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Comparison of sea urchin and human mtDNA: Evolutionary rearrangement

(recombinant DNA/hybridization/gene organization)

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ABSTRACT **Clones of full-length mtDNA have been isolated from a *Strongylocentrotus franciscanus* recombinant DNA library by screening a cDNA clone of cytochrome oxidase subunit 1 mRNA. Restriction fragment cross-hybridization analysis shows the following difference in gene arrangement between sea urchin and human mtDNA. The 16S rRNA and cytochrome oxidase subunit 1 genes are directly adjacent in sea urchin mtDNA. These two genes are separated in human and other mammalian mtDNAs by the region containing unidentified reading frames 1 and 2. In spite of the difference in gene order, gene polarity appears to have been conserved. We conclude that the difference in gene order reflects a rearrangement that took place in the sea urchin lineage since sea urchins and mammals last shared a common ancestor.**

Animal mtDNA has a fairly constant size of approximately 16 kilobase pairs (kb). The complete nucleotide sequences of human (1), bovine (2), and mouse (3) mtDNA reveal a compact organization of genetic information with no intervening sequences and little intergenic spacing. The overall gene order is identical in human, bovine, and mouse mtDNAs (1–3). Less is known about mtDNA organization in animals other than mammals, although similar tandem arrangements of the two rRNA genes have been reported for amphibian (4) and *Drosophila* mtDNAs (5, 6).

In spite of the constancy of size and gene order of mammalian mtDNA, there is evidence for the involvement of rearrangement and transposition in mtDNA evolution. Gene order surrounding the origin of replication in *Drosophila* mtDNA, for example, differs from that in mammalian mtDNA, apparently reflecting a translocation and inversion of a segment of mtDNA (6). Jacobs *et al.* (7) isolated a region from the nuclear genome of *S. purpuratus* that contains divergent copies of at least portions of two mitochondrial genes, those for 16S rRNA and cytochrome oxidase subunit 1 (*CO I*) mRNA. These sequences apparently were transferred from the mitochondrial to the nuclear genome before the evolutionary differentiation of the genus *Strongylocentrotus*. Other examples of sequence transfer between the nuclear and mitochondrial genomes are known. *Neurospora* mtDNA, for example, contains a sequence related to the functional nuclear gene for a subunit of the proton-translocating ATPase. No translation product of the mitochondrial gene has been detected (8). Similarly, yeast nuclear DNA contains sequences homologous to the mitochondrial *var1* and *cob* genes (9). In addition, Gellissen *et al.* (10) have reported the isolation of locust nuclear DNA clones containing mitochondrial rRNA gene sequences. Analogous gene transfer between plastid genomes can occur in plants. Maize mtDNA, for example, contains a 12-kb sequence homologous to a portion of the chloroplast genome containing the 16S rRNA gene and genes for two transfer RNAs (11).

We report the isolation and characterization of recombinant clones containing the entire mtDNA of the sea urchin *Strongylocentrotus franciscanus* and its comparison with human mtDNA revealing differences in gene arrangement.

MATERIALS AND METHODS

DNA Isolation. Sea urchin DNA was isolated by a modification of the sperm DNA isolation procedure of Britten *et al.* (12). Minced testis tissue in approximately 50 ml of 0.1 M NaCl/50 mM EDTA, pH 7–8, was filtered through a 50-μm pore size Nitex screen, frozen at −70°C, homogenized to a powder with dry ice, and then lysed and processed exactly like a sperm DNA preparation. Care was exercised to avoid shearing of the DNA during isolation.

Recombinant Charon 4A phage were grown and purified, and their DNA was extracted as described by Anderson *et al.* (13). Recombinant plasmid DNA was prepared from chloramphenicol-amplified cultures (14) by the method of Katz *et al.* (15).

Construction and Isolation of Recombinant Phage and Plasmids. The construction, isolation, and characterization of plasmid cDNA clones SpG30 and SpP389 have been described (7, 16).

Bacteriophage λ recombinants containing the entire *S. franciscanus* mitochondrial genome were isolated from a partial *Eco*RI library constructed from testis DNA of a *S. franciscanus* individual using Charon 4A as vector (17) as described by Anderson *et al.* (13). Screening of the library with radiolabeled SpG30 DNA (specific activity, approximately 10^8 cpm/μg) was carried out essentially as described by Anderson *et al.* (13) except that all hybridization and washing steps were carried out at 60°C, and the filters were subjected to three final washes of 0.3× SET (SET = 0.15 M NaCl/0.03 M Tris·HCl, pH 8.0/2 mM EDTA)/0.1% $NaDodSO_4$/1.5 mM sodium pyrophosphate/25 mM sodium phosphate. Subclones of restriction fragments from bacteriophage recombinants were prepared by standard methods using plasmid pBR322 as vector.

Restriction Mapping. Restriction endonuclease digestions were carried out under conditions recommended by the enzyme supplier. Restriction fragments were separated by electrophoresis on 0.5–2.5% agarose gels or 6% polyacrylamide gels using *Hin*dIII fragments of bacteriophage λ, *Hin*fI fragments

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Abbreviations: kb, kilobase pair(s); CO I, cytochrome oxidase subunit 1; bp, base pair(s); URF, unidentified reading frame.

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of plasmid pBR322, and *Xba* I fragments of human mtDNA as size markers.

Blot Hybridization. Methods for gel electrophoresis of DNA fragments and their transfer to nitrocellulose filters have been described (13, 18). Restriction fragments and subcloned DNAs used as hybridization probes were labeled to a specific activity of 0.2–2.0 × 10^8 cpm/μg by nick-translation (19).

All intraspecies hybridization and washing was carried out exactly as described for library screening. Hybridization of blots of human mtDNA with sea urchin tracers was carried out at open criterion to maximize cross reaction of divergent sequences. Hybridization was overnight at 55°C in 6× SET/5× Denhardt's solution (20)/0.1% $NaDodSO_4$ containing denatured calf thymus DNA at 50 μg/ml. After hybridization the filters were washed four to six times for 1 hr each at 55°C in the same solution lacking calf thymus DNA.

Nucleotide Sequence Analysis. Nucleotide sequence analyses of SpP389 fragments were carried out by using the chemical cleavage procedure of Maxam and Gilbert (21).

RESULTS

Isolation and Restriction Mapping of *S. franciscanus* mtDNA Clones. Recombinant plasmid SpG30 is a cDNA clone of approximately 700 nucleotides from the 3′ end of *CO I* mRNA, which is encoded in the mitochondrial genome (7, 16). Radiolabeled SpG30 DNA was used as a hybridization probe to screen a recombinant library constructed from a partial *Eco*RI digest of *S. franciscanus* DNA. Twenty recombinants that reacted with SpG30 were obtained. Three recombinants that yielded the same six *Eco*RI digestion products but different *Hin*dIII digestion patterns were chosen for further characterization. Restriction maps for these recombinants (Fig. 1) showed that they contain as inserts circular permutations of the same 15.5 ± 1 kb sequence, with the insert boundaries defined by three different natural *Eco*RI sites. Thus, each recombinant was generated by cleavage of a circular mtDNA molecule at a different *Eco*RI site followed by ligation of the linear full-length mtDNA molecule to the λ vector arms.

Fig. 1 also shows the map locations of the *Eco*RI, *Hin*dIII, and *Eco*RI/*Hin*dIII fragments of *S. franciscanus* mtDNA that were subcloned in plasmid pBR322 and the map locations of the *CO I* and 16S rRNA genes (see below).

Mapping of the 16S rRNA and *CO I* Genes in Sea Urchin mtDNA. The 16S rRNA gene was mapped in sea urchin mtDNA by using recombinant plasmid SpP389, which, according to its nucleotide sequence, carries most of the 16S rRNA gene of *S. purpuratus* (7). Fig. 2*A* is a restriction map of the SpP389 insert showing the four fragments that were used as hybridization probes on blots of *Hin*dIII digests of subclone R1 DNA. The results are shown in Fig. 2*B*, lanes 1–4. Note that the terminal fragments of the SpP389 insert react with *Hin*dIII fragments H2 and H4 (lanes 1 and 4). The polarity of the region of the 16S rRNA gene carried by SpP389 was determined by direct nucleotide sequence analysis (see below). The 3′ end of the 16S rRNA gene is, therefore, located in fragment H2 and the 5′ end is in fragment H4.

The location of the *CO I* gene in *S. franciscanus* mtDNA was determined by blot hybridization as shown in Fig. 2*B* (lane 5). Radioactive SpG30 tracer was allowed to react with a blot of an *Eco*RI/*Bam*HI digest of Fλ8. The predominant reaction was with a 1.2-kb fragment that extends to the left of the *Eco*RI site marked by the vertical arrow in Fig. 1. In some experiments, weak hybridization of SpG30 was detected to the 0.3-kb *Eco*RI fragment mapping directly to the right of the marked *Eco*RI site. The orientation of the *CO I* gene is given by the reaction of radiolabeled subclone R6 with a *Hin*cII fragment of SpG30 known from nucleotide sequence data to be from the 3′ end of the *CO I* gene (7). The sea urchin *CO I* gene is probably about 1.6 kb long, similar to that in human mtDNA, since SpG30 reacts with an approximately 1.6-kb RNA species from sea urchin eggs and embryos (7, 16). Thus, we conclude that the 3′ end of the *CO I* gene lies within the 0.3-kb *Eco*RI fragment (R6) and the 5′ end probably lies to the left of the *Bam*HI site as indicated in Fig. 1.

As shown in Figs. 1 and 2, the *CO I* and 16S rRNA genes are almost directly adjacent in sea urchin mtDNA. In contrast, the human *CO I* and 16S rRNA genes are separated by a greater than 2.4-kb segment of mtDNA containing unidentified reading frame (URF)-1 and URF-2 (1) (see Fig. 4). Thus, there appears to be a major difference in sequence organization between sea urchin and human mtDNAs.

Comparison of the Overall Sequence Organization of Sea Urchin and Mammalian mtDNA. To compare overall mtDNA sequence organization, radioactive subclones or isolated restriction fragments from sea urchin mtDNA were used as hy-

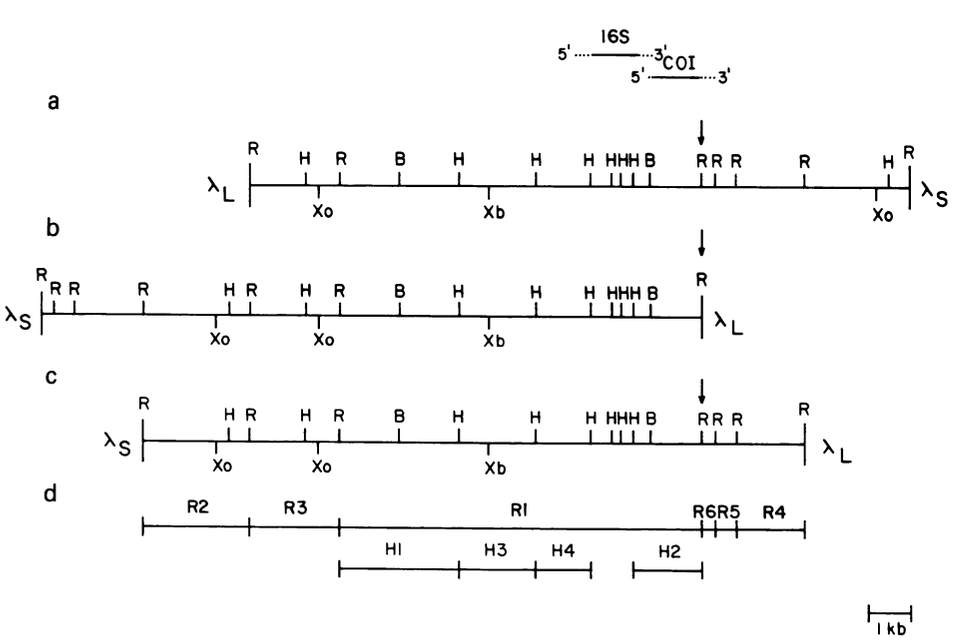

FIG. 1. Restriction endonuclease maps of *S. franciscanus* mtDNA clones Fλ9 (*a*), Fλ14 (*b*), and Fλ8 (*c*) and Fλ8 subclones (*d*). The maps are aligned on the basis of common restriction sites. An *Eco*RI site discussed in the text is indicated by a vertical arrow. The order of the four *Hin*dIII sites in the cluster mapping approximately 2 kb to the left of the marked *Eco*RI site is uncertain. Also indicated are the locations and polarities of the 16S rRNA and *CO I* genes and the *Eco*RI, *Hin*dIII, and *Eco*RI/*Hin*dIII fragments of Fλ8 that were subcloned in plasmid pBR322 and used as probes in cross-hybridization experiments. Abbreviations are: λ_L, the 19.8-kb arm of the Charon 4A vector; λ_S, the 10.9-kb arm of the Charon 4A vector; B, *Bam*HI; H, *Hin*dIII; R, *Eco*RI; Xb, *Xba* I; Xh, *Xho* I.

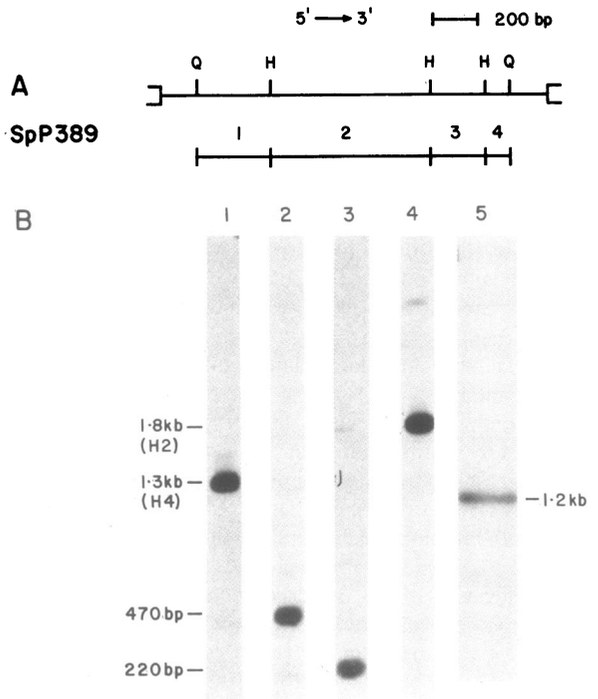

FIG. 2. Locations of 16S rRNA and *CO I* genes in sea urchin mtDNA. (*A*) Restriction map of the SpP389 insert showing the locations of the *Rsa* I (S) and *Hin*dIII (H) sites used to generate fragments 1–4. (*B*) Blots of restriction digests of mtDNA clones hybridized with radiolabeled cDNA clones or cDNA clone fragments. Lanes: 1–4, blots of *Hin*dIII digests of subclone R1 DNA hybridized to radioactive fragments 1–4 from the SpP389 insert; 5, blot of an *Eco*RI/*Bam*HI digest of Fλ8 DNA hybridized to radioactively labeled subclone SpG30 DNA.

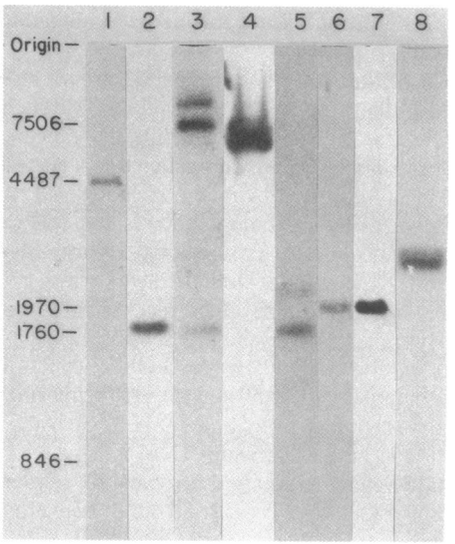

FIG. 3. Cross-hybridization of sea urchin and human mtDNA fragments. Blots of restriction digests of human mtDNA were hybridized to radiolabeled sea urchin mtDNA fragments. Lanes: 1, blot of an *Xba* I digest of human mtDNA hybridized to sea urchin fragment H3; 2, blot of an *Xba* I digest of human mtDNA hybridized to sea urchin fragment H4; 3, blot of an *Xba* I digest of human mtDNA hybridized to sea urchin fragment H1; 4, blot of an *Xho* I/*Eco*RI digest of human mtDNA hybridized to sea urchin fragment H1; 5, blot of an *Xba* I/*Eco*RI digest of human mtDNA hybridized to sea urchin fragment H2; 6, blot of an *Xba* I digest of human mtDNA hybridized to sea urchin fragment R4; 7, blot of an *Xba* I digest of human mtDNA hybridized to sea urchin fragment R2; 8, blot of an *Xho* I/*Eco*RI digest of human mtDNA hybridized to sea urchin fragment R3. At the left are the positions of the *Xba* I digestion products of human mtDNA used as size markers.

bridization probes on blots of appropriate restriction digests of human mtDNA. All hybridization and washing steps were carried out at substantially reduced criterion to maximize cross-reaction of divergent sequences. Some examples are shown in Fig. 3, and the results of the analysis are diagrammed in Fig. 4.

Figs. 3 and 4 confirm a difference in organization between human and sea urchin mtDNA involving the regions contained in sea urchin fragments H3 and H4. Fragment H3 reacts with the 4.5-kb *Xba* I fragment extending from position 2.9 to position 7.4 in human mtDNA (Fig. 3, lane 1). The extent of the crossreaction can be more precisely defined by the predominant reaction of H3 with a 1,465-base-pair (bp) *Hpa* II fragment extending from position 3.3 to position 4.7 in human mtDNA (data not shown). This region of human mtDNA contains URF-1 and a portion of URF-2. Fragment H4, which maps to the right of H3 in sea urchin mtDNA, reacts with the 1.76-kb *Xba* I fragment that extends between positions 1.2 and 2.9 in human mtDNA (Fig. 3, lane 2). This region of human mtDNA contains most of the 16S rRNA gene and a portion of the 12S rRNA gene. Thus, it appears that the 16S rRNA gene and the region containing URF-1 and URF-2 have exchanged positions in sea urchin mtDNA relative to their positions in human mtDNA.

The 12S rRNA gene does not appear to map directly to the left of the 16S rRNA gene in sea urchin mtDNA. Neither sea urchin fragment H3 nor H4 react with human mtDNA fragment to the left of the *Xba* I site at position 1.2. If the 12S rRNA

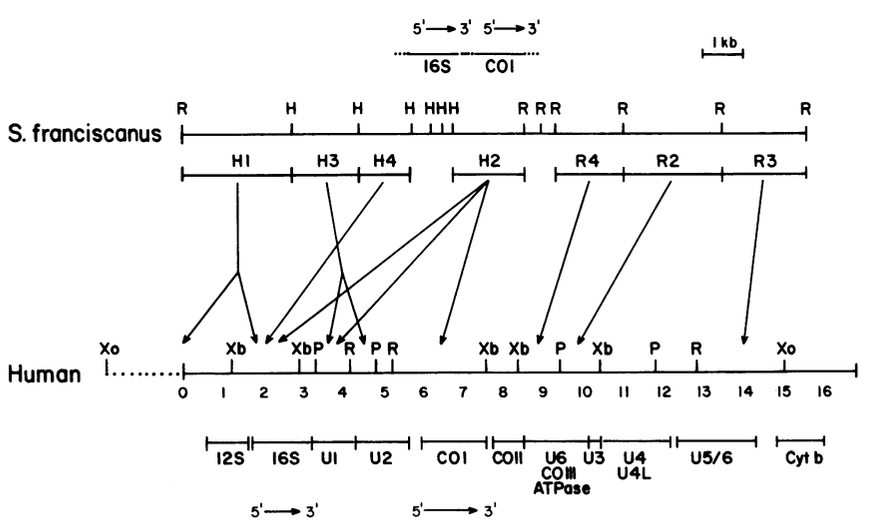

FIG. 4. Comparison of the sequence organization of sea urchin and human mtDNA. The upper line is a linear map of sea urchin mtDNA showing the locations of the 16S rRNA and *CO I* genes and of the *Eco*RI, *Hin*dIII, and *Eco*RI/*Hin*dIII fragments used as hybridization probes in cross-hybridization experiments. The lower line is a map of human mtDNA indicating *Eco*RI (R), *Xba* I (Xb), and *Xho* I (Xh) sites. Some *Hpa* II sites discussed in the text are abbreviated by P. Note that the human mtDNA has been linearized and is numbered in kb from the zero position of Anderson *et al.* (1). Gene order is given below the human mtDNA map (U, unidentified reading frame). Diagonal and vertical arrows denote cross-hybridization of a sea urchin fragment with a region of human mtDNA.

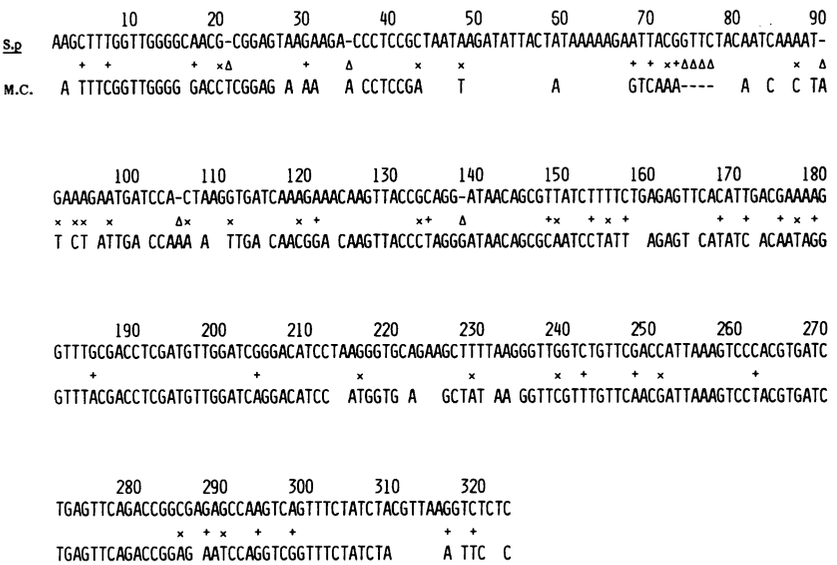

FIG. 5. Nucleotide sequence of a region of the sea urchin 16S rRNA gene. The nucleotide sequences of SpP389 fragments 3 and 4 (Fig. 2*A*) are shown in the upper line. The lower line is a mammalian unanimity sequence indicating positions occupied by an identical nucleotide in mouse, human, and bovine mtDNA (1–3). Gaps in the mammalian unanimity sequence indicate positions not occupied by an identical nucleotide in the three mammalian sequences. Differences between the sea urchin and mammalian unanimity sequences are indicated as follows: +, difference due to a transition; ×, difference due to a transversion; Δ, difference due to an insertion or deletion.

gene were directly to the left of the 16S rRNA gene in sea urchin mtDNA, these reactions would be expected. This conclusion is further supported by the reaction of sea urchin fragment H1 with human mtDNA. Fragment H1 reacts with the 1.76-kb *Xba* I fragment extending between positions 1.2 and 2.9 (which contains the 3′ end of the 12S rRNA gene) and with the 7.5-kb *Xba* I fragment extending from position 10.2 to position 1.2 (which contains the 5′ end of the 12S rRNA gene) in human mtDNA (Fig. 3, lane 3); the upper band in this lane does not correspond to any normal *Xba* I fragment of human mtDNA and therefore probably represents a partial digestion product. The region of reaction of fragment H1 can be limited to the right of the *Xho* I site at position 15.0, since H1 reacts only with a 5.7-kb *Eco*RI/*Xho* I fragment that extends from position 15.0, through the zero position, to position 4.1 (Fig. 3, lane 4). In addition, fragment H1 reacts with a 0.95-kb species of *S. purpuratus* egg RNA that closely matches the size of human 12S rRNA (data not shown).

The complex pattern of reaction of sea urchin fragment H2 with human mtDNA reflects the difference in sequence organization between the two mtDNAs. Fragment H2 reacts with a 2.3-kb *Xba* I/*Eco*RI fragment extending from position 5.2 to position 7.5 (which contains the entire *CO I* gene) and the 1.76-kb *Xba* I fragment extending between positions 1.2 and 2.9 (which contains most of the 16S rRNA gene). Weak reaction was also detected to the 1.1-kb *Eco*RI/*Xba* I fragment extending from position 2.9 to position 4.1 (which contains the 3′ end of the 16S rRNA gene) in human mtDNA (Fig. 3, lane 5).

With the exceptions noted above, the overall organization of sea urchin mtDNA appears to be colinear with that of human mtDNA. Sea urchin fragments R4 and R2 react with the 1,970-bp *Xba* I fragment extending from position 8.3 to position 10.3 in human mtDNA (Fig. 3, lanes 6 and 7). This segment of human mtDNA contains the 3′ end of the gene for cytochrome oxidase subunit 2, the genes for ATPase 6 and cytochrome oxidase subunit 3, URF-6L, and URF-3. The reaction of fragment R2 can be confined to the right-hand portion of this segment since R2 reacts exclusively with a 2.4-kb *Hpa* II fragment extending between positions 9.3 and 11.7 (data not shown). Finally, sea urchin fragment R3 reacts with the 2.4-kb *Eco*RI/*Xho* I fragment that extends from position 12.6 to position 15.0 in human mtDNA (Fig. 3, lane 8).

None of the *S. franciscanus* fragments used in this study reacted with the segment of human mtDNA bounded by the *Xba* I site at position 10.2 and the *Eco*RI site at position 12.6. This region of human mtDNA contains all of URF-4 and portions of URF-3 and URF-5/6. These regions may be so divergent that crossreaction is not detectable, even at the reduced criterion used in this study.

Nucleotide Sequence Analysis of a Region of the Sea Urchin 16S rRNA Gene. Recombinant plasmid SpP389 was initially isolated as a cDNA clone of a highly prevalent pluteus stage RNA in *S. purpuratus* (16). The sequence cloned in SpP389 was identified as a portion of 16S rRNA by several criteria, including direct nucleotide sequence analysis.

Fig. 2*A* shows a restriction map of the insert in SpP389. The fragments labeled 3 and 4 were isolated and their sequences were determined by the chemical cleavage method of Maxam and Gilbert (21). The sea urchin sequence is shown in Fig. 5, along with a mammalian unanimity sequence indicating positions occupied by the same nucleotide in human, bovine, and mouse 16S rRNA genes (1–3). The sequences have been visually aligned for maximum sequence similarity.

For the sequence shown in Fig. 5, 76.5% of the positions are occupied by the same nucleotide in the three mammals and, of these mammalian unanimity positions, 77.7% are occupied by an identical nucleotide in the sea urchin sequence. Thus, 59.4% of the positions in this sequence are occupied by the same nucleotide in all four species.

DISCUSSION

Sequence Divergence in Sea Urchin mtDNA. Comparison of a segment of the sea urchin 16S rRNA gene with the three-mammal unanimity sequence gives some insight into mtDNA evolution (Fig. 5). When the 68 nucleotides of the most highly divergent region (positions 43–110) are aligned for maximum similarity, 28 (41%) of the positions are occupied by an identical nucleotide in the three mammals and only 13 (19%) of the positions are occupied by an identical nucleotide in all four animals. There is a relatively high frequency of insertions/deletions, particularly in the region between positions 73 and 110, making alignment difficult, even for the mammalian sequences. In the following highly conserved region (positions 111–310), 76% of the positions are occupied by the same nucleotide in all four species. Baer and Dubin (22) have proposed a secondary structure involving much of this highly conserved region that may be a component of the ribosome-binding site for the 3′ terminus of an aminoacyl-tRNA. Of particular interest is the region between positions 243 and 257, which con-

stitutes the highly conserved "UmGmU hairpin." Analogous hairpins consisting of a 5-bp stem and an invariant 5-base loop sequence of U-G-U-U-C are located near the 3′ ends of *E. coli* 23S rRNA (23), yeast and *Drosophila* mitochondrial 16S rRNA (6, 24), and mammalian 16S rRNA (Fig. 5). The invariant U-G-U-U-C sequence is present in sea urchin 16S rRNA (positions 243–247). The sea urchin stem region (positions 238–242 and 248–252) differs from the mammalian unanimity sequence by two pairs of compensating base substitutions; thus, 4 of the 15 nucleotides in this region differ between sea urchins and mammals but the overall secondary structure has been conserved.

Rearrangement in mtDNA Evolution. Comparison of the overall sequence organization of sea urchin and human mtDNA reveals the major difference in gene arrangement shown in Fig. 4. In mouse, human, and bovine mtDNA, the 12S rRNA and 16S rRNA genes are adjacent, and the 16S rRNA gene is separated from the *CO I* gene by an approximately 2.5-kb segment containing URF-1 and URF-2 (1–3). In the sea urchin mtDNA clones, in contrast, the 16S rRNA gene is directly adjacent to the *CO I* gene and is apparently separated from the 12S rRNA gene by an approximately 3-kb region that may contain URF-1 and URF-2. It is unlikely that this difference in sequence organization is due to a cloning artifact, since three independent mtDNA clones with identical restriction maps were screened from the *S. franciscanus* DNA library (Fig. 1). In addition, the six *Eco*RI restriction fragments produced from these clones match in size those obtained from purified *S. franciscanus* mtDNA (data not shown).

In all mammalian mtDNA molecules for which the sequences have been determined, the 16S and 12S rRNA genes are adjacent (1–3). Although gene order is different in *Drosophila* mtDNA, the two rRNA genes are likewise adjacent (6). The rearrangement event responsible for the difference in organization of sea urchin mtDNA, therefore, probably occurred in the sea urchin lineage after sea urchins and mammals last shared a common ancestor, more than 500 million years ago. The relative positions of the 16S rRNA gene and the *CO I* gene are probably identical in *S. purpuratus* and *S. franciscanus*, since restriction fragment data (W. Brown and S. Hechtel, personal communication) do not indicate major differences in mtDNA sequence organization between these two species of sea urchins. Thus, the rearrangement of sea urchin mtDNA probably occurred prior to the evolutionary differentiation of the genus *Strongylocentrotus*, 15–20 million years ago (25). It is interesting that, in a segment of the nuclear genome of *S. purpuratus* containing regions of mtDNA homology, a portion of the 16S rRNA sequence is adjacent to at least part of the *CO I* gene, apparently reflecting the organization of the mitochondrial genome. Analysis of sequence divergence suggests that this insertion of a segment of mtDNA into the nuclear genome may have taken place as recently as 25 million years ago (7).

Little can be said about the events involved in the rearrangement of sea urchin mtDNA. The polarities of the 16S rRNA genes in sea urchin and mammalian mtDNA rule out a simple inversion of the 4-kb region containing URF-1, URF-2, and the 16S rRNA gene (Fig. 4). Conservation of 16S rRNA gene polarity may be of functional significance, since in mammals, all mitochondrial rRNA and poly(A)-containing RNA may be produced by endonucleolytic cleavage of a single polycistronic H-strand transcript (26, 27). An inverted 16S rRNA gene would require L-strand transcription for functional expression. Determination of the nucleotide sequence of sea urchin mtDNA will be required to precisely define all differences in mitochondrial genome organization. The region between the *CO I* and 16S rRNA genes in sea urchin mtDNA is of particular interest, because the origin of L-strand replication maps directly to the left of the *CO I* gene in mammalian mtDNA (1, 2).

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