Evolution of the 28S Ribosomal RNA Gene in Anurans: Regions of Variability and Their Phylogenetic Implications[1]

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Fifteen restriction sites were mapped to the 28S ribosomal RNA gene of individuals representing 54 species of frogs, two species of salamanders, a caecilian, and a lungfish. Eight of these sites were present in all species examined, and two were found in all but one species. Alignment of these conserved restriction sites revealed, among anuran 28S rRNA genes, five regions of major length variation that correspond to four of 12 previously identified divergent domains of this gene. One of the divergent domains (DD8) consists of two regions of length variation separated by a short segment that is conserved at least throughout tetrapods. Most of the insertions, deletions, and restriction-site variations identified in the 28S gene will require sequence-level analysis for a detailed reconstruction of their history. However, an insertion in DD9 that is coextensive with frogs in the suborder Neobatrachia, a *Bst*EII site that is limited to representatives of two leptodactylid subfamilies, and a deletion in DD10 that is found only in three ranoid genera are probably synapomorphies.

Introduction

Frogs constitute a relatively speciose order of vertebrates (comprising almost 3,500 described species; Frost 1985) that first diversified ~200 Myr ago in the Triassic (Duellman and Trueb 1985). However, morphological divergence of anurans has been minor compared to that of other tetrapod orders, most of which diversified more recently and contain fewer species (Wilson et al. 1974; Larson et al. 1984). Partially because of the paucity of morphological characters of anurans, considerable disagreement exists concerning the phylogeny and classification of frogs (Lynch 1973; Savage 1973; Starrett 1973; Duellman 1975; Sokal 1975, 1977; Laurent 1979; Dubois 1983, 1984; Duellman and Trueb 1985). Comparative studies of chromosomes and proteins have provided considerable information on phylogenetic relationships within many genera of frogs but have contributed little to an understanding of higher-level anuran phylogeny (see review in Duellman and Trueb 1985).

Ribosomal RNA genes and associated spacer regions (rDNA) have been sequenced and studied extensively in the anuran *Xenopus laevis* (Boseley et al. 1979; Hall and Maden 1980; Salim and Maden 1981; Ware et al. 1983), but comparative data are unavailable for other frogs. Clark et al. (1984) identified nine "expansion segments" (ES) of 28S rDNA that accounted for the major differences in secondary structure

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between *E. coli* and *X. laevis*. These ES correspond to nine of 12 "divergent domains" (DD) proposed by Hassouna et al. (1984) to account for changes in secondary structure between *E. coli* 23S rRNA and mouse 28S rRNA. Much of the change in rRNA genes through time is a result of insertions that may serve as useful markers of evolutionary history.

Because ribosomal RNA genes are evolutionarily highly conserved (Elwood et al. 1985), data on the structure of rDNA should provide information on evolutionary relationships of anurans. In addition, rDNA evolves in a concerted fashion (Dover and Coen 1981), so the rDNA array of a single individual is largely representative of the species. Therefore, we surveyed, by restriction-site mapping, 28S rDNA of representatives of most of the anuran families and subfamilies to determine the extent of variation of this region among frogs and to assess the ability of this technique to provide phylogenetic information over this time scale.

Material and Methods

Tissue samples (liver, muscle, or whole blood) were obtained from 61 individuals representing 58 species (table 1), including 54 species of frogs, two species of salamanders, one species of caecilian, and one species of lungfish. Seventeen of 21 recognized families of frogs (Duellman and Trueb 1985) were represented; several diverse families (Pelobatidae, Leptodactylidae, and Hylidae) were represented by species from more than one subfamily.

Table 1
Classification of Species Examined

Species	Classification
Lepidosiren paradoxa (a lungfish)	Dipnoi: Lepidosirenidae
Dermophis mexicanus (a caecilian)	Gymnophiona: Caecilidae
Siren intermedia (a salamander)	Caudata: Sirenidae
Plethodon glutinosus (a salamander)	Caudata: Plethodontidae
Ascaphus truei	Anura: Leiopelmatidae
Bombina orientalis	Anura: Discoglossidae
Xenopus laevis	Anura: Pipidae
Rhinophrynus dorsalis	Anura: Rhinophrynidae
Scaphiopus multiplicatus	Anura: Pelobatidae: Pelobatinae
Megophrys nasutus	Anura: Pelobatidae: Megophryinae
Limnodynastes salmini	Anura: Myobatrachidae
Bufo woodhousii	Anura: Bufonidae
Ceratophrys ornata	Anura: Leptodactylidae: Ceratophryinae
Leptodactylus wagneri	Anura: Leptodactylidae: Leptodactylinae
Telmatobius niger	Anura: Leptodactylidae: Telmatobiinae
Pseudis paradoxa	Anura: Pseudidae
Gastrotheca riobambae	Anura: Hylidae: Hemiphractinae
Hyla chrysoscelis	Anura: Hylidae: Hylinae
Centrolenella (n. sp.)	Anura: Centrolenidae
Glossostoma aequatoriale	Anura: Microhylidae: Microhylinae
Colostethus (n. sp.)	Anura: Dendrobatidae
Hyperolius tuberilinguis	Anura: Hyperoliidae
Nesomantis thomasetti	Anura: Sooglossidae
Rana (32 species)	Anura: Ranidae
Pyxicephalus adspersus	Anura: Ranidae

Approximately 1 g of tissue was pulverized to fine powder in liquid nitrogen with a prechilled mortar and pestle. This powder was suspended in STE (0.1 M NaCl, 0.05 M Tris [tris(hydroxymethyl)aminomethane], 0.001 M ethylenediaminetetraacetic acid, disodium salt [EDTA], pH 7.5) in a 1:20 tissue:buffer ratio. Cells were lysed by the addition of 20% sodium dodecyl sulfate in water (0.05 ml/ml), and proteins were digested with 100 U/ml proteinase K (from *Tritirachium album*) for 2 h at 55 C. After digestion, the preparation was extracted twice with an equal volume of a 25:25:1 solution of phenol:chloroform:isoamyl alcohol. Samples were centrifuged for 5 min at 7,000 *g* during each extraction to facilitate separation of the layers. The supernatant was then extracted twice with chloroform, again with 5 min centrifugation at 7,000 *g*. DNA was precipitated from the supernatant by addition of a 0.1 vol 2 M NaCl and 2 vol 95% ethanol. Precipitated DNA was recovered by centrifuging for 10–20 s at 7,000 *g* and then was dissolved in TE (0.001 M Tris, 0.0001 M EDTA, pH 7.2). Skeletal muscle yielded ~50 μg of high-molecular-weight DNA/100 mg of tissue; the yield from liver and blood was ~150 μg DNA/100 mg tissue.

Sixteen restriction endonucleases (New England Biolabs) were used to digest 0.5–1.0 μg of DNA in 50-μl reactions according to the manufacturer's specifications. All endonucleases used had a recognition sequence that included six specific nucleotides as follows: *Apa*I, GGGCC/C; *Bam*HI, G/GATCC; *Bcl*I, T/GATCA; *Bgl*II, A/GATCT; *Bst*EII, G/GTNACC; *Dra*I, TTT/AAA; *Eco*RI, G/AATTC; *Hin*dIII, A/AGCTT; *Kpn*I, GGTAC/C; *Nco*I, C/CATTG; *Pst*I, CTGCA/G; *Pvu*II, CAG/CTG; *Sac*I, GAGCT/C; *Stu*I, AGG/CCT; *Xba*I, T/CTAGA; and *Xmn*I, GAANN/NNTTC. Digestion times normally ranged from 4 to 6 h.

Double digests were used as necessary to locate specific restriction sites or to size specific insertions within the 28S gene. Samples to be subjected to double digestions were first cleaved with one enzyme under normal conditions and then ethanol precipitated overnight. The cleaved DNA was then recovered by centrifugation at 8,500 *g* for 10 min. Residual ethanol was removed by drying briefly under vacuum, and the DNA was resuspended in an appropriate volume of TE. The cleaved DNA samples were stored at −20 C until needed and then digested with a second restriction enzyme. This allowed easy handling of samples when a series of double digestions were performed using the same primary enzyme.

Cleaved DNA was electrophoresed at ~5 V/cm for 15 h in 0.8%, 1.0%, or 1.2% agarose gels (buffer system: 0.04 M Tris, 0.02 M sodium acetate, 0.018 M sodium chloride, 0.001 M EDTA, pH 8.0). Lambda c1857 phage DNA cut with *Hin*dIII and/or *Eco*RI was included on each gel as a standard. After electrophoresis, DNA was stained with ethidium bromide and then viewed and photographed under 302-nm UV light.

DNA in the agarose gels was denatured in 1.5 M NaCl, 0.5 N NaOH for 1 h and then neutralized in 3 M NaCl, 0.5 M Tris for 1.5 h. DNA fragments were transferred to nitrocellulose paper (Scheicher and Schuell BA85) using a modification of the method of Southern (1975). Southern blots in the initial mapping survey were probed with a radioactively labeled clone that contained the mouse 28S gene (p I19); this segment had been subcloned by Arnheim from the λgtWES clones described by Arnheim (1979).

Because of limited similarity with the mouse 28S gene, problems arose when we probed small fragments from double digestions. To avoid this difficulty, DNA from *Rana catesbeiana* was partially cleaved with *Sau*3A1 and cloned into the phage vector

EMBL3. The library thus generated was screened for rDNA positives with pI19, and several recombinant phage-bearing rDNA inserts were isolated. The *Eco*RI fragment that contains the internal transcribed spacers, the 5.8S rRNA gene, and most of the 28S rRNA gene (Hillis and Davis 1986) was subcloned from one of these phage into pUC18. The resulting plasmid, designated pE2528, was used to prepare a radioactive probe for blots with small restriction fragments.

After hybridization, filters were washed and exposed to X-ray film for 12–48 h at −80 C. Hybridization conditions and washes were identical to those described by Sytsma and Schaal (1985). After autoradiography, DNA fragments were sized by fitting migration distances to a least-squares regression line of lambda C1857 phage DNA–fragment migration distances (excluding fragments >9 kb) using the algorithm of Schaffer and Sederoff (1981). Mapping of sites was accomplished by double digestions, using as a reference point the *Eco*RI site that is uniformly present in vertebrates near the 5′ end of the 28S gene (Cortadas and Pavon 1982).

To compare the regions of variability among our 28S-gene restriction maps with sequenced 28S rRNA genes, we aligned the published sequences for the genes of *Xenopus* (Ware et al. 1983) and *Mus* (Hassouna et al. 1984). By comparing the aligned sequences, we identified specific regions of substitution and length divergence for comparison with our restriction maps.

Results

Restriction maps of the 28S gene were similar throughout amphibians and lungfish (fig. 1). Of 15 restriction sites within the 28S gene, eight were found in all 58 species surveyed. Two sites (a *Pvu*II site and an *Nco*I site) were found in all but one species, and two others (*Bst*EII and *Stu*I) were located in many species. One site (*Bst*EII) occurred in only two of the species studied (*Leptodactylus* and *Telmatobius,* both of the Leptodactylidae). The remaining sites were found in single species.

Alignment of the conserved restriction sites revealed five regions of major length variation among the species (fig. 1), corresponding to several of the ES reported by Clark et al. (1984) and to DD reported by Hossouna et al. (1984). Specifically, these regions are DD3 (ES3), two distinct regions of DD8 (ES7), DD9 (ES8), and DD10 (no corresponding ES). Insertions of different sizes in DD3 and DD8 may not be at the same nucleotide position.

Comparatively little restriction-site variation was observed within divergent domains, and no restriction sites were found to be exclusively associated with particular insertions. However, of the seven restriction sites that vary among genera, five were found within divergent domains (fig. 1).

Discussion

Cortadas and Pavon (1982) attributed the considerable length conservation of the 28S rDNA coding region within vertebrate classes to the functional necessity of particular rRNA secondary structures. Nevertheless, several regions of the gene clearly have undergone many insertion and/or deletion events (fig. 1).

The regions of greatest divergence among the 28S genes of amphibians generally correspond closely to the regions of greatest divergence between the 28S genes of *Xenopus* and *Mus* (fig. 2). Although we could not detect insertional/deletional events in the first 1,000 bp of the 28S gene among the species in our study because of the

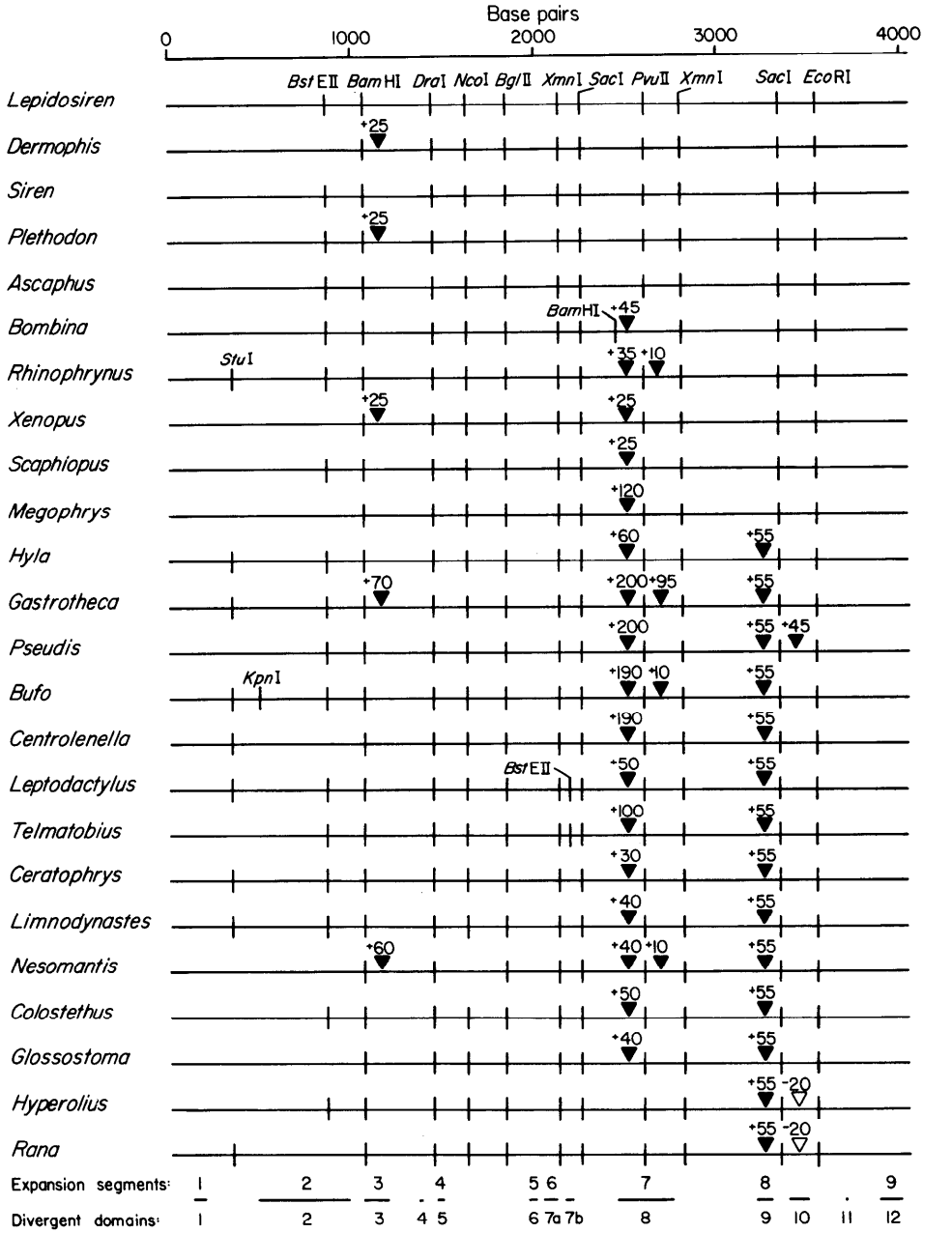

FIG. 1.—Restriction maps of amphibian and lungfish 28S rDNA. ES of Clark et al. (1984) and DD of Hassouna et al. (1984) are indicated at the bottom of the figure. Length variants are shown as insertions (black triangles) or deletions (unfilled triangles) relative to the 28S gene of *Lepidosiren*. The *Rana* map is also representative of the map for *Pyxicephalus*.

lack of conserved restriction sites in that region, most of the widely variable restriction sites occurred in that area (fig. 1). This is also the region of greatest substitutional divergence between the 28S rRNA genes of *Mus* and *Xenopus* (fig. 2; also see Hillis

and Davis 1986). The DD8 region, highly variable in amphibians, is also divergent between *Mus* and *Xenopus* (fig. 2).

Variation in DD3 is not extensive, and the insertions, being of different sizes or from different orders, are probably not synapomorphic. In contrast, DD8 is the most variable portion, in size, of the 28S gene in amphibians, but we hesitate to treat these size classes as evolutionary markers because of the high degree of variation in this region. Sequence analysis of this region would be highly informative; such an analysis could identify a transition series of insertions, as well as distinguish homoplasious insertions.

DD8 appears to contain two distinct regions of length variation separated by a conserved *Pvu*II site (fig. 1). These two distinct subregions are also apparent in the alignment of the *Mus* and *Xenopus* sequences through DD8 (fig. 3). Among amphibians, DD8a (in the *Sac*I-*Pvu*II fragment) is much more variable than is DD8b (in the *Pvu*II-*Xmn*I fragment).

In the lungfish, caecilian, and salamanders, as well as in six genera of frogs, DD9 is 55 bp shorter than it is in the remaining anurans. Because this shorter fragment is present in the nonfrogs as well as in some frogs, we interpret this to be the ancestral condition for anurans. The remaining frog species that share the 55-bp insertion correspond to the suborder Neobatrachia (Duellman 1975; = Ranoidei of Dubois [1983]). This insertion, therefore, is probably a synapomorphy characteristic of the suborder.

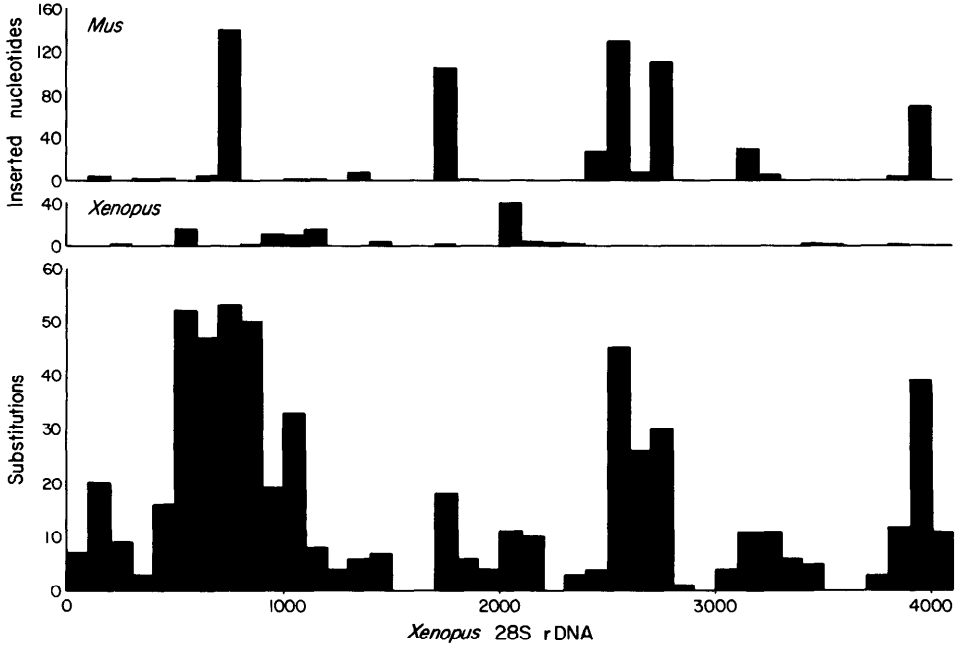

FIG. 2.—Divergence due to substitutions and insertions between the 28S rRNA genes of *Mus* and *Xenopus* in 100-bp intervals. Divergence is shown as percent difference in noninserted regions. The *X*-axis represents the nucleotide positions of the *Xenopus* gene, so insertions within the *Mus* sequence >100 bp are possible within the 100-bp blocks.

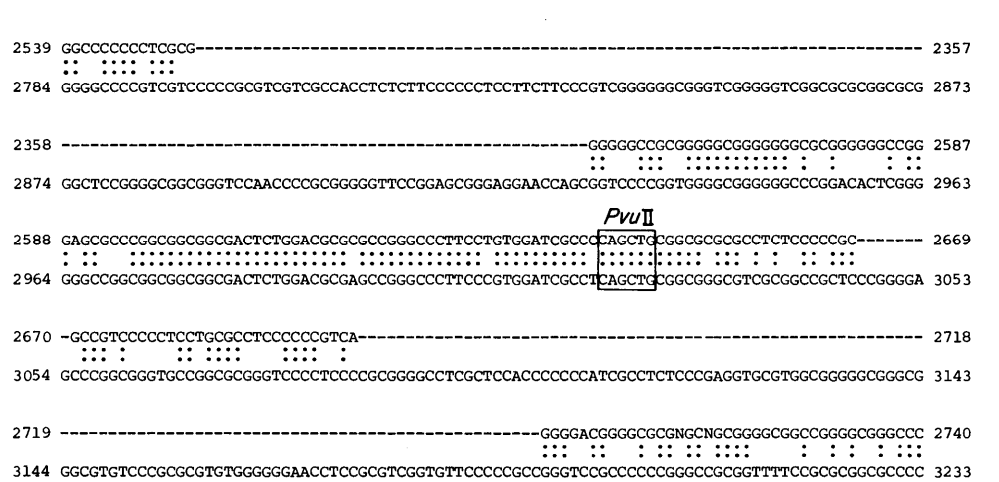

FIG. 3.—Manually aligned sequences through DD 8 of *Mus* (lower) and *Xenopus* (upper) 28S rRNA genes. Starting and ending nucleotide positions in the 28S gene are shown for each line. The conserved region that contains the *Pvu*II site is shown flanked by insertions in the *Mus* gene.

A 20-bp deletion in DD10, shared by all of the ranids (*Pyxicephalus* and 32 species of *Rana*) and the single hyperoliid (*Hyperolius*), is probably a synapomorphy, since these two families are considered closely related on the basis of morphological criteria (Liem 1970). The phylogeny of ranoids is not clear (Drewes 1984; Duellman and Trueb 1985), and further study of the taxonomic distribution of this DD10 deletion may help clarify the relationships within this group.

The *Bst*EII-site gain in DD7b of *Leptodactylus* and *Telmatobius* is a possible synapomorphy for two of the three subfamilies of leptodactylids studied (Telamatobiinae and Leptodactylinae); the third subfamily of leptodactylids represented in our analysis (Ceratophryinae: *Ceratophyrs*) lacks this site. The Leptodactylidae is the largest family of frogs, and relationships within the family are poorly understood (Lynch 1971; Duellman and Trueb 1985). This *Bst*EII site provides a character that may help elucidate higher relationships among leptodactylids.

Although restriction mapping of the 28S gene provided relatively few synapomorphies among amphibians, the location of regions of variability within the 28S gene of amphibians should facilitate investigations of phylogeny based on sequencing of rDNA. Such sequence-level analysis probably is necessary in order to tap effectively the hundreds of millions of years of phylogenetic information contained in rDNA genes.

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