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The number, physical organization and transcription of ribosomal RNA cistrons in an archaebacterium: Halobacterium halobium

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ABSTRACT

Because it is now clear that *archaebacteria* may be as distinct from *eubacteria* as either group is from eukaryotic cells, and because a specifically archaebacterial ancestry has been proposed for the nuclear-cytoplasmic component of eukaryotic cells, we undertook to characterize, for the first time, the ribosomal RNA cistrons of an archaebacterium (*Halobacterium halobium*). We found these cistrons to be physically linked in the order 16S-23S-5S, and obtained evidence that they are also transcribed from a common promoter(s) in the order 5'-16S-23S-5S-3'. We showed that, although slightly larger immediate precursors of 16S and 23S are readily seen, no common precursor of both 16S and 23S can be easily detected *in vivo*. In all these respects the archaebacterium *H. halobium* is like a eubacterium and unlike the nuclear-cytoplasmic component of eukaryotic cells. We found, however, that it differs from eubacteria of comparable (large) genome size in having only one copy of the rRNA gene cluster per genome.

INTRODUCTION

In the past two decades we have come to believe that "the line of demarcation between eukaryotic and prokaryotic cellular organisms is the largest and most profound single evolutionary discontinuity in the contemporary biological world (1)". During the last decade, it has also become generally accepted that the properties of eukaryotic cells are themselves the result of cooperation between two (in animals) or three (in plants) distinct genomes with separate evolutionary histories; those of nuclei, mitochondria and plastids. Plastids are almost certainly the descendants of once free-living, oxygen-evolving, photosynthetic prokaryotes which became entrapped in permanent cytoplasmic endosymbioses (2,3), and mitochondria are perhaps similarly descended from aerobic bacteria (4). The evolutionary origin of the nuclear genome is still obscure.

In the last two years, it has become apparent that prokaryotes are themselves divided by an evolutionary discontinuity as profound as that separating them from eukaryotes. Sequence analyses of 16S ribosomal RNAs (rRNAs) first

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indicated (5), and many other kinds of analyses now confirm, that the *"eubacteria"* (most bacteria, all blue-green algae, all chloroplasts and mitochondria) and the *"archaebacteria"* (composed so far of methanogenic bacteria, halobacteria, and the thermoacidophiles *Thermoplasma* and *Sulfolobus*) comprise two quite distinct assemblages of prokaryotic organisms, whose evolutionary divergence may have been very ancient (6,7,8).

There are several reasons to suppose that the nuclear genomes of eukaryotic cells derive from archaebacteria. Searcy *et al.* (9) argue that *Thermoplasma acidophilum*, which shows histone and actin-like proteins and an energy metabolism like that postulated for the first eukaryotic cells, is a contemporary prokaryotic survivor of the eukaryotic (nuclear) lineage. Stoeckenius (10) and Bayley and Morton (11) have summarized features of halobacteria which seem remarkably eukaryotic. These include the presence of membrane glycoproteins, amino-acid transport mechanisms, ribosomal proteins and initiator methionyl-tRNAs all more like those of eukaryotes than those of eubacteria.

One character which seems to distinguish nuclear genomes from those of *eubacteria* is the physical and transcriptional organization of rRNA cistrons. In all nuclei, "18S", "5.8S" and "28S" rRNA cistrons are linked, and transcribed in the order 5'-18S-5.8S-28S-3' to produce a single easily detectable common precursor whose subsequent processing gives rise to the mature species (12,13). 5S genes are separately transcribed and usually, but not always (14,15), physically unlinked. In the diverse eubacteria *Escherichia coli* (16), *Bacillus subtilis* (17) and the blue-green alga *Anacystis nidulans* (J.D. Hofman and W.F. Doolittle, unpublished), multiple rRNA cistrons are linked in the order 16S-23S-5S and are (at least in the first two) transcribed in that order from a single promoter or cluster of promoters. Precursor processing begins before transcription is complete, and only the immediate precursors (p23, p16 and p5) of the mature species can be detected in wild-type cells (18).

Archaebacteria have rRNAs described as 16S, 23S and 5S (5,6,7) but the number and physical and transcriptional organization of the cistrons coding for them is unknown. If claims for the archaebacterial origin of the nucleus are correct and if, as the data of Wrede and Erdmann (19) suggest, eukaryotic 5.8S evolved from prokaryotic 5S, then one might expect archaebacterial rRNA cistrons to be linked in the order 16S-5S-23S and perhaps to produce a readily-detectable common transcript. Here we present data bearing on the physical and transcriptional organization of rRNA cistrons in the archae-

bacterium *Halobacterium halobium*, and on the number of such cistrons borne by the halobacterial genome.

MATERIALS AND METHODS

Culture conditions

Halobacterium halobium strain HR1 (of W. Stoeckenius) was obtained from R.D. Simon, and grown on a medium containing, per liter, 250 gm NaCl, 20 gm $MgSO_4.7H_2O$, 3 gm sodium citrate, 2 gm KCl, 0.2 gm $CaCl_2.2H_2O$ and 100 ml of a separately autoclaved and neutralized solution of 3.0% Bacto-yeast extract and 5.0% Bacto-Tryptone. For ^{32}P-orthophosphate-labelling, medium was completely dephosphorylated by the method of Nazar *et al.* (20). Growth was at 39-40°C, with vigorous shaking.

Preparation of DNA

Cells at a turbidity of 0.5 A_{550} units were pelleted by centrifugation at 8000 x g for 5 min and lysed by dispersion in 2.3 ml TE (10 mM Tris [Tris (hydroxymethyl) aminomethane]-HCl, 1 mM EDTA [ethylenediaminetetraacetate] pH 7.3) per 100 ml culture. After addition of sodium N-lauroyl sarcosine to 0.2%, DNA was extracted by the method of Zaslof *et al.* (21) and stored as an ethanolic suspension at - 20°C. ^{3}H-DNA was obtained from cells grown in 4 µCi/ml ^{3}H-5,6-uracil (New England Nuclear, 40-50 Ci/mmol).

Isolation and labelling of RNA for hybridization experiments

Cells at 0.6 A_{550} units were pelleted and lysed by dispersion in 10 mM Tris-HCl, pH 7.4. After addition of $MgCl_2$ to 10 mM and sarkosyl to 0.4%, deoxyribonuclease I (ribonuclease-free, Worthington) was added to 10 µg/ml and the lysate was incubated at 22°C for 2 hr. After addition of SDS (sodium dodecylsulfate) to 1.0% and EDTA to 25 mM, RNA was isolated by three extractions with TE-saturated redistilled phenol and precipitated by the addition of sodium acetate to 0.2 M and ethanol to 70%. Precipitated RNA was collected by centrifugation, dissolved in TE, extracted with ether (2-3 times) and sparged with N_2.

In vivo-labelled RNA was extracted from cells grown in dephosphorylated medium containing 15 µCi/ml ^{32}P-orthophosphate. 16S and 23S rRNAs were prepared by two cycles of 2.0 M NaCl precipitation and sucrose gradient centrifugation (22) and further purified by electrophoresis on 4.0% polyacrylamide gels containing 7.0 M urea (23). 5S rRNA was precipitated with ethanol from the supernatants remaining after salt-precipitation of 16S and 23S rRNAs and fractionated on 10% polyacrylamide gels containing 7.0 M urea (23). RNAs were extracted from gels as described by Maxam and Gilbert (24) and further puri-

fied by passage through columns of Whatman CF-11 cellulose powder (14).

High-specific-activity *in vitro*-labelled 5S, 16S and 23S rRNAs were prepared from the purified *in vivo*-labelled products. Following partial hydrolysis in 50 mM Tris-HCl, pH 9.5 (at 85°C for 5 min for 5S and at 95°C for 5 min for 16S and 23S rRNAs) rRNAs were 5'-end labelled by the method of Maxam and Gilbert (24) with polynucleotide kinase (Boehringer-Mannheim) and γ-^{32}P-ATP (> 5000 Ci/mmol) synthesized by the method of Walseth and Johnson (25).

Hybridization

Solution hybridizations using *in vivo*-labelled RNA (3.2 x 10^5 cpm/μg) and 50 μg DNA in 150 μl were performed at 50°C as described by Casey and Davidson (26). Filter hybridizations using *in vivo* labelled rRNA and ^{3}H-labelled DNA (*ca* 5 μg/filter) were performed as described by Gillespie (27).

"Southern" hybridizations were performed with 100 μg of DNA digested to completion in a volume of 120 μl containing 9 mM Tris-HCl, pH 7.4, 4.4 mM $MgCl_2$, 50 mM NaCl, 100 μg/ml autoclaved gelatin (Sigma) and 25 units of *Eco* R1 (New England Biolabs). The digested DNA, and ^{32}P-labelled *Eco* R1-digested λ DNA (28) as marker, were resolved on 0.7% agarose gels made in 50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 20 mM sodium acetate, 18 mM NaCl and transferred to nitrocellulose filters (29). After transfer, the filters were cut into 1 cm strips (each carrying *ca* 4 μg DNA). Strips were hybridized with 15 to 750 ng of *in vitro*-labelled rRNAs (each at 2 x 10^6 cpm/μg) in 200 μl of 50% deionized formamide, 4xSSC (0.6 M NaCl, 0.06 M sodium citrate) and 60 μg/ml *E.coli* tRNA (Schwartz-Mann), degassed before use. Hybridizations were performed in sealed plastic bags at 42°C for 16 hr. Strips were washed three times for 5 min in 5xSSC, once for 2 hr in 50% formamide:4xSSC at 42°C, three times for 5 min in 5xSSC, and twice for 20 min in 3 mM Tris base. After mounting and drying the strips on glass plates, radioactive bands were detected by autoradiography at -70°C with Cronex Lightning Plus intensifying screens (Du Pont) and Kodak X-Omat XRP-1 film.

Kinetic labelling experiments

Logarithmically growing cells (generation time *ca* 4 hr) were harvested and resuspended in phosphate-free medium 2-3 hr before labelling with ^{32}P-orthophosphate (New England Nuclear). Labelling with ^{3}H-5,6-uracil was effected in medium of normal phosphate content. For lysis and RNA extraction, 1.0 ml samples were removed and cells were harvested by centrifugation (5 sec at 15,000 x g in an Eppendorf 5412 microcentrifuge). Lysis was effected by vigorous suspension in 0.4 ml of TE containing 1% SDS and 20 μg/ml *E.coli*

tRNA. Lysates were extracted 3-4 times with TE-saturated redistilled phenol, and RNA was precipitated with sodium acetate (to 0.2 M) and 2 volumes of ethanol. After 10 min at - 70°C, RNAs were resuspended in loading buffer and subjected to electrophoresis on 2.8 or 10.0% polyacrylamide gels, as described previously (16,31). Gel slices were dissolved in toluene-Protosol-Omnifluor (New England Nuclear) and analysed in a Nuclear Chicago Mark II Liquid Scintillation spectrometer.

RESULTS

The number of ribosomal RNA cistrons in *H. halobium*

RNA:DNA hybridization experiments were performed in two different ways. (A) *Solution hybridization.* 16S and 23S rRNAs labelled *in vivo* with ^{32}P-orthophosphate to a specific activity of 3.2 x 10^5 cpm/μg were separately eluted from 4.0% polyacrylamide gels containing 7M urea, and hybridized with unlabelled *H. halobium* DNA in solution, under conditions (26) which minimize or eliminate DNA reannealing (Fig. 1). In 48 hr, saturation by 23S rRNA was achieved with RNA at 100 ng/150 μl and an RNA:DNA ratio of 0.002. Approximately 0.017% of the added DNA was involved in hybrid formation. The 16S rRNA, although gel-purified, was clearly contaminated with fragments of 23S. (An inflection in the 16S saturation curve corresponding to hybridization with 0.010% of the DNA *was* observed at a concentration of 50 ng/150 μl.) With pooled 16S and 23S rRNAs, approximately 0.021% of the added DNA was hybridized at saturation (at about 150 ng RNA/150 μl). (B) *Filter hybridization.* Gel-purified 23S rRNA from the same *in vivo*-labelled preparation was hybridized by the method of Gillespie (27) to nitrocellulose-filter bound DNA which had been labelled *in vivo* with ^{3}H-5,6-uracil to a specific activity of 1.08 x 10^3 cpm/μg (Fig. 2). Saturation was achieved (after 16 hr at 66°C in 2xSSC) at 50 ng RNA/400 μl and an RNA:DNA ratio of 0.01, and corresponded to hybridization of 0.024% of the DNA.

The values for 23S hybridization obtained by these two methods (0.017 and 0.024%) were in reasonable agreement, and the lower value (*ca* 0.01%) obtained with 16S rRNA suggests that the halobacterial genome has equal numbers of 23S and 16S rRNA genes. These values are, however, surprisingly low. If we assume that 0.02% of the halobacterial genome codes for 23S rRNA, and that this genome comprises 2.7 x 10^9 daltons (30), then only 0.54 x 10^6 daltons of (single-stranded) DNA can code for the approximately 1.1 x 10^6 dalton 23S molecule. The most reasonable interpretation is that there is in fact but a single copy of the 23S (or 16S) rRNA cistron in the *H. halobium* genome, and that the values

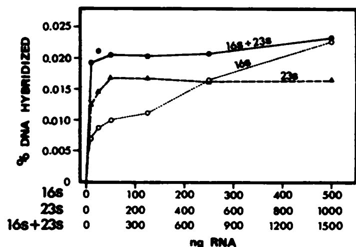

Fig. 1. Solution hybridization of ^{32}P-labelled *H. halobium* rRNAs to *H. halobium* DNA.

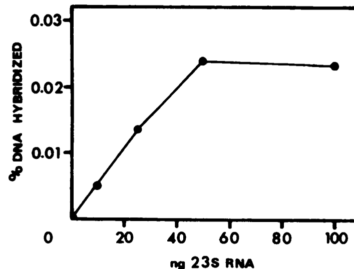

Fig. 2. Filter hybridization of ^{32}P-labelled *H. halobium* 23S rRNA to *H. halobium* DNA.

for gene dosage determined from Figs.1 and 2, the genome size previously determined by Moore and McCarthy from renaturation kinetics (30), or both, represent underestimates within the limits of sensitivity of these methods. It is possible to test this interpretation by "Southern" hybridization experiments, and such hybridization experiments indicate that there is indeed no more than a single gene for each rRNA (16S, 23S and 5S) on the halobacterial chromosome (see below).

Physical linkage of 16S, 23S and 5S rRNA genes

Eco R1 digests of total halobacterial DNA were resolved on 0.7% agarose gels (with *in vitro*-^{32}P-labelled *Eco* R1 λ fragments as markers) and transferred to nitrocellulose filters by a modification of the method of Southern (29). Identical strips cut from the filters were separately hybridized (in 200 μl of 50% formamide:4xSSC at 42°C for 16 hr) with *in vitro* ^{32}P-labelled, partially hydrolyzed, gel-purified 5S, 16S and 23S rRNAs, present at 15, 30, 75, 150, 375 and 750 ng/200 μl (Fig. 3). 5S rRNA gave hybridization signals with a fragment of approximately 3.1 md (million daltons) even at the lowest concentrations. At the highest concentrations, contamination by degradation products of 16S or 23S rRNA produced hybridization signals with an 11.0 md fragment. (It is possible to determine the extent of this contamination by measuring, in a liquid scintillation counter, radioactivity contained in each band of each filter strip. The amount of radioactivity bound to the 11.0 md fragment in the presence of 750 ng of the 5S preparation was equivalent to that bound in the presence of 10-20 ng of the 16S or 23S preparations, so contamination was less than 3.0%.) 16S rRNA gave hybridization signals only with an 11.0 md fragment except, again, at the highest concentrations. 23S

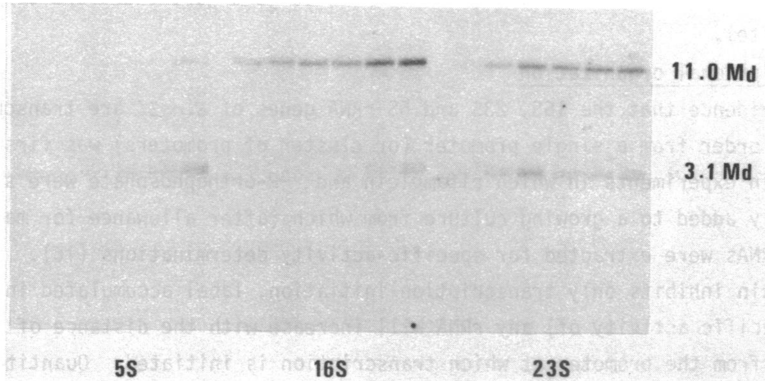

Fig. 3. "Southern" hybridization of purified ^{32}P-*in vitro*-labelled *H. halobium* 5S,16S and 23S rRNAs to nitrocellulose filter-bound *Eco* R1 fragments of *H. halobium* DNA. Successive strips correspond (from left to right) to RNA concentrations of 15, 30, 75, 150, 375 and 750 ng/200 µl.

rRNA gave signals of different absolute intensities with the two *Eco* RI fragments, but similar *relative* intensities with both the 3.1 md and the 11.0 md fragment at all concentrations, and we conclude that portions of its sequence are present on each DNA fragment. It is thus apparent that 23S and 5S-specific sequences occur together on the 3.1 md fragment and that the separation between them cannot be any *more* than about 4000 base pairs (assuming that about one-third of the 23S cistron is present on this fragment). Other 23S-specific sequences are present on the 11.0 md fragment together with all detectable 16S-specific sequences. The physical order is therefore 16S-23S-5S, and there is an *Eco* R1 site within the 23S cistron. No other fragments showed signals with any of these probes and thus either (i) there is more than one cistron for each rRNA, but all *Eco* R1 sites lie within conserved regions of the rRNA genes themselves, and 16S and 23S genes are separated by spacers all of which are the same size and very large (> 12,000 base pairs), (ii) there is more than one cistron for each rRNA but flanking sequences at some distance (as much as 12,000 base pairs) are all conserved, or (iii) as the quantitative hybridization experiments discussed earlier independently indicate, there is only one copy of each cistron. The last possibility seems again the most reasonable, and gains further support from the observation (not illustrated) that *Xho* I-digested DNA shows only a single (> 11 md) fragment which hybridizes to 5S and 23S rRNAs, and only this fragment and a much

smaller one which hybridizes to 16S rRNA (whose gene must therefore contain an *Xho* I site).

Transcriptional organization

Evidence that the 16S, 23S and 5S rRNA genes of *E.coli* are transcribed in that order from a single promoter (or cluster of promoters) was first obtained in experiments in which rifampicin and ^{32}P-orthophosphate were simultaneously added to a growing culture from which (after allowance for maturation) rRNAs were extracted for specific-activity determinations (16). Since rifampicin inhibits only transcription initiation, label accumulated in (and thus specific activity of) any rRNA will increase with the distance of its cistron from the promoter at which transcription is initiated. Quantitative predictions can be made and tested if there is no lag in rifampicin inhibition or phosphate-pool equilibration (11,16). Even if these conditions are not met, drastic differences in 5S rRNA specific activity are expected depending on the position of its gene with respect to the promoter from which it is transcribed. If the 5S gene is independently transcribed from a nearby promoter, or is promoter-proximal in a common transcriptional unit, then the specific activity of 5S rRNA will be very much less than that of 16S or 23S rRNA (because so many fewer RNA polymerase molecules can reside on the 5S gene at the time of rifampicin addition). If the 5S rRNA gene lies between 16S and 23S genes in a common transcriptional unit, 5S specific activity will be between those of 16S and 23S rRNA, while if the 5S gene is promoter distal to both 16S and 23S genes, 5S specific activity will be higher than 16S or 23S specific activities.

Preliminary experiments showed that rifampicin action is rapid but phosphate pool equilibration is slow in *H. halobium*; no label incorporation is detected if ^{32}P-orthophosphate is added simultaneously with rifampicin. For the experiment shown in Fig. 4, a culture grown for 24 hr in 1 mM phosphate and 5 μCi/ml ^{3}H-5,6-uracil was washed and resuspended in phosphate-free medium. (Control experiments showed that phosphate deprivation, although increasing incorporation of ^{32}P-orthophosphate, has no effect on growth for two generations [9 hr].) After 3 hr, ^{32}P-orthophosphate was added (to 0.5 mCi/ml). Rifampicin was added 5 min later (to 200 μg/ml). After 85 min incubation, RNA was extracted and resolved on polyacrylamide gels (Fig. 4). Maturation was not complete and ^{32}P-incorporation into 16S and 5S (although reproducibly observable) was low. Specific activities, determined as the ratio of cpm above background in peaks of ^{32}P-radioactivity to cpm above background in the corresponding (but not coincident) peaks of ^{3}H-radioactivity, should be taken as only approximate. Observed

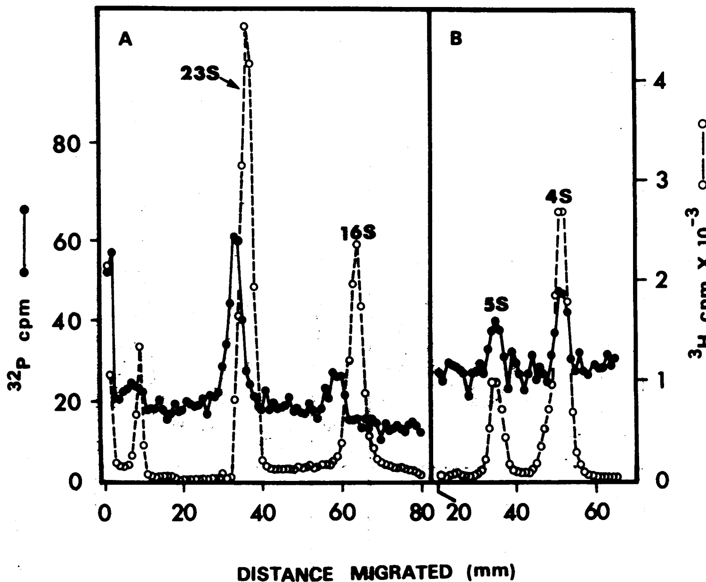

Fig. 4. Effect of addition of rifampicin on accumulation of label in 23S, 16S and 5S rRNAs. Panel A: 2.8% polyacrylamide gel. Panel B: 10.0% polyacrylamide gel. Closed circles: ^{32}P-radioactivity, from label added 5 min before rifampicin. Open circles: ^{3}H-radioactivity from 24 hr prelabelling in ^{3}H-5,6-uracil.

specific activities for 16S, 23S and 5S rRNA were in the ratio 1:1.8:1.9 (*versus ca* 1:1:1 for the uninhibited culture). This result eliminates models (consistent with the restriction mapping data) in which all three genes are transcribed independently, or transcribed together in the order 5'-5S-23S-16S-3'. It favors a model in which transcription from a common promoter occurs in the order 16S-23S-5S, but does not eliminate the possibility that 23S and 5S are transcribed together in that order, while 16S is independently transcribed.

Post-transcriptional processing

Figs. 5 and 6 show the results of an experiment in which ^{32}P-orthophosphate was added to a logarithmically growing culture in phosphate-free medium at zero time, and samples were harvested for extraction of RNA at 10, 30, 90 and 270 min. RNA was resolved on 2.8% (Fig. 5) or 10.0% (Fig. 6) polyacrylamide gels with ^{3}H-5,6-uracil-labelled *Anacystis nidulans* rRNAs as markers. (This blue-green alga exhibits partial post-maturational cleavage of

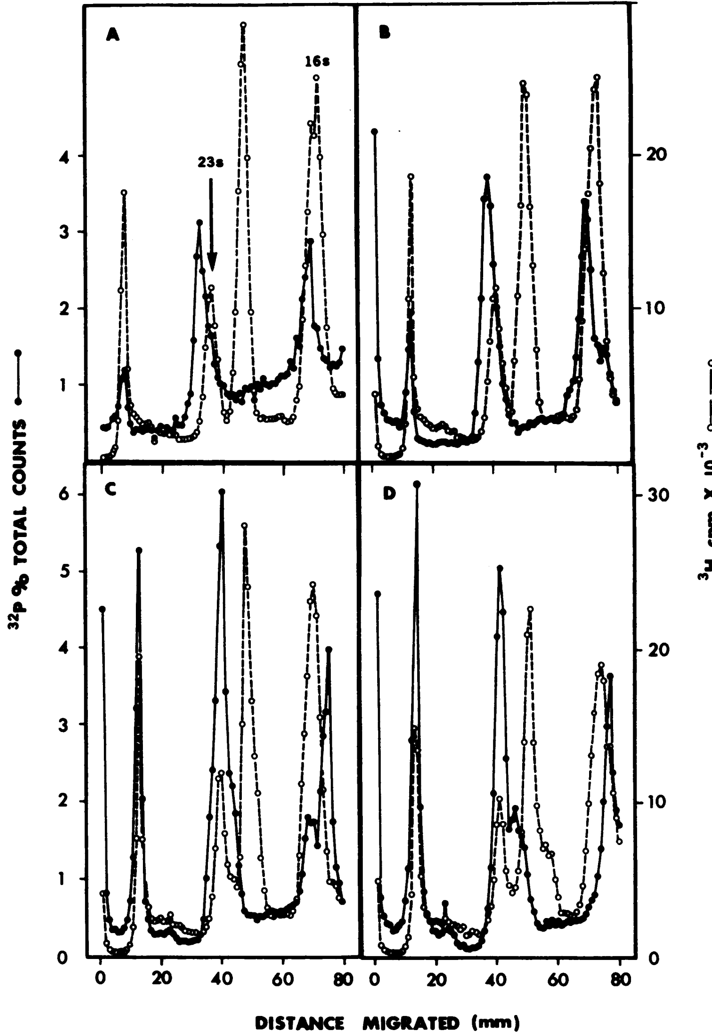

Fig. 5. Accumulation of ^{32}P-radioactivity in high-molecular-weight RNA after 10(A), 30(B), 90(C) and 270(D) min of exposure. Closed circles: ^{32}P-radioactivity. Open circles: ^{3}H-radioactivity from added *A. nidulans* marker RNA. Results presented as percent of total radioactivity in gel slices 1-80.

its 23S rRNA, and the [0.88 md] species migrating between 23S and 16S corresponds to the larger of the two fragments produced [31].) Species of decreasing mobility are increasingly prominent in *H. halobium* RNAs labelled for

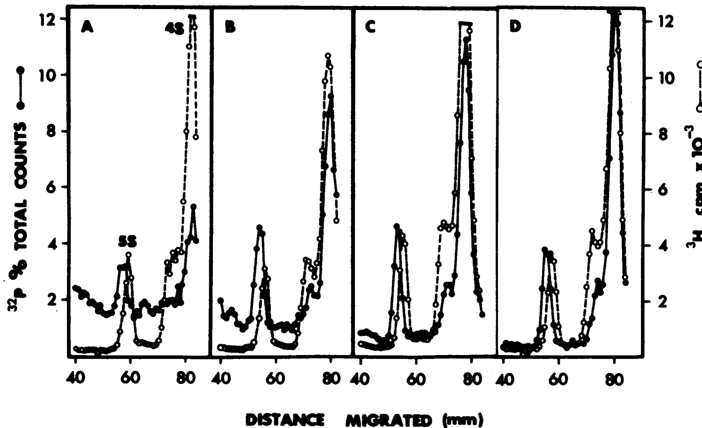

Fig. 6. Accumulation of ^{32}P-radioactivity in low-molecular-weight RNA after 10(A), 30(B), 90(C) and 270(D) min of exposure. Closed circles: ^{32}P-radioactivity. Open circles: ^{3}H-radioactivity from added *A. nidulans* marker RNA. Results presented as percent of total radioactivity in gel slices 40-85.

increasingly long periods of time (Fig. 5). The simplest interpretation is that mature *H. halobium* 23S and 16S rRNAs (apparent molecular weights 1.05-1.10 and 0.50-0.52 md) are derived from immediate precursors ("p23" and "p16") of respective molecular weights 1.10-1.15 and 0.57-0.59 md. The same pattern was observed with RNAs heated for 3 min at 100°C prior to loading, and so these mobility differences are unlikely to reflect only conformational changes. In no sample was an RNA of the mobility expected of a common precursor to 16S and 23S rRNAs detected. (The material migrating between 10 and 15 mm does not behave kinetically like a precursor and is insensitive to ribonuclease; we presume it to be DNA.) No higher-molecular-weight precursor to halobacterial 5S rRNA was observed (Fig. 6), although this species, which may have a unique secondary structure (20), migrated consistently more slowly than *A. nidulans* 5S rRNA. Although it is never possible to prove that a very transient common precursor to 16S, 23S and 5S rRNAs does not exist, the pattern observed here is typically "prokaryotic" (18) and not "eukaryotic" (12).

DISCUSSION

We have shown that the 16S, 23S and 5S rRNA cistrons of an archaebacterium, *Halobacterium halobium*, are physically linked in the order 16S-23S-

5S. They are also probably transcribed in that order from a common promoter (or cluster of promoters) on the 5' side of the 16S rRNA cistron, but we cannot exclude the possibility that only 23S and 5S rRNA genes are transcribed together (in that order) with 16S rRNA genes being independently transcribed. The only detectable precursors are molecules of mobilities slightly less than those of mature 23S and 16S rRNAs. In all these respects *H. halobium* resembles a typical "eubacterium" such as *E.coli* (16), *B. subtilis* (17) or *A. nidulans* (31) more than it does the nucleus of eukaryotic cells. This would appear to weaken arguments (10,11) for the halobacterial ancestry of the eukaryotic nuclear-cytoplasmic lineage, although no single piece of evidence such as this can demolish such arguments.

What is perhaps more surprising is the finding, by three essentially independent methods, that *H. halobium* contains only one cluster of rRNA cistrons. Eubacteria of comparable genome size (*E. coli*, *B. subtilis* and *A. nidulans* being good examples representing a broad phylogenetic spectrum [5])characteristically have multiple rRNA gene-clusters. Only the small genomed (0.5-1.0 x 10^9 dalton) mycoplasmas are otherwise known to contain but a single set of rRNA cistrons (32).

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