J Mol Evol (1986) 24:94–102

Journal of Molecular Evolution

Nucleotide Sequence and Evolution of the Orangutan ϵ Globin Gene Region and Surrounding Alu Repeats*

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Summary. We have mapped and sequenced the ϵ globin gene and seven surrounding Alu repeat sequences in the orangutan β globin gene cluster and have compared these and other orangutan sequences to orthologously related human sequences. Noncoding flanking and intron sequences, synonymous sites of α, γ, and ϵ globin coding regions, and Alu sequences in human and orangutan diverge by 3.2%, 2.7%, and 3.7%, respectively. These values compare to 3.6% from DNA hybridizations and 3.4% from the ψη globin gene region. If as suggested by fossil evidence and "molecular clock" calculations, human and orangutan lineages diverged about 10–15 MYA, the rate of noncoding DNA evolution in the two species is 1.0–1.5 × 10^{-9} substitutions per site per year. We found no evidence for either the addition or deletion of Alu sequences from the β globin gene cluster nor is there any evidence for recent concerted evolution among the Alu sequences examined. Both phylogenetic and phenetic distance analyses suggest that Alu sequences within the α and β globin gene clusters arose close to the time of simian and prosimian primate divergence (about 50–60 MYA). We conclude that Alu sequences have been evolving at the rate typical of noncoding DNA for the majority of primate history.

Key words: Epsilon globin — Alu repeats — Orangutan — Nucleotide sequences

Introduction

The α and β globin gene clusters in mammals arose from a duplication in early vertebrates followed by a translocation of one gene to another chromosome and a series of subsequent duplications (Collins and Weissman 1984; Hardison and Gelinas 1986). Each cluster, whether α type, or β type, contains at least one gene expressed in embryonic life and at least one postnatally expressed gene. In the β globin cluster of some species, a gene expressed only during fetal life also occurs. Generally, the 5′ globin genes are expressed early in development and the 3′ genes are expressed after birth. Scattered within the α and β globin gene clusters of primates are numerous short interspersed Alu-like repeat sequences.

The fact that many Alu repeats are scattered throughout the globin gene clusters of primates enables one to compare the evolution of Alu repeats with the evolution of adjacent coding and noncoding globin gene regions (Sawada et al. 1985). Within the globin gene clusters, for example, concerted evolution has been demonstrated in human and orangutan α^1 and α^2 globin genes (Marks et al. 1986) as well as human and orangutan γ^1 and γ^2 globin genes

Offprint requests to: Ben F. Koop
* Presented at the FEBS Symposium on Genome Organization and Evolution, held in Crete, Greece, September 1–5, 1986

(Slightom et al. 1986). In each case, intraspecific variation is less than interspecific divergence. Evolution in the absence of sequence specific selection has also been demonstrated in the human and orangutan $\psi\eta$ globin genes (Koop et al. 1986). Coding and noncoding sequence comparisons of the human and orangutan ϵ globin gene presented in this paper suggests that the ϵ gene is the only functional globin gene within primates that has not been duplicated or subjected to gene conversions. It therefore provides the most straight-forward example of independent functional globin gene evolution. Thus for both human and orangutan, there are six globin genes, each with a slightly different evolutionary history with which to compare human and orangutan Alu sequences.

Higher primate Alu repeats (Schmid and Shen 1985; Weiner et al. 1986) typically are short dimeric sequences (300 bp in length) probably derived by retroposition of a sequence that was originally derived from the 7SL RNA gene. They contain several internal RNA polymerase III promoter elements and they appear to be transposed passively (Weiner et al. 1986). In humans they constitute 3–5% of the genome and differ from their consensus sequence by about 14% (Weiner et al. 1986). The galago, a prosimian primate, contains a Type 1 Alu repeat that is very similar to that of higher primates plus a Type 2 Alu-like repeat of which only part resembles the Alu repeat of higher primates (Daniels and Deininger 1983). Galago Type 2 Alu sequences differ from their consensus by about 7–9% (Deininger and Daniels 1986). Rodent Alu-like repeats (B1), on the other hand, are typically monomers and differ from their consensus by about 8% (Kalb et al. 1983; Deininger and Daniels 1986).

Some repeat sequences that appear to be shared among several major mammalian lineages exhibit greater similarities within specific lineages (Britten and Kohne 1968). This has, in the past, led to several hypotheses invoking concerted evolution to explain purported species specific "homogenization" of Alu sequences (Deininger and Schmid 1979; Daniels and Deininger 1983, 1985; Sawada et al. 1985; Schmid and Shen 1985; Weiner et al. 1986). More recently however Deininger and Daniels (1986) have suggested that these "species specific" Alu repeats are the result of independent amplifications from 7SL RNA-like retropseudogenes. The two hypotheses predict different interspecific divergence values as well as different phylogenetic relationships among similarly positioned Alu sequences in different species (see Results and Discussion). To distinguish between the two hypotheses, we have examined seven similarly (orthologously) positioned Alu sequences in the β globin cluster of human (Shen et al. 1981; Collins and Weissman 1984; Li et al. 1985a) and orangutan (sequences determined in this study) and compared Alu sequences to adjacent coding and noncoding globin gene sequences with respect to patterns of inter- and intraspecific divergences. This study complements that of Sawada et al. (1985) on the Alu sequences of chimpanzee and human globin-gene regions.

Methods and Materials

High molecular weight DNA from orangutan (*Pongo pygmaeus*) was isolated from liver tissue (Maniatis et al. 1982) and partially digested with Mbol. DNA fragments 15–25 kb in length were separated in a NaCl gradient and subsequently ligated to BamHI arms of Charon 35 lambda phage (Loene and Blattner 1983). Recombinant phage DNA was packaged in vitro (Hohn 1979), and propagated on Rec A$^-$ *E. coli* K12 strain ED8767 (Murray et al. 1977). The genomic library was then screened (Benton and Davis 1977) using a human ϵ probe (0.7 kb BamHI fragment) and a gorilla γ probe (2.7 kb EcoRI fragment). Three recombinant phage clones were isolated and mapped. EcoRI fragments from these lambda clones were further subcloned into pUC 8, transformed and grown in *E. coli* JM 83. All gene-containing EcoRI fragments were confirmed using genomic blot hybridizations (Southern 1975). Nucleotide sequences were obtained by the chemical cleavage method of Maxam and Gilbert (1980). Where both strands were not sequenced, multiple sequences were obtained for the same strand. A more detailed account of the procedures used are presented by Slightom et al. (1986). Phylogenetic reconstruction procedures are described by Goodman et al. (1984).

Results and Discussion

Human and Orangutan ϵ Globin Gene Comparisons

In Fig. 1, we present the organization of orangutan ϵ, γ^1, γ^2, $\psi\eta$, and δ globin genes as determined from three recombinant Charon 35 clones and three recombinant Charon 32 clones (Ppy CH32 13.5 and Ppy CH32 14.2 are from Slightom et al. [1986]). The EcoRI restriction map of orangutan differs at only 3 of the 18 sites in human ($3/(18*6) = 3.0\%$). The small arrows above the human and orangutan maps indicate the position and orientation of Alu sequences whose identities have been confirmed by sequence analysis. Recombinant plasmid subclones were named according to the size of the parent lambda clone insert, the size of the subcloned region, and the restriction site. More detailed restriction enzyme maps as well as the sequencing strategy of recombinant plasmids are indicated in Fig. 1.

In Fig. 2 the orangutan ϵ sequence and its 0.3 kb 3′ and 3.3 kb 5′ flanking sequences are aligned with the corresponding human sequences (Baralle et al. 1980; Li et al. 1985a). The overall organization and sequence of the orangutan ϵ gene region is very similar to that of the human.

The human and orangutan ϵ coding regions differ

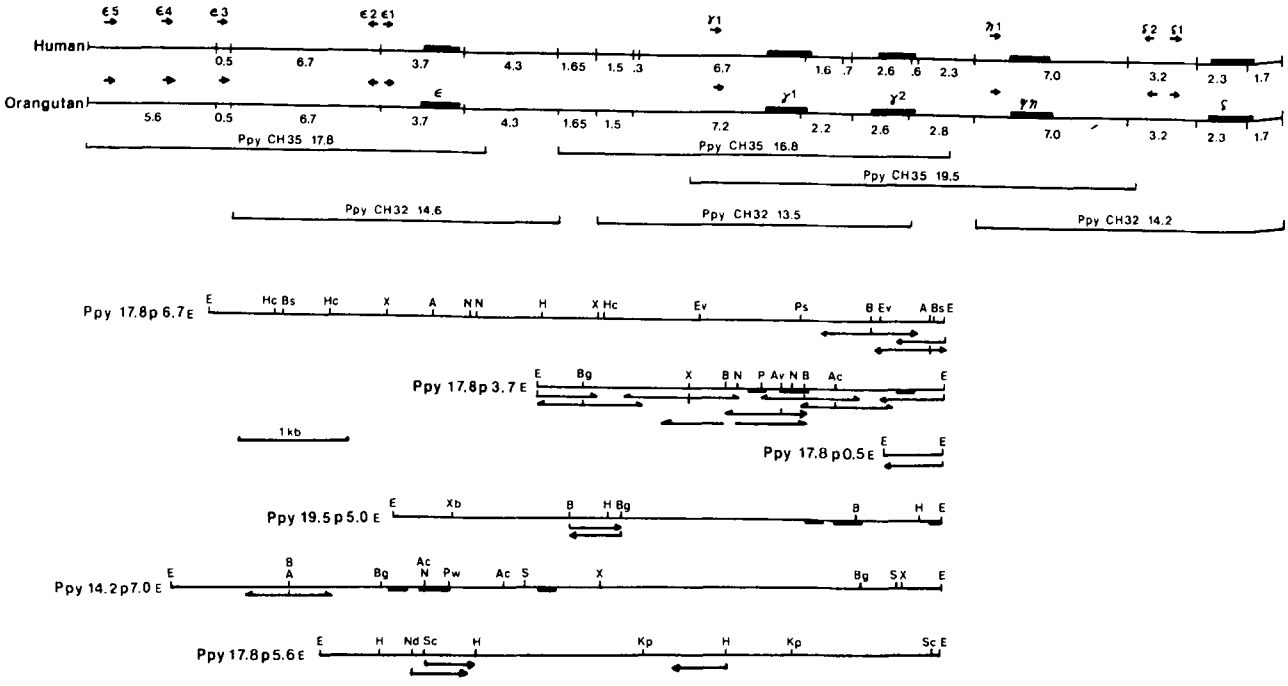

Fig. 1. Restriction map and strategy used to locate and sequence the orangutan β globin gene region and Alu sequences $\epsilon 1$, $\epsilon 2$, $\epsilon 3$, $\epsilon 4$, $\epsilon 5$, $\eta 1$, and $\gamma 1$. The top two lines show the organization of the human and orangutan ϵ, γ^1, γ^2, $\psi\eta$, and δ genes in the β globin cluster. The locations of EcoRI restriction enzyme sites and number of kilobases (kb) between sites are indicated below each line. The small labeled arrows above each line show the positions of Alu sequences. Below the human and orangutan maps are 6 recombinant lambda clone inserts used to derive the orangutan map. The length and position of the lambda clone inserts correspond directly to the orangutan linkage map. Below the lambda clone inserts are 6 recombinant plasmid inserts on which restriction enzyme sites are indicated. Directly below each insert is the sequencing strategy used. Ac = AccI; Av = AvaI; B = BamHI; Bs = BstEII; Bg = Bgl II; Bn = Bst NI; E = EcoRI; Ev = EcoRV; H = Hind III; Hc = Hinc II; Kp = Kpn I; N = Nco I; Nd = Nde I; P = Pvu II; Ps = Pst I; Sc = Sca I; S = Stu I; and X = Xba I.

at 5 of the 438 positions. At codons 83 and 84, (positions 3697 and 3700), the human amino acid sequence has a proline and an alanine, whereas the orangutan sequence has two threonine residues. Proline is a strong α-helix destabilizer, and threonine is a weaker α-helix destabilizer (Chou and Fasman 1974). It is interesting to note that codons 83 and 84 occur near the bend between the F and F′ helices of the β-globin molecule, and that such amino acid changing mutations would produce an α-helix destabilizing environment which in turn could stabilize a bend.

The human and orangutan ϵ genes also differ by 3 synonymous substitutions (positions 3540, 3597, 4746). Divergence between the silent sites of orangutan and human ϵ is approximately 3.1%, while divergence between amino acid changing sites is approximately 0.6%. These values compare to 7.3% and 1.0% between human and orangutan α^1 and α^2 genes (Marks et al. 1986), and to 3.1% and 1.5% between human and orangutan γ^1 and γ^2 genes (Slightom et al. 1986). Divergence values were calculated as per Nei and Gojobori (1986).

The noncoding regions of the human and orangutan ϵ gene (Fig. 2) show 144 differences. Of these, 86 are transitions, 40 are transversions, and 18 are gaps. The divergence between human and orangutan in the 122 positions in intron 1, 864 positions in

Fig. 2. Aligned nucleotide sequences of the human and orangutan ϵ gene and flanking regions. Those orangutan positions that differ from the human sequences appear below the human sequence. Gaps (*) are placed to maximize sequence homology. The nucleotide sequence for human is from Li et al. (1985a). Both the human and orangutan ϵ genes are divided into three exons separated by two introns each obeying the GT-AG splicing rule. The amino acids are indicated above their respective codons. The immediate 5′ flanking regions contain identical CCACCC and CCAAT promoter elements, and a RNA polymerase II binding site (AATAAA). Both sequences share the same initiation and terminator sequences as well as the 3′ poly (A) addition signal. In the extended 5′ region, two Alu sequences are labeled with their flanking direct repeats (solid arrows). Also present is a possible stem loop structure (solid line labeled LOOP is the loop and the flanking inverted repeats indicate the stem). Of special note is the mutational difference at positions 1584 to 1596 in orangutan. This short orangutan insert is almost identical to the inverted opposite strand in human spanning positions 1619–1608.

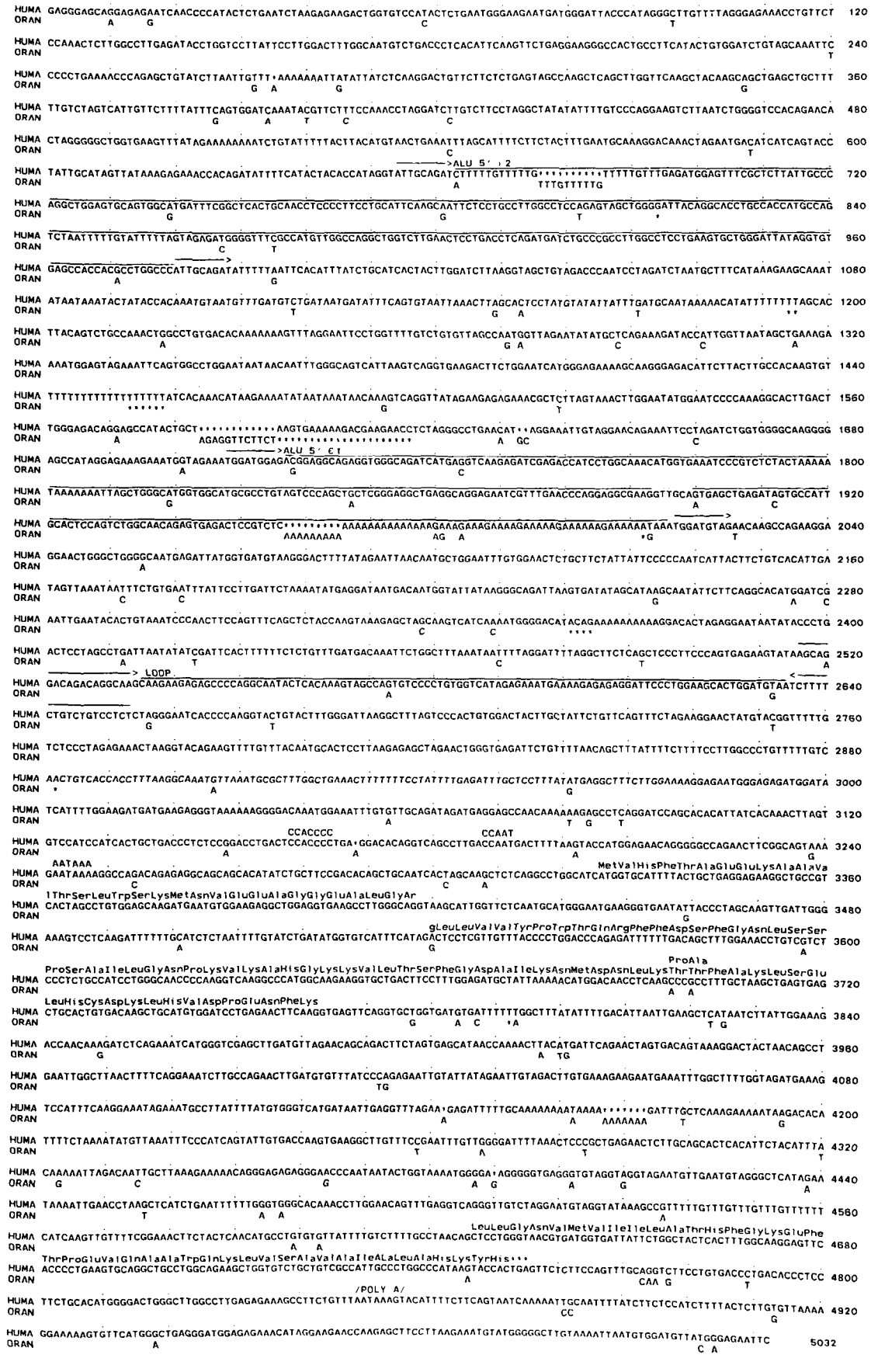

Table 1. Rates of evolutionary substitution since the divergence of human (H) and orangutan (O) in the α, γ, $\psi\eta$, and ϵ genes and flanking regions. The separation of the two lineages probably occurred 10–15 MYA (Andrews and Cronin 1982; Delson 1985). AA Chg = amino acid changing substitutions in the coding region. Syn Chg = changes in the coding region that do not change the amino acid composition. Non-Cod means that differences are obtained from outside the translated region.

	Rates of substitutions/site/year ($\times 10^{-9}$)			
	AA Chg	Syn Chg	Total	Non-Cod
H-O α^1	0.3–0.5	2.6–4.0	1.3–1.9	2.2–3.3*
H-O α^2	0.3–0.5	2.3–3.4	1.2–1.8	2.2–3.3*
H-O γ^1	0.4–0.6	0.7–1.0	0.5–0.8	1.4–2.1**
H-O γ^2	0.6–0.9	1.3–2.0	0.8–1.1	1.1–1.7**
H-O $\psi\eta$	1.0–1.5	1.2–1.8	1.1–1.6	1.1–1.7
H-O ϵ	0.2–0.3	1.0–1.6	0.4–0.6	1.0–1.5

* Taken from the unconverted 3′ 150 bases and then averaged for both α^1 and α^2

** Taken from the unconverted 3′ 260 bases

Table 2. Orthologous and paralogous comparisons of the human (H), chimpanzee (C), and orangutan (O) Alu sequences and of each Alu sequence to the overall consensus (Schmid and Shen 1985). "n" = the number of sequences, $\bar{x}$ = the average percent, and SD is the standard deviation

	n	$\bar{x}$	SD	Range
Paralogous comparisons				
H vs H	14	20.9	2.4	13.9–25.9
C vs C	7	22.6	2.1	18.8–26.0
O vs O	7	20.0	3.0	15.6–24.6
Overall	28	21.0	2.4	13.9–25.9
Orthologous comparisons				
H vs C	7	2.2	1.4	1.0–5.2
H vs O	7	3.7	1.9	2.4–6.9
Consensus comparisons				
Con vs H	14	13.8	1.6	11.6–15.9
Con vs C	7	14.4	1.2	12.5–15.6
Con vs O	7	14.0	1.8	12.4–16.6
Con vs All	24	14.0	1.5	11.6–16.6

intron 2, 277 positions in the 3′ flanking region, and the 3325 positions in the 5′ flanking region are 1.6%, 4.2%, 4.3%, and 2.9%, respectively. The average divergence for all noncoding regions is 3.2% (each gap was treated as a single position irrespective of gap size). This value corresponds closely to the 3.4% divergence (over 2.2 kb) found between the human and orangutan $\psi\eta$ genes (Koop et al. 1986) and the 3.6% divergence estimated from DNA hybridization results (Sibley and Ahlquist 1984). The close correlation between the divergence obtained from DNA hybridizations and noncoding DNA sequences supports the suggestion that the majority of the genome is not subject to conserving selection.

Orangutan and human lineages are thought to have diverged 10–15 MYA (Andrews and Cronin 1982; Delson 1985; Koop et al. 1986). Over this period the coding portions of the ϵ gene have diverged by an average of 1.1% or by a rate of 0.4–0.6 $\times$ 10^{-9} substitutions per site per year. The rate of synonymous substitutions is 1.0–1.6 $\times$ 10^{-9}, and the rate of amino acid changing substitutions is 0.2–0.3 $\times$ 10^{-9}. These rates of change are compared with human and orangutan α^1, α^2, γ^1, γ^2, and $\psi\eta$ genes in Table 1. The ϵ gene appears to be one of the slower evolving globin genes (Table 1). Nearly all of the conservation is at the amino acid changing sites, with little conservation observed in the silent sites.

The overall rate of divergence between human and orangutan in noncoding regions as well as for the synonymous sites within coding regions is approximately 1.0–1.5 $\times$ 10^{-9} subs./site/year. This value is very close to the 1.3 $\times$ 10^{-9} obtained for primates (Britten 1986) but different from the 5.0 $\times$ 10^{-9} subs./site/year determined from other species and gene comparisons (Li et al. 1985b). Such differences in rates of evolution could be the result of different generation times (Wu and Li 1985) or perhaps differential effectiveness of DNA repair processes (Neel 1983; Goodman 1985; Britten 1986).

Human and Orangutan Alu Sequences

The question of why Alu-like repeat sequences can be more similar within species than between distant species has been the subject of several recent studies (Schmid and Shen 1985; Deininger and Daniels 1986; Weiner et al. 1986). Four hypotheses have been advanced to explain these patterns: (1) Extensive gene conversion, (2) coevolution by reciprocal recombination, (3) specific replacement of old sequences by new ones (Steady State), and (4) independent recent expansions from a few progenitors followed by neutral drift. A recent study involving paired human and chimpanzee Alu sequences (Sawada et al. 1985) suggested that neutral drift was the major factor in determining sequence divergence between human and chimpanzee Alu pairs. The authors further speculated that low levels of replacement could also be operating. Orthologous Alu comparisons between human and orangutan provide a time frame that is large enough to observe more accurate patterns of divergence yet small enough to test whether patterns of divergence are different from that expected for concerted evolution.

Human and orangutan pairs of Alu sequences presented in Fig. 3 show an average divergence of 3.7% (Table 2). The large range of divergences (2.4–6.9%) between pairs of Alu sequences is expected because each sequence comparison involves less than 300 bases. The average divergence between orangutan and human Alu sequences is approximately equal

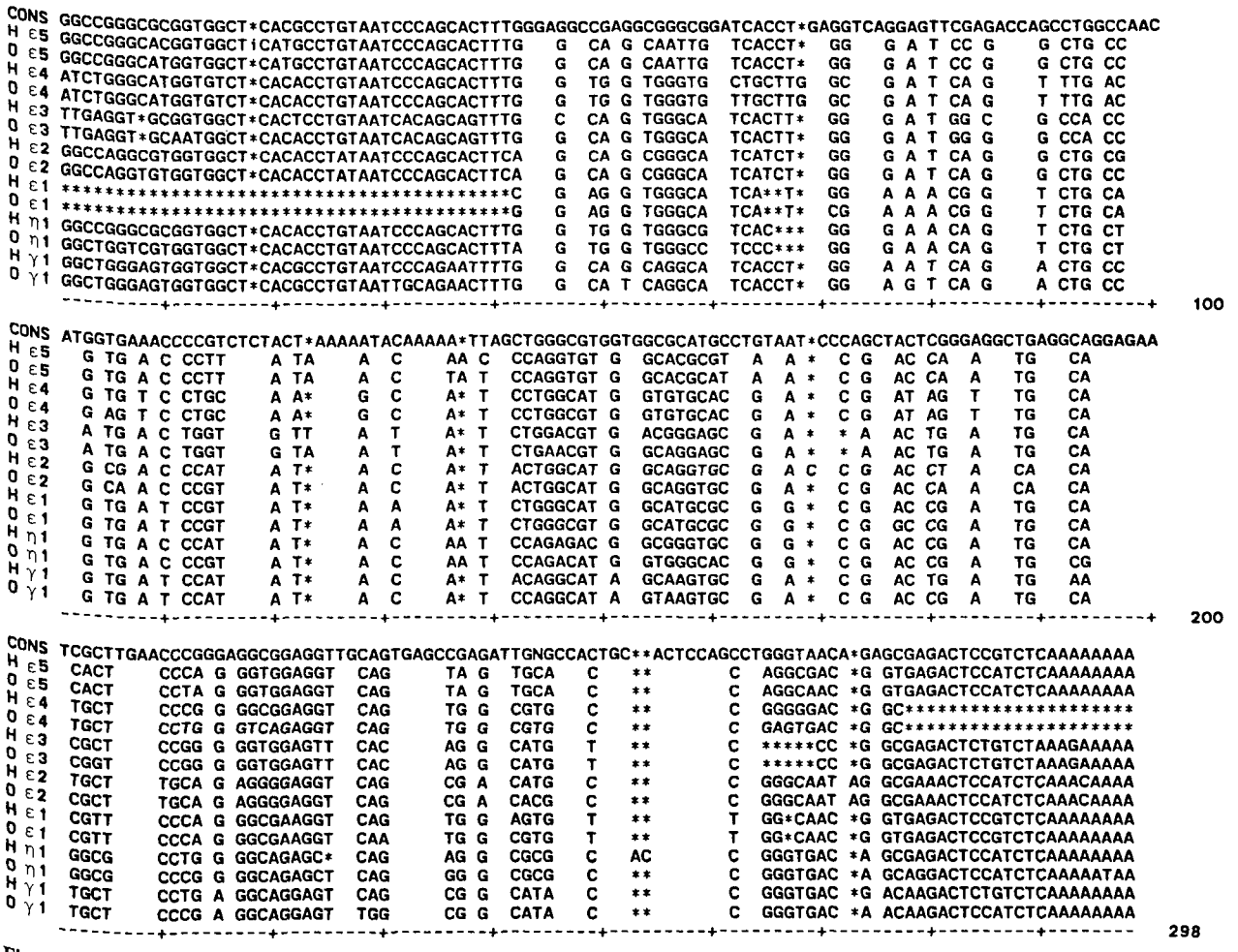

Fig. 3. Nucleotide sequences of human and orangutan Alu sequences (ϵ1, ϵ2, ϵ3, ϵ4, ϵ5, γ1, and η1, see Fig. 1) as aligned with the consensus Alu sequence (Schmid and Shen 1985). Only those positions which vary from the consensus are listed; all other positions are the same as the consensus. Gaps (*) were inserted to maximize sequence homology. "i" indicates an insertion.

to that of noncoding sequences (3.2–3.6%), indicating that divergence is accumulating at a rate expected from neutral drift. Though the Alu sequence pairs shown in Fig. 3 have accumulated mutations over a combined evolutionary period of about 20–30 million years, these sequences show no evidence of concerted evolution either by conversion or replacement. If either of these mechanisms were operating, we would not expect the percent difference between orthologously positioned Alu sequences of human and orangutan to be similar to that expected from random drift. If conversions were occurring we would expect the divergence between orthologously positioned pairs of Alu sequences to be close to that of paralogous sequences (21%, Table 2), or that the divergence between Alu sequences within species would be very low, much lower than the 13.9–25.9% found (Table 2). Neither of these possibilities appears to be supported. If concerted evolution had occurred through replacement of Alu sequences by a few nonconserved progenitors, then we would also expect to see a much greater similarity between different Alu sequences within species and at least one case where the divergence between orthologous Alu sequences approximates that of paralogous Alu sequences. In the unlikely event that these progenitor sequences were conserved throughout primate evolution, we would expect to find little or no divergence between paralogous Alu sequences or, alternatively, little or no divergence between the consensus and modern Alu sequences. In fact comparisons with the consensus range from 11.6–16.6% (Table 2). If coevolution by reciprocal recombination had occurred then we would expect little similarity in linkage maps. As indicated in Fig. 1, this is certainly not the case. Clearly the levels of divergence between the Alu sequences we have examined are not consistent with explanations involving concerted evolution.

The phylogenetic reconstruction presented in Fig. 4 combines the results of 7 human and chimpanzee Alu comparisons (Sawada et al. 1985) with the results of 7 human and orangutan Alu comparisons. The consensus Alu sequence (Sawada et al. 1985) was also added to the analysis. The tree was then rooted with the highly conserved functional human

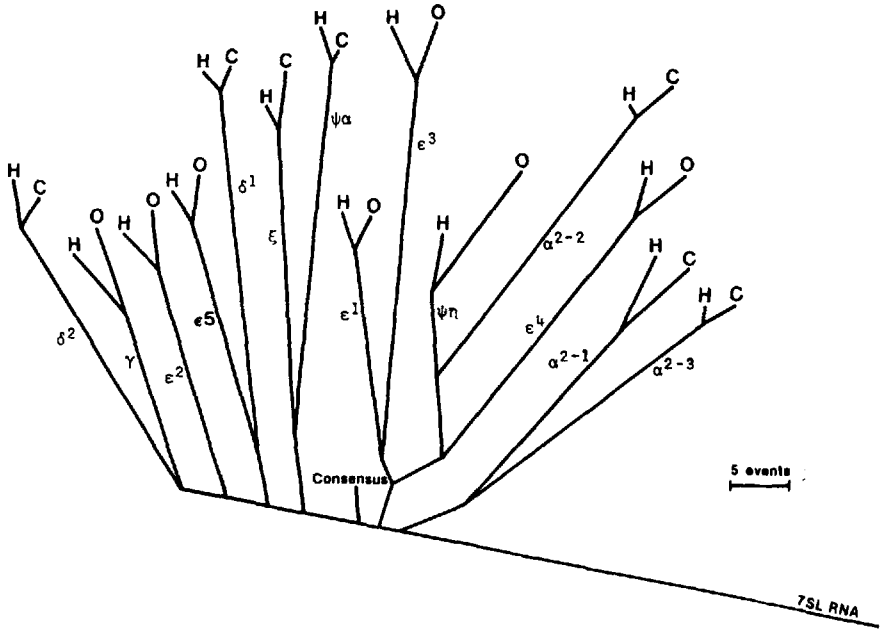

Fig. 4. Phylogenetic relationships of 28 different Alu sequences, the consensus Alu sequence and the 7SL RNA gene sequence. The 7SL RNA gene sequence was chosen to root the network because it is proposed to have given rise to the Alu sequences (Ullu et al. 1982). H indicates human, C indicates chimpanzee, and O indicates orangutan Alu sequences. A total of 567 changes over 185 variable positions were used to determine this branching arrangement.

7SL RNA gene sequence (Ullu et al. 1982). In each case, pairs of orthologously positioned Alu sequences remain very strongly associated. This suggests that each pair, either human and chimpanzee or human and orangutan, defines a distinct Alu lineage, clearly separated from other such lineages. This further emphasizes the lack of concerted evolution among the specific Alu sequences examined, either between the Alu sequences examined or between the Alu sequences examined and other Alu sequences.

In addition, it should be noted that the 14 Alu lineages (Fig. 4) within the α and β globin clusters are separated from one another by a large number of unique mutations or events. On average, two thirds (the range is from 50–83% of the changes) occurring since the most recent common ancestral sequence of all Alu sequences examined occur in the individual orthologous Alu lineages. The relatively large number of events occurring on these lineages (average = 29) as compared to that for human and orangutan Alu lineages (average = 5.6) suggests that the Alu lineages have been separated for about 5.2 times (29/5.6) longer than the human and orangutan Alu lineages. This places the separation of Alu lineages present within the α and β globin clusters at about 52–78 MYA (assuming a linear molecular clock).

The time of the apparent Alu radiation can more accurately be estimated by comparing the divergence of hominid and prosimian $\psi\eta$ sequences (23.9%; Koop et al. 1986) with the divergence of Alu sequences within each species (21%). That the two values are similar suggests that the average appearance of the 14 Alu lineages occurred about the same time as or a little after the divergence of simians and prosimians (50–60 MYA [Gingerich 1986]). Given this information, the 14% divergence between the Alu sequences and their consensus could, in part, represent the divergence between an ancestral Alu and its modern counterparts; this is about half the divergence between paralogous Alu sequences. The ancestral nature of the consensus sequence is further supported by its placement near the base of the phylogenetic reconstruction presented in Fig. 4.

The greater similarity of Alu-like families within human and galago lineages could be the result of amplification from uniquely different but similar progenitors after the separation of human and galago lineages. After amplification, Alu sequences evolved as noncoding DNA. This scenario is consistent with the model proposed by Deininger and Daniels (1986).

Clearly, since we have examined only 14 of the 500,000 (Rinehart et al. 1981) to 900,000 (Hwu et al. 1986) Alu sequences in humans, the question of whether Alu sequences have progressively amplified over the course of simian evolution remains to be answered. Amplification results in the presence of Alu sequences in regions where none were previously present. The finding of approximately twice as many Alu sequences in human as in either the gorilla, chimpanzee, or orangutan has led Hwu et al. (1986) to conclude that many interspersed copies of Alu sequences have been added in the last few million years. That orthologous Alu sequences are present in corresponding locations in the α and β globin clusters of human and orangutan and human and chimpanzee however argues against a recent amplification event giving rise to the Alu sequences examined in this study. Perhaps, as suggested by

Hwu et al. (1986) there may be some selection against changes in the sequence organization of the two globin clusters. However, among the 25 human Alu sequences reviewed by Schmid and Shen (1985) plus the 23 Alu sequences reviewed by Slagel et al. (1986) pairwise divergences are much greater than the 2% indicative of a recent (5–7 MYA) amplification event in the human lineage. In fact, divergence between Alu sequences varies from 10–34.8% with most values being between 20 and 30% (Slagel et al. 1986). It will be interesting to find out from additional studies why these values differ from the 15% average divergence among Alu sequences determined from Hwu et al. (1986). Though the presence of some subgroups of Alu sequences exhibiting an average divergence of 9% from the consensus indicates that amplification may have occured 25–30 MYA (Slagel et al. 1986), the majority of sequence divergences seem to indicate a much earlier appearance.

We have examined 14 different Alu pairs (7 human and orangutan, and 7 human and chimpanzee) and found no examples where intraspecific divergence is less than interspecific divergence between orthologously positioned Alu sequences. Instead, what we found was that divergence among Alu sequences within a species was much greater than interspecific divergence between orthologous Alu sequences. This clearly contrasts with examples of conversion found in the α^1 and α^2 globin genes and the γ^1 and γ^2 globin genes. We also found that the divergence between human and orangutan and human and chimpanzee Alu pairs is very close to that found for typical noncoding sequences and pseudogenes, thus suggesting that Alu sequences are evolving at the rate expected for typical noncoding DNA. Using divergence values between simians and prosimians as a reference, the primate Alu lineages examined have existed as independent entities for nearly 50–60 MY. While we can not dismiss the role concerted evolution might have had prior to the radiation of simian and prosimian primates, we think it is likely that the majority of Alu-like sequences within primates resulted from amplification of a few ancestral progenitors in early primates after which Alu sequences evolved as noncoding DNA.

Acknowledgments. We thank Drs. R.J. Britten and A.J. Mitchell for their helpful comments, and Dr. P.L. Deininger for providing us with prepublication manuscripts and suggestions. This study was supported by grants from NIH HL33940, NSF BSR82-07336, and Alfred P. Sloan Foundation.

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