Patterns of Ribosomal RNA Evolution in Salamanders[1]

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Sequence comparisons are presented for four segments of the large subunit of ribosomal RNA, including divergent domains D7a and D7b, portions of the large divergent domains D2, D3, and D8, and evolutionarily conservative sequences flanking divergent domains. These results resolve phylogenetic relationships among exemplars of seven families of salamanders and the three amphibian orders. Phylogenetic analysis confirms the prediction that divergent domains feature the highest relative rates of base substitution and length variation within the ribosome, but the divergent domains evolve more slowly than nuclear noncoding DNA and the silent sites of structural genes. Base substitutions demonstrate approximately twice as many transitions as transversions and an uneven distribution among sites within the divergent domains but no apparent bias in base composition. Length mutations are primarily small insertions and deletions, with deletions predominating. The divergent domains appear to be a good source of phylogenetic information for evolutionary events occurring approximately 100–200 million years ago.

Introduction

Ribosomal RNA demonstrates an unusual evolutionary pattern featuring the interspersion of rapidly evolving segments among highly conserved regions that contain some of the most evolutionarily stable macromolecular sequences known (Gerbi 1985). The highly conserved regions show considerable sequence identity among prokaryotic ribosomes and both the nuclear-encoded and organellar ribosomes of eukaryotes (Ware et al. 1983; Huysmans and De Wachter 1986). These segments have been critically important for resolving the early phylogenetic history of life (Fox et al. 1980; Küntzel and Köchel 1981; Spencer et al. 1984; Hasegawa et al. 1985; Lane et al. 1985; Pace et al. 1985; Field et al. 1988; Lake 1988). The most evolutionarily variable segments of the ribosomal RNA, termed "divergent domains" (Hassouna et al. 1984), are found primarily in the large subunit of the nuclear-encoded eukaryotic ribosome. The divergent domains may differ extensively both in sequence and length among even closely related genera, but they occupy homologous positions relative to the evolutionarily conserved regions (Hassouna et al. 1984; Gerbi 1985; Clark 1987).

The hypotheses advanced to explain this evolutionary pattern suggest that selective constraints are strong within the evolutionarily conserved regions but that the divergent domains evolve free of strong structural and functional constraints. One hypothesis views the divergent domains as the remnants of mobile elements that inserted into the transcribed portions of the ribosomal genes (Clark et al. 1984); another hypothesis views the divergent domains as remnants of linkers that connected different "functional

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segments" during the evolutionary assembly of the ribosome and that were subsequently eliminated from all but the nuclear-encoded eukaryotic ribosomes (Clark 1987; see also Gonzalez et al. 1985; Spencer et al. 1987).

Detailed resolution of ribosomal evolutionary pattern is required to evaluate hypotheses of the evolutionary forces that direct ribosomal evolution. We present a phylogenetic analysis of ribosomal evolution, focusing on divergent domains of the large ribosomal subunit and the regions flanking them. Sequences comprising ~800 bases are reported for 13 salamanders and are compared with homologous sequences from a frog, a caecilian (amphibian order Gymnophiona), and two mammals. This is the first study to report a detailed phylogenetic analysis of these particular sequences, testing for departures from the patterns expected for sequences experiencing only weak structural and functional constraints.

Material and Methods

Specimens Analyzed

Ribosomal RNA sequences were obtained from 13 salamanders (amphibian order Caudata) representing the families Ambystomatidae [one specimen of *Ambystoma californiense* (Santa Cruz Co., Calif.; Museum of Vertebrate Zoology #FC 13156)], Amphiumidae [one specimen of *Amphiuma means* (Alachua Co., Fla.; Museum of Vertebrate Zoology # FC 10981)], Cryptobranchidae [one specimen of *Andrias davidianus* (Hong Kong; Museum of Vertebrate Zoology #FC 13424)], Dicamptodontidae [one specimen of *Rhyacotriton olympicus* (Cathlamet, Wash.; collected by David A. Good, #DAG 1514)], Plethodontidae [seven specimens, one each of *Aneides hardii* (Sierra Blanca, N.M.; collected by James Kezer and Stanley K. Sessions) *A. ferreus* (Mendocino Co., Calif.; collected by David Good and Thomas A. Wake), *A. flavipunctatus* (Sonoma Co., Calif.; collected by Charles Brown), *Batrachoseps attenuatus* (Del Norte Co., Calif.; collected by David A. Good and Thomas A. Wake), *Desmognathus ochrophaeus* (Highlands, Macon Co., N.C.; Museum of Vertebrate Zoology #FC 10775), *Ensatina eschscholtzii* (Del Norte Co., Calif.; collected by David A. Good and Thomas A. Wake), and *Plethodon dunni* (collected by David A. Good)], Proteidae [one specimen of *Necturus beyeri* (Texas; Museum of Vertebrate Zoology #FC 13770)], and Salamandridae [one specimen of *Notophthalmus viridescens* (Brunswick Co., N.C.; Museum of Vertebrate Zoology #FC 12755)]. In addition, one specimen of the amphibian order Gymnophiona (caecilians) was examined [*Typhlonectes compressicauda* (Barranquilla, Colombia; Museum of Vertebrate Zoology #FC 12006)].

The evolutionary time scale spanned by these comparisons is known approximately, although few divergence events are precisely dated. The most recent common ancestry of the amphibian orders probably exceeds 200 million years ago (Mya), and at least the earlier divergences within the salamanders exceed 100 Mya (Duellman and Trueb 1986). Several divergence events within the family Plethodontidae have been dated using a combination of molecular and paleontological data. The separation of *Aneides ferreus* and *A. flavipunctatus* occurred ~14 Mya, and the separation of this lineage from *Aneides hardii* occurred ~20 Mya (Larson et al. 1981). The separation of the genus *Aneides* from the western species of *Plethodon* is estimated to have occurred ~35 Mya (Larson et al. 1981), and the separation of *Ensatina* from the clade containing *Aneides* and *Plethodon* occurred ~65 Mya (Maxson et al. 1979; Larson et al. 1981). The two mammalian outgroups (genera *Mus* and *Rattus*) separated from each other ~22 Mya (Sarich 1985).

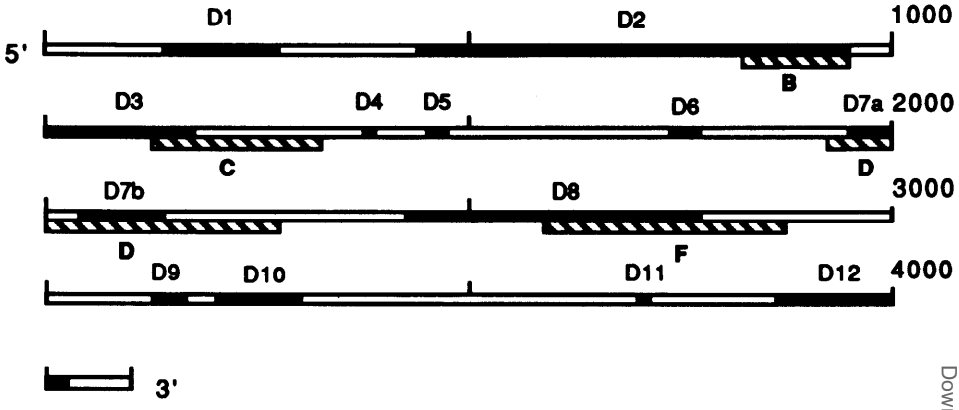


FIG. 1.—Schematic diagram of the large ribosomal subunit. The positions of 12 divergent domains (D1–D12) are indicated by shading, and the positions of sequences analyzed in this study (B–F) are indicated below the appropriate ribosomal segment. Base numbers corresponding to the sequence of *Xenopus laevis* (Ware et al. 1983) are given at the right.

Ribosomal Segments Sequenced

Four distinct segments of the large ribosomal subunit (fig. 1) were sequenced utilizing synthetic oligonucleotide primers complementary to sequences flanking the segments of interest toward the 3′ end of the RNA molecule. The oligonucleotide sequences chosen are complementary to segments of the molecule known, on the basis of previous studies (summarized by Gerbi 1985), to demonstrate high levels of evolutionary conservation in eukaryotes; this maximizes their utility for examining phylogenetically diverse specimens.

The first oligonucleotide primer (28SB) is complementary to a segment consisting of bases numbered 952–984 in the *Xenopus laevis* sequence (Ware et al. 1983) located adjacent to "divergent domain 2" of the large ribosomal subunit (as identified by Hassouna et al. 1984); the sequence of this primer is 5′CGTTAGACTCCTTGGTCCGTGTTTCAAGACGGG3′. This primer is used to sequence a segment of ~130 bases located in the 3′ end of divergent domain 2, homologous to the bases numbered 820–950 in the *X. laevis* sequence (Ware et al. 1983). Sequence information obtained for this segment was confirmed in part by using another primer (28SB2) synthesized after sequence information had been collected for the salamanders; this primer is complementary to bases 919–940 of the consensus salamander sequence (numbered in accord with the *X. laevis* sequence) and contains the sequence 5′ACCGACATCGCCGCAGACCCCT3′. The ribosomal segment sequenced using primers 28SB and 28SB2 is denoted "segment B."

The second oligonucleotide primer (28SC) is complementary to the bases numbered 1329–1350 in the *X. laevis* sequence (Ware et al. 1983), located between divergent domains D3 and D4 (Hassouna et al. 1984), adjacent to the 5′ end of domain D4. It consists of the sequence 5′GCTATCCTGAGGGAAACTTCGG3′. This primer is used to sequence a segment of ~200 bases corresponding to the bases numbered 1121–1325 in the *X. laevis* sequence. This includes (*a*) most of the relatively conserved segment located between divergent domains D3 and D4 and (*b*) the 3′ end of divergent domain D3. The segment sequenced using oligonucleotide primer 28SC is denoted "segment C."

The third oligonucleotide primer (28SD) is complementary to the bases

numbered 2327–2344 in the *X. laevis* sequence, located between divergent domains D7b and D8 (Hassouna et al. 1984). It consists of the sequence 5′CTTGGAGACCTGCTGCGG3′. This primer is used to sequence a segment corresponding to bases numbered 1920–2320 in the *X. laevis* sequence. This includes (*a*) much of the relatively conserved segment located between divergent domains D7 and D8, (*b*) divergent domain D7, and (*c*) a short part of the relatively conserved segment between divergent domains D6 and D7. This sequence was confirmed in part by using another primer (28SD2) complementary to the consensus salamander sequence corresponding to the segment numbered 2119–2142 in the *X. laevis* sequence. The sequence of this primer is 5′CTCCCGCCGGCTTCTCCGGGATC3′. The ribosomal segment sequenced using oligonucleotide primers 28SD and 28SD2 is denoted "segment D."

The fourth oligonucleotide primer (28SF) is complementary to the bases numbered 2851–2872 in the *X. laevis* sequence, located toward the 3′ end of divergent domain D8. It consists of the sequence 5′CAGAGCACTGGGCAGAAATCAC3′. This primer is used to read the segment corresponding to bases numbered 2595–2770 in the *X. laevis* sequence, which comprise the 3′ portion of divergent domain D8. The ribosomal segment sequenced with oligonucleotide primer 28SF is denoted "segment F."

Extraction of Cellular RNA

Total cellular RNA was extracted from specimens by using a modification of the guanidinium/hot-phenol method of Maniatis et al. (1982). Tissue (generally internal organs; but, for small specimens, the entire animal was used) was ground in liquid nitrogen with a porcelain mortar and pestle. Guanidinium isothiocyanate solution (Maniatis et al. 1982, p. 189) containing $^1/_{100}$ v 2-mercaptoethanol (added immediately before use) was added to the frozen, powdered tissue and was mixed until the solution froze. The mixture was placed in a water bath at 60°C until thawed and then was drawn into a polypropylene syringe (fitted with an 18-gauge needle) and ejected repeatedly to reduce viscosity. An equal volume of phenol (preheated to 60°C) was then added and mixed by continued passage through the syringe. The mixture was then extracted by adding a solution containing 0.1 M sodium acetate (pH 5.2), 10 mM Tris-HCl [tris(hydroxymethyl)aminomethane-HCl; pH 7.4], and 1 mM ethylenediaminetetraacetic acid (disodium salt) (5 ml solution/g tissue) and then a 24:1 solution of chloroform and isoamyl alcohol (5 ml/g tissue) followed by shaking for 10 min at 60°C, cooling on ice for 10 min, and spinning for 10 min at 2,350 relative centrifugal force in a swinging-bucket rotor at 4°C.

The aqueous (top) phase was collected with a siliconized Pasteur pipette and reextracted twice (as described above), first at 60°C and then at room temperature. The final extract was precipitated in 2–2.5 v absolute ethanol overnight at −20°C. The pellet was dried under vacuum and resuspended in a solution containing 50 mM sodium chloride, 0.1 M Tris-HCl (pH 7.4), 10 mM ethylenediaminetetraacetic acid (disodium salt) and 0.2% sodium dodecyl sulfate (5 ml solution/g tissue, in starting material). A solution of proteinase K (10 mg/ml) was added (20 µl/ml, starting volume) and incubated for 1–2 h at 37°C to eliminate contaminating protein. The proteinase reaction was terminated by heating to 60°C, followed by extraction in a mixture of phenol, chloroform, and isoamyl alcohol (as described above) at 60°C. The aqueous phase was reextracted twice with chloroform and isoamyl alcohol and was precipitated as described above.

The precipitate was dried under vacuum and suspended in distilled water (pretreated with diethylpyrocarbonate and autoclaved). Samples were adjusted, on the basis of optical density readings, to a concentration of ~0.5–1 mg/ml for use in sequencing reactions and were stored at −70°C.

Radioactive Labeling of the Sequencing Primers

Synthetic oligonucleotides were purified and suspended in distilled water at a concentration of 2 pmol/μl. They were labeled with ^{32}P shortly before use by mixing 10 μl of the oligonucleotide solution, 5 μl of gamma-ATP^{32} [tetra(triethylammonium) salt, ~6,000 Ci/mmol], 1 μl of T4 polynucleotide kinase (Bethesda Research Laboratories enzyme, 8 units), 2 μl of 10 × kinase buffer, and 2 μl distilled water and then incubating the mixture at 37°C for 45 min. Kinase buffer (10 ×) is 0.5 M Tris-HCl (pH 7.6), 0.1 M $MgCl_2$, 50 mM dithiothreitol, 1 mM spermidine, and 1 mM ethylenediaminetetraacetic acid (disodium salt). Incorporation was tested by precipitation with trichloroacetic acid according to the method described by Maniatis et al. (1982, p. 473). Labeled oligonucleotides were purified by precipitation in ammonium acetate according to the method described by Maniatis et al. (1982, p. 462).

Sequencing Reactions

The RNA sequencing procedure used was modified from previously published methods utilizing primer extension by reverse transcriptase with dideoxyribonucleotide termination (Hamlyn et al. 1978; Youvan and Hearst 1979; Qu et al. 1983; Lane et al. 1985). All stock solutions used in this procedure were made from diethylpyrocarbonate-treated distilled water unless otherwise noted. Sequencing reactions were performed on 6-μl aliquots of the RNA samples described above. The aliquots to be sequenced were first heated to >90°C for 5 min on a heating block (to denature secondary structure) and were cooled on ice. The aliquot was then mixed with 1 μl 20 × reverse transcription buffer, 2 μl labeled primer (0.5 pmol/μl working stock) and 1.5 μl RNasin (Promega Biotec, 2,000 units/ml) and was incubated at 42°C for 30 min to allow the primer to anneal to the RNA template. Reverse transcription buffer (20 ×) is 400 mM Tris-HCl (pH 8.3 at assay temperature), 150 mM $MgCl_2$, 150 mM KCl, and 40 mM dithiothreitol.

For each aliquot of RNA to be sequenced, four separate reactions were run with termination specific for bases A, C, G, and T. To each tube was added 2 μl of "reaction mixture 1," 2.12 μl of RNA with annealed primer (from above), and 1 μl of the appropriate dideoxyribonucleotide solution (8 mM ddATP, 2 mM ddCTP, 1.5 mM ddGTP, or 5 mM ddTTP, all suspended in 10 mM Tris-HCl, pH 7.9). Reaction mixture 1 consists of equal volumes of dNTP mix (5 mM each dATP, dCTP, dGTP, and TTP, in 10 mM Tris-HCl, pH 7.9), reverse transcriptase (avian myeloblastosis virus reverse transcriptase; Life Sciences, ~20,000 units/ml), and distilled water. The sequencing reaction is incubated at 48°C for 40 min (the expected duration of enzyme activity under the assay conditions). This is followed by a "chase" reaction produced by adding to each tube 1 μl of "reaction mixture 2" (equal parts dNTP mix and reverse transcriptase, as above) followed by incubation at 48°C for 40 min.

Following the chase reaction, 4 μl of loading buffer (prepared as described by Maniatis et al. 1982, p. 476) are added to the reactions, which are then heated to >90°C for 5 min (to separate the extended primers from the RNA template) and are then cooled on ice. Sequencing reactions were loaded on an 8% polyacrylamide gel (0.4 mm thick) by using a shark's-tooth comb. The polyacrylamide was prepared

according to the method described by Maniatis et al. (1982, p. 478). Each set of four reactions was loaded twice in equal amounts (4.5 μl/lane) in four adjacent lanes; the first set loaded was run ~6 h, and the second set was run for ~3 h. Gels were dried prior to exposure of X-ray film to produce an autoradiogram.

Results

Aligned sequences for four segments of the large ribosomal subunit appear in figure 2. Salamander and caecilian sequences are aligned with previously published sequences for *Xenopus laevis* (Ware et al. 1983), *Mus domesticus* (Hassouna et al. 1984), and *Rattus norvegicus* [Hadjiolov et al. 1984; a sequence published earlier by Chan et al. (1983) from an unspecified species of rat differs slightly from the *Rattus* sequence used here]. No length polymorphism was evident in the ribosomal RNA of any sample. For phylogenetic analyses, the segment between bases numbered 2049 and 2095 is omitted because it did not resolve clearly on the sequencing gels. Also omitted were segments labeled E, F, I, and J plus positions 910–910r. Because the alignment of the amphibian and mammalian sequences for positions numbered 2700–2728 is uncertain, only the amphibian sequences from this region are included in the analyses. Numbering of bases follows the convention of Ware et al. (1983). Number of differences between each pair of sequences is given in table 1 for the 871 sites aligned in figure 2. A site is considered informative if the number of substitutions thereat differs among the three trees in figure 5. There are 17 informative sites (table 2).

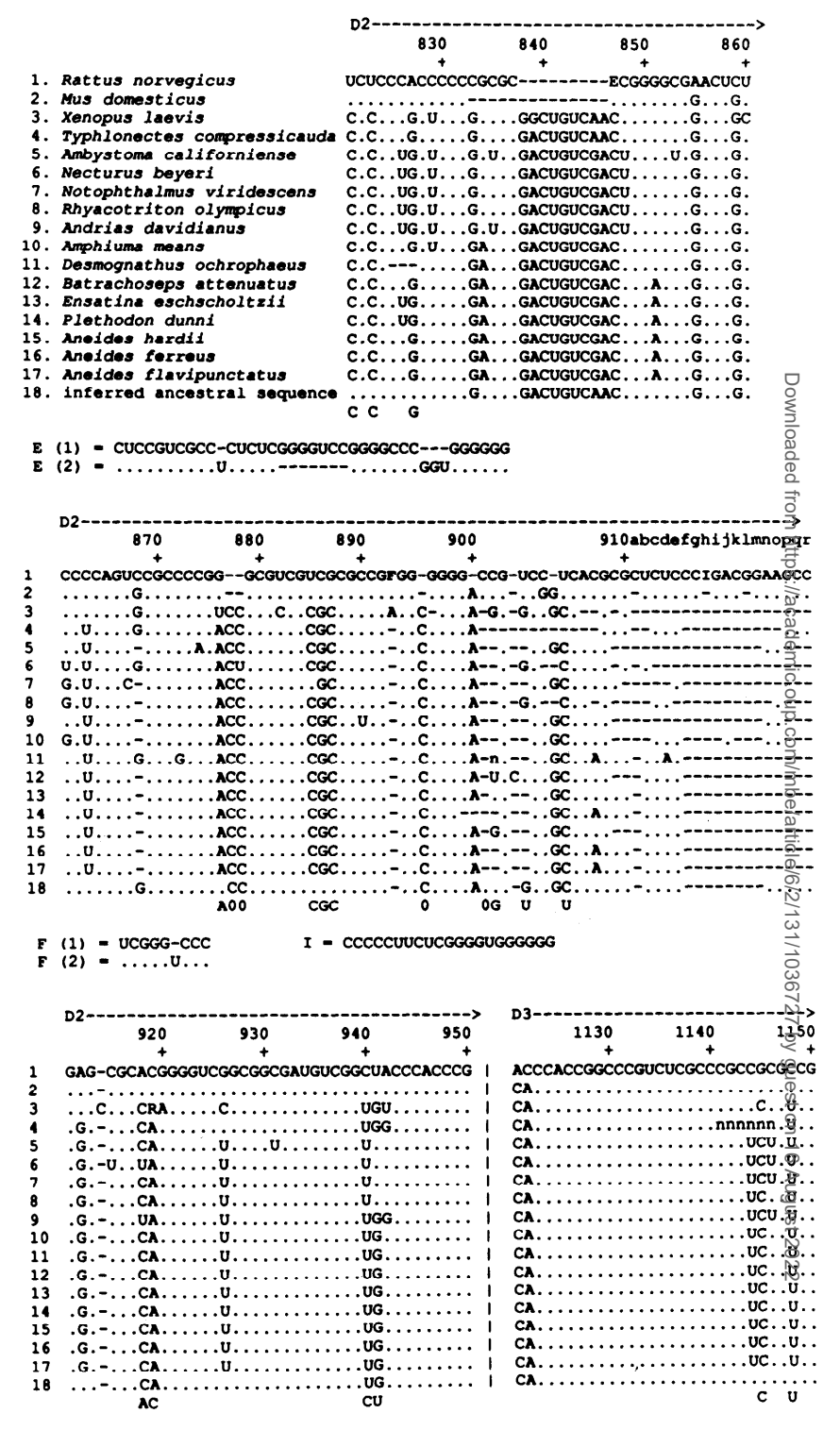
Samples of the autoradiograms are given in figures 3 and 4.

Phylogenetic Reconstruction

A tree was constructed using the standard maximum parsimony method (Nei 1987, pp. 313–320) for representatives of seven families of salamanders (tree 1 in fig. 5) by using the informative sites. A gap was considered as a fifth character. This tree was tested against two alternative topologies by using the criterion of parsimony and by using *Typhlonectes* and *Xenopus* as outgroups. The first alternative (tree 2 in fig. 5) is the topology suggested by an earlier parsimony analysis of morphological and chromosomal data (Duellman and Trueb 1986); the other alternative (tree 3 in fig. 5) corresponds to the morphological tree, except for relocation of the plethodontid genus *Aneides* to the placement indicated by the rRNA data. The maximum parsimony tree constructed from the rRNA data requires 28 changes at the 17 sites listed in table 2, whereas the alternatives require 50 and 38 changes, respectively. The rRNA tree is significantly more parsimonious than the others (table 2) when the criteria of Templeton (1983) are used, although the validity of this test has been disputed for comparisons involving more than three taxa (Felsenstein 1985; Nei and Tajima 1987).

Relationships among representatives of the three amphibian orders (caecilians, frogs, and salamanders) are evaluated using five sites that are informative at this level (sites 863, 912, 1177, 1213, and 2656; although there is an ambiguity for the *Xenopus* sequence at site 1213). The *Ambystoma californiense* and *Aneides flavipunctatus* samples represent the salamander lineage. The rat and mouse sequences (which are identical at these positions) are used as the outgroup. All five sites support the inference that salamanders and caecilians form a sister group relative to the frogs and mammals, which is a statistically significant result (Felsenstein 1985).

A composite tree relating all amphibians studied here is shown in figure 6. This tree is based on the topologies resolved above, except for the relationships of the plethodontid genera. The rRNA data indicate monophyly of the plethodontids relative



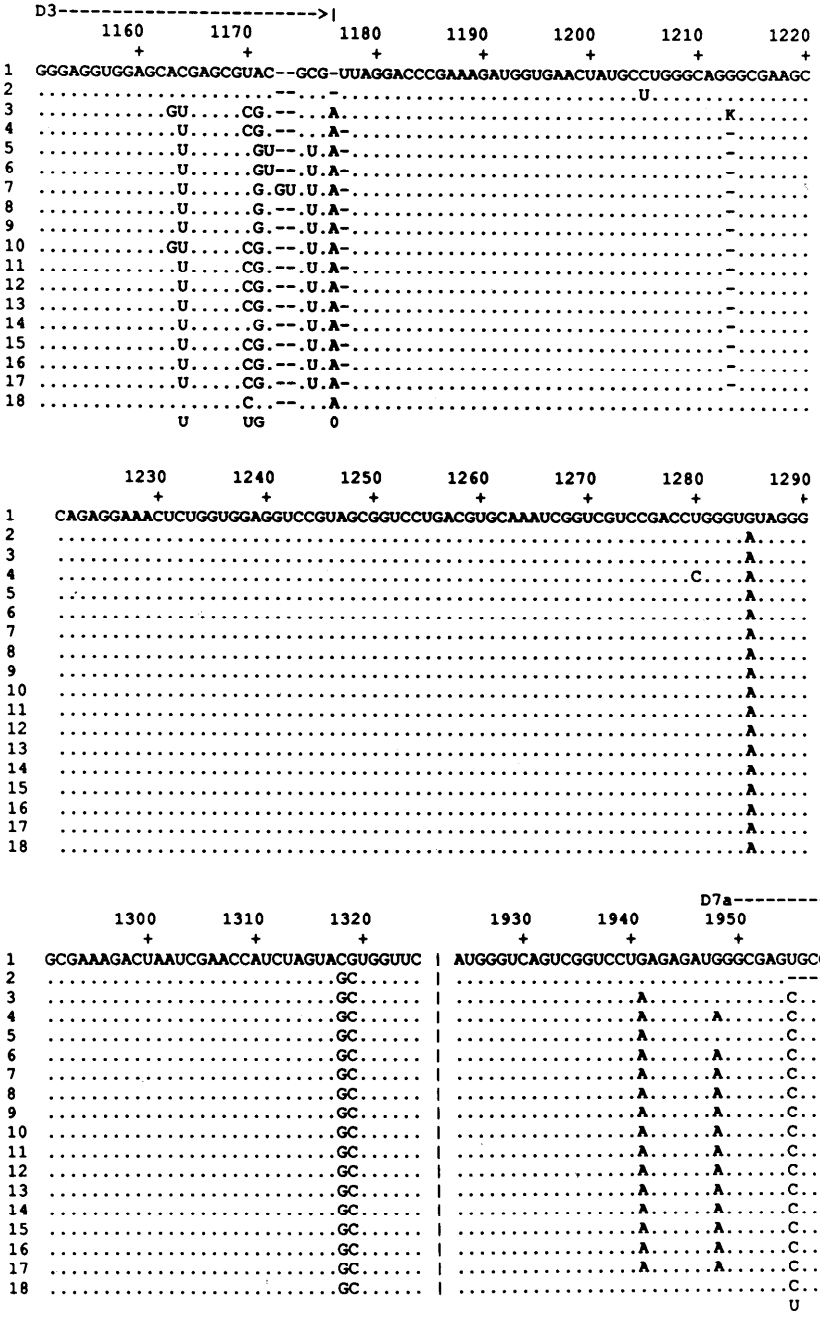


FIG. 2 (*Continued*)

to the other specimens studied, but the plethodontid relationships shown in figure 6 are based on previous molecular and morphological data (Maxson et al. 1979; Larson et al. 1981; Larson 1984; Lombard and Wake 1986) because the rRNA data do not resolve these relationships. Evolutionary changes in ribosomal RNA are partitioned on the inferred topology by using the method of Fitch (1971), and the numbers of changes partitioned on each branch are noted on figure 6. Summaries of the changes

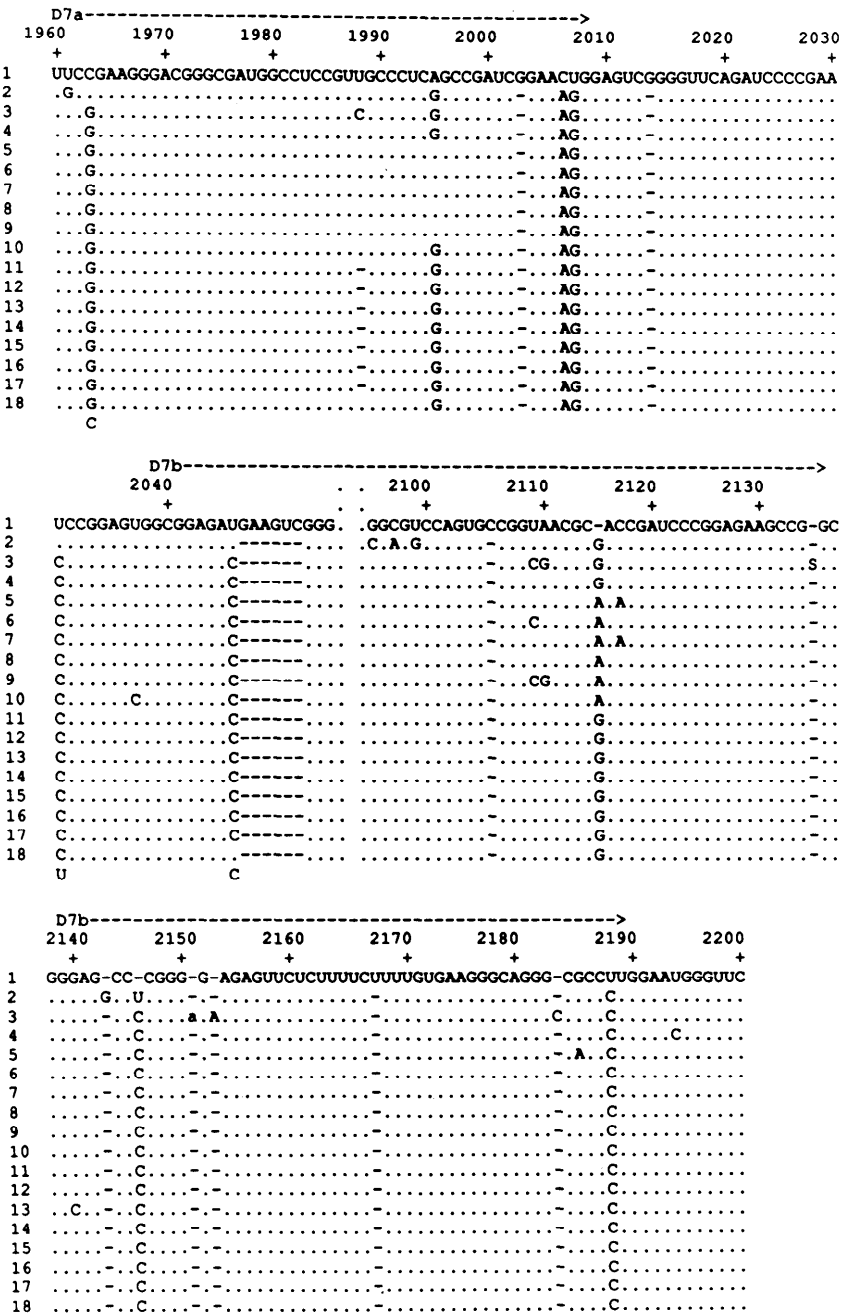


FIG. 2 (*Continued*)

partitioned on this tree and on the *Mus/Rattus* lineages are given in table 3 and figure 7.

Patterns of Molecular Evolution

Estimates of substitution rates are obtained using the portions of the tree for which divergence times are available from previous studies (the *Mus/Rattus* com-

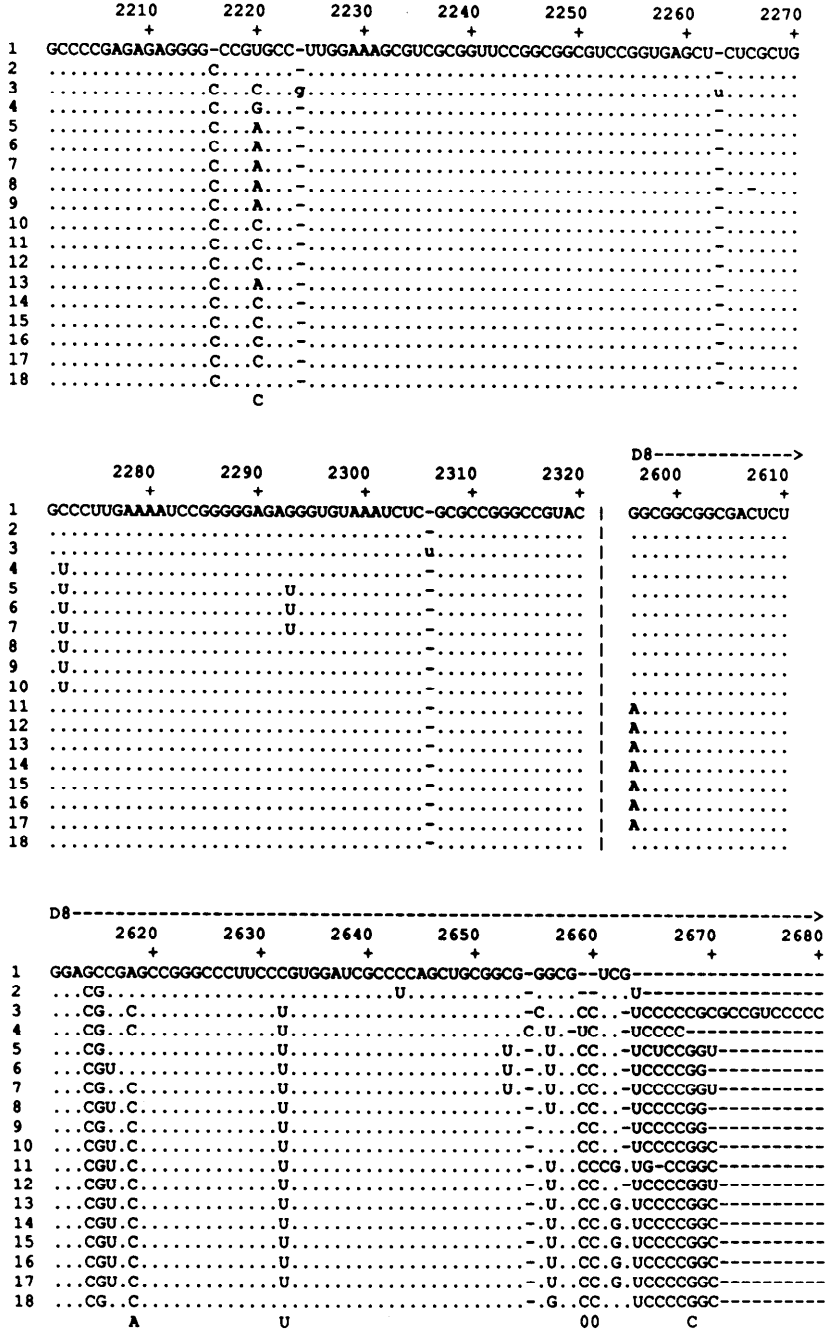


FIG. 2 (*Continued*)

parisons and the *Aneides, Ensatina,* and *Plethodon* comparisons). Rates of substitution are expressed as percentage divergence between paired taxa (including changes accumulated on both lineages derived from their most recent common ancestor) per site per million years (Myr). For all four ribosomal segments together, the substitution rate for the salamanders is 0.01%–0.02% divergence/Myr; for mammals, it is ~0.1%

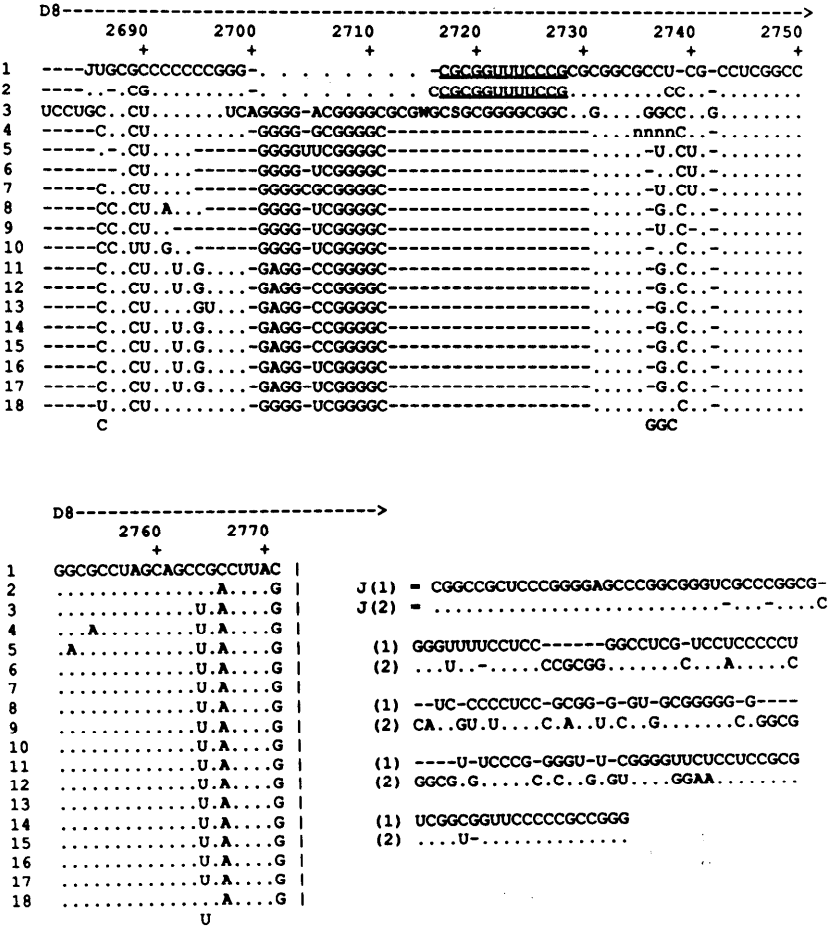


FIG. 2.—Aligned base sequences for four segments of the large ribosomal subunit. Base designations follow IUB recommendations: A = adenine; C = cytosine; G = guanine; U = uracil; K = guanine or uracil; S = cytosine or guanine; and lowercase letters denote uncertain positions. Letters E, F, I, and J denote long inserts in the aligned sequence (given below the corresponding block of aligned sequences). Numbers at the tops of the columns are the standard base numbers for the published *Xenopus* sequence (Ware et al. 1983) given on line 3. Positions not present in the *Xenopus* sequence are designated alphabetically. Dots denote identity to the sequence in row 1. Dashes denote gaps in the aligned sequence. Ambiguous positions in the inferred amphibian ancestral sequence are indicated by alternative bases below line 18—or by 0, where sites might have been absent in the ancestor. Segments corresponding to divergent domains D2, D3, D7, and D8 are marked above the appropriate columns. Vertical bars separate the four different segments sequenced (see fig. 1). The break between positions 2049 and 2095 denotes a segment that did not resolve clearly. All sequences are original with this study, except for those of *Rattus norvegicus* (Hadjiolov et al. 1984), *Mus domesticus* (Hassouna et al. 1984), and *X. laevis* (Ware et al. 1983).

divergence/Myr. When only the sequences from large divergent domains D2 and D8 are considered, the divergence due to accumulated substitutions is 0.02%/Myr for the salamanders and 0.16%/Myr for the mammals. There is some indication in the data, however, that the rate calculated for the salamanders is anomalously low. The salamander lineages for which divergence times are available demonstrate relatively few

Table 1
Differences between Paired Ribosomal RNA Sequences

	SAMPLES																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1.....		58	156	120	140	136	137	132	137	131	129	128	127	127	128	127	127
2.....	6.7		137	103	122	120	123	118	122	114	110	110	109	112	110	109	109
3.....	17.9	15.7		78	92	87	87	82	80	80	79	75	77	80	74	75	75
4.....	13.8	11.8	9.0		62	54	51	44	51	48	46	41	41	43	41	38	38
5.....	16.1	14.0	10.6	7.1		26	21	31	26	37	54	47	48	49	48	50	50
6.....	15.6	13.8	10.0	6.2	3.0		25	24	29	38	49	44	43	44	45	45	45
7.....	15.7	14.1	10.0	5.9	2.4	2.9		25	26	31	45	38	37	39	39	39	39
8.....	15.2	13.5	9.4	5.1	3.6	2.8	2.9		26	23	42	32	34	34	34	34	34
9.....	15.7	14.0	9.2	5.9	3.0	3.3	3.0	3.0		28	50	42	43	43	42	44	44
10.....	15.0	13.1	9.2	5.5	4.2	4.4	3.6	2.6	3.2		37	28	31	33	26	29	29
11.....	14.8	12.6	9.1	5.3	6.2	5.6	5.2	4.8	5.7	4.2		18	15	14	13	10	10
12.....	14.7	12.6	8.6	4.7	5.4	5.1	4.4	3.7	4.8	3.2	2.1		13	14	6	9	9
13.....	14.6	12.5	8.8	4.7	5.5	4.9	4.2	3.9	4.9	3.6	1.7	1.5		10	8	7	7
14.....	14.6	12.9	9.2	4.9	5.6	5.1	4.5	3.9	4.9	3.8	1.6	1.6	1.0		9	5	5
15.....	14.7	12.6	8.5	4.7	5.5	5.2	4.5	3.9	4.8	3.0	1.5	0.6	0.9	1.0		4	4
16.....	14.6	12.5	8.6	4.4	5.7	5.2	4.5	3.9	5.1	3.3	1.1	1.0	0.8	0.6	0.4		0
17.....	14.6	12.5	8.6	4.4	5.7	5.2	4.5	3.9	5.1	3.3	1.1	1.0	0.8	0.6	0.4	0	

NOTE.—Numbers of divergent sites for all pairs of aligned rRNA sequences (fig. 2) are given above the diagonal. Percentages of sites differing between the paired sequences are given below the diagonal. Samples are identified in fig. 2. Unalignable segments and segments present only in the mouse and rat rRNA are not included.

Table 2
Wilcoxon Test of Alternative Trees

Site	Tree 1 Changes	Tree 2			Tree 3		
		Changes	Score	Rank	Changes	Score	Rank
826.....	1	3	−2	−11.5	2	−1	−8.5
848.....	1	3	−2	−11.5	2	−1	−8.5
861.....	4	3	+1	+3.5	3	+1	+8.5
941.....	1	3	−2	−11.5	2	−1	−8.5
1146.....	2	4	−2	−3.5	3	−1	−8.5
1170.....	1	3	−2	−11.5	2	−1	−8.5
1172.....	1	2	−1	−3.5	2	−1	−8.5
1995.....	1	3	−2	−11.5	2	−1	−8.5
2115.....	1	2	−1	−3.5	1	0	
2117.....	2	2	0		1	+1	+8.5
2220.....	1	2	−1	−3.5	2	−1	−8.5
2293.....	1	3	−2	−11.5	2	−1	−8.5
2616.....	4	3	+1	+3.5	3	+1	+8.5
2618.....	1	2	−1	−3.5	2	−1	−8.5
2653.....	1	3	−2	−11.5	2	−1	−8.5
2687.....	3	4	−1	−3.5	4	−1	−8.5
2740.....	2	4	−2	−11.5	3	−1	−8.5

Tree 1 vs. tree 2: $T_s = 7$, n = 16, $P < 0.01$
Tree 1 vs. tree 3: $T_s = 25.5$, n = 16, $P < 0.05$

NOTE.—The three alternative trees are shown in fig. 5. Only those informative sites that discriminated among these three trees are shown.

substitutions, compared with others. When substitutions are counted over all lineages (those shown in fig. 6 plus the mouse and rat lineages), the mean number of substitutions per site is 0.32 within the divergent domains and only 0.05 outside the divergent domains. If we assume that the segments outside the divergent domains evolve at approximately the rate reported for small subunit ribosomal RNA (Wilson et al. 1987), these ratios predict that evolutionary rates within the divergent domains approximate the values obtained above for the mammals. These rates are considerably lower than those reported for nuclear, noncoding DNA [0.34%–0.40%/Myr (Helm-Bychowski and Wilson 1986; Wilson et al. 1987)] and for synonymous sites of coding regions [0.7%–1%/Myr (Wilson et al. 1987)].

Variance:mean ratios for substitution rates for the four ribosomal segments were estimated by comparing the inferred changes for the paired branches of each node (fig. 6). The average variance:mean ratio for substitution rate is 2.7. When length mutations are included in rate calculations, this ratio drops to approximately 2.4. These values are very similar to those reported elsewhere for protein and nucleic acid sequences (Wilson et al. 1977; Gillespie 1984, 1986; Ochman and Wilson 1987).

Only 2 of 89 inferred length-mutational events occur outside the divergent domains. Rates of length-mutational events in the divergent domains are 0.16%/Myr for mammals and 0.01%/Myr for salamanders when using the same comparisons as above. These values are obtained by dividing the minimum number of length-mutational events (estimated using the tree in fig. 6) by the total length of the divergent domains studied and then converting to percentages. As noted above, however, the rate for salamanders is probably underestimated. The ratio of length mutations to

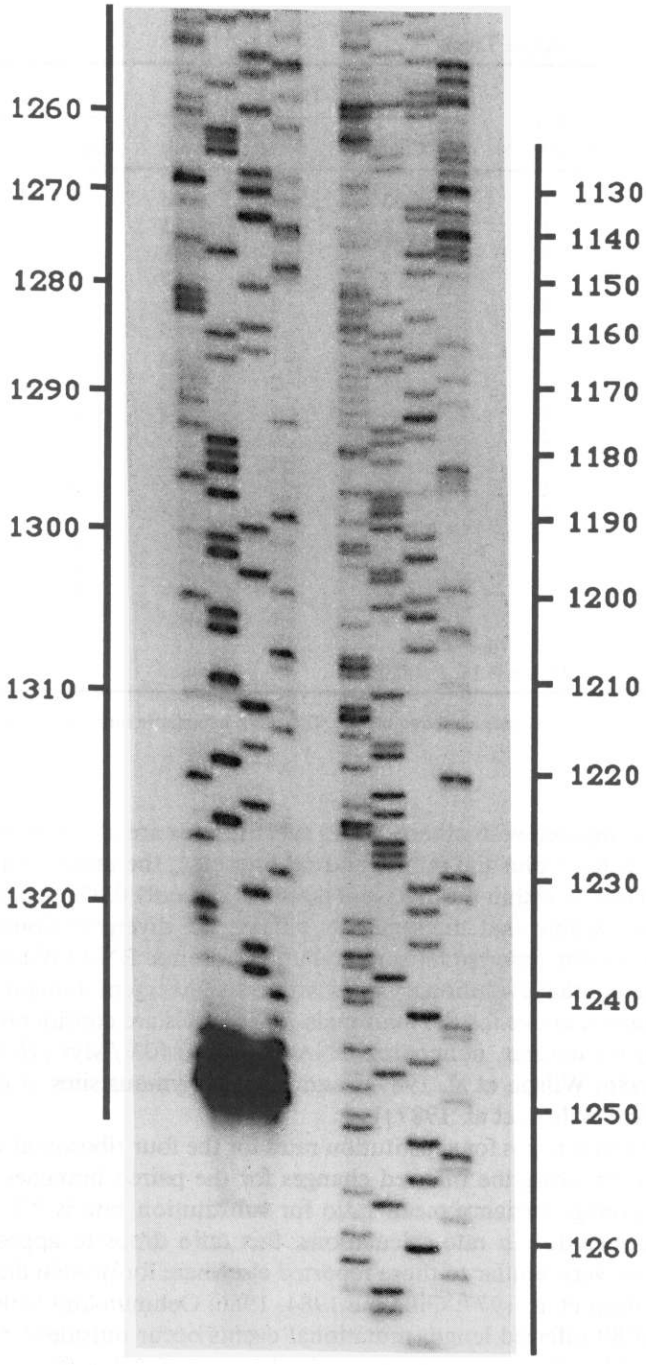


FIG. 3.—Autoradiogram showing results of sequencing reactions from the *Plethodon dunni* sample for the segment numbered 1121–1325. Bases are read in the order GAUC (left to right). The sequence is read in the reverse order of the numbers beginning at the lower left. The two lanes are separate loadings of the same set of sequencing reactions, with the column on the right having been run twice as long as the one on the left.

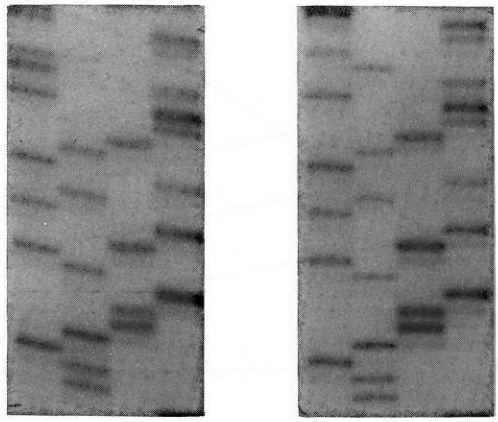


FIG. 4.—Autoradiograms showing homologous sequences from a portion of the divergent domain D8 (2748–2771, top to bottom, with the last two bases omitted from the published sequence) for specimens of *Notophthalmus viridescens* (*left*) and *Ambystoma californiense* (*right*). These sequences differ by a transition substitution at position 2752. Sequences are read in the order GAUC (left to right).

base substitutions is 0.68 for the D2 and D8 regions, a result placing the rate of length-mutational events slightly below that of base substitutions. Small deletions and insertions are more common than large ones (fig. 7); the majority of inferred deletions and insertions involve one or two bases (fig. 7), and the maximum number of bases likely to have been associated with a single deletion event is 9 (in divergent domain D2). An apparent insertion of 21 bases is observed within the most highly variable portion of divergent domain D2 on the rat lineage. The remaining large differences in length occur between amphibian and mammalian sequences and require a more extensive sampling of the vertebrates to evaluate the magnitudes of incremental changes.

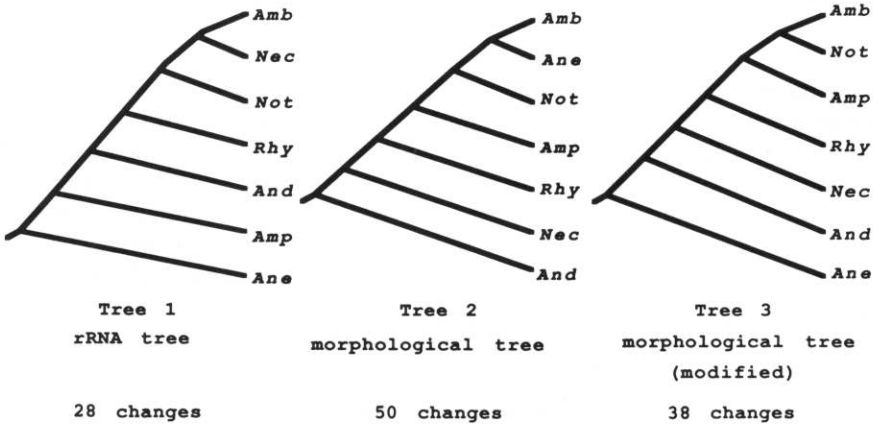


FIG. 5.—Three alternative trees predicted by different hypotheses of the relationships among seven families of salamanders. Genera are abbreviated as follows: *Amb* = *Ambystoma; Amp* = *Amphiuma; And* = *Andrias; Ane* = *Aneides; Nec* = *Necturus; Not* = *Notophthalmus; Rhy* = *Rhyacotriton. Typhlonectes* and *Xenopus* are used as outgroups on all trees (not shown). The numbers of changes required at 17 informative sites (see table 2) are given for each tree.

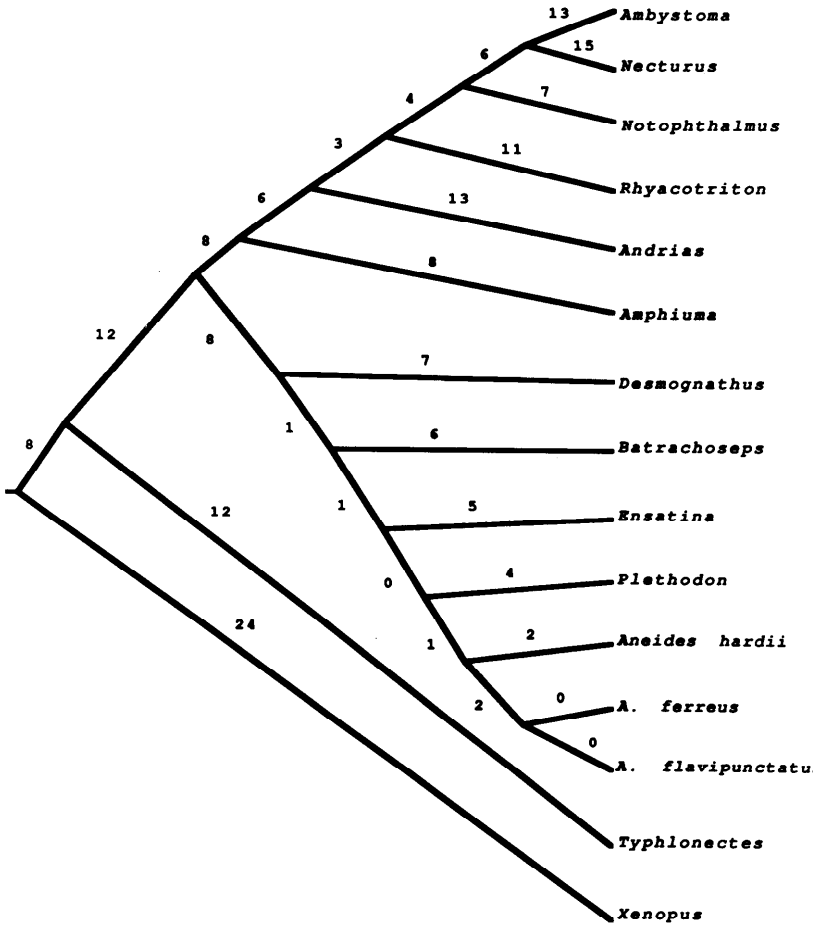


FIG. 6.—A maximum parsimony tree showing the numbers of changes (71 length mutations and 116 base substitutions) inferred for each lineage.

The method of Brown and Clegg (1983) was used to test for bias in the observed substitution types (table 4). The null hypothesis is that six categories of substitution occur with equal frequency relative to the base composition of the sequence. (The base composition of the inferred ancestral sequence is 15% A, 32% C, 38% G, and 15% U, and the specimens compared do not differ from this composition by more than a few percentage points in any cateogory.) The null hypothesis is falsified ($P < 0.005$) with transition substitutions (types 1 and 2 in table 4) occurring at approximately twice the expected frequency and all transversion types (types 3–6 in table 4) occurring at lower than expected frequencies.

The distribution of variable sites along the sequence is evaluated using a statistical test for clustering of variable sites (Brown and Clegg 1983), and the density distribution of substitutions is evaluated using the negative binomial (Bliss and Fisher 1953; Uzzell and Corbin 1971; Holmquist et al. 1983). Significant clustering of mutable sites is detected for segments C and F (table 5, fig. 2). The negative binomial distribution is defined by the mean (m) and a parameter, k (also denoted r), which is estimated as $m^2/(s^2 - m)$, where s^2 is the variance (Bliss and Fisher 1953) [a similar parameter,

Table 3
Summary of Evolutionary Changes by Ribosomal Segment

Segment	T	V	I	Del	L	S	Total	*N*	Total/*N*
B (820–950)	36	24	12	34	6	4	116	154	0.8
C (1121–1325)	17	5	1	2	1	0	26	207	0.1
D (1980–2320)	24	13	9	6	0	0	52	299	0.2
F (2596–2771)	28	27	7	18	5	2	87	149	0.6
Total	105	69	29	60	12	6	281	809	0.3

NOTE.—Changes are inferred from the lineages shown on the tree in fig. 6 plus the mouse and rat lineages. T = transitions; V = transversions; Del = deletions; I = insertions; L = ambiguous-length variants; S = ambiguous base substitutions; Total = sum of mutational events; *N* = number of bases per segment. Base numbers corresponding to each segment are given in parentheses beside the segment designations.

alpha = m^2/s^2, has been used by Nei and Li (1979) and by Carr et al (1987)]. The Poisson distribution is a special case of the negative binomial in which k is very large. Values of k for the D2 and D8 domains are 0.85 and 0.67, and the values for regions C and D are 0.17 and 0.31, respectively. These are lower than values of k observed for protein sequences (approximately 1–2) but comparable in magnitude to the alpha value used by Carr et al. (1987) for mitochondrial DNA (0.45). These results indicate that some molecular sites are much more likely than others to undergo base substitution.

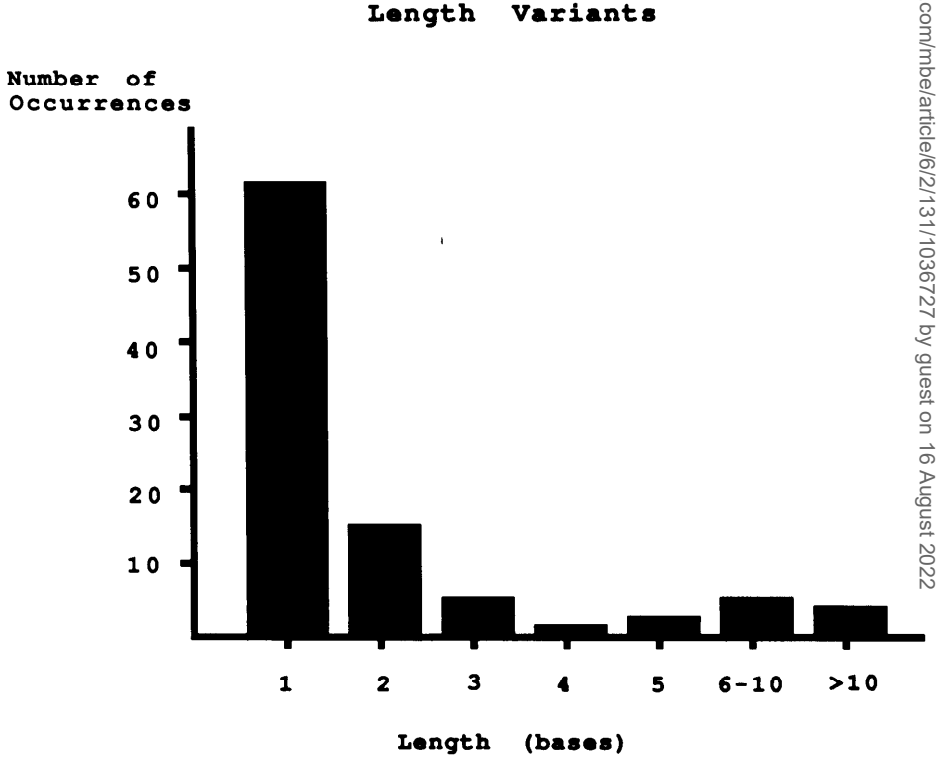


FIG. 7.—Distribution of the sizes of 101 length mutations (as inferred from the tree in fig. 6 plus the mouse and rat lineages).

Table 4
Statistical Tests for Random Distribution of Substitution Types

	Substitution Type[a]						
	1	2	3	4	5	6	Total
Expected	13.8	32.2	13.8	32.2	13.8	32.2	138
Observed	26	57	2	26	7	20	138
$\chi^2 = 49.01$ ($P < 0.005$), df = 4							

[a] 1 = A → G, U → C; 2 = C → U, G → A; 3 = A → U, U → A; 4 = C → G, G → C; 5 = A → C, U → G; 6 = C → A, G → U.

Sequence information from the divergent domains indicates that the genes encoding functional ribosomal RNA do not demonstrate significant intragenomic variation. Unlike previous studies that obtained sequence information from clones of single ribosomal genes, this methodology sequences the products of many genes simultaneously, and salamander genomes typically contain several thousand ribosomal genes (Long and Dawid 1980). The result—that a single, relatively unambiguous sequence is read through the divergent domains by using this methodology—demonstrates that there is not extensive length polymorphism among functional ribosomal genes. This demonstrates strong concerted evolution (Zimmer et al. 1980; Dover 1982) and implies that periods of transient polymorphism comprise only a small portion of the evolutionary history of the functional ribosomal genes.

Table 5
Test for Clustering of Variable Sites

	Region							
	B		C		D		F	
Run Length	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.
0	17	17.6	9		6	5.2	16	8.1
1	10	10.2	3	5.7	7	8.4	7	6.0
2	7	5.9	0		3		0	5.7
3	5		0		3	6.4	0	
4	1		1	5.3	3		2	
5	1	8.2	1		2		0	
6	0		0		6	6.8	0	
7	0		2		1		0	
8–9	1		0	5.2	1	5.6	3	9.3
10–11			0		0		1	
12–13			0		0		1	
14–16			1		2	7.6	0	
17–20			0	4.8	3		1	
21+			4		3		0	
χ^2 (df)	0.2 (2)		11.0 (2)		4.9 (4)		15.8 (2)	
Significance	NS		$P < 0.005$		NS		$P < 0.005$	

Note.—The variable sites:total sites ratios for the four regions are as follows: B, 42:100 = 0.42; C, 21:211 = 0.10; D, 40:307 = 0.13; F, 31:117 = 0.26. Several large length variants were scored as single sites for this test. Brackets denote classes of run length that were pooled to avoid small expected values.

Discussion

Evolutionary Properties of Ribosomal Divergent Domains

Hypotheses that the ribosomal divergent domains are remnants of linkers or transposable elements predict rapid evolution and a neutral pattern of change. Observed rates of base substitution within the divergent domains are lower than those of nuclear noncoding DNA by a factor of two; however, the rate observed for nuclear noncoding DNA is approximated when length-mutational events are included. This evolutionary rate is less than that observed for synonymous substitutions in coding regions by a factor of two to three. The distribution of mutable sites and the density distribution of base substitutions are nonrandom within divergent domains, suggesting that at least parts of the divergent domains experience structural or functional constraints.

One potential source of evolutionary constraint is selection acting to maintain a particular base composition. This is believed to be an important force in the evolution of nucleic acids (Bernardi et al. 1985; Bernardi and Bernardi 1986; Sueoka 1988), one involving directional mutation and fixation pressures. It has been suggested that an excess of G and C bases is expected for ribosomal regions where secondary structure requires the formation of strong, double-stranded stems, because the base pairs formed by G and C feature stronger hydrogen bonding. Although the base composition of the ribosomal sequences studied here is highly skewed toward an excess of G and C bases, there is no evidence that this composition is being maintained by substitutional bias. The excess of G and C bases in ribosomal RNA is accompanied by a proportionately larger number of substitutions that replace G and C bases with A or U bases (table 4). Over all segments and lineages, there is, in A and U bases, a net increase of 46 positions. Furthermore, there is no evidence that length mutation is acting to maintain a skewed base composition. Both insertions and deletions feature an approximately threefold excess of G/C over A/U bases, consistent with the base composition of the sequence. Because deletions predominate, there is a net loss of ~40 G/C positions. The amount of evolution observed in the taxa studied here has not been sufficient, however, to remove the G/C bias or to produce significant divergence in base composition among the specimens compared.

The observation that transitions are approximately twice as frequent as transversions represents a major nonrandom pattern of evolution. This transition bias is a standard observation for the genomic DNA of eukaryotes and prokaryotes (Fitch 1980; Jukes 1980; Nichols et al. 1980). It is smaller than the transition bias observed for the evolution of animal mitochondrial DNA (see review by Moritz et al. 1987) and falls outside the causal mechanisms proposed to explain the mitochondrial transition bias (Wilson et al. 1985). Because of its generality, this transition bias is not likely to reflect functional or structural constraints specific to the ribosomal divergent domains.

Formulation of precise hypotheses of structural and functional constraint for the ribosomal divergent domains requires knowledge of their secondary-structural features. On the basis of primary structures, secondary-structural models have been proposed for the large ribosomal subunits of *Mus* (Michot et al. 1984), *Rattus* (Hadjiolov et al. 1984), and *Xenopus* (Clark et al. 1984), but these models show little agreement even for positions showing unambiguous homology among these three vertebrates. Discrimination of alternative models requires both direct experimental probing of ribosomal secondary structure and examination of the possibility that secondary struc-

ture may change during ribosomal function and that alternative structures therefore may coexist within a cell (Noller 1984).

Systematic Information Content of Ribosomal Divergent Domains

Ribosomal divergent domains are useful for evaluating evolutionary events that occur on a time scale that is intermediate relative to (*a*) the very early divergences investigated using the highly conserved ribosomal segments and (*b*) the much more recent ones studied using noncoding nuclear DNA, silent sites of structural genes, and mitochondrial DNA. Although precise dating is available only for the most recent evolutionary events investigated here, this study spans a time scale of ~14–250 Mya, with most divergences presumably >100 Mya. Of the 17 sites that discriminate alternative relationships among the salamander families, all except 2 are from divergent domains. Only 1 of the 5 sites informative for the relationships among the amphibian orders is outside the divergent domains. In several parts of the divergent domains where evolutionary change involving length mutations has been unusually abundant, phylogenetic information is obscured; however, the sites of extensive length variation appear to be very localized even within the divergent domains.

The observation that some molecular sites have a much higher density of substitution than others indicates that saturation (multiple substitution at the same molecular site) may be a problem if divergent domains are used to study evolutionary events much older than the ones studied here. The effects of saturation can be evaluated by measuring the transition bias that occurs when molecular sequences separated by successively older divergences are compared. Multiple substitution causes the measured transition bias to diminish as successively more divergent sequences are compared (DeSalle et al. 1987). Holmquist (1983) proposes that the transition:transversion ratio is expected to approach an asymptotic value determined by the equilibrium base composition of the molecular sequences as their divergence increases. The expected asymptote for the ribosomal sequences compared in this study [using formula (11) of Holmquist (1983)] is a transition:transversion ratio of 0.42, which is not attained. For example, comparison of the *Ambystoma* ribosomal sequence with those most distantly related to it (*Xenopus* and *Mus*) gives a transition:transversion ratio of 2.2. Saturation therefore appears not to be a serious problem at the level of this study.

The ribosomal divergent domains are used here to resolve evolutionary relationships that have been highly controversial from the perspective of morphological and chromosomal systematics and that are outside the range of resolution of the commonly used methods for protein comparison. Although the evolutionary relationships among salamander families have been highly controversial and not enjoyed a strong consensus, the relationships specified by the ribosomal RNA data conflict strongly with hypotheses derived from morphological and chromosomal data (fig. 5; see also Salthe and Kaplan 1966; Edwards 1976; Hecht and Edwards 1976; Estes 1981; Milner 1983; Duellman and Trueb 1986). Ribosomal RNA sequences from additional salamanders and from different parts of the ribosome (including the other divergent domains) are being collected to test these alternative hypotheses further and to examine in more detail the conflicts occurring between the alternative studies.

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