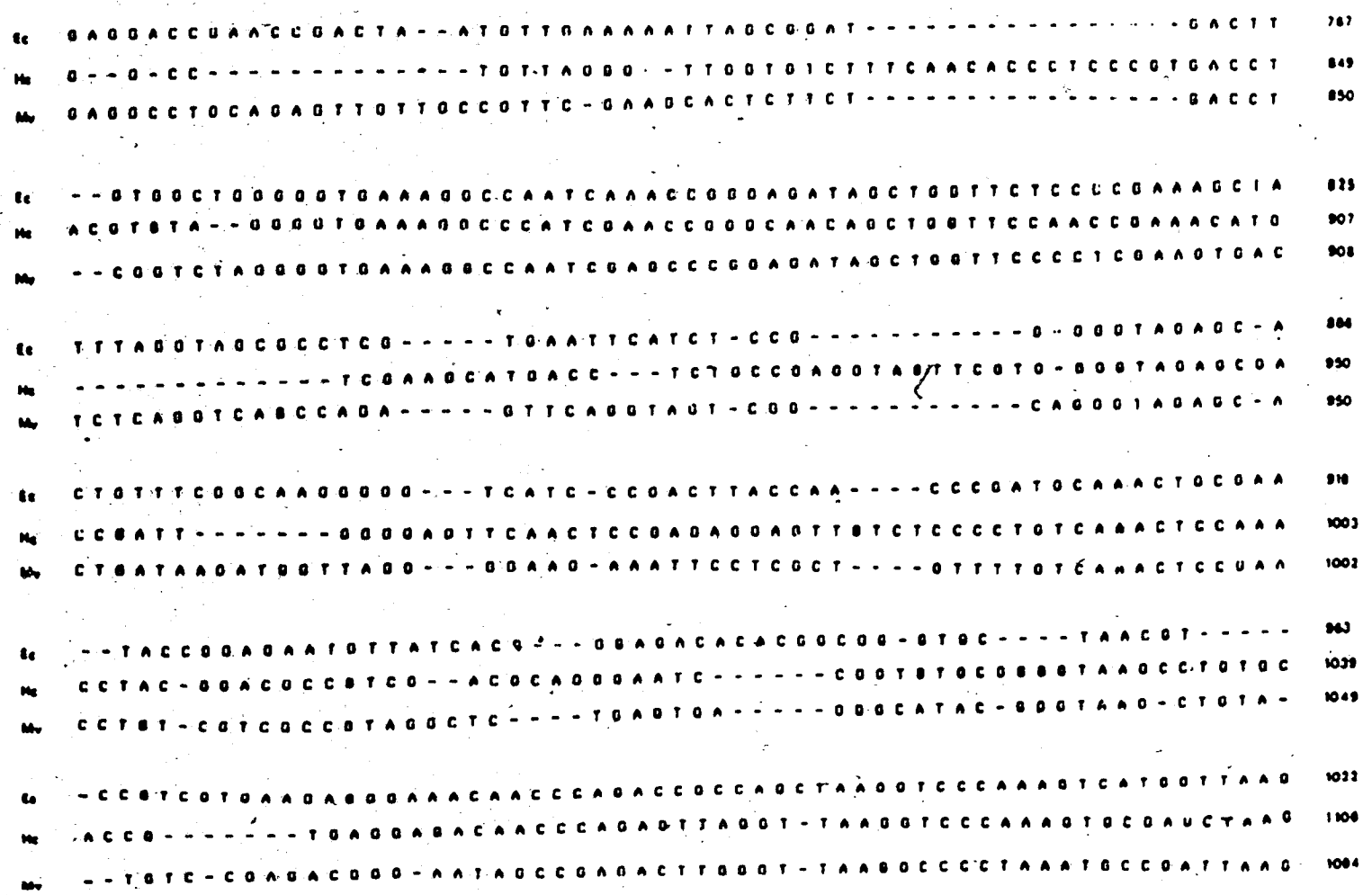
Figure 4. Nucleotide sequence of the 16S/23S rRNA gene spacer (region b in figure 2). This sequence is compared to the coressponding region in *M. vannielii* (Jarsch and Bock, 1985a). Arrows (→) define the direction of transcription. The pentanucleotide direct repeats are indicated by (→).

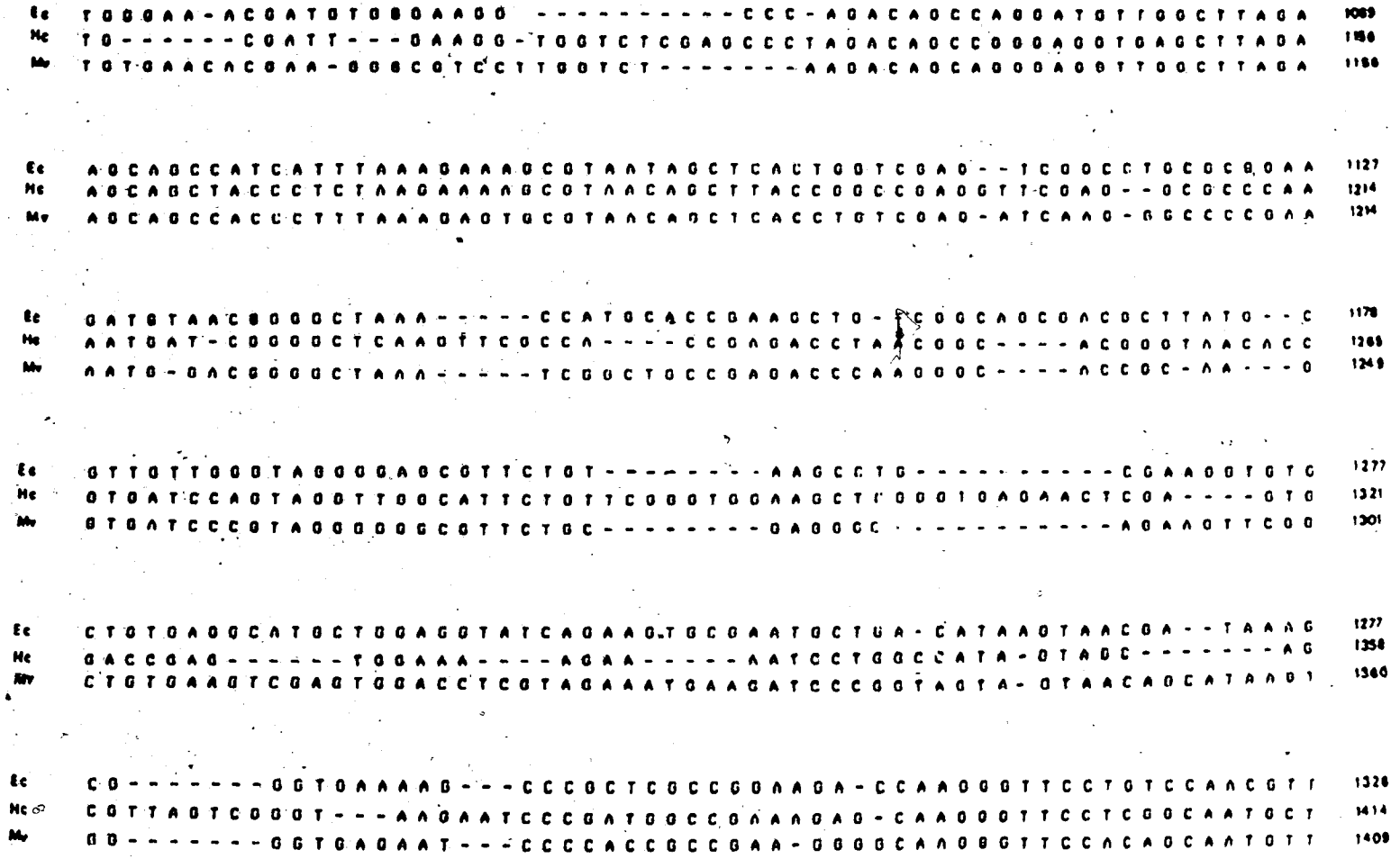

3' end of 16S rRNA gene
alanine tRNA gene
5' end of 23S rRNA gene

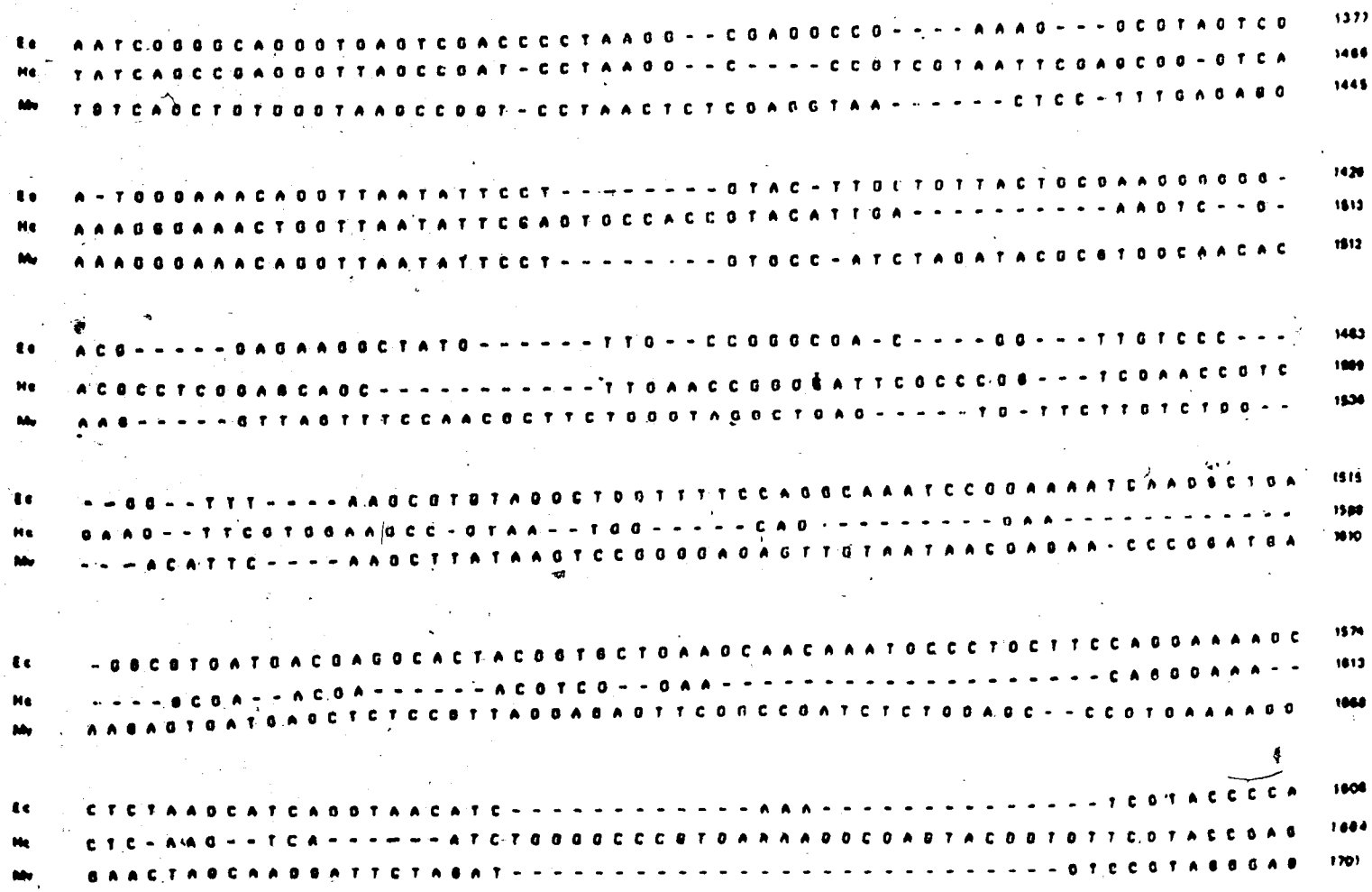
Figure 5. Nucleotide sequence of the non-coding strand of the *H. cutirubrum* 23S rRNA gene (region c in figure 2). This sequence (middle line) is compared to that of *E. coli* (top line) and *M. vannieii* (bottom line). See table 1 for the source of gene sequences.

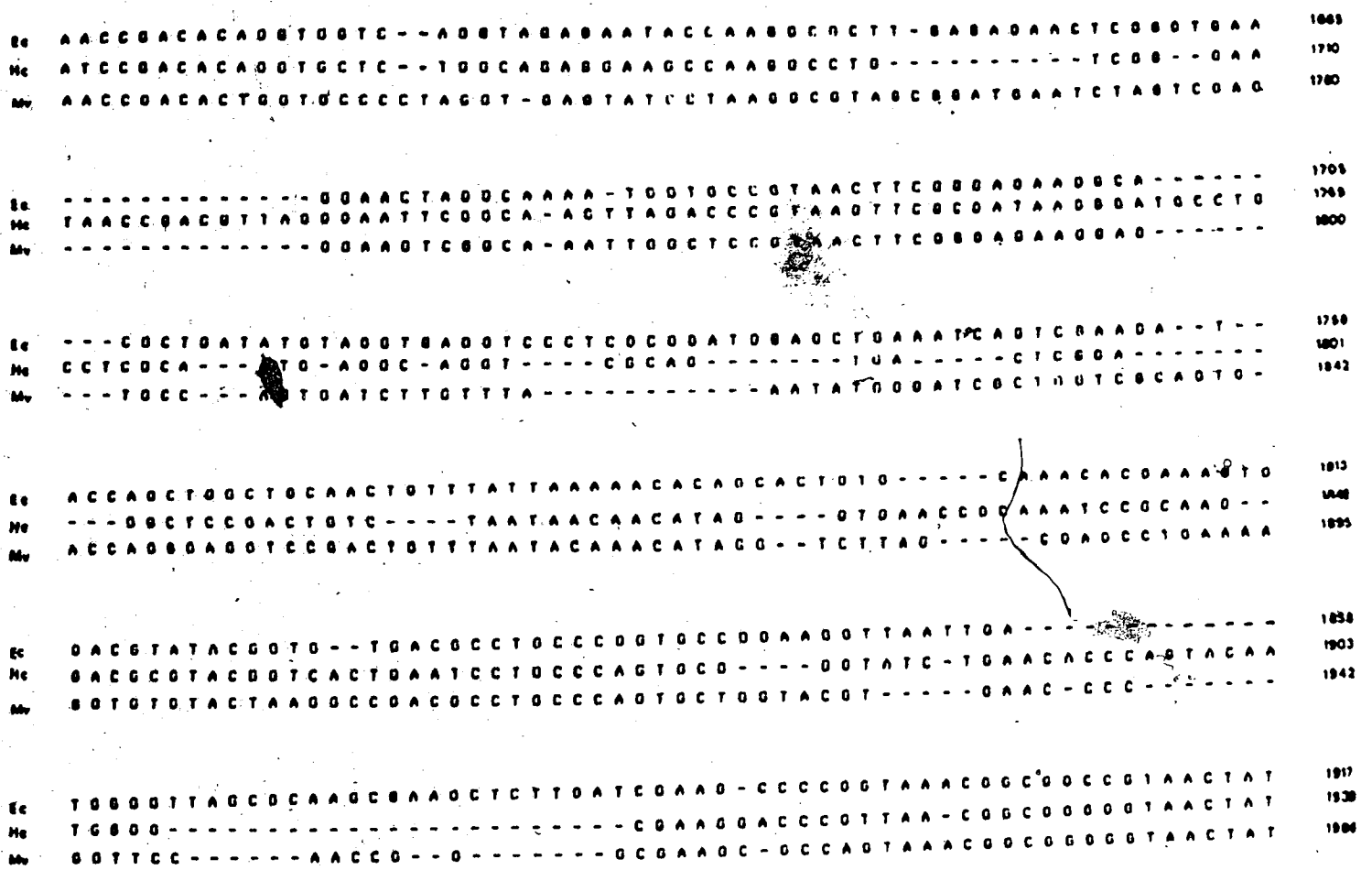


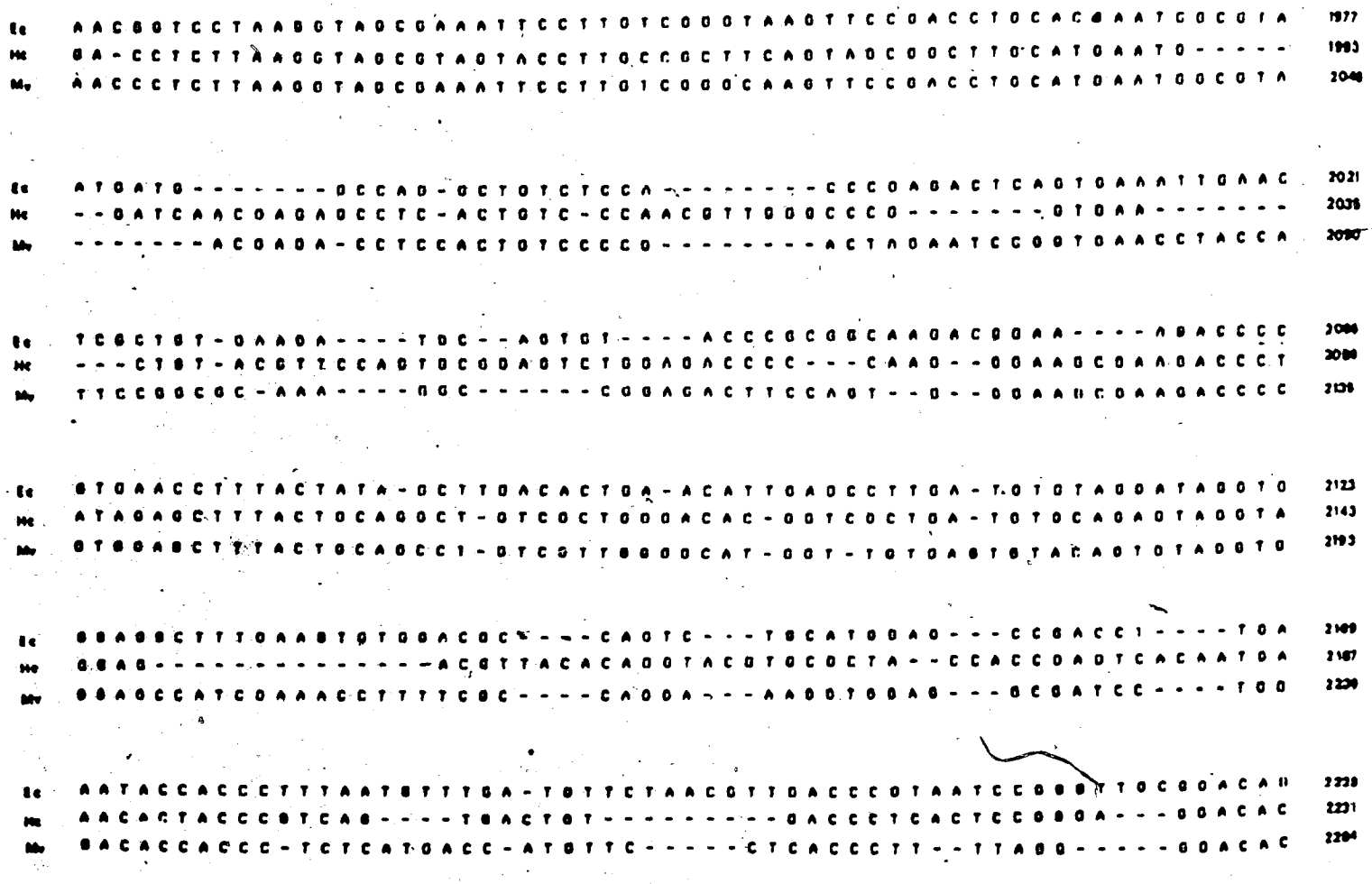


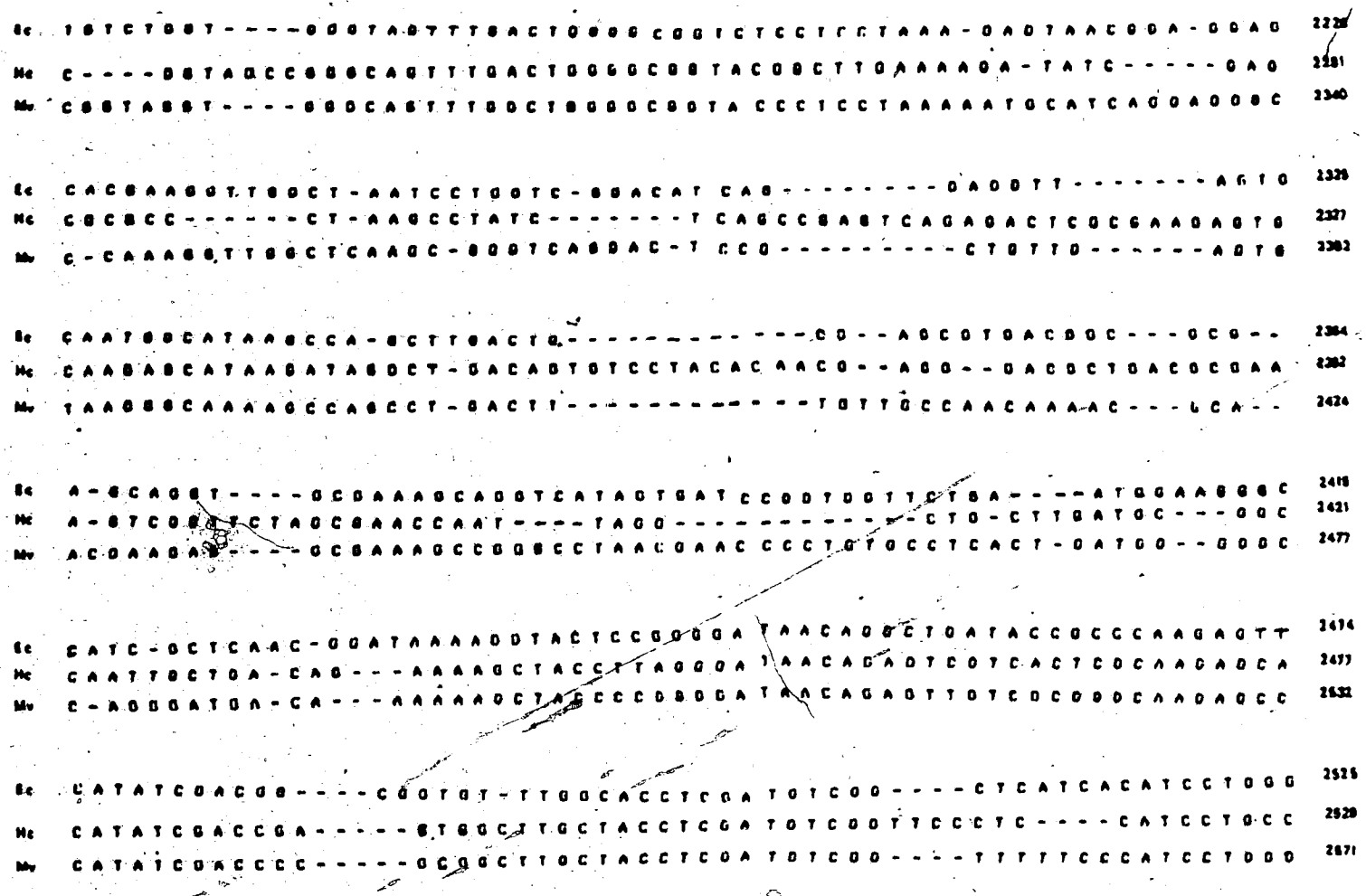


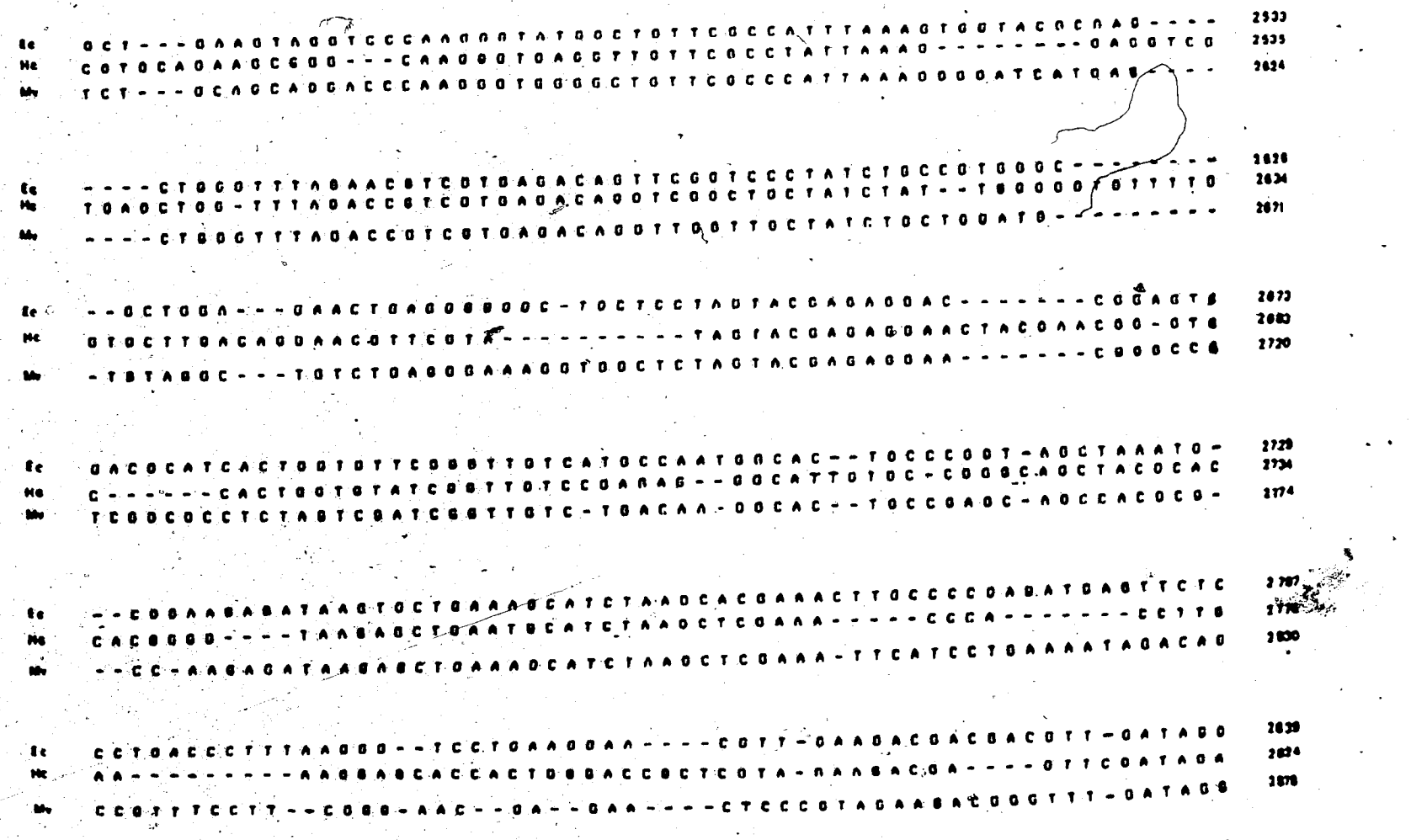












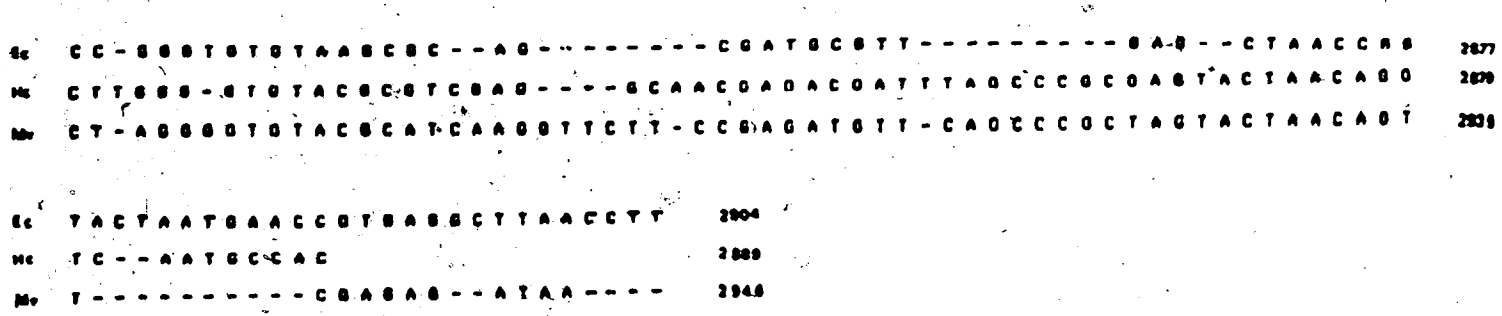
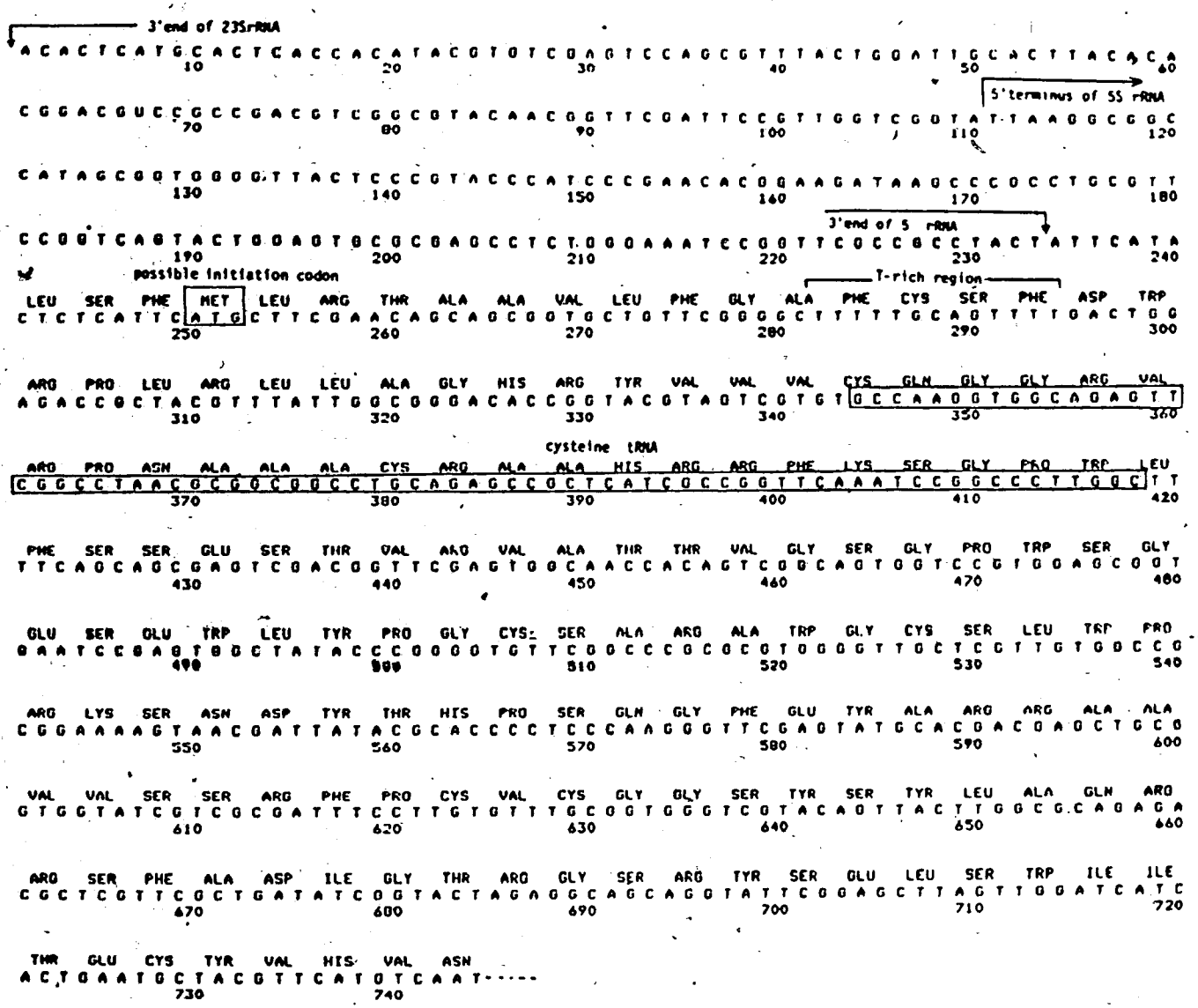

Sc CC-GGGTGTGTAAGCGC--AG---------CGATGCGTT---------GA-G--CTAACCAG 2877
Hc CTTGGG-GTGTACGCGTCGAG----GCAACGAGACGATTTAGCCCGCGAGTACTAACAGG 2879
Mv CT-AGGGGTGTACGCATCAAGGTTCTT-CCGAGATGTT-CAGCCCGCTAGTACTAACAGT 2835
Sc TACTAATGAACCGTGAGGCTTAACCTT 2904
Hc TC--AATGCCAC 2889
Mv T----------CGAGAG--ATAA---- 2848

Figure 6. Nucleotide sequence of the 3'-flanking region of the 23S rRNA gene. The locations of the 3'-terminus of the 23S rRNA gene, the 5S rRNA gene, the $tRNA^{Cys}$ gene are indicated. The amino acid sequence of the open reading frame is superimposed.


3'end of 23SrRNA
5'terminus of 5S rRNA
3'end of 5 rRNA
possible initiation codon
T-rich region
cysteine tRNA

The sequences of the rRNAs and tRNAs were derived from their DNA sequences. The RNA sequences were then analysed for regions capable of intra-molecular Watson and Crick basepairing. The stem and loop structures formed were then arranged according to the existing secondary structral models for eubacterial rRNAs and tRNAs. The secondary structures for the 23S rRNA, the 5S rRNA and the two tRNAs are given in figure 9, 15 and 7 respectively.

Gene expression

In vitro transcription of pHcr1 in a eukaryotic system (HeLa cell extract) showed some high molecular weight transcripts as well as a tRNA sized product (see figure 21A). The transcription of this tRNA sized product was not inhibited by a-amanitin. This transcript hybridized to the Sma I/ Sal I, 1.2 kb fragment (see figure 21C), locating its coding sequence to the intercistronic spacer between the 16S and the 23S rRNA genes. Using this tRNA sized transcript as a probe, no hybridization to the $tRNA^{Cys}$ coding region, downstream from the 5S rRNA gene, was observed. The identity of this tRNA transcript was analysed by two dimensional fingerprinting (data not shown).

There was no functional in vitro transcription system for *Halobacterium* avaliable. The expression of the cloned rRNA genes in *H. cutirubrum* was demonstrated by northern hybridizations. Figure 22 shows the result of the hybridization of pHcr1 to the northern transfers of total cellular nucleic acids obtained from (i) *H. cutirubrum* (ii) *E. coli* HB101 habouring pHcr1 and (iii) *E. coli* HB 101 carrying pHt4-11 as a control. The recombinant plasmid pHt4-11, has a 620 basepair Hind III fragment inserted (Roy et al.,1982). Both pHt4-11 and pHcr1 are pAT 153 derivatives. In pHt4-11, the insert contains a human leucine tRNA gene rather than *Halobacterium* DNA. Hybridizing RNA bands corresponding to the sizes of *H. cutirubrum* rRNAs were observed only in the sample of total *H. cutirubrum* RNAs.

Hybridization of a *H. cutirubrum* tRNA mixture to the coding regions for both the

$tRNA^{Ala}$ and the $tRNA^{Cys}$ on pHcr1 was observed. The reciprocal experiment, using pHcr1 to probe the northern transfer of *H. cutirubrum* low molecular weight RNAs also yielded hybridization. In order to demonstrate the expression of both tRNAs in *H. cutirubrum* unambiguously, the Southern cross experiment was employed. This experiment gave a two-dimensional hybridization pattern of (i) [5'-^{32}P]- pCp labelled low molecular weight RNAs from *H. cutirubrum* to (ii) DNA fragments generated by an Mlu I digestion of pHcr1. The DNA fragment containing both the 5S rRNA gene and the cysteine tRNA gene showed hybridizing crosses corresponding to the locations of the two RNA species. The DNA fragment containing the alanine tRNA gene showed one hybridizing cross at the tRNA region (see figure 20).

DISCUSSION

Gene Organization

The organization of the single cluster of rRNA genes in *H. cutirubrum* conforms to the eubacterial arrangement, which is also true for all the halophilic archaebacteria studied so far. This result is consistent with that described by Hui and Dennis (1985). The association of tRNA genes with the rRNA gene cluster is also consistent with the eubacterial scheme. The location of these rRNA genes in the *H. cutirubrum* genome (in relation to other loci such as the replicative origin) remains unknown.

Primary sequence

The comparison of *H. cutirubrum* 23S rRNA sequence with the *E. coli* sequence (Brosius et al., 1980) showed significant divergence. The alignment of the two sequences is given in figure 5. A number of deletions and insertions in the *H. cutirubrum* sequence have to be proposed to allow reasonable alignment with the *E. coli* sequence. A 59% homology is shared by the two sequences. Interestingly, the *M. vannielii* 16S and 23S rRNA both share 60% sequence homology with the *E. coli* sequences as well (Jarsch and Bock, 1985a; 1985b). The 23S rRNA from the halophile is 53 % homologous with that of the methanogen. *E. coli* 23S rRNA shares 79%, 75% and >70% sequence homologies with its eubacterial counterparts in *A. nidulans* (Douglas and Doolittle, 1984), *B. stearothermophilus* (Kop et al., 1984b) and *B. subtilus* (Green et al., 1985) respectively. The consistency in the % sequence homology in their 23 rRNA genes with *E. coli* (i) supports sequence homology as an accurate measure of phylogenetic relatedness (ii) reflects the evolutionary divergence between eubacteria and the archaebacteria. The low degree of sequence homology (53%) between the methanogen and the halophile may reflect evolutionary divergence within the archaebacteria.

16S/23S spacer

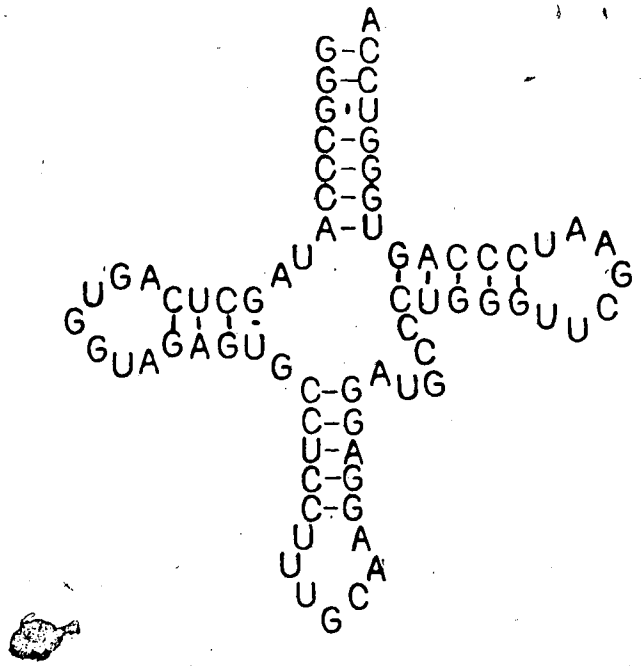
The 16S and 23S rRNA coding sequences are separated by a 508 base pair spacer. The spacer sequence is presented in Figure 4. In the overall transcript, the spacer sequence contains the descending strand of the 16S rRNA stem duplex and the ascending strand of the 23S stem duplex. These duplexes are formed by the long inverted repeats bracketing the sequences for the two mature rRNAs.

The coding sequence for an alanine tRNA has been located in this spacer. Alanine and isoleucine tRNAs are often found in such spacers in eubacteria and plastids (Young et al., 1979; Williamson and Doolittle, 1983; Loughney et al., 1982; Grey and Doolittle, 1982; Takaiwa and Sugiura, 1982; Koch et al. 1981; Fournier and Ozeki, 1985). The sequence of this tRNA gene is indicated in figure 4. Figure 7A shows the secondary structure of the $tRNA^{Ala}$. The sequence and the structure of this tRNA is consistent with those presented by Hui and Dennis (1985).

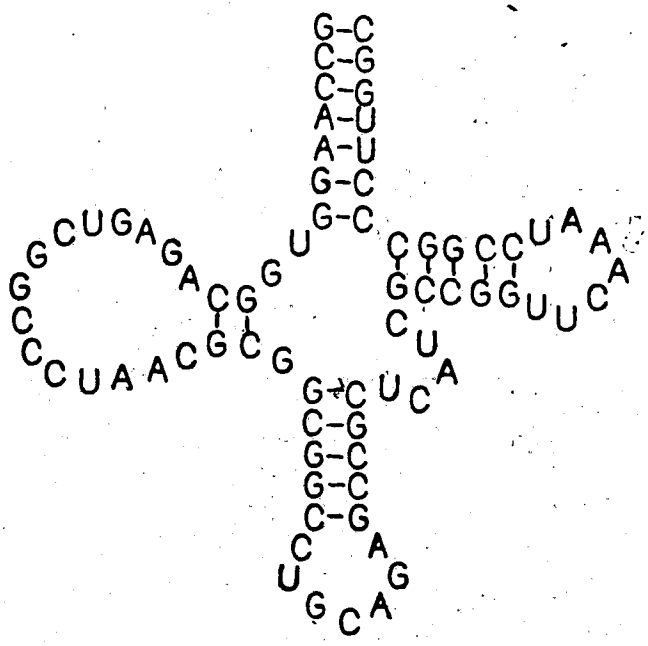
Comparison of the *H. cutirubrum* spacer sequence with its counterpart in *M. vannielii* reveals no significant homology, execpt for the tRNA coding region. Figure 4 shows the alignment of the spacers. Jarsch and Bock (1983b) observed a pentanucleotide, CTTTA, five basepairs before and immediately after the *M. vannielii* spacer $tRNA^{Ala}$ gene. Such direct repeats are found adjacent to the *H. cutirubrum* spacer tRNA gene as well. The sequence TCGGG is located one basepair before and three basepairs after the alanine tRNA gene. Pentanucleotide direct repeats are not identified in the sequences surrounding the $tRNA^{Cys}$ gene, located 110 basepairs down stream from the rRNA gene cluster.

Examination of the tRNA gene-containing spacer sequences from a number of eubacteria has revealed the occurrence of such pentanucleotide direct repeats. The *E. coli* rrn D and rrn X operons both contain an isoleucine tRNA gene and an alanine tRNA gene in their 16S/23S spacer (Young et al. 1979). The pentanucleotide CTACA overlaps the first nucleotide of the $tRNA^{Ile}$ gene and occurs again 31 basepairs downstream from the

Figure 7. Secondary structures of two *H. cutirubrum* tRNAs: The sequences and secondary structures of the alanine tRNA and the cysteine tRNA were derived from the two tRNA genes on pHcr1.



tRNA^{Ala}



tRNA^{Cys}

gene. Similarly the sequence GTTTT is found 13 basepairs upstream and 58 basepairs downstream from the $tRNA^{Ala}$ gene. Another sequence, GCAAA is located 27 basepairs before and 40 basepairs after this $tRNA^{Ala}$ gene. In *Bacillus subtilis* , direct repeats of the sequences TATAA and TCTTA are found near the termini of the isoleucine spacer tRNA gene. Another pentanucleiotide, ATAAC, is situated immediately before and twenty basepairs after the spacer $tRNA^{Ala}$ gene (see Loughney et al. 1982 for the complete spacer sequence). Examination of the spacer sequence in *A. nidulans* (sequence determined by Williamson and Doolittle 1983) revealed the pentanucleotide sequence TTTTC which lies 34 basepairs before the spacer $tRNA^{Ile}$ gene and 43 basepairs downstream from the adjacent $tRNA^{Ala}$ gene. This sequence also occurs in between these two tRNA genes, six basepairs from the 3'-terminus of the $tRNA^{Ile}$ gene and 22 basepairs from the 5'-terminus of the $tRNA^{Ala}$ gene.

Five or nine nucleotide direct repeats are often found as terminal sequences of insertion sequences and transposons (Kleckner, 1981; Berg and Berg, 1983). The implication of having pentanucleotide direct repeats bracketing spacer tRNA genes in tRNA gene organization is open for speculation.

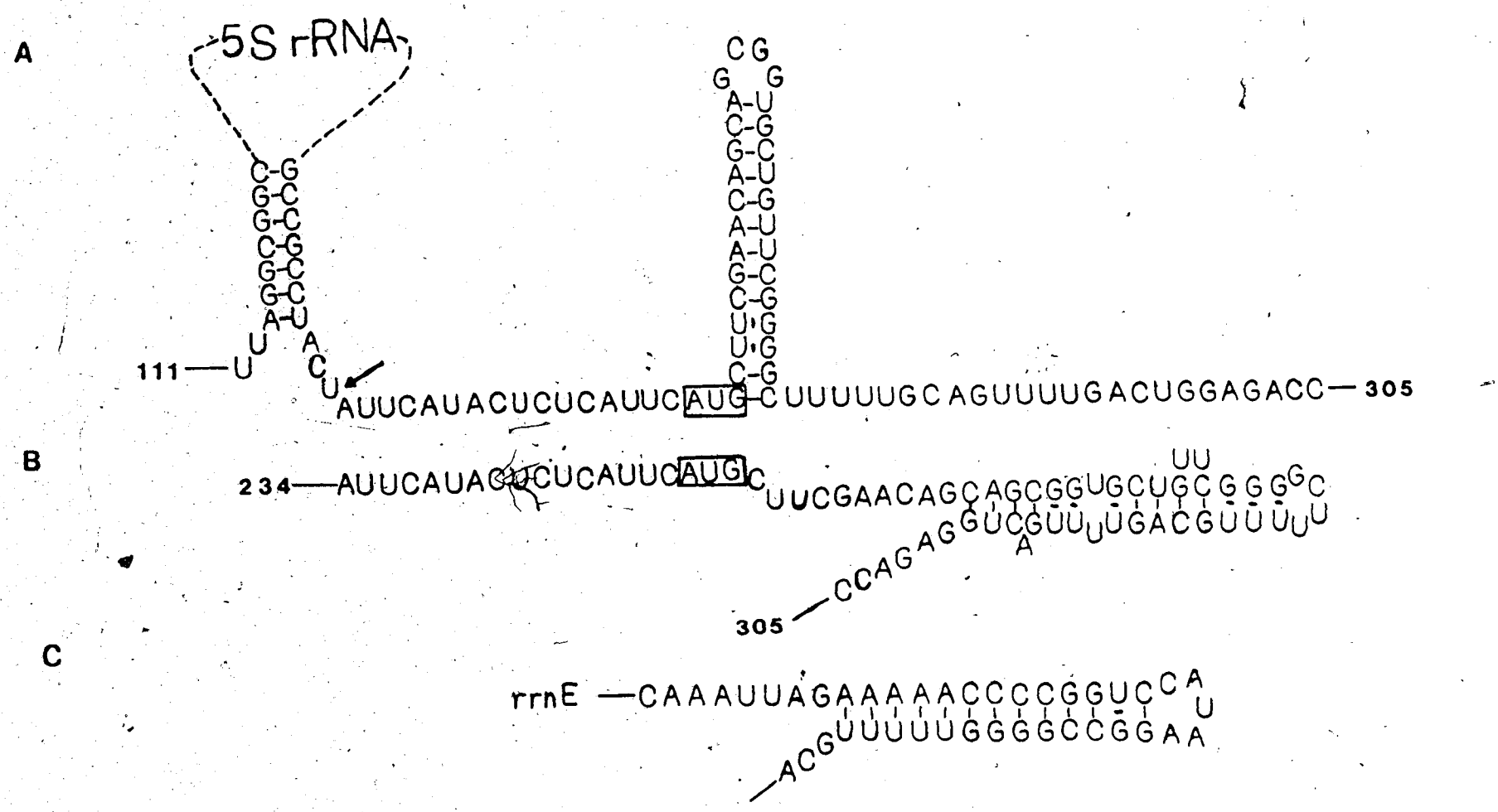
3'-flanking region

Hui and Dennis (1985) described a structure similar to a rho- independent terminator downstream from the *H. cutirubrum* 5S rRNA coding sequence. The secondary structure of this terminator is deduced and is shown in figure 8A. This structure is followed by a run of T's. This type of termination signal has also been described in a similar location following the ribosomal RNA operons in *H. volcanii* (Daniels et al., 1985b). It has been documented that a regular rho-independent termination signal is not sufficient to terminate transcripts originated from an rrn promoter. Genes fused to rrn promoters showed this anti-termination effect (Holben and Morgan, 1984). RNA polymerase transcription originated from such an anti-terminator promoter will read

through templates with complicated secondary structures. We have located another termination signal, centred at 34 nucleotides downstream from the 5S rRNA sequence, overlapping the rho-independent terminator. This terminator has dyad symmetry. It resembles the recently identified rrn terminators common in the *E. coli* rrn operons (Liebke and Hatfull 1985). However, the composition of this structure is quite diverged from that of *E. coli*. Figures 8B and 8C give the comparison. The *E. coli* rrn terminator dyad symmetry has a G/C rich stem, extended by A/U pairings, giving a bimodal hairpin. The *H. cutirubrum* dyad symmetry does not have this feature. The 15 basepair long stem contains two stretches of weak G-U pairing. This structure is less stable than the terminator proposed by Hui and Dennis (1985). The sequence immediately preceding the dyad symmetry (from position 234 to position 263 in figure 6 and in figure 8B) is A/T rich (62%). The role of such a structure in the regulation of anti-terminated transcription is unclear. The involvement of the downstream $tRNA^{Cys}$ sequence in transcription termination is possible since a complicated cloverleaf secondary structure can be formed. This $tRNA^{Cys}$ is transcribed and processed as demonstrated by the Southern cross experiment described below.

Examination of sequences distal to the 5S rRNA coding region revealed an open reading frame (ORF). The AUG initiator codon is located 17 nucleotides downstream from the 5S rRNA. Surprisingly, the ORF runs through the $tRNA^{Cys}$ coding sequence. This ORF may encode a peptide of over a hundred amino acids. Whether this ORF is actually translated is unknown. There is no typical Shine-Dalgarno sequence preceeding the start codon complementary to the 3'-terminus of the 16S rRNA sequence (AUCACCUCC), which is involved in mRNA binding. The amino acid sequence deduced from this ORF is shown in figure 6. If this ORF did represent a run-on transcript originated from the ribosomal RNA operon promoter, then it would likely encode a protein produced in synchrony with variations in rRNA production. Examination of the DNA sequences of the *H. volcanii* 5S rRNA genes and their flanking

Figure 8. Possible terminator structures downstream from the 5S rRNA. (A) and (B) show the two terminator structures postulated. (C) is the terminator structure downstream from the *E. coli* rrn E operon (Liebke and Hatfull, 1985). An arrow indicates the 3'-terminus of the 5S rRNA. The potential initiation codon of the open reading frame described in the text is boxed. Sequence positions correspond to those given in figure 6.


A
5S rRNA
111
305
B
234
305
C
rrnE

sequences revealed downstream open reading frames. However, no start codon was identified. The amino acid sequences deduced bear no significant homology with the sequence found in *H. cutirubrum*.

Secondary structure

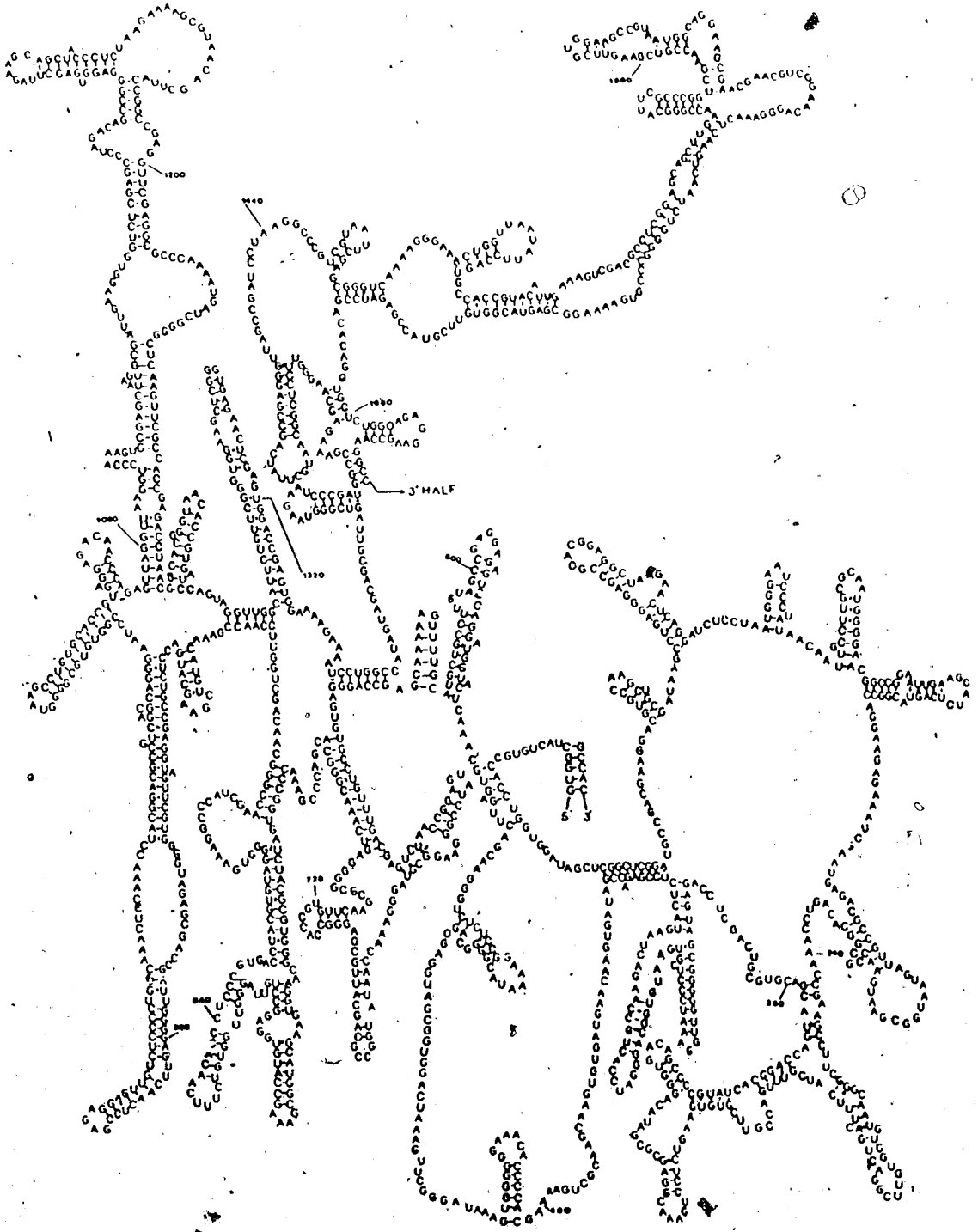
The sequence of the *H. cutirubrum* 23S rRNA is derived from its primary DNA sequence. The secondary structure is deduced by first searching for intra-molecular Watson and Crick basepairing to form basic stem and loop structures, then arranging these substructures according to the existing prokaryotic 23S rRNA secondary structure models. The secondary structure proposed for the *H. cutirubrum* 23S rRNA is shown in figure 9. The RNA molecule is divided into structural domains for discussion purposes (see figure 9).

The danger of derivation of secondary structures according to existing models (such as the *E. coli* model) is to conform without seeking alternate interpretations. These hypothetical structures have to be proven experimentally. Out of the four 23S rRNA secondary structures published so far, only the *E. coli* model has been partially confirmed experimentally (for review see Branlant et al. 1981; Noller, 1984). The *Bacillus stearo-thermophilus* model was derived through free energy calculation to give the most stable secondary structure (Kop et al. 1984). The models for *M. vannielii* and *A. nidulans* were constructed following the *E. coli* structure (Jarsch and Bock, 1985a; Douglas and Doolittle, 1984).

The overall secondary structures of 23S rRNAs from examples of eubacteria, eukaryotes and archaebacteria are compared in figure 10. These secondary structures all conform to a general scheme but at the same time show obvious differences in substructures, features which are unique to each phylogenetic group. This is indicative of all rRNA being related through a common ancestry.

Modified nucleotides and mismatched basepairings cannot be identified from the

Figure 9. The 23S rRNA secondary structure model of *H. cutirubrum* . The molecule is divided in half purely for ease of presentation. Roman numerals define structural domains.


3'HALF

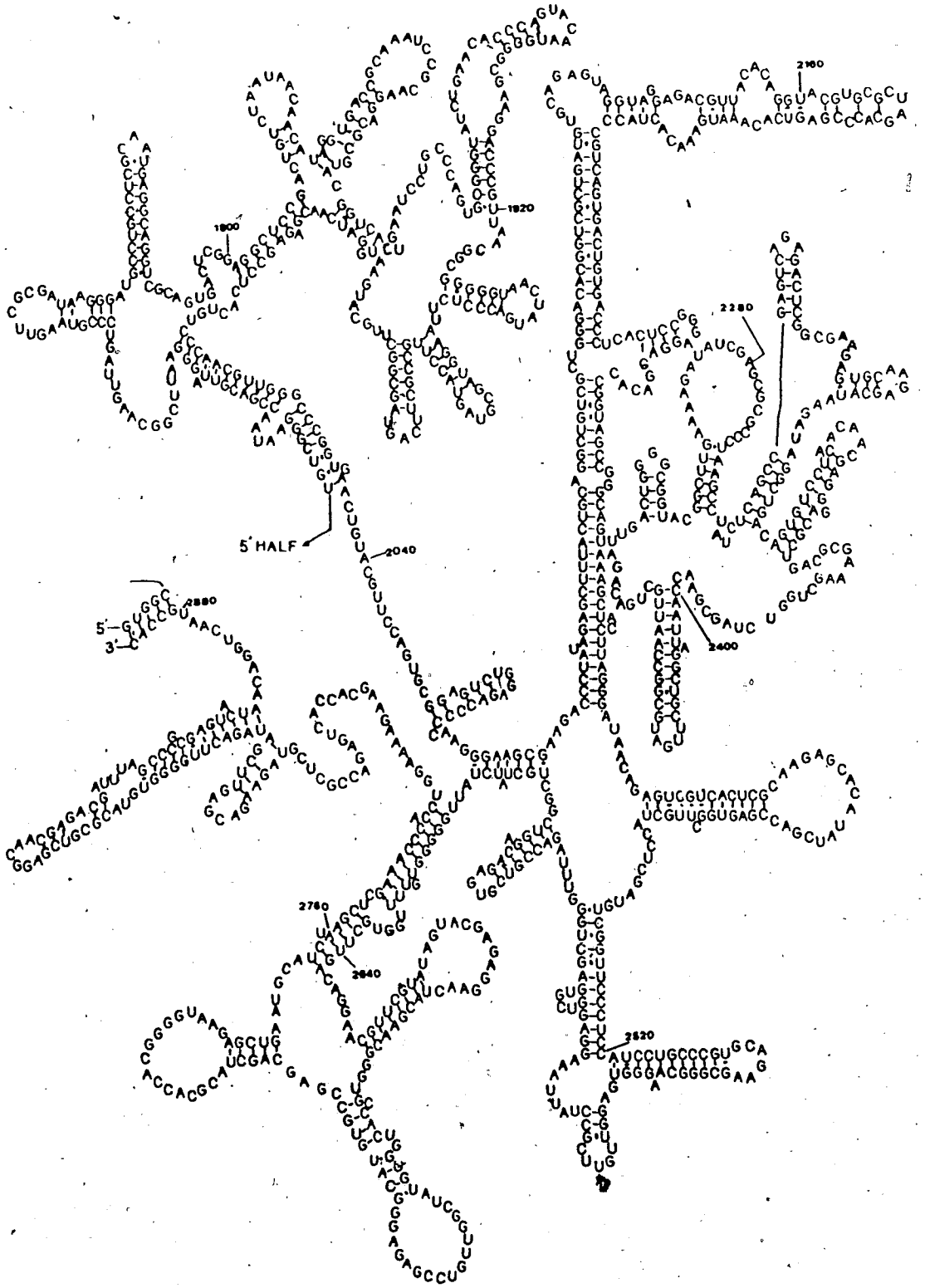
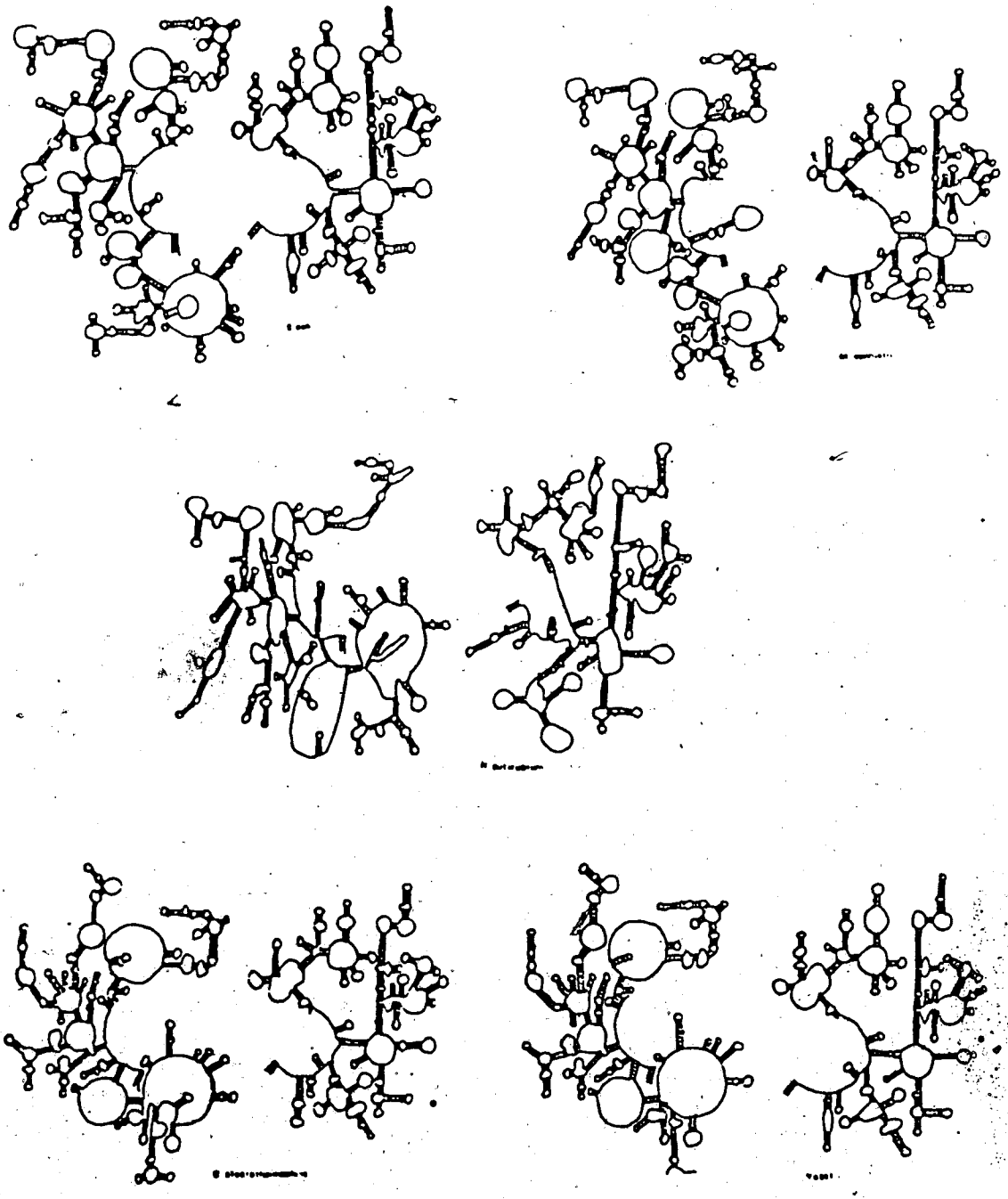

5' HALF
1800
1920
2040
2160
2280
2400
2520
2640
2760
2880

Figure 10. Comparisons of overall secondary structure of 23S rRNAs from *E. coli* (Noller 1984), *B. stearothermophilis* (Kop et al. 1984), *M. vannielli* (Jarsch and Bock, 1985a), *H. cutirubrum* and yeast 26S rRNA (Hogen et al., 1984).



primary DNA sequence. There are identical bases in *H. cutirubrum* at positions corresponding to some of the modified bases on the *E. coli* 23S rRNA (identified by Branlant et al., 1981). For example, the uridine (which is methylated at the 7 position in *E. coli*) near the peptide transfer center is found in all the examples shown in figure 11 as well. There are mismatches in a number of helices. However mismatched basepairing has not been experimentally demonstrated in this work.

Substructures on the large subunit ribosomal RNA

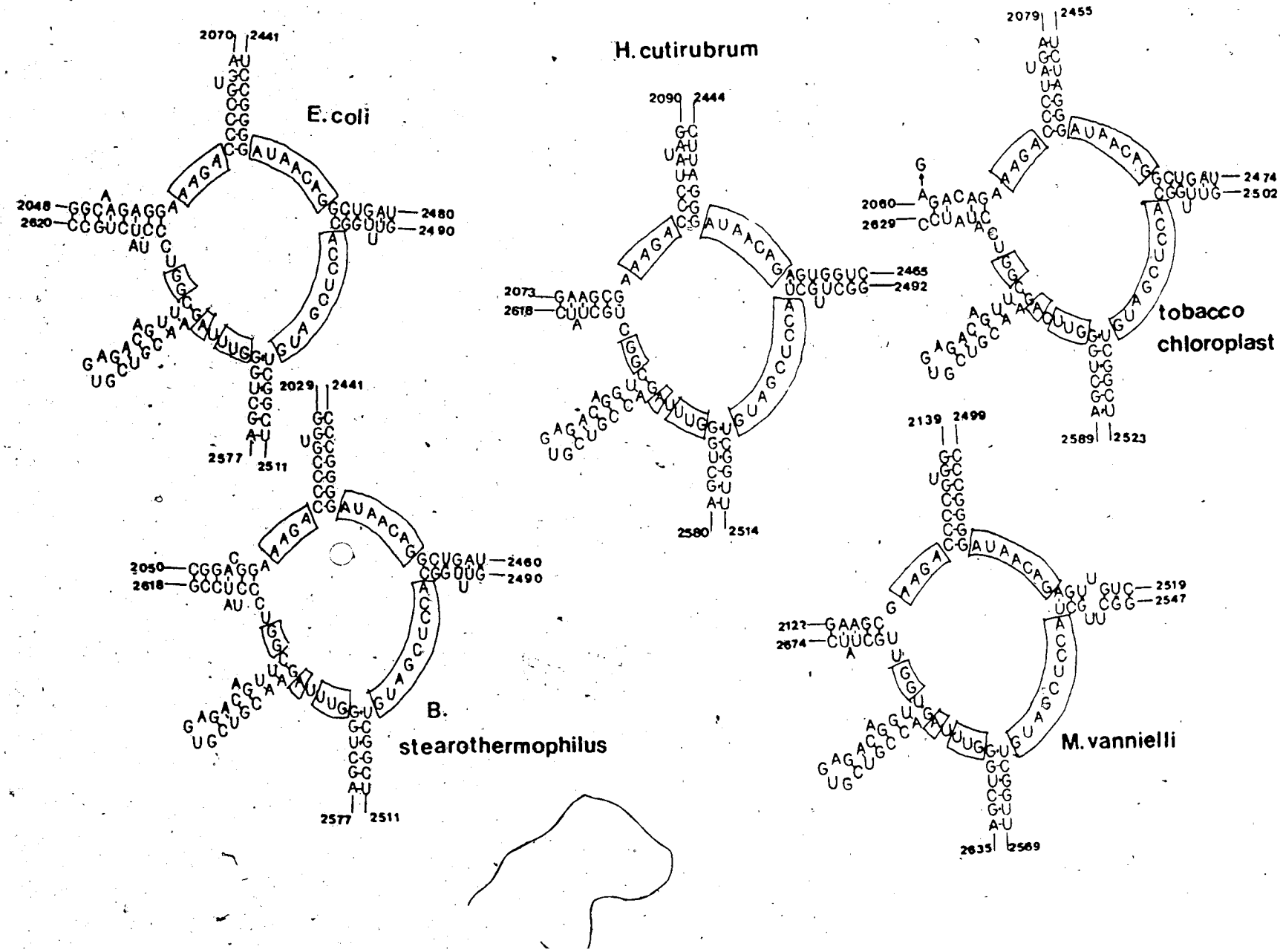
Examination of the secondary structure of the *H. cutirubrum* large subunit RNA reveals substructures which resemble functional sites on the *E. coli* 23S rRNA model. Structural homologs are not necessarily formed by a common sequence. Regions of conserved sequences within these substructures may imply their functional significance.

Peptide transfer centre

The peptidyl transferase activity is an essential function of the ribosome. A structure that resembles the *E. coli* peptide transfer centre is located in domain V of the *H. cutirubrum 23S* rRNA. This loop structure is formed by five non-contigious sequences (see figure 11). Most unpaired bases in the loop are highly conserved in diverged organisms. Figure 11 shows the comparison of the presumed peptide transfer centre from *H. cutirubrum, M. vannielii, E. coli, B. stearothermophilus, A. nidulans* and the tobacco chloroplast.

Genetic studies of resistance to chloramphenicol and erythromycin in *E. coli* and in yeast and mammalian mitochondria have mapped mutations to the central loop of the peptide transfer centre (Sigmund et al., 1984; Dujon et al., 1980; Kearsey et al., 1980; Sor et al., 1982; Blanc et al., 1981; Blanc et al.,1983). The sequence AAAGA is found in this central loop in wild type *E. coli* . Erythromycin resistant mutants have the sequence UAAGA instead (Sigmund et al., 1984). The sequence GAAGA is found in

Figure 11. Peptide transfer centres from *E. coli* (Barta et al., 1984), *B. stearothermophilus* (Kop et al., 1984), *A. nidulans* (Douglas and Doolittle, 1984; Kumano et al., 1983), *M. vannielii* (Jarsch and Bock, 1985a), tobacco chloroplast (Takaiwaa and Sugiura, 1982) and *H. cutirubrum*. The structure from *A. nidulans* is identical to that of tobacco chloroplast with the execption of a single base change (from A to G) which is indicated on the tobacco chloroplast structure.


E. coli
H. cutirubrum
tobacco chloroplast
B. stearothermophilus
M. vannielli

H. cutirubrum , identical to the ones found in the other erythromycin resistant archeabacteria studied so far (they are *M. vannielii, H. volcanii and S. solfataricus* ; see Jarsch and Bock, 1985a for details).

Transfer RNA binding sites

One of the interacting sites between amino-acylated tRNA and 23S rRNA on the *E. coli* ribosome has been identified using crossing-linking agents (Barta 1984). The terminal CCA of the tRNA interacts with a specific UGG sequence in domain II on the large subunit rRNA. The sequence UGG is found in the corresponding location on the *H. cutirubrum* 23S rRNA at position 889 to 891. A comparison of the structures, responsible for tRNA-rRNA interaction in these two organisms is shown in figure 12. The size of this structure in *H. cutirubrum* is significantly larger than that of *E. coli* . Sequence conservation between these two structures is limited to two small areas in the open loop region. One of these two conserved areas contains the site for CCA base-pairing.

Based on the *E. coli* model (Dalberg et al.,1978; Edwards and Kossel, 1981), two binding sites for the initiator $tRNA^{Met}$ in domain IV and domain V are proposed. These possible tRNA-rRNA interactions are summarized in figure 14. The nucleotide sequence of the *H. cutirubrum* initiator $tRNA^{Met}$ is not available. The tRNA sequence shown (in figure 13) is derived from the *Halobacterium volcanii* and *Halococcus morrhuae* initiator $tRNA^{Met}$ (Kuchino et al., 1982; Gupta, 1984). The 23S rRNA-interacting region on the $tRNA_i^{Met}$s from these two organisms are identical. The sequence is likely to be conserved in the *H. cutirubrum* initiator tRNA as well. Initiator $tRNA^{Met}$ binding regions have been identified in similar locations on the large subunit RNA of *Anacystis nidulans* and the tobacco chloroplast as well (Kumano et al,1983; Douglas and Doolittle,1984; Kumano et al., 1983).

Figure 12. The tRNA binding sites in domain II of the 23S rRNA from *H. cutirubrum* and from *E. coli* (Barta et al., 1984). Conserved regions are indicated by a box. The sequence UGG (indicated by arrows) has been proposed to basepair with the sequence CCA at the 3'-terminal of transfer RNAs.

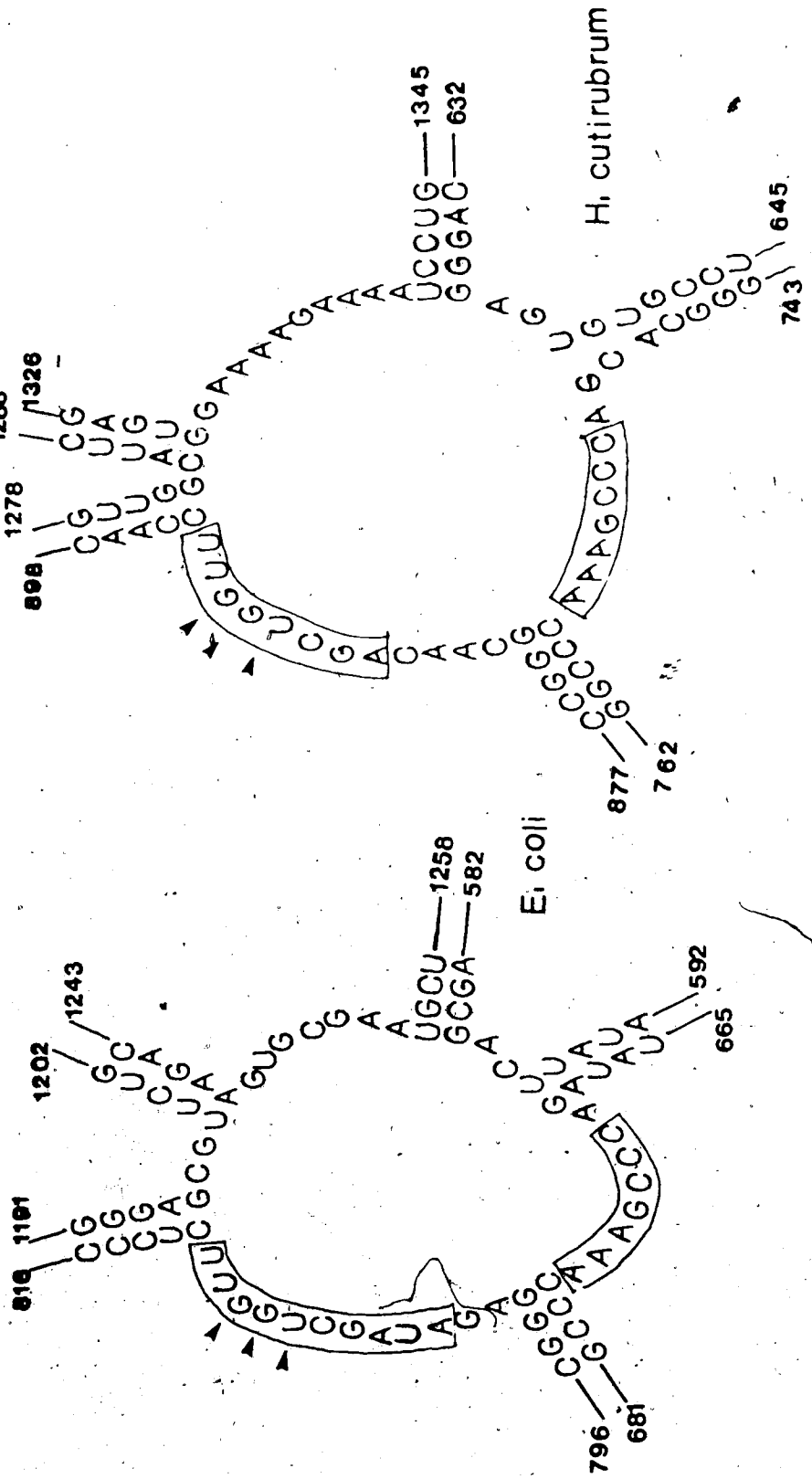
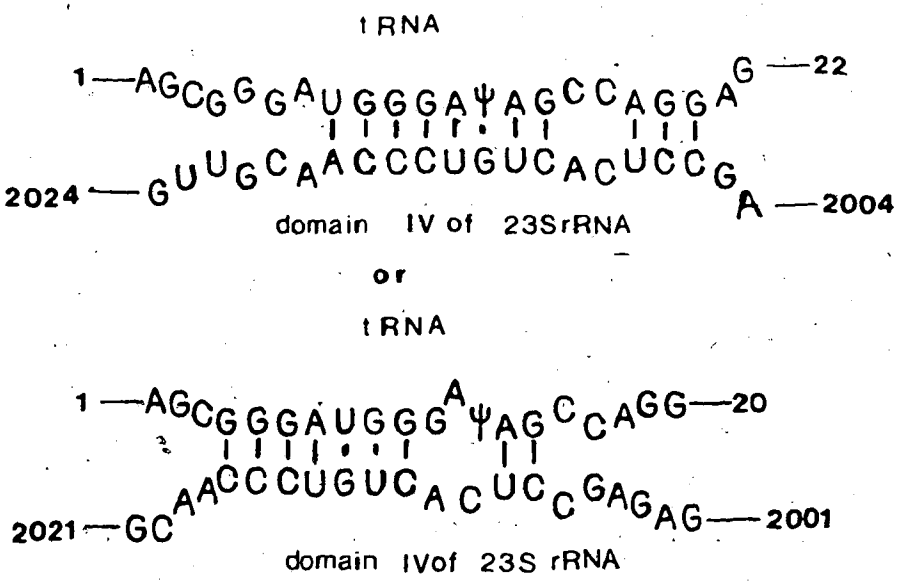

H. cutirubrum
Ei coli

Figure 13. Possible basepair interactions between the initiator $tRNA^{Met}$ and domain V of the *H. cutirubrum* 23S rRNA. The tRNA sequence shown was derived from the *Halobacterium volcanii* (Gupta, 1984) and the *Halococcus morrhuae* (Kuchino et al., 1982) intiator tRNA sequences, which were identical in this region of interest.

A


tRNA
1—AGCGGGAUGGGAΨAGCCAGGAG—22
2024—GUUGCAACCCUGUCACUCCGA—2004
domain IV of 23SrRNA
or
tRNA
1—AGCGGGAUGGGAΨAGCCAGG—20
2021—GCAACCCUGUCACUCCGAGAG—2001
domain IVof 23S rRNA

B

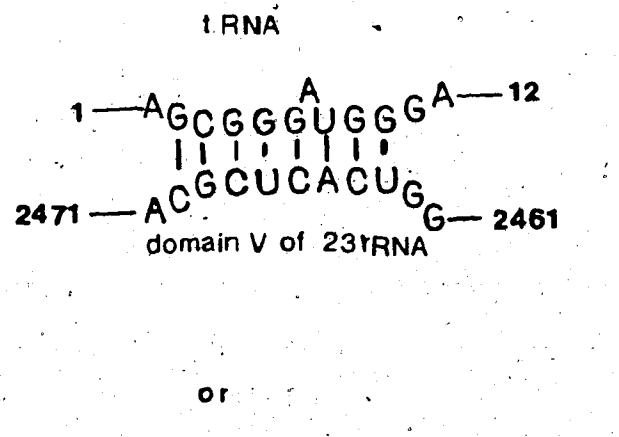
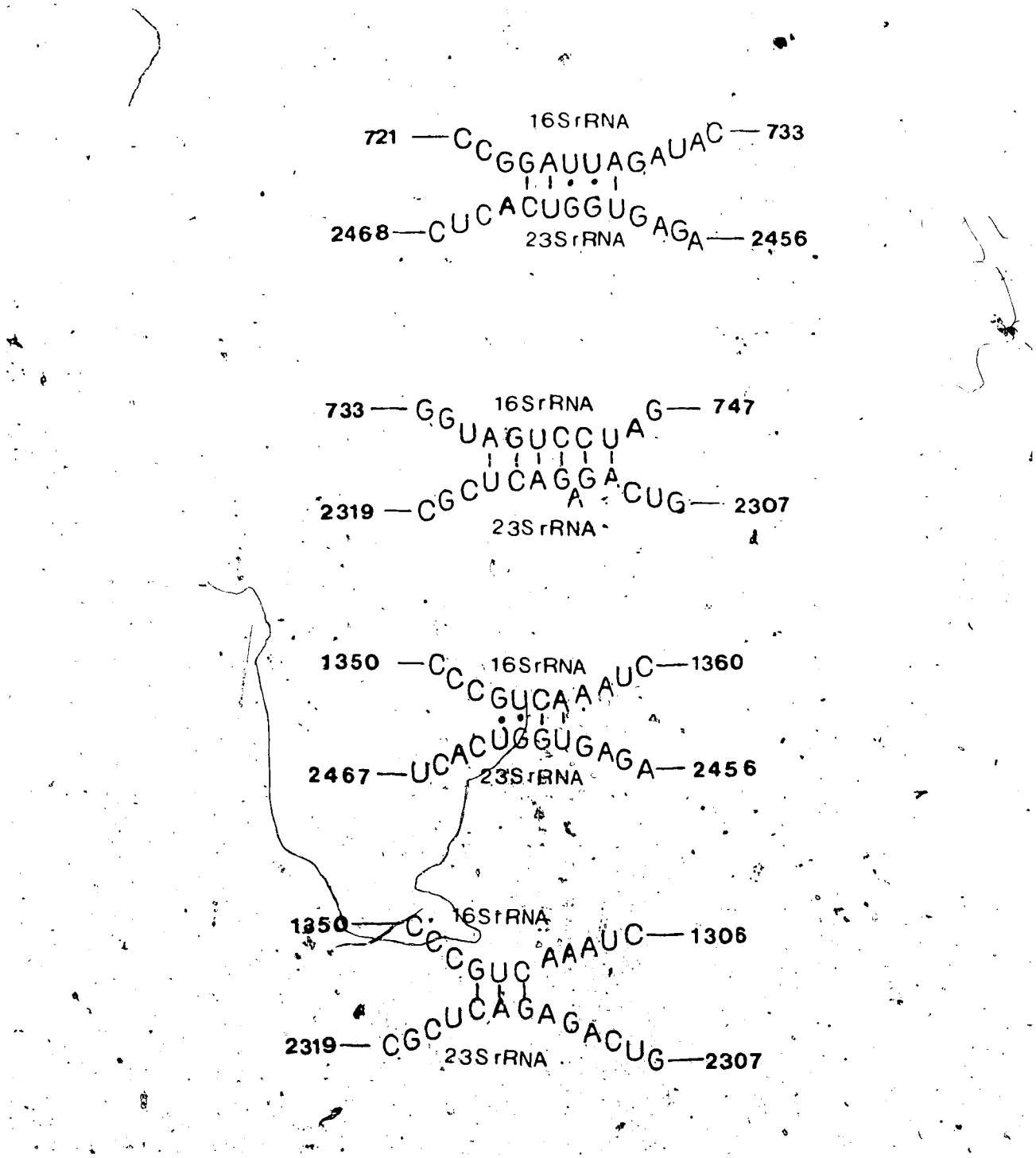

tRNA
1—AGCGGGUGGGA—12
2471—ACGCUCACUGG—2461
domain V of 23rRNA
or
tRNA
5—GGAUGGGAΨGACCAGG—20
2471—ACGCUCACUGGUGA—2458
domain V of 23S rRNA

Figure 14. Possible 16S/23S rRNA interaction sites.


16SrRNA
721 — C C G G A U U A G A U A C — 733
2468 — C U C A C U G G U G A G A — 2456
23SrRNA
733 — G G U A G U C C U A G — 747
16SrRNA
2319 — C G C U C A G G A A C U G — 2307
23SrRNA
1350 — C C C G U C A A A U C — 1360
16SrRNA
2467 — U C A C U G G U G A G A — 2456
23SrRNA
1350 — C C C G U C A A A U C — 1306
16SrRNA
2319 — C G C U C A G A G A C U G — 2307
23SrRNA

Sites for rRNA-rRNA interaction

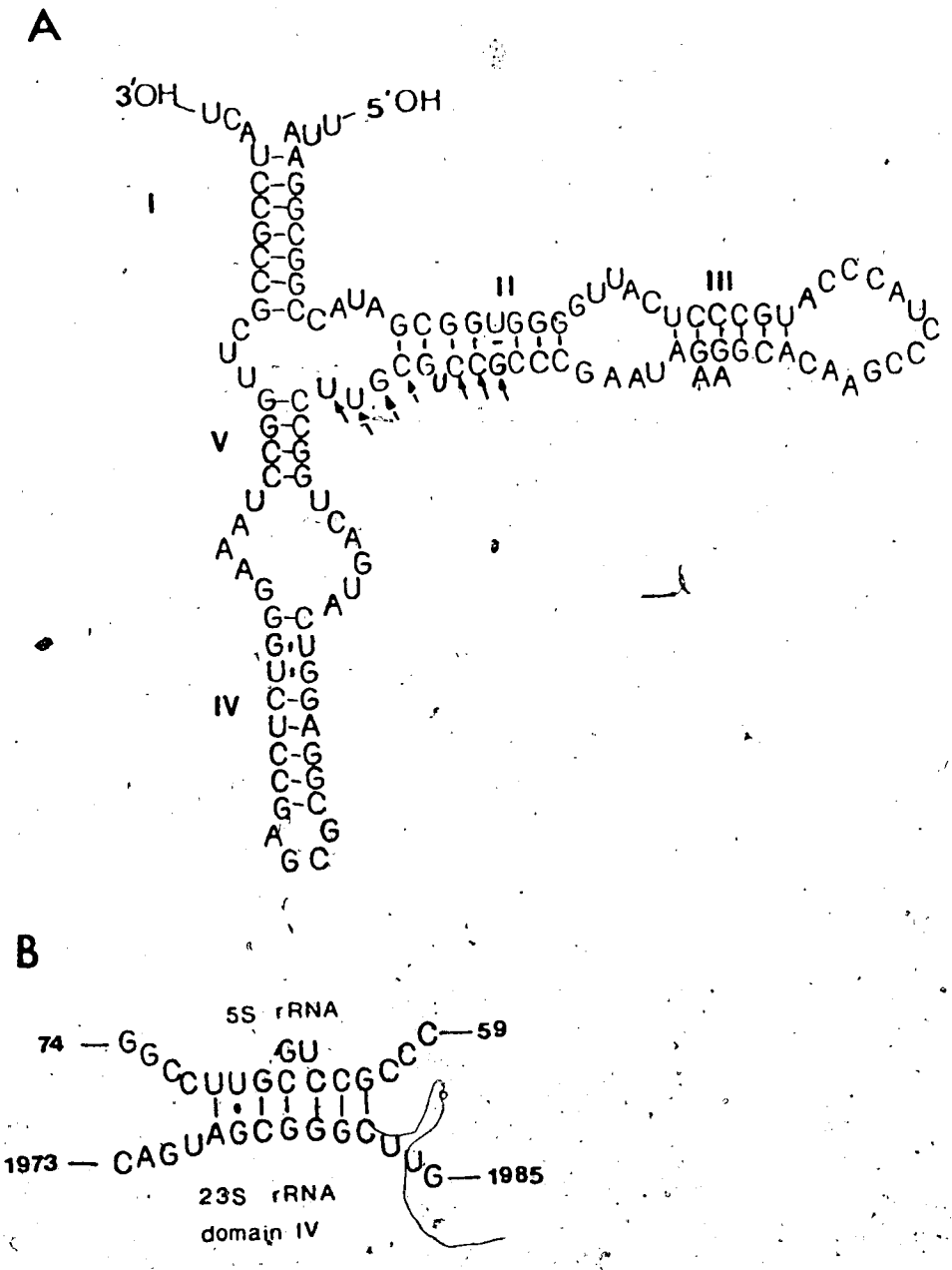
The binding site for 5S rRNA on the *E. coli* 23S rRNA has been determined (Branlant et al., 1976; Branlant et al., 1981). The sequence GCCUUG in domain IV of the 23S RNA can basepair with a sequence on the loop between helix II and IV of the 5S rRNA. All prokaryotic 23S rRNA secondary structures published to date contain this sequence (*A. nidulans* by Douglas and Doolittle, 1984; *B. stearothermophilus* by Kop et al., 1984b; *E. coli* by Branlant et al., 1981; *M. vannielii* by Jarsch and Bock, 1985a). In *H. cutirubrum* , this binding site has been identified by structural comparison. However, the sequence GCCUUG is replaced by GCGAUG. The sequence 3'-UUGCGUCCG-5' in domain IV of the 23S rRNA can interact with the sequence 5'-AGCG--GGC-3' on the loop at the base of helix II on the 5S rRNA (see figure 15).

Sites for 16S-23S rRNA interaction (responsible for ribosomal subunit association) are proposed. Sequences from two separate regions on the 16S rRNA can each basepair with two sites in domain V of the 23S rRNA. One such site domain V overlaps a binding site for the initiator tRNA mentioned above. The four possible interactions are summarized in figure 14. The *H. cutirubrum* sequences involved in such interactions are very similar but not identical to those found in eubacteria or chloroplasts (Edwards and Kossel, 1981; Douglas and Doolittle, 1984)..

Ribosomal protein binding sites

A number of ribosomal protein binding sites on the *H. cutirubrum* large subunit rRNA have been identified by comparative analysis of the secondary structure models. Like the other functionally significant regions discussed above, these protein-binding structures show higher sequence conservation than the rest of the molecule. These binding sites all share greater than 60% sequence homology with their *E. coli* counterparts. The L8/11 protein complex association region is a very good example. The

Figure 15. Secondary structure of the *H. cutirubrum* 5S rRNA (A). The arrows indicate nucleotides capable of interacting with the 23S rRNA by basepairing with a specific sequence located in domain IV (B). The helices of the 5S rRNA are denoted by Roman numerals.


A
3'OH
5'OH
I
II
III
IV
V
B
5S rRNA
74
59
1973
1985
23S rRNA
domain IV

H. cutirubrum sequence in this region is 79% homologous to that of *M. vannielii* and is 77% homologous to the *E. coli* sequence. This region also shows 70% and 65% homology to its counterparts in yeast and rat liver. The yeast sequence and the rat liver sequence share 98% sequence homology. The sequences used for comparison were obtained from Brosius et al., 1980; Raue et al., 1985; Jarsch and Bock, 1985a; Hogen et al., 1982 and Beauclerk et al., 1985.

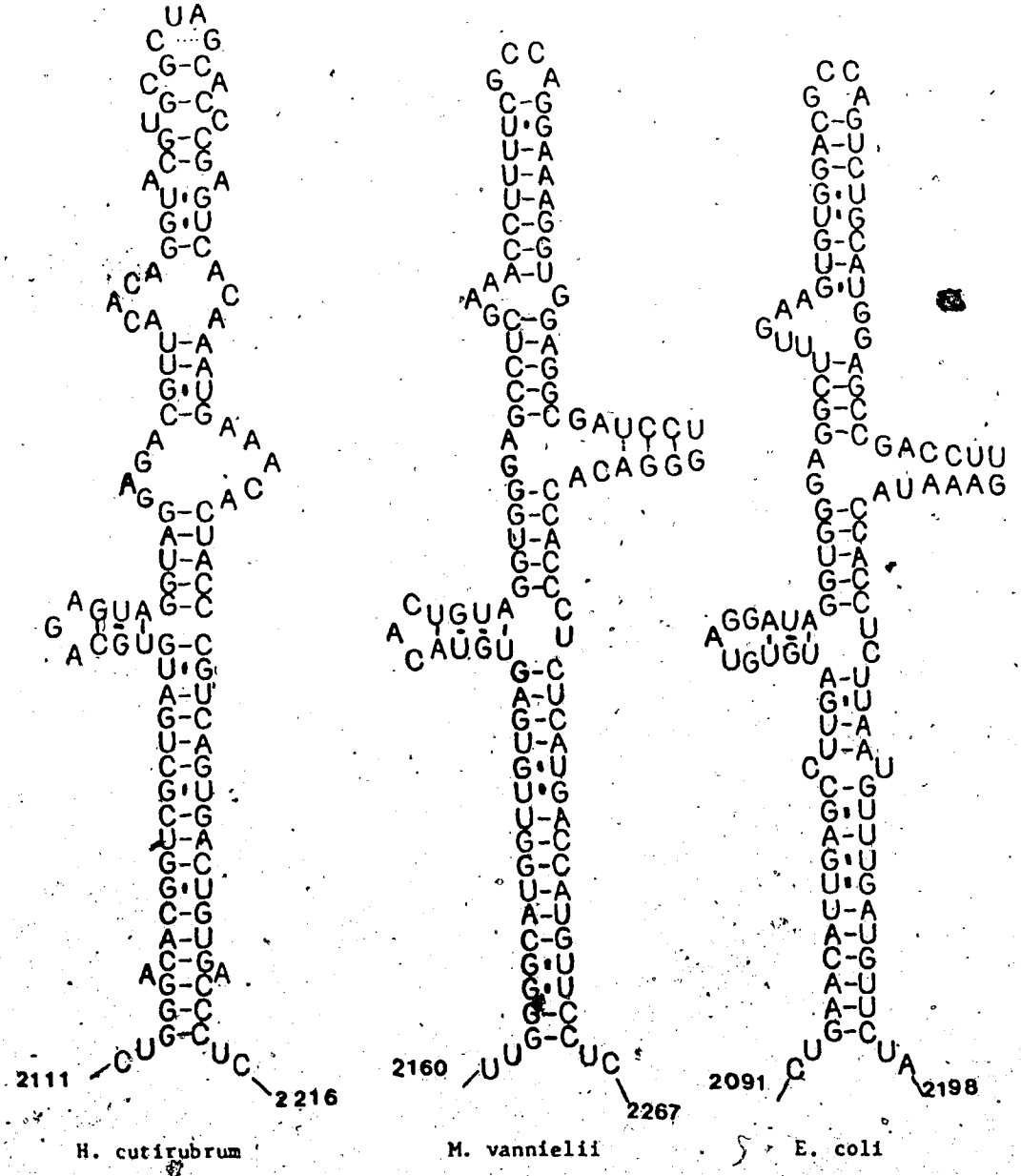
The phenomenon of site-specific sequence conservation points out a flaw in using overall sequence homology of the large subunit RNA as the phylogenetic measurement. Changes in sequence in the functional regions have greater evolutionary significance than changes found in less essential regions.

Although the general structures of many ribosomal protein binding regions from various organisms are conserved, differences in details are obvious. Figure 16 shows the secondary structure comparison of L1 ribosomal protein binding sites from *H. cutirubrum, M. vannielii and E. coli.* Figure 17 shows the structure of the L8/L11 complex association site of *H. cutirubrum* as well as its counterparts in *E. coli* and in yeast. These comparisons illustrate the point clearly.

Alpha-sarcin cleavage site

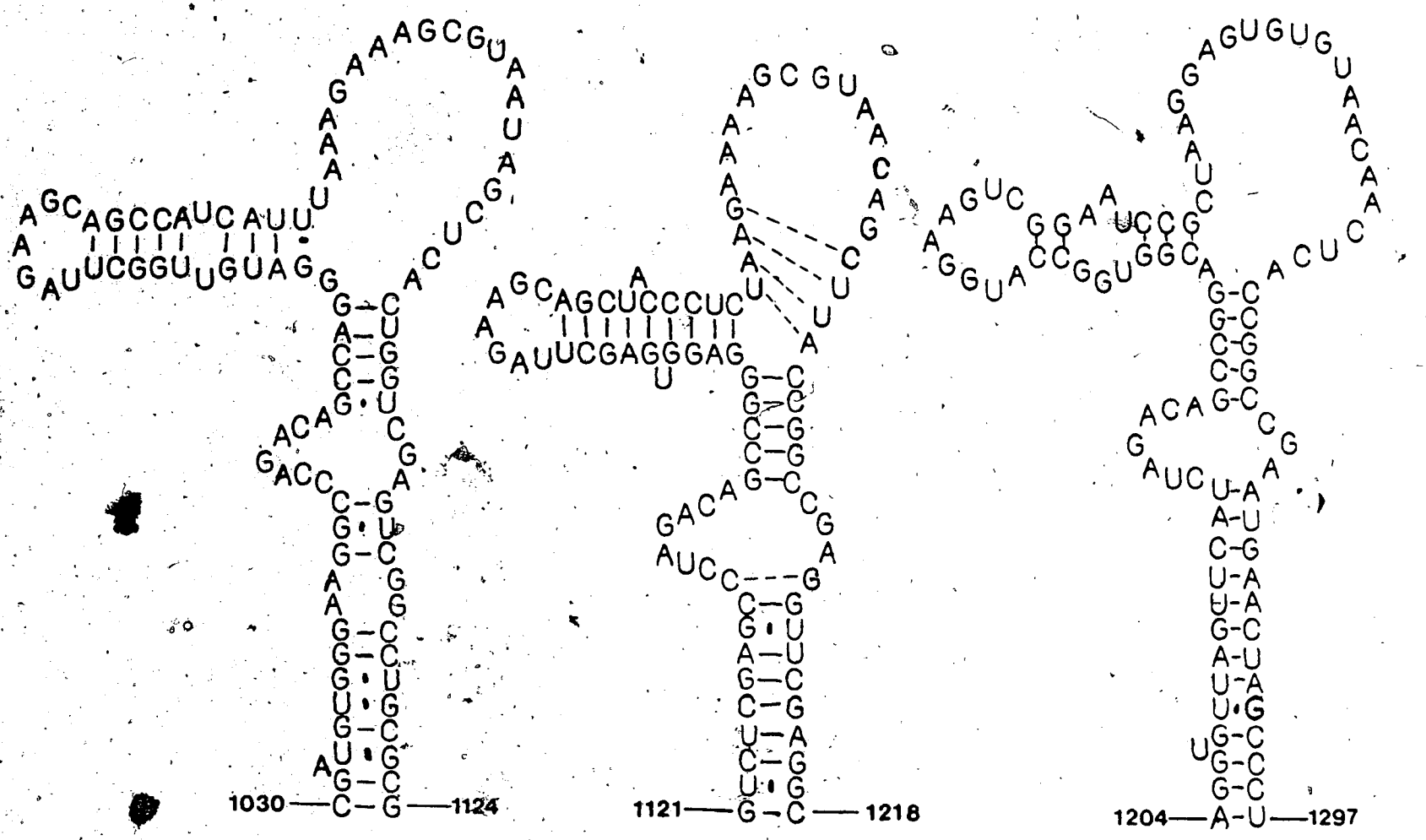
Alpha-sarcin is an inhibitor of a number of energy dependent steps in protein synthesis. This enzyme inactivates ribosomes by cleaving a site at an open loop in domain VI of the large subunit rRNA (see figure 18). The nucleotide sequence of this loop is highly conserved in eukaryotes. The homologous loop in *M. vannielii* conforms to this eukaryotic sequence (Jarsch and Bock, 1985a). The loop in *H. cutirubrum* is located from position 2658 to 2671 in domain IV of the 23S rRNA. Like *M. vannielii* the loop sequence is identical to that of the eukaryote and is slightly different from the eubacterial sequence. Although the stems from the two archaebacterial structures are of the same length, they show no significant sequence homology. Figure 18 shows

Figure 16. Comparison of secondary structures of the L1 ribosomal protein binding region in *H. cutirubrum* , *M. vannielii* (extracted from Jarsch and Bock, 1985a) and *E. coli* (Branlant et al., 1976a; Branlant et al., 1976b; Branlant et al., 1981; Noller, 1984).



H. cutirubrum M. vannielii E. coli

Figure 17. Secondary structures for the L8/11 ribosomal protein complex association region on the large subunit ribosomal RNAs of *H. cutirubrum* , *E. coli* and yeast. The structures for *E. coli* and yeast were modified from those described by Hogen et al., 1984; Branlant et al., 1981 and Raue et al.,1985.



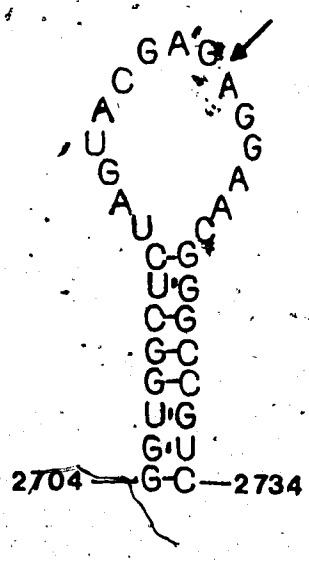
E. coli

H. cutirubrum

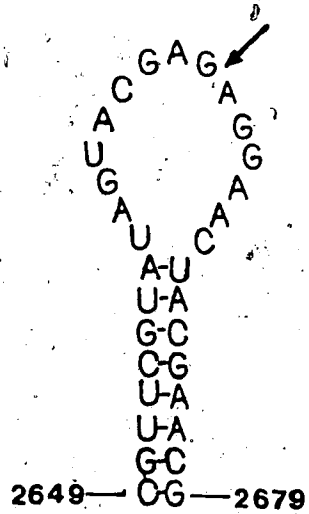
Yeast

Figure 18. Regions on the 23S rRNA of *H. cutirubrum* and *M. vannielii* (shown in B) susceptable to a-sarcin activity. Comparison of the *H. cutirubrum* loop sequence with the eukaryotic, eubacterial and *M. vannielii* sequences (Jarsch and Bock, 1985a) is presented in (A). The cleavage site is indicated by an arrow.

eubacteria	AGUACGAGAGGACC
M. vannielii	AGUACGAGAGGAAC
H. cutirubrum	AGUACGAGAGGAAC
eucaryote	AGUACGAGAGGAAC



M. vannielii



H. cutirubrum

comparisons of the structures and the loop sequences.

3'-terminus

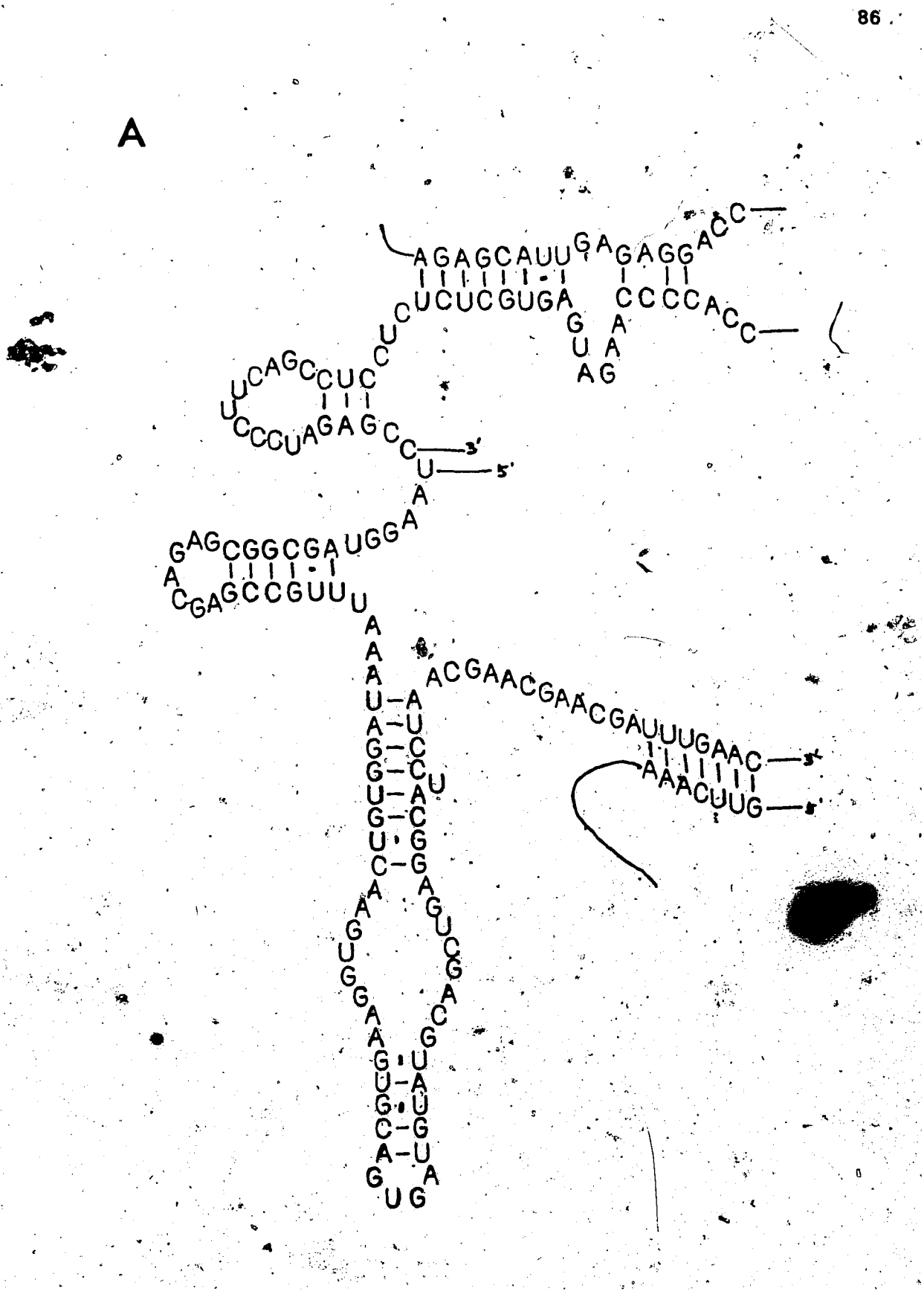
Figure 19A shows the structure formed by the 4.5S rRNA and the 3'-terminus of the 23S rRNA from maize chloroplast (Branlant et al., 1981; Glotz et al., 1981). This structure is highly homologous to the 3'-terminal region of the *E. coli* 23S rRNA (Branlant et al., 1981). The 3'-terminal region of the *H. cutirubrum* 23S rRNA also, but to a lesser extent, bears resemblence to the structure in the maize chloroplast. Figures 19A and 19B show the comparison.

Gene expression

The in vivo expression of each gene in this cluster has been demonstrated. Hybridization of pHcr1 to northern transfers of total *H. cutirubrum* RNA yields the expected result (see figure 22). Hybridizing RNA bands corresponding to each of the rRNAs as well as the tRNA mixture were observed. Hybridizing RNA bands migrating slower than the 23S rRNA may represent partially processed or unprocessed RNA transcripts. A very low level of hybridization of pHcr1 to *H. cutirubrum* rRNA size products were observed when northern blots of the total RNA isolated from *E. coli* harbouring pHcr1 was analysed.

Hybridization of a *H. cutirubrum* tRNA mixture to the coding regions for both the alanine tRNA and the cysteine tRNA are observed. This result is indicative, but not conclusive, of the expression of both tRNAs since (i) transfer RNA preparations are often contaminated with a trace amount of 5S rRNA and its break down products and (ii) the 5S rRNA gene and the cysteine tRNA gene are located in the same region and cannot be conveniently separated by restriction endonucleases. The results from the Southern cross experiment provide some clarification (see figure 20). Both the alanine tRNA gene and the cysteine tRNA gene appear to be expressed in *H. cutirubrum* . Furthermore,

Figure 19. Comparison of the secondary structure of the 3'-terminus of the *H. cutirubrum* 23S rRNA (B) to the structure formed by the 4.5S rRNA and the 3'-terminal sequence of the 23S rRNA from maize chloroplast (A). The 3'-terminal structure of maize chloroplast was derived from a figure used by Branlant et al., 1981.


A
AGAGCAUUGAGAGGACC
UCUCGUGA CCCCACC
G A
U A
AG
UCAGCCUCC
UCCCUAGAGCC 3'
U 5'
A
GAGCGGCGAUGGA
ACGAGCCGUUU
A
A
A
U-A
A-U
G-C
G-CU
U-A
G-C
U G
C-G
A AGUCG
A A
G C
U G
G A
A G
G U
U-A
G U
C-G
A-U
G A
U G
ACGAACGAACGAUUUGAAC 3'
AAACUUG 5'

B

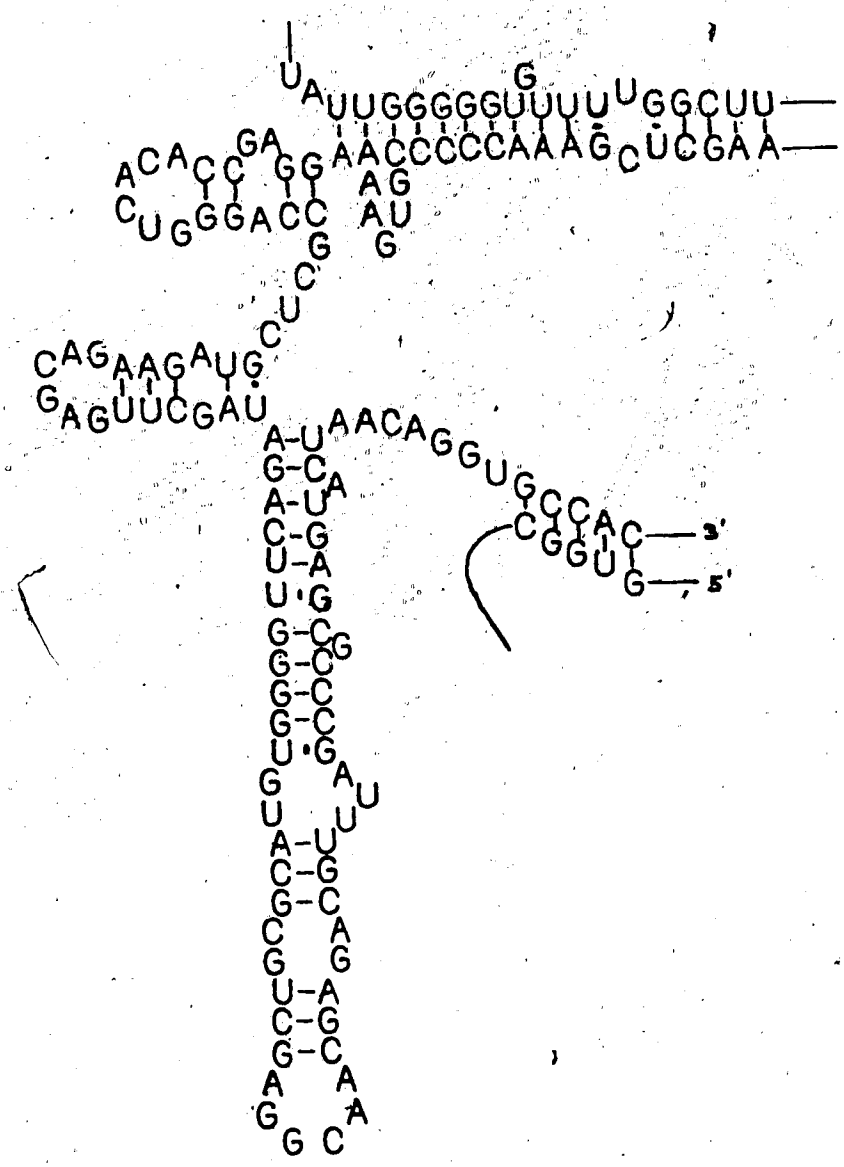
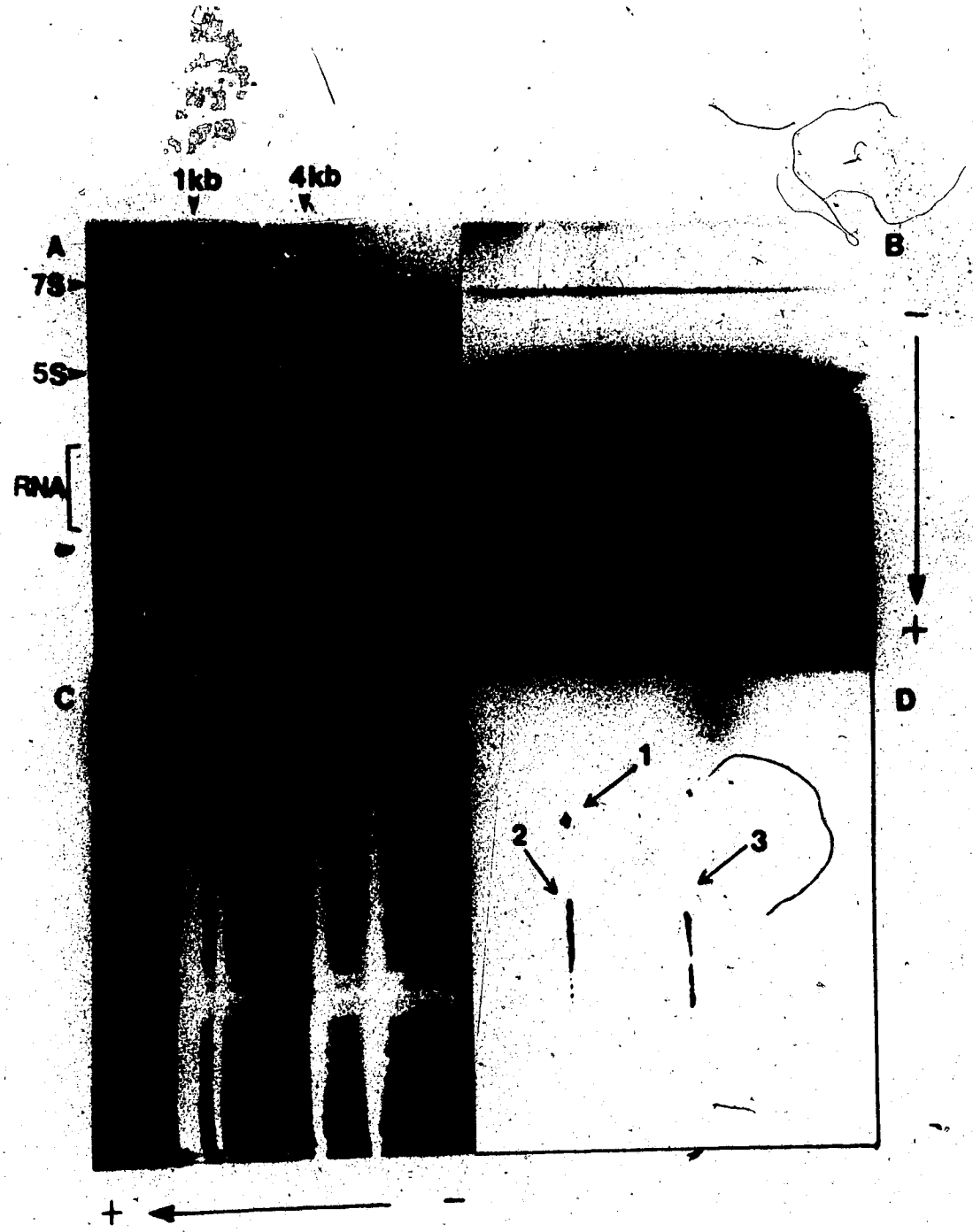


Figure 20. Radioautograph (A) showing the Southern cross hybridization between ^{32}P labelled *H. cutirubrum* RNAs (B) and Mlu I digested pHcr1 (C). DNA fragments generated by a complete Mlu I digest of 50 µg of pHcr1 was separated by electrophoresis (on a 0.75% agarose gel in TEA buffer at 5 volts per cm). The separated DNA fragments were transferred onto nitrocellulose paper by the method of Southern. The RNA probe was prepared by labelling freshly isolated *H. cutirubrum* total RNA with [^{32}P]pCp using RNA ligase. The ^{32}P-RNA was then separated on a 10% polyacrylamide gel (acryl:bis, 29:1) in TEA buffer at 10 volts per cm. The radioactive RNA was transferred onto the same piece of nitrocellulose paper in the dimension perpendicular to the initial transfer of DNA fragments. The transfer was carried out in 5X SSC and 50% formamide at 37 °C for 48 hours. The nitrocellulose paper was then washed twice in 2X SSC, 0.2% SDS at 45°C and once in 0.2X SSC and 0.2% SDS at 45°C. The radioautograph of a ten day exposure with intensifying screen at -90°C is shown (A). (D) is a schematic representation of (A). Spot 1 represents hybridization of 5S rRNA to the 1 kb Mlu I fragment. Spot 2 shows hybridization of $tRNA^{Cys}$ to the same Mlu I fragment. Spot 3 shows hybridization of the $tRNA^{Ala}$ and its breakdown products to the separate 3 kb Mlu fragment.


1kb
4kb
A
7S
5S
RNA
C
B
D
1
2
3
+
−
−
+

tRNAs with sequences nearly identical to the ones derived from the cloned tRNA genes have been found in *H. volcanii* (Gupta, 1984).

The expression of *H. cutirubrum* tRNA genes in eukaryotic and eubacterial systems has been analyzed. In vitro transcription of pHcr1 in the eukaryotic system (HeLa cell lysate) shows only one tRNA sized product, the $tRNA^{Ala}$. The $tRNA^{Cys}$ may be transcribed at an undetectable level or it may exist as part of unprocessed run-on transcripts. High molecular weight transcripts were observed but not characterized.

Transcription of pHcr1 in *E. coli* was analysed by northern hybridization. No detectable tRNA-sized products have been observed hybridizing to pHcr1, indicating little or no transcription of *H. cutirubrum* tRNA genes in *E. coli* (see figure 22). The weak hybridization of pHcr1 to *E. coli* rRNAs is expected since there are highly conserved regions in rRNAs.

The unidentified stable RNA species, roughly 300 nucleotides long, seen in figure 20B, did not hybridize to pHcr1, therefore is not encoded by the 11 kb of cloned *H. cutirubrum* DNA sequence. It is not a partially processed product generated from the overall RNA transcript. This stable RNA is probably the 7S RNA found in a number of halophilic archaebacteria by Moritz and Goebel (Moritz and Goebel, 1985; Moritz et al., 1985). The *H. halobium* 7S RNA has only 50% sequence homology to human or rat 7S-L RNA, which represents part of the "signal recognition particle" involved in the transport of proteins across membranes. Surprisingly, the *H. halobium* and the human 7S RNA show remarkable homology in their secondary structure (Moritz and Goebel, 1985).

Evolutionary implications

The sequence homology (53%) between the *H. cutirubrum* and *M. vannielii* 23S rRNAs provides evidence for the phylogenetic divergence between the two organisms. This implies the divergence within the archaebacteria. Uncommon features in the

Figure 21. In vitro transcription of pHcrl in a HeLa cell lysate and hybridization of the product to pHcrl. (A) Radioautograph showing the in vitro transcription of pHcr1 in HeLa cell extract. (B) A Sal I/Sma I digestion of pHcr1 was separated on a 1% agarose gel . The restriction fragment containing the alanine tRNA gene is indicated by (▶). (•) indicates the DNA fragment containing the cysteine tRNA gene. (C) is the corresponding radioautograph showing hybridization of the tRNA sized transcriptional product to (B).

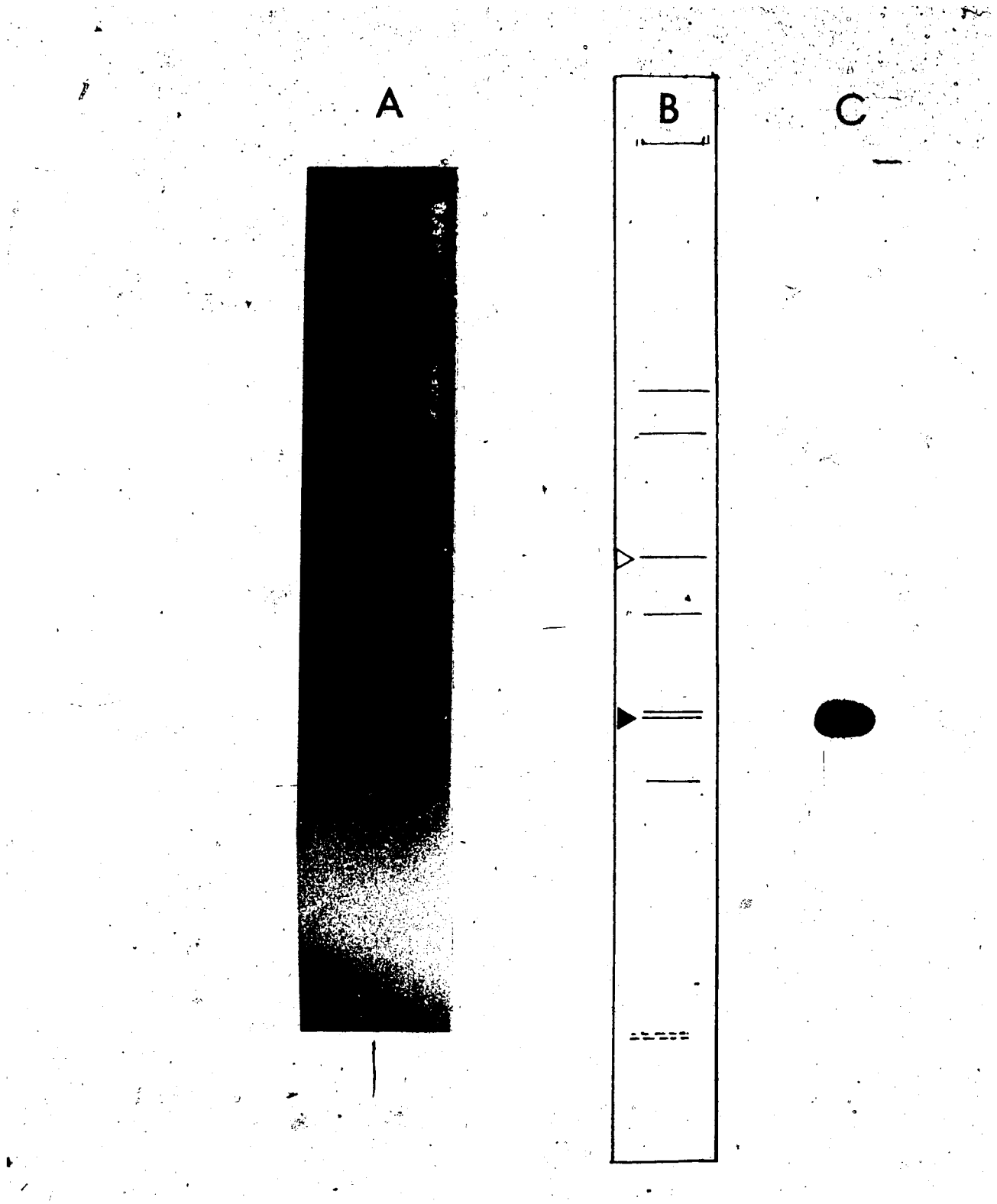
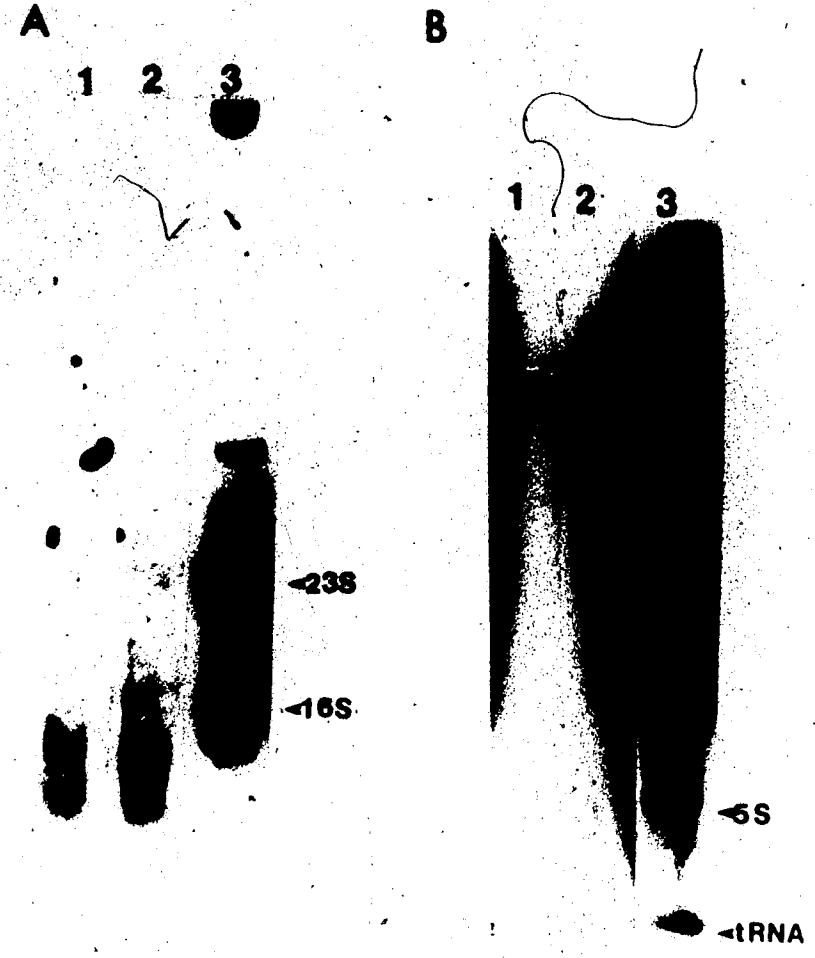

A
B
C

Figure 22. Hybridization of pHcr1 to northern transfers of total RNA. Total RNA was prepared from each of (lane 1) E. coli HB101 habouring pHt4-11, (lane 2) E. coli HB101 habouring pHcr1 and (lane 3) H. cutirubrum. The amount of RNA loaded on lane 1 and 2 are 25 times that in lane 3. Nick translated pHcr1 is hybridized to northern transfers of (A) glyoxylated RNA samples separated on a 1% agarose gel in 0.01 M phosphate buffer and (B) RNA samples separated on a 6% polyacrylamide gel (acryl bis, 29:1) in TEA buffer.


A
1 2 3
23S
16S
B
1 2 3
5S
tRNA

substructures and the functional sites described above support the uniqueness of archaebacteria. The *M. vannielii* sequence is the only archaebacterial 23S rRNA sequence avialable for comparison, therefore a strong statement on the subdivision within the archaebacteria cannot be made. The nucleotide sequences of the 23S rRNAs from *Sulfolbus solfataricus, Halobacterium volcanii* and *Desulfurococcus mobilis* are being determined by independent research groups (this information is obtained from Garrett, 1985 and Jarsch and Bock, 1985 a).

The high degree of conservation in secondary structures, and to a lesser extent sequences, at the functional sites on the large subunit ribosomal RNA implies the conservation of these biological functions. The translational apparatus is, in general, universal to all organisms. This supports the arguement of a common ancestry in translational machineries. Divergent evolution is probably responsible for specific changes unique to each phylogenetic group.

Further studies on the regulation, the assembly and the specific functions of the ribosomal RNAs are undoubtedly needed to provide understanding of the process of translation in archaebacteria.

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APPENDIX

Complete nucleotide sequence of the H. cutirubrum 23S rRNA gene

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      10         20         30         40         50         60         70         80         90        100        110        120
GTGGCTACTG TGCCACCTGG TGGATAGCTC GGCTCGGATG CCGACGAAGG ACGTGCCAAG CTGCGATAAG CCTGAGGGAG CCGCACGGAG GCTAAGAACT CAGGATCTCC TAATGGGAAT
CACCGATGAC ACGGTGGACC ACCTATCGAG CCGAGCCTAC GGCTGCTTCC TGCACGGTTC GACGCTATTC GGACTCCCTC GGCGTGCCTC CGATTCTTGA GTCCTAGAGG ATTACCCTTA

     130        140        150        160        170        180        190        200        210        220        230        240
CCCTATAACA ATTGCCTTGC CGAATGGGGA ACGGCCGGAA TTGAAGCATC TCAGTACGGC CAGGAAGAGA AATCGAATGA GACGCCGTTA GTAATGGCGA ATGAACGCGG CACAGTCCAA
GGGATATTGT TAACGGAACG GCTTACCCCT TGCCGGCCTT AACTTCGTAG AGTCATGCCG GTCCTTCTCT TTAGCTTACT CTGCGGCAAT CATTACCGCT TACTTGCGCC GTGTCAGGTT

     250        260        270        280        290        300        310        320        330        340        350        360
ACCGAAGCCT TCGGGCAATG TGGTGTTCGG ACTGACTTTC ATCGTTTGAC CGTTCGTGTG AAGTCTCCTG AAACGGAGCG CGATACAGGG TGACAGCCCC GTATCACGGA CCAGTACGAC
TGGCTTCGGA AGCCCGTTAC ACCACAAGCC TGACTGAAAG TAGCAAACTG GCAAGCACAC TTCAGAGGAC TTTGCCTCGC GCTATGTCCC ACTGTCGGGG CATAGTGCCT GGTCATGCTG

     370        380        390        400        410        420        430        440        450        460        470        480
GTGCGTCAGC TCCAGAGTAG CGGGGGTTGG AAATCCCTCG TGAATGTGGC AGGCATCGAC TGCCAAGACT AAGTACTCTC CGAGACCGAT AGTGAACAAG TAGTGTGAAC GAACGTGAAA
CACGCAGTCG AGGTCTCATC GCCCCCAACC TTTAGGGAGC ACTTACACCG TCCGTAGCTG ACGGTTCTGA TTCATGAGAG GCTCTGGCTA TCACTTGTTC ATCACACTTG CTTGCACTTT

     490        500        510        520        530        540        550        560        570        580        590        600
AGCACCCCAC AAAGGGGGGT GAAATAGGGC TTGAAATCAG GTGGCGATGG AGCGACGGGG CATAAAAGGC CTCTCTGGGA ACGACTTGAG TGCAAACTCA TGGTAGGACC TGAGAGGAGC
TCGTGGGGTG TTTCCCCCCA CTTTATCCCG AACTTTAGTC CACCGCTACC TCGCTGCCCC GTATTTTCCG GAGAGACCCT TGCTGAACTC ACGTTTGAGT ACCATCCTGG ACTCTCCTCG

     610        620        630        640        650        660        670        680        690        700        710        720
CGATGTTCCG TCGTACGTTT TGAAAAACGA GGCCAGGGAG TGTGCCTGTT TGACGAGTCT AACCGGAGTA TCCGGGAAGG CGTAGGGAAA CCAATATGGC CGCAGCATTG CGAGGGCCAC
GCTACAAGGC AGCATGCAAA ACTTTTTGCT CCGGTCCCTC ACACGGACAA ACTGCTCAGA TTGGCCTCAT AGGCCCTTCC GCATCCCTTT GGTTATACCG GCGTCGTAAC GCTCCCGGTG

     730        740        750        760        770        780        790        800        810        820        830        840
CGTGTTCAAG CGCGGGGAGT CAAACGGGCA CGACCCGAAA CCCGGTGATC TACGCGTGGG CAAGGTGAAG CATGGCGAAA GCCATGTGGA GGCCTGTTAG GGTTGGTGTC TTTCAACACC
GCACAAGTTC GCGCCCCTCA GTTTGCCCGT GCTGGGCTTT GGGCCACTAG ATGCGCACCC GTTCCACTTC GTACCGCTTT CGGTACACCT CCGGACAATC CCAACCACAG AAAGTTGTGG

     850        860        870        880        890        900        910        920        930        940        950        960
CTCCCGTGAC CTACGTGTAG GGGTGAAAGG CCCATCGAAC CGGGCAACAG CTGGTTCCAA CGGAAACATG TCGAAGCATG ACCTCTGCCG AGGTAGTTCG TGGGGTAGAG CGACCGATTG
GAGGGCACTG GATGCACATC CCCACTTTCC GGGTAGCTTG GCCCGTTGTC GACCAAGGTT GGCTTTGTAC AGCTTCGTAC TGGAGACGGC TCCATCAAGC ACCCCATCTC GCTGGCTAAC

     970        980        990       1000       1010       1020       1030       1040       1050       1060       1070       1080
GGGAGTTCAA CTCCGAGAGG AGTTGTCTCC CCTGTCAAAC TCCAAACCTA CGGACGCCGT CGACGCAGGG AATCCGGTGT GCGGGGTAAG CCTGTGCACC GTGAGGAGAC AACCCAGAGT
CCCTCAAGTT GAGGCTCTCC TCAACAGAGG GGACAGTTTG AGGTTTGGAT GCCTGCGGCA GCTGCGTCCC TTAGGCCACA CGCCCCATTC GGACACGTGG CACTCCTCTG TTGGGTCTCA

     1090       1100       1110       1120       1130       1140       1150       1160       1170       1180       1190       1200
TAGGTTAAGG TCCCAAAGTG CGAGCTAAGT GCGATTGAAG GTGGTCTCGA GCCCTAGACA GCCGGGAGGT GAGCTTAGAA GCAGCTACCC TCTAAGAAAA GCGTAACAGC TTACCGGCCG
ATCCAATTCC AGGGTTTCAC GCTCGATTCA CGCTAACTTC CACCAGAGCT CGGGATCTGT CGGCCCTCCA CTCGAATCTT CGTCGATGGG AGATTCTTTT CGCATTGTCG AATGGCCGGC

     1210       1220       1230       1240       1250       1260       1270       1280       1290       1300       1310       1320
AGGTTCGAGG CGCCCAAAAT GATCGGGGCT CAAGTTCGCC ACCGAGACCT AACGGCACGG GTAACACCGT GATCCAGTAG GTTGGCATTC TGTTCGGGTG GAAGCTCGGG TGAGAACTCG
TCCAAGCTCC GCGGGTTTTA CTAGCCCCGA GTTCAAGCGG TGGCTCTGGA TTGCCGTGCC CATTGTGGCA CTAGGTCATC GAACCGTAAG ACAAGCCCAC CTTCGAGCCC ACTCTTGAGC

     1330       1340       1350       1360       1370       1380       1390       1400       1410       1420       1430       1440
AGTGGACCGA GTGGAAAAGA AAATCCTGGC CATAGTAGCA GCGTTAGTCG GGTAAGAATC CCGATGGCCG AAAGAGCAAG GGTTCCTCGG CAATGCTTAT CAGCCGAGGG TTAGCCGATC
TCACCTGGCT CACCTTTTCT TTTAGGACCG GTATCATCGT CGCAATCAGC CCATTCTTAG GGCTACCGGC TTTCTCGTTC CCAAGGAGCC GTTACGAATA GTCGGCTCCC AATCGGCTAG

     1450       1460       1470       1480       1490       1500       1510       1520       1530       1540       1550       1560
CTAAGGCCCG TCGTAATTCG AGCGGGTCAA AAGGGAAACT GGTTAATATT CCAGTGCCAC CGTACATTGA AAGTCGACGC CTCGGAGCAG CTTGAACCGG GCATTCGCCC GGTCGAACCG
GATTCCGGGC AGCATTAAGC TCGCCCAGTT TTCCCTTTGA CCAATTATAA GGTCACGGTG GCATGTAACT TTCAGCTGCG GAGCCTCGTC GAACTTGGCC CGTAAGCGGG CCAGCTTGGC
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