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EVOLUTION OF THE SARAFOTOXIN/ENDOTHELIN SUPERFAMILY OF PROTEINS

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G. LANDAN, A. BDOLAH, Z. WOLLBERG, E. KOCHVA and D. GRAUR. Evolution of the sarafotoxin/endothelin superfamily of proteins. *Toxicon* **29,** 237–244, 1991.—Sixteen protein and nucleic acid sequences from the vasoconstrictor sarafotoxin/endothelin/endothelin-like superfamily of peptides were studied, and the evolutionary relationships between the sarafotoxin and endothelin gene families as well as the phylogenetic topology within each gene family and the three endothelin subfamilies was reconstructed. The endothelin gene family has diverged from an ancestral gene that has experienced an exon duplication event followed by two gene duplication events. The sarafotoxins' lineage diverged from the ancestral gene prior to the first endothelin gene duplication event. Analysis of the resulting phylogenetic trees revealed that in several lineages, the peptides have independently accumulated identical replacements in position 2, therefore supporting the hypothesis that residue 2 is crucial to their activity.

INTRODUCTION

THE SARAFOTOXINS (SRTXs), the venom toxins of *Atractaspis engaddensis*, are 21 amino acid long peptides which are highly toxic: they have powerful effects on the heart and strong vasoconstriction activity (WOLLBERG *et al.*, 1988, 1989; BDOLAH *et al.*, 1989*a*, *b*). The sarafotoxins and their mammalian homologues, the endothelins (see below), bind specifically to the brain, heart, blood vessels and other smooth muscle systems. These peptides also activate the phosphoinositide transduction system (KLOOG *et al.*, 1988; KLOOG and SOKOLOVSKY, 1989).

The endothelins (ETs) are mammalian vasoactive peptides originally isolated from porcine endothelial cells (YANAGISAWA *et al.*, 1988*a*). The mature ET is a 21 amino acid long peptide, with vasoconstriction activity in cardiovascular systems and various other pharmacological activities (see YANAGISAWA and MASAKI, 1989*a*, *b* for review). ET was found to exist in mammals as a gene family consisting of three duplicate genes, named ET-1, ET-2 and ET-3. The presence of these genes has been demonstrated in humans,

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pigs, rats and mice (INOUE *et al.*, 1989; SAIDA *et al.*, 1989). Mature ET is produced from a prepropeptide about 200 amino acids long in two stages: first by proteolytic cleavage to produce a so-called big-ET which is then processed by a putative 'endothelin converting enzyme' to produce the mature ET peptide (YANAGISAWA *et al.*, 1988*a*). In ET-1 and ET-3, where the preproendothelin sequence is known, a 15 codon long sequence homologous to the mature ET is found about 55 codons downstream to the mature ET. This segment, named the endothelin-like (ET-like) sequence, is probably the product of an exon duplication in an ET ancestral gene (BLOCH *et al.*, 1989*b*). Relative to its surrounding sequence, the ET-like segment is highly conserved, therefore it has been suggested by BLOCH *et al.* (1989*a*) that the ET-like sequence possesses some important physiological function.

The SRTXs and the mature ET peptides exhibit high sequence similarities (52–67%). In particular, four Cys residues, Cys-1, Cys-3, Cys-11 and Cys-15, that form two disulfide bonds, are preserved in all the SRTXs and ETs, as well as in the ET-like sequences. A preliminary evolutionary study (GRAUR *et al.*, 1988/1989) of SRTX-a, SRTX-b, SRTX-c, ET-1 and ET-3, demonstrated that these sequences are homologous, that is, that they have been derived from a common ancestral gene. In the present study we reconstruct the evolutionary relationships between the sarafotoxin and endothelin gene families, as well as the phylogenetic topology within each gene family and the three endothelin subfamilies.

MATERIALS AND METHODS

The analyses are based on the amino acid sequences of four sarafotoxin isopeptides, SRTX-a, SRTX-b, SRTX-c (TAKASAKI *et al.*, 1988) and SRTX-d (BDOLAH *et al.*, 1989*b*) and on the nucleic acid sequences of nine endothelin genes: human ET-1 (ITOH *et al.*, 1988; BLOCH *et al.*, 1989*b*; INOUE *et al.*, 1989), human ET-2 (INOUE *et al.*, 1989), human ET-3 (BLOCH *et al.*, 1989*a*; INOUE *et al.*, 1989), mouse ET-1 (SAIDA *et al.*, 1989), mouse ET-2 (referred to as VIC, SAIDA *et al.*, 1989), canine ET-1 (KIMURA *et al.*, 1989), porcine ET-1 (YANAGISAWA *et al.*, 1988*a*), rat ET-3 (YANAGISAWA *et al.*, 1988*b*) and rabbit ET-1 (OHKUBO *et al.*, 1990, see Fig. 1). The predicted amino acid sequences of the ET-1 peptide of human, mouse, dog and porcine are identical, as are those of the ET-3 peptides from human, rat and rabbit. The ET-2 peptides from mouse (VIC) and human differ from one another by a single amino acid residue. The complete prepropeptide sequence is available only for the human ET-1 and ET-3 and the porcine ET-1 genes. In all these genes a 45 nucleotide long ET-like sequence is present downstream of the mature ET sequence (Fig. 1).

RESULTS

The amino acid sequences of the sarafotoxins and endothelins exhibit a high degree of sequence similarity (71–95% within the endothelins, 81–95% within the sarafotoxins and 52–67% between the SRTXs and ETs, see Table 1).

Using SAITOU and NEI's (1987) neighbor-joining method with the estimated number of replacements $d = -\ln(p)$ where p is the percentage similarity (Table 1), we obtained an unrooted tree for the four SRTX and the four distinct ET peptides (Fig. 2). The SRTX and ET families are clearly distinguished from each other. The root was placed at the midpoint of the longest path, i.e. from SRTX-c to ET-3 (arrow in Fig. 2) and is located between the SRTX and ET groups so that the SRTXs can be taken as an outgroup to the ETs and vice versa.

Protein maximum parsimony analysis (FELSENSTEIN, 1982) of these eight peptides resulted in two equally parsimonious trees, each requiring a total of 24 amino acid replacements (Fig. 3). The location of the root was determined by taking the SRTXs and ETs as outgroups to each other. One of these trees (Fig. 3a) is identical to the neighbor-

	1	5	10	15	20
SRTX-a	Cys Ser Cys Lys Asp Met Thr Asp Lys Glu Cys Leu Asn Phe Cys His Gln Asp Val Ile Trp				
SRTX-b	Cys Ser Cys Lys Asp Met Thr Asp Lys Glu Cys Leu Tyr Phe Cys His Gln Asp Val Ile Trp				
SRTX-c	Cys Thr Cys Asn Asp Met Thr Asp Glu Glu Cys Leu Asn Phe Cys His Gln Asp Val Ile Trp				
SRTX-d	Cys Thr Cys Lys Asp Met Thr Asp Lys Glu Cys Leu Tyr Phe Cys His Gln Asp Ile Ile Trp				
Hum ET-1	TGC TCC TGC TCG TCC CTG ATG GAT AAA GAG TGT GTC TAC TTC TGC CAC CTG GAC ATC ATT TGG				
Dog ET-1	TGC TCC TGC TCT TCC CTG ATG GAT AAA GAG TGT GTC TAC TTC TGC CAC CTT GAC ATC ATC TGG				
Pig ET-1	TGC TCC TGC TCT TCC CTG ATG GAT AAA GAG TGT GTC TAC TTC TGC CAC CTG GAC ATC ATC TGG				
Mus ET-1	TGT TCC TGT TCT TCC TTG ATG GAC AAG GAG TGT GTC TAC TTC TGC CAC CTG GAC ATC ATC TGG				
	Cys Ser Cys Ser Ser Leu Met Asp Lys Glu Cys Val Tyr Phe Cys His Leu Asp Ile Ile Trp				
Hum ET-2	TGC TCC TGC AGC TCC TGG CTC GAC AAG GAG TGC GTC TAC TTC TGC CAC TTG GAC ATC ATC TGG				
	Cys Ser Cys Ser Ser Trp Leu Asp Lys Glu Cys Val Tyr Phe Cys His Leu Asp Ile Ile Trp				
Mus ET-2 (VIC)	TGC TCC TGC AAC TCC TGG CTT GAC AAG GAA TGT GTG TAC TTC TGC CAC CTG GAC ATC ATC TGG				
	Cys Ser Cys Asn Ser Trp Leu Asp Lys Glu Cys Val Tyr Phe Cys His Leu Asp Ile Ile Trp				
Hum ET-3	TGC ACG TGC TTC ACC TAC AAG GAC AAG GAG TGT GTC TAC TAT TGC CAC CTG GAC ATC ATT TGG				
Rat ET-3	TGC ACG TGC TTC ACT TAT AAG GAC AAG GAG TGT GTC TAC TAC TGC CAC CTG GAC ATC ATC TGG				
Rab ET-3	TGC ACC TGC TTC ACC TAC AAA GAC AAA GAG TGC GTC TAC TAC TGC CAC CTG GAC ATC ATC TGG				
	Cys Thr Cys Phe Thr Tyr Lys Asp Lys Glu Cys Val Tyr Tyr Cys His Leu Asp Ile Ile Trp				
Hum ET-like-1	TGC CAA TGT GCT AGC CAA AAA GAC AAG AAG TGC TGG AAT TTT TGC				
	Cys Gln Cys Ala Ser Gln Lys Asp Lys Lys Cys Trp Asn Phe Cys				
Pig ET-like-1	TGC CAG TGT GCC AGC CAA AAA GAC AAG AAG TGC TGG AGT TTC TGC				
	Cys Gln Cys Ala Ser Gln Lys Asp Lys Lys Cys Trp Ser Phe Cys				
Hum ET-like-3	TGC GCT TGT GTG GGG AGA TAT GAC AAG GCC TGC CTG CAC TTT TGC				
	Cys Ala Cys Val Gly Arg Tyr Asp Lys Ala Cys Leu His Phe Cys				

FIG. 1. AMINO ACID SEQUENCES OF FOUR SARAFOTOXIN ISOPEPTIDES AND NUCLEIC ACID SEQUENCES OF NINE MATURE ENDOTHELINS AND THREE ENDOTHELIN-LIKE SEGMENTS, ALONG WITH THEIR DEDUCED AMINO ACID SEQUENCES

Data from TAKASAKI et al., 1988; BDOLAH *et al.*, 1989; ITOH *et al.*, 1988; BLOCH *et al.*, 1989*a*, *b*; INOUE *et al.*, 1989; SAIDA *et al.*, 1989; KIMURA *et al.*, 1989; YANAGISAWA *et al.*, 1988*a*, *b*; OHKUBO *et al.*, 1990.

TABLE 1. AMINO ACID SEQUENCE SIMILARITY BETWEEN THE EIGHT DISTINCT SRTX AND ET PEPTIDES

Peptide		A	B	C	D	1	2	VIC
SRTX-a	(A)							
SRTX-b	(B)	0.952						
SRTX-c	(C)	0.857	0.810					
SRTX-d	(D)	0.857	0.905	0.810				
ET-1	(1)	0.619	0.667	0.524	0.667			
Hum ET-2	(2)	0.619	0.667	0.524	0.667	0.905		
Mus ET-2	(VIC)	0.619	0.667	0.571	0.667	0.857	0.952	
ET-3	(3)	0.524	0.571	0.524	0.667	0.714	0.714	0.714

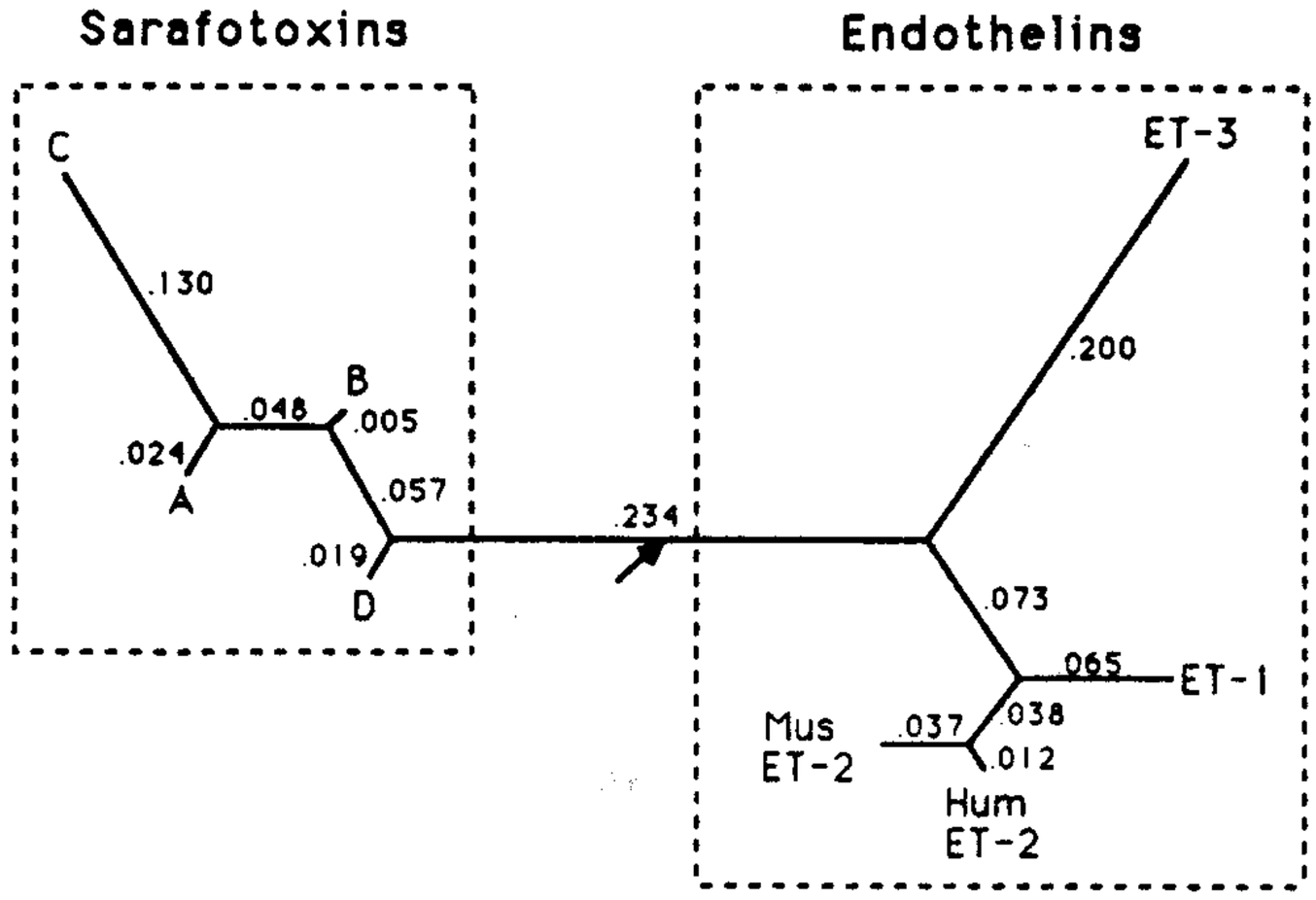
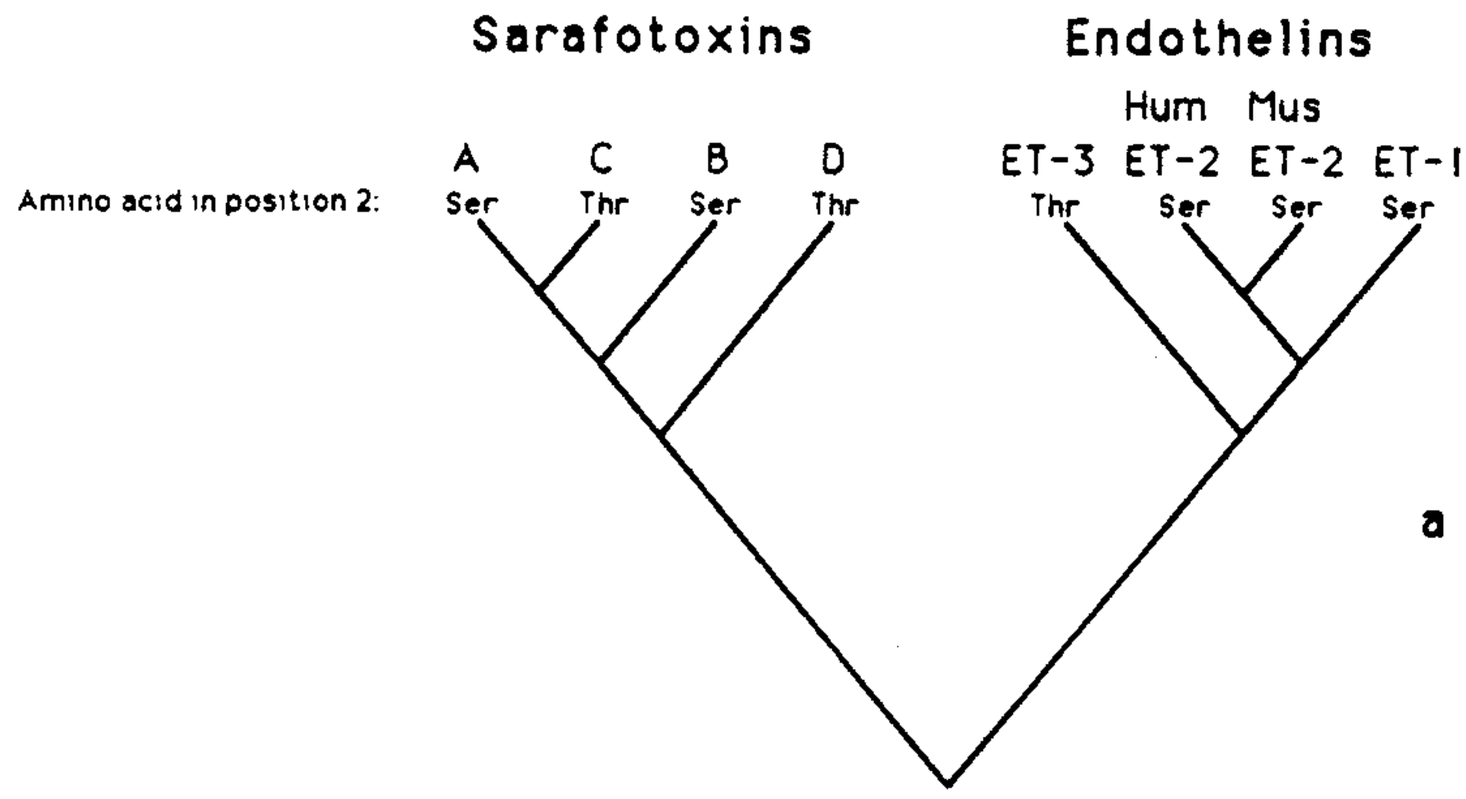


FIG 2. UNROOTED PHYLOGENETIC TREE OF THE EIGHT DISTINCT SRTX AND ET PEPTIDES, OBTAINED BY THE NEIGHBOR-JOINING METHOD (SAITOU AND NEI, 1987).
The branch length is proportional to the number of replacements. Arrow points to the deduced root position.



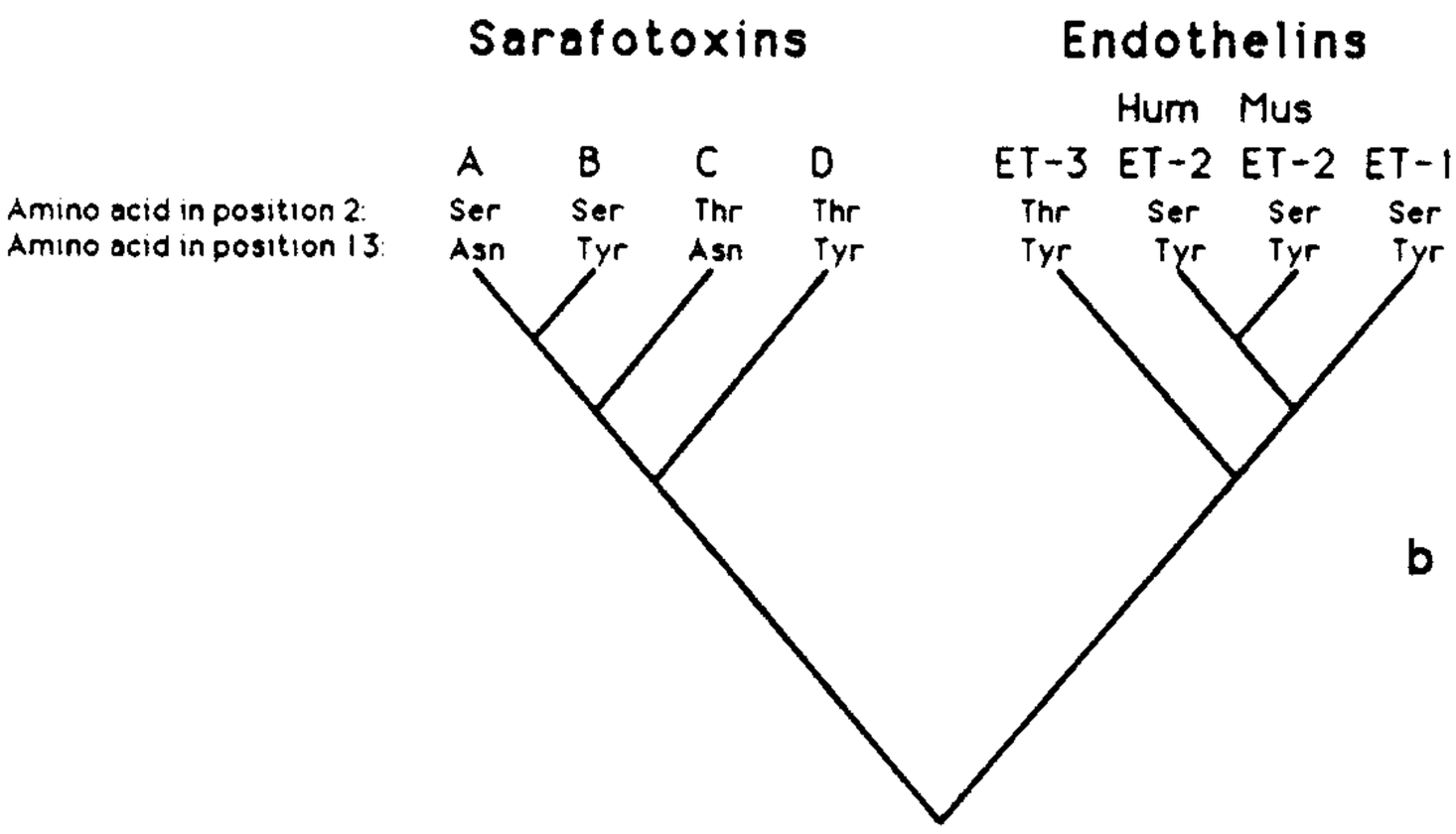


FIG. 3. TWO MOST PARSIMONIOUS TREES OF THE EIGHT SRTX AND ET PEPTIDES, OBTAINED BY THE PROTEIN MAXIMUM PARSIMONY METHOD (FELSENSTEIN, 1982).
Both trees require 24 amino-acid replacements. The amino acids in positions 2 and 13 are shown. The root was placed by taking the SRTXs and the ETs as outgroups to each other.

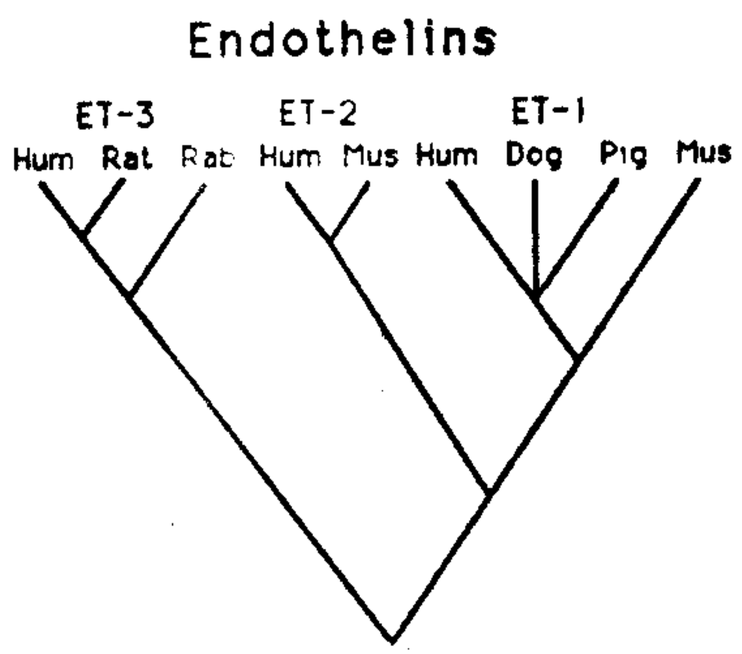


FIG. 4. CONSENSUS TREE FOR THE NINE MATURE ETS, OBTAINED BY MAXIMUM PARSIMONY ALGORITHM (FITCH 1977).
The tree requires 35 nucleotide substitutions. The root was placed by taking either the SRTX or the ET-like segment as outgroups.

joining tree while the other differs in the branching order of SRTX-b and SRTX-c. The topology of the ET sub-tree is the same in both trees as well as in the neighbor-joining tree.

In order to reconstruct the phylogenetic relationships within the ETs and to maximize available data, we applied the DNA maximum parsimony method (FITCH, 1977) on the nucleic acid sequences of nine ET genes. Three unrooted trees were obtained, each requiring a total of 35 nucleotide substitutions, and differing only in the branching order of the porcine and canine ET-1 genes. A consensus tree was constructed (Fig. 4), in which the three ET gene subfamilies are easily distinguished from one another.

To ascertain that the root of the ET tree is indeed located between the ET-3 subfamily and the ET-1 and ET-2 subfamilies, we used the 45 nucleotide long sequence of the paralogous ET-like segment as an outgroup. Maximum parsimony analysis of the nine mature ET and 3 ET-like sequences yielded six unrooted trees differing in the branching order within the ET subfamilies, but identical with respect to the branching order among the subfamilies, that is, in all trees the ET-3 subfamily diverged from the other ET subfamilies prior to the split between ET-2 and ET-1.

DISCUSSION

The various maximum parsimony sequence analyses of the SRTXs and ETs clarify the topology of the ET gene family (Fig. 4) and its relationship to the SRTX gene family, yet the results are ambiguous with respect to the branching order within the SRTXs. Therefore two phylogenetic trees of the SRTXs and ETs are possible (Figs 3a and 3b).

Close inspection of the mutational pathways of the reconstructed trees for the SRTXs and ETs reveals that the peptides experienced parallel amino acid replacements, i.e. the same type of replacement occurred independently in different lineages. The replacements that are needed to explain the phylogenetic trees are identical for trees 3a and 3b, with the exception of the replacements in positions 2 and 13 of the peptides. In tree 3a, position 2 of the peptide, being Ser-2 in SRTX-a, SRTX-b ET-1 and ET-2 and Thr-2 in SRTX-c, SRTX-d and ET-3, underwent three parallel replacements. It is not possible to determine unambiguously which was the ancestral amino acid in position 2, Ser or Thr, but either

way the same replacements must have occurred independently in three different lineages. In tree 3b, on the other hand, only two parallel replacements are required in position 2, with Thr-2 as the ancestral residue, but another pair of parallel replacements (or alternatively a back replacement) is required in position 13, which is Asn-13 in SRTX-a and SRTX-c and Tyr-13 in all the other peptides. Given that only 24 amino acid replacements occurred over the whole SRTX-ET tree, and that parallel and back replacements are very rare, it is likely that the above instances of parallel replacements were selectively advantageous rather than due to chance alone.

Several hypotheses were raised to explain the differences in activity of the SRTX and ET peptides. KLOOG and SOKOLOVSKY (1989) suggested a possible effect of the net charge of the peptides on their activity. On the basis of the comparison of ET-1, ET-2 and ET-3, INOUE *et al.* (1989) argued that the activity is correlated to the hydrophobicity of the entire peptide. GRAUR *et al.* (1988/89) suggested that a comparison between the activity and sequences of SRTX-a, SRTX-b, SRTX-c ET-1 and ET-3 point to the possibility that position 2 is crucial to the activity of these peptides. We find the latter hypothesis to be more plausible for several reasons. (a) Classification of the peptides characterized to date according to activity and according to the amino acid in position 2 yields the same grouping. Namely, higher activity and Ser-2 residue in SRTX-a, SRTX-b, ET-1 and ET-2, and lower activity and Thr-2 residue in SRTX-c, SRTX-d and ET-3. No other residues correlate as perfectly with activity. (b) Given the phylogenetic relationships among the peptides (Fig. 3), position 2 has undergone two, and perhaps three, parallel replacements. As mentioned above, this can hardly be due to chance alone, and is likely to represent some selective pressure on this particular residue. (c) The functional importance of residue 2 can also be argued for on structural grounds: the Cys-1 and Cys-3 residues create two disulfide bonds with Cys-15 and Cys-11, respectively, so it seems that the amino acid residue residing between them should have a pronounced influence on the resulting structure of the peptide, although the secondary structure of ET-3 has been found to be similar to that of ET-1 (NAKAJIMA *et al.*, 1989). (d) The four SRTX peptides have the same hydrophobicity, and yet they exhibit different levels of activity. Moreover, the SRTXs are less hydrophobic than ET-3, yet SRTX-a and especially SRTX-b are generally as active as ET-1.

Although there is as yet no conclusive way to decide which of the two possible trees 3a or 3b should be preferred, several considerations point to tree 3a as the more plausible one. (a) Neighbor-joining reconstruction yields topology 3a. However, this result should not be taken as decisive since the peptides are relatively short for this method to yield conclusive results. (b) Since the parallel replacements are probably reflecting selective advantageous replacements, it is more parsimonious to assume that they occur at only one amino acid position, as in tree 3a, than to assume parallel replacements at two amino acid positions, as required by tree 3b. (c) The amino acid replacements in tree 3a are more conservative than those in tree 3b (GRANTHAM 1974; GRAUR, 1985).

The prepropeptide structure of the ET-1 and ET-3 genes, along with the observation that the gene duplication leading to the ET-2 family took place after the ET-3 gene duplication, leads us to propose the following scenario for the evolution of the endothelins: the ET-like sequence is a product of an early exon duplication event in an ancestral ET gene (BLOCH *et al.*, 1989*b*). This ancestral gene subsequently underwent a complete gene duplication resulting in the ET-3 gene and an ET gene, which underwent yet another gene duplication leading to the creation of the ET-1 and ET-2 genes (Fig. 5). All these stages took place before the main mammalian radiations, so that all mammals possess

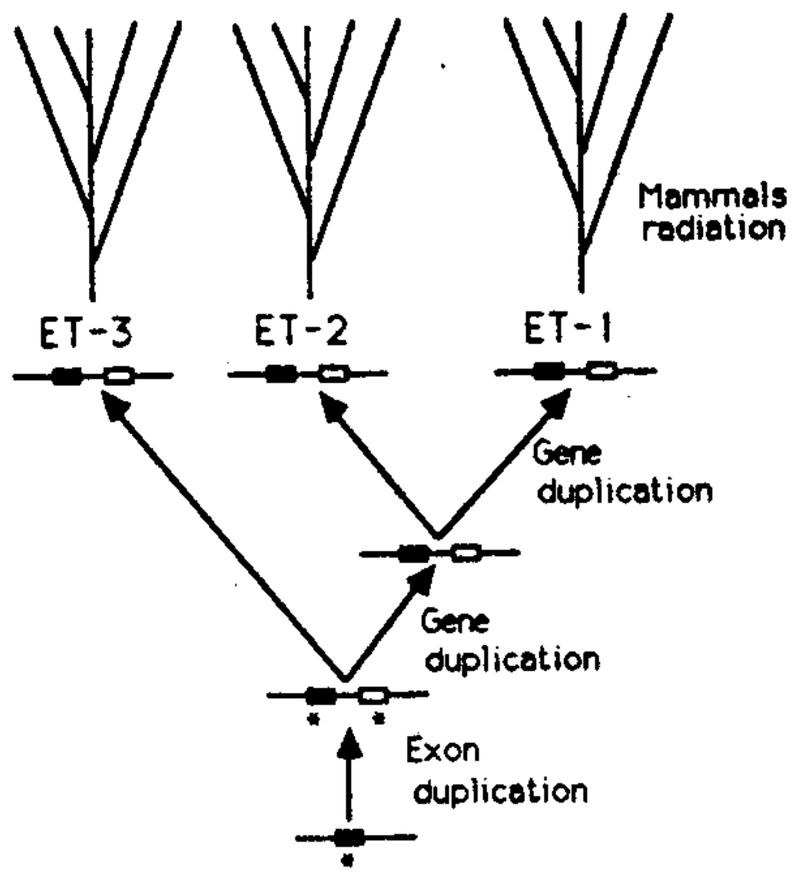


FIG. 5. PUTATIVE EVOLUTIONARY HISTORY OF THE ENDOTHELIN GENES.
Black boxes indicate the mature ET segment; open boxes represent the ET-like segment. Asterisks mark the possible sites of the SRTX lineage branching.

three ET genes. The four SRTX peptides, on the other hand, are clearly the result of an independent divergence process.

Further analysis was carried out to reconstruct the phylogenetic history of the three major classes under study, that is: the SRTXs, mature ETs and the ET-likes, but the results were ambiguous allowing for several scenarios (see asterisks in Fig. 5). (a) The SRTX branching took place before the ET/ET-like exon duplication. (b) The SRTX evolved from the mature ET segment after the ET/ET-like exon duplication but before the ET-3 branching. (c) The SRTX evolved from the ET-like segment after the ET/ET-like exon duplication but before the ET-3 branching. Further work on the nucleotide sequence, gene structure and genomic distribution of the SRTX genes may clarify the true scenario of the overall evolution of the three major families.

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