Rapid evolution of a unique family of primate ribonuclease genes

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We have traced the rapid molecular evolution of eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP), two host defense proteins that are members of the mammalian ribonuclease gene family. The EDN/ECP gene pair arose from a recent duplication event that occurred after the divergence of New World and Old World monkeys. Since duplication, the genes encoding EDN and ECP have accumulated non-silent mutations at rates exceeding those of all other functional coding sequences studied in primates, while retaining both the structural and catalytic components required for ribonuclease activity. These results suggest that both EDN and ECP may be responding to unusual evolutionary constraints, which has prompted a reexamination of their physiologic function.

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The study of molecular evolution is based on the premise that currently functional genes are related to and derived from gene sequences found in evolutionary predecessors. In accordance with this theory, quantitative comparisons of homologues from divergent species may provide insight into the evolutionary selection process. Mammalian ribonucleases provide an interesting subject for this type of study, as they are a unique group of proteins that share a distinct secondary structure and enzymatic activity, yet have diverged from one another to promote a variety of otherwise unrelated cellular functions. Ribonuclease A is the prototype of this gene family, which includes pancreatic and brain ribonucleases, as well as eosinophil-derived neurotoxin (EDN), eosinophil cationic protein (ECP) and angiogenin.

Human EDN (hEDN) and ECP (hECP) are two host defense proteins found in the large specific granules of eosinophilic leukocytes[1,2]. A variety of functions have been attributed to human ECP, including helminthotoxicity and antibacterial activity. Human EDN has little or no activity against pathogens, and in fact has no known physiologic function. Structural similarities between hECP and hEDN, and between these proteins and pancreatic ribonuclease were initially discovered from N-terminal peptide sequences[3], and purified hEDN and hECP were later shown to have ribonuclease activity[4,5]. The cDNAs encoding hEDN and hECP (coding regions, 85% identical; 5' and 3' untranslated regions, 96% identical) provide the complete primary amino acid sequences, which include the characteristic eight cysteines as well as histidine and lysine residues corresponding to those found in the active-site crevice of ribonuclease A[6–9]. Genomic analysis has indicated nearly complete identity of the putative promoter regions (95%), and the presence of a single intron situated immediately 5' to the translational start site[10]. Intronless coding sequences have also been found in other mammalian ribonuclease genes[11,12].

In this study, we have characterized several primate homologues of EDN and ECP which show a remarkaby high degree of sequence divergence from their human counterparts.

Homologues of human EDN and ECP

Fig. 1 shows Southern blots containing *Pst*I-digested genomic DNA from several mammalian species hybridized at low stringency with a radiolabelled hEDN and human pancreatic ribonuclease (hPR)[13] probes. As anticipated, four distinct bands corresponding to *Pst*I-digests of both hEDN and hECP were detected in the lanes containing human (*H. sapiens*) DNA when hybridized with hEDN (Fig. 1*b*, lanes 1 and 2). Homologous sequences were detected in all primate species examined, including chimpanzee (*P. troglodytes*, lanes 3–6), gorilla (*G. gorilla*, lane 7), orangutan (*P. pygmaeus*, lane 8), macaque (*M. fascicularis*, lane 9) and marmoset (*L. saguinus*, lane 10); duplicate lanes represent independent individual animals. In contrast, non-primate homologues, if they exist, have nucleotide sequence homologies insufficient for detection by this method. By contrast, sequences homologous to hPR were detected in both the primate and non-primate genomes (Fig. 1*c*), indicating that the primate-specific pattern obtained with the hEDN probe is not characteristic of all mammalian ribonuclease genes. The rat (*R. norvegicus*) pancreatic ribonuclease gene (lane 12) is easily detected

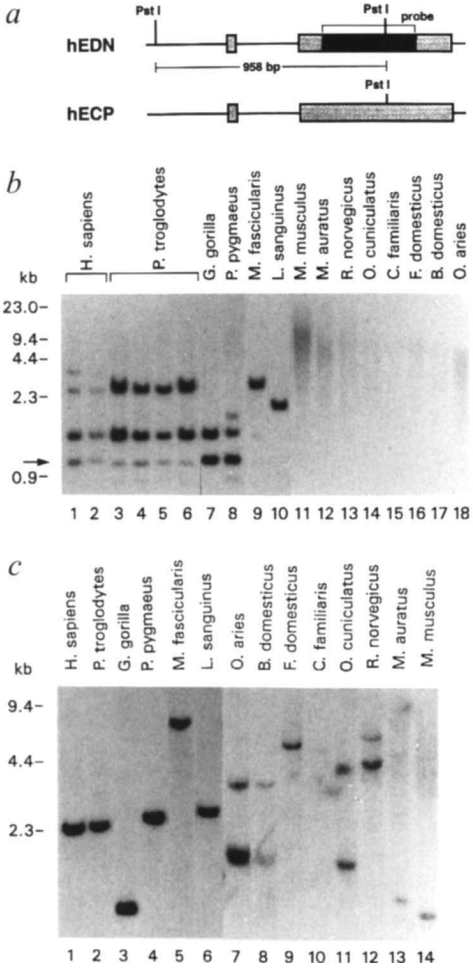

Fig. 1 *a*, Restriction maps of hEDN and hECP[10]. The intronless coding sequence (black box) was used as the probe in the blot shown in (*b*). Shaded boxes indicate transcribed sequences (exons 1 and 2) of both genes, which are 89% identical[6,7]. *b*, Mammalian genomic DNAs (10 μg/lane) digested with *Pst*I, electrophoresed in 1% agarose/TBE, transferred and hybridized at 37 °C to ^{32}P-radiolabelled hEDN probe shown in the black box in (*a*). The genus/species identification is noted above each lane; lanes 1–10 are from the Order Primata; lanes 11–13 from the Order Rodentia; lane 14 from the Order Legomorpha; lanes 15–16 from the Order Carnivora: lanes 17–18 from the Order Artiodactyla. Multiple lanes with DNA from a single species are samples from independent individuals. The arrow denotes the 958 bp *Pst*I fragment of the hEDN gene as noted in panel (*a*). *c*, Genomic blots probed with ^{32}P-radiolabelled (intronless) coding sequence of hPR[13]. The genus/species identification is noted above each lane; Orders as described in (*b*).

with the human pancreatic ribonuclease probe despite 25% nucleotide sequence divergence[14].

Characterization of homologues

Complete coding sequences of EDN and ECP were amplified using gene-specific oligonucleotide primers and purified genomic DNA templates (Table 1). Sequences corresponding to both EDN and ECP were isolated from four non-human primate species, while only one sequence could be isolated from the marmoset (*L. saguinus*); no sequences were isolated from non-primate mammalian species. Each gene isolated was shown to be more closely related to either EDN or ECP, with the exception of the single marmoset sequence, which was equally homologous to both human genes. All genes contained complete open reading frames with no introns or aberrant stop codons. Nucleotide sequence divergence increased with evolutionary distance in accordance with the accepted phylogenetic order of these primate species.

Amino acid sequence comparisons (Fig. 2) indicate points of dissimilarity between hEDN and hECP and their respective primate homologues. All primate genes encode polypeptides containing the structural prerequisites for ribonuclease activity[15]. Dissimilarities occur throughout, with no apparent 'mutation-prone' regions. All primate EDN genes encoded polypeptides with isoelectric points (pIs) of ~8–9, while the ECP genes encoded polypeptides with pIs of ~11 (Table 1). The single gene isolated from the marmoset genome (mmEDN) encodes a polypeptide with a pI of 8.25.

Nonsynonymous and synonymous substitutions

A quantitative analysis of mutation rates is presented in Table 2. Nonsynonymous substitutions per nonsynonymous site and synonymous substitutions per synonymous site (Nd/N=Ka and Sd/S=Ks, respectively; units=substitutions/site) were calculated as described[16,17] for 65 human/non-human primate coding sequence pairs from 26 independent sets of gene homologues, including EDN and ECP. These ratios were plotted against total years of divergent evolution, 2T[18], derived from consensus dates for species divergence[19]; the slope of each line, Ka versus 2T or Ks versus 2T (units = substitutions/site/year (s/s/yr)) was determined by least-squares method; correlation coefficients (r^2) were included. The highest rates of nonsynonymous substitution were those calculated for EDN and ECP at 1.9×10^{-9} and 2.0×10^{-9} s/s/yr, respectively. These rates are more than two-fold greater than the average rate of nonsynonymous substitution for this series, calculated to be 0.82×10^{-9} s/s/yr. Other coding sequences with high rates of nonsynonymous substitution include interleukin-3 (1.7×10^{-9} s/s/yr), the cell surface antigen CD59 (1.7×10^{-9} s/s/yr), the sex-determination locus SRY (1.4×10^{-9} s/s/yr), and the sperm proteins, protamine P1 and P2 (both at 1.7×10^{-9} s/s/yr).

Also shown in Table 2 (in parentheses) are values for Ka/Ks for the same human/non-human primate coding sequence pairs. Interestingly, most of the coding sequences identified as having rapid rates of nonsynonymous substitution also include pairs with Ka/Ks ratios greater than 1.0, suggesting the existence of selection pressure favoring alterations in the amino acid sequence[18]; these include pairs within *SRY*, protamines 1 and 2 and ECP. In contrast, despite its sequence similarity to ECP and its rapid rate of nonsynonymous substitution, the Ka/Ks ratios determined for human/non-human primate pairs of EDN are all significantly less than 1.0.

Values obtained for Ks versus 2T, are also shown in Table 2. The average rate of synonymous substitution for these primate sequences is 1.9×10^{-9} s/s/yr, which is 2.3 times greater than the average rate of nonsynonymous substitution (0.82×10^{-9} s/s/yr).

Table 1 Analysis of nucleotide and predicted amino acid sequences of non-human primate EDN and ECP genes

Name	Genus/species	Abbrev.	Nucleotide sequences			Predicted polypeptide sequences		
			Length (nt)	% identity to human homologue	% identity to ECP of same sp.	Isolectric point (pI)	% divergence from human EDN	% divergence from human ECP
Eosinophil derived neurotoxin								
Human	*H. sapiens*	hEDN	483	–	85.3	8.86	–	33.9
Chimpanzee	*P. troglodytes*	chEDN	483	99.2	85.6	8.86	0.8	33.9
Gorilla	*G. gorilla*	gEDN	483	99.0	85.4	9.17	0.8	33.1
Orangutan	*P. pygmaeus*	oEDN	483	95.5	86.7	8.87	6.8	32.4
Macaque	*M. fascicularis*	mcEDN	480	89.0	81.8	8.43	16.5	37.9
Marmoset	*L. saguinus*	mmEDN	474	82.3	–	8.25	26.8	31.3
Eosinophil cationic protein								
Human		hECP	480	–		11.37	33.9	–
Chimpanzee		chECP	480	99.2		10.94	33.9	2.3
Gorilla		gECP	480	99.1		11.17	34.6	2.3
Orangutan		oECP	480	94.6		10.70	29.4	15.1
Macaque		mcECP	480	94.6		11.31	36.1	13.6
Marmoset		mmEDN	474	82.3		8.25	31.3	31.3

The EDN and ECP genes were isolated by PCR using gene specific primer pairs and genomic DNA templates. Lengths of nucleotide sequences were determined as number of nucleotides from the beginning of the coding sequence (ATG) to (but not including) the stop codon (TAA). Nucleotide sequences of human EDN and ECP are as published[6,7]. Polypeptide analysis included predicted sequences of mature peptides only (without signal sequences). Percentage identity, percentage divergence, and isoelectric points were determined using the BESTFIT and ISOELECTRIC algorithms of the Wisconsin Genetics Computer Group Program.

Discussion

We have traced the sequence lineages of EDN and ECP, two distinct members of the mammalian ribonuclease gene family back to a duplication event occurring after the divergence of the Old World from the New World monkeys. Since duplication, these two genes have accumulated non-silent mutations a rapid rate while retaining structural and catalytic residues necessary for ribonuclease activity. The rates of amino acid substitution determined for both EDN and ECP (1.9 and 2.0×10^{-9} s/s/yr, respectively) are the most rapid among the primate coding sequences available for study, exceeding those calculated for proteins such as SRY[20] and protamines P1 and P2 (refs 21,22), which are well-known for their evolutionary sequence diversity. It is interesting to compare these rates to those calculated by Li and colleagues[23] for 40 human/rodent coding pairs. The two genes with the most rapid rates of nonsynonymous substitution in the human/rodent series were interferon-gamma (2.8×10^{-9} s/s/yr) and relaxin (2.4×10^{-9} s/s/yr). In contrast, the rates calculated for these genes in the human/non-human primate series presented here are not particularly dramatic (0.48 and 0.63×10^{-9} s/s/yr, respectively), suggesting that individual coding sequences evolve at varying rates during their respective evolutionary histories.

The ratio of nonsynonymous substitution per site versus synonymous substitution per site (Ka/Ks) has been used as a measure of evolutionary selection pressure[18]; a Ka/Ks ratio greater than 1.0 (that is, the rate of non-silent mutation exceeding that of the silent) implies that the sequences involved are responding to a directed evolutionary selection rather than to random drift. Many of the sequences with elevated non-synonymous substitution rates also tend toward high Ka/Ks ratios (Table 2); this group includes SRY, protamines P1 and P2 and ECP. In contrast, the Ka/Ks ratios calculated for the EDN pairs are quite low. These results suggest that while ECP may be responding to selection pressure, EDN appears

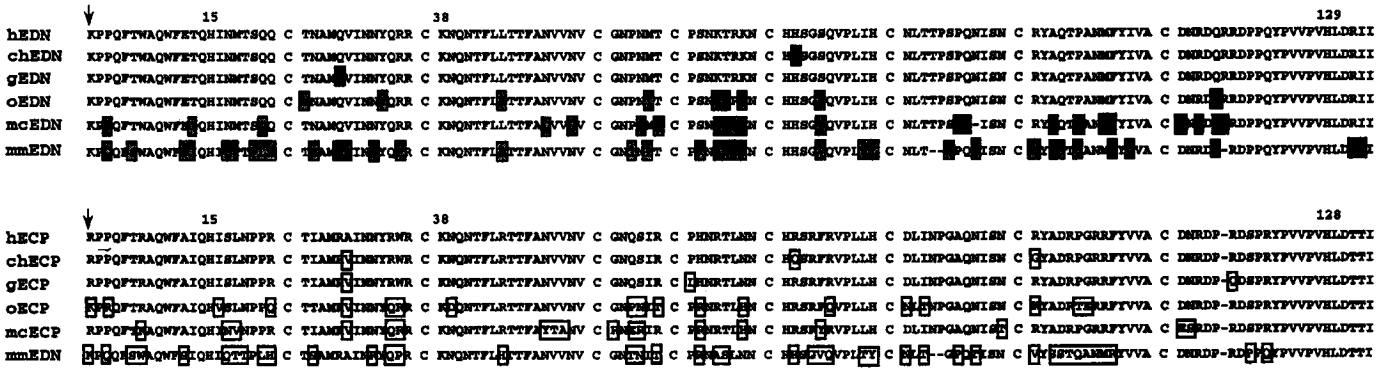

Fig. 2 Alignment of the amino acid sequences encoded by the primate genes described in Table 1. Abbreviations identifying the primate sequences are also described in Table 1. Shaded boxes enclose the amino acids encoded by the EDN isolates that differ from those of hEDN; open boxes enclose the amino acids encoded by the ECP isolates that differ from those of hECP; the mmEDN (*L. saguinus*) sequence has been included in both sets of analyses. The catalytic residues are numbered from the N termini of the mature polypeptides (at arrows). Analysis of the polypeptide sequences can be found in Table 1.

Table 2 Evolutionary analysis of primate coding sequences

Coding sequence	Ka × 10² (Ka/Ks) \ chimp	\ gorilla	\ orangutan	\Old World monkey	\ New World monkey	Ka vs. 2T	r²	Ks vs. 2T	r²
ECP	0.97 (0.88)	0.97 (0.88)	6.5 (3.0)	6.2 (0.63)	20.0 (1.3)	2.0	0.92	1.7	0.97
EDN	0.31 (0.09)	0.31 (0.09)	3.1 (0.32)	9.3 (0.34)	17.7 (0.72)	1.9	0.98	2.9	0.80
Interleukin-3 (IL-3)	0.32 (0.14)			6.7 (0.86)	16.4 (0.86)	1.7	0.98	1.9	0.97
CD59					16.7 (0.81)	1.7	–	2.1	–
Protamine P2	4.6 (1.8)	4.6 (0.92)		4.6 (0.92)[a] 8.3 (1.1)	18.7 (1.2)	1.7	0.90	1.5	0.93
Protamine P1	6.4 (2.0)	6.4 (2.0)	7.2 (1.1)		18.0 (0.66)	1.6	0.93	2.8	0.998
SRY[b]	1.5 (1.9)	0.8 (0.32)	2.5 (1.0)		12.9 (0.47)	1.4	0.99	2.9	0.98
Metallothionein I				6.5 (0.36)		1.2	–	3.3	–
Metallothionein II				5.7 (0.19)		1.1	–	5.4	–
Erythropoeitin				5.1 (0.5)[c]		1.0	–	1.9	–
Relaxin	0.76 (0.40)					0.63	–	1.6	–
CD4	0.5 (0.38)			3.4 (0.44)		0.63	0.995	1.4	0.997
β-2-microglobulin	0	0	1.7 (0.35)			0.61	0.60	1.9	0.70
δ globin			0.29 (0.08)	3.1 (0.15)		0.57	0.84	3.9	0.99
Insulin (a-chain)	0			0[c]	5.9 (0.28)	0.55	0.73	1.7	0.49
Interferon-γ					4.8 (0.60)	0.48	–	0.8	–
Apolipoprotein AI				2.3 (0.29)		0.42	–	1.5	–
P-53 tumour antigen				2.0 (0.22)[c]		0.37	–	1.6	–
α globin	0			1.9 (0.29)		0.37	0.96	0.99	0.72
TCR, ε subunit	0.51 (–)			2.0 (0.61)		0.36	0.99	0.60	–
Complement C4a	2.3 (0.72)		2.6 (0.45)	3.8 (0.62)	4.1 (0.32)	0.35	0.72	1.1	0.90
Somatotropin (GH)				1.6 (0.12)		0.29	–	0.24	–
Insulin (b-chain)	0			0[c]	3.1 (0.13)	0.29	0.73	2.4	0.92
C-mos protooncogene				1.4 (0.17)		0.26	–	1.5	–
C-myc protooncogene	0.093 (0.28)				2.1 (0.15)	0.22	0.995	1.5	0.99
Factor IX	0.92 (1.2)			1.3 (0.28)		0.20	0.73	0.83	0.997
EGF-like growth factor					1.2 (0.15)	0.12	–	0.80	–

Nonsynonymous substitutions per nonsynonymous site (Ka, units = substitutions/site) and synonymous substitutions per synonymous site (Ks, units = substitutions/site) were calculated for 65 coding sequence pairs (human/chimp, human/gorilla, human/orangutan, human/Old World monkey and human/New World monkey) from sequences of 26 independent sets of homologous genes as reported to GenBank. The values calculated for Ka are listed in each lane followed by Ka/Ks in parentheses. The slopes for plots of Ka vs 2T and Ks vs 2T (units = substitutions/site/year × 10^9) were calculated by least squares; correlation coefficients r^2 are included for those with three or more points. The value 2T represents the total years of divergent evolution, derived from the consensus dates of species divergence[19] (see Methods). [a]Two distinct species of Old World monkey yielded two values for Ka and Ks. [b]Values taken from ref. 20. [c]Identical values obtained from two distinct species of Old World monkeys. [d]GenBank accession numbers: IL-3: M14743, X51890; CD59: X16447, L22862; protamine P2: X07862, X71334, X71336, X71337, X71338, X71340, X71335; protamine P1: Y00443, L14590, L14587, L14589, L14592; metallothionein I: X64177, K00484; metallothionein II: X76717, K00485; erythropoeitin: X02157, M18189, M15818; relaxin: A06925, Z27245; CD4: M12807, X73323, M31134; β-2-microglobulin: J00105, M30683, M30682; δ globin: V00505, M21825, M19061; insulin: V00565, X61089, J00336, X61092, J02989; interferon-γ: M29383, X74875, X74876, X64659, X74877; apolipoprotein AI: X02162, M15411; p53: X02469, X16384, L20442; alpha globlin: V00493, X00226, J04495; TCR β chain: S51397, S51400, S51401; complement C4: K02403, Z31605, Z22724, Z22721, Z22715; somatotropin: V00519, L16556; c-mos: J00119, X12449; c-myc: V00568, M38057, M88116; factor IX: J00136, X65473; EGF-like factor: M60278, M93012.

to be simply drifting at an elevated rate. The nature of the evolutionary constraints that may be affecting the mutation rates observed for ECP and EDN are not known.

Perhaps the most significant finding of our study is that, in spite of the rate at which these sequences have accumulated mutations, all divergent evolutionary variants of EDN and ECP retain the eight spaced cysteines and catalytic histidine and lysine residues that are prerequisites for ribonuclease activity[15]. This suggests the need to re-evaluate the relationship between ribonuclease activity and biological function. The importance of ribonuclease activity to the known functions of hECP and hEDN is unclear; while two independent groups provided evidence suggesting that ribonuclease activity is essential for (non-physiologic) neurotoxicity[24,25], others have determined that ribonuclease activity is not essential for ECP's anti-parasitic[26] or antibacterial activities[27]. These conflicting and somewhat counter-intuitive results suggest that additional, perhaps more crucial physiologic functions of both ECP and EDN remain to be discovered. Of particular interest in this regard are recent reports describing a variety of ribonucleases with anti-neoplastic activity[28–32].

The large sequence divergences found between closely related evolutionary variants may provide a clue to physiologic function. In an extensive comparison of human and rodent coding sequences, Murphy[33] has shown that the largest sequence divergences are found among peptide ligands and receptors involved in host defense. Interleukin-3 and CD59, which are both involved in host defense, are among the most divergent sequences in this primate series. The possibility that secreted ECP and/or EDN could function as peptide ligands mediating signal transduction has not to our knowledge been addressed.

Jermann and colleagues[34] recently applied the principle of maximun parsimony to generate plausible ancestral sequences from existing members of the artiodactyl pancreatic ribonuclease gene family; functional analysis of the polypeptides encoded by these ancestral sequences has provided insight into evolutionary adaptation at the molecular level. Our work has created a similar database for primate members of a variant branch of the ribonuclease gene family; functional analysis of these existing primate homologues along with any plausible ancestral sequences may assist in identifying the constraints

directing the rapid evolutionary rates observed for this unique primate gene family.

Methods

Genomic Southern blots. 10 μg mammalian genomic DNAs (BIOS) were digested with *Pst*I, electrophoresed in 1% agarose/TBE, and transferred to a nylon membrane (Hybond-N, Amersham). Blotted DNA was fixed by UV crosslinking (Stratagene) and prehybridized and hybridized at 37 °C in a formamide/dextran solution (6× SSPE, 50% formamide, 0.5% SDS, 50 ug ml^{-1} sheared ssDNA) with ^{32}P-radiolabelled probe (Random Priming kit, Boehringer Mannheim, Indianapolis, IN). Blots were washed at very low stringency (5×SSPE/0.1% SDS at 37 °C to 45 °C) and autoradiograms were developed after three days exposure at –80 °C. The blots were stripped in 0.2 ×SSPE/0.1% SDS at 80 °C for 1 h and checked by autoradiography between probings. The hEDN coding sequence probe was prepared by PCR using an original cDNA isolate as template and oligonucleotide primers corresponding to nts 46 to 69 (5' to 3') and 528 to 505 (3' to 5') of published sequence[5]. The hPR coding sequence (305 base pairs) was isolated by PCR using human genomic DNA as template with oligonucleotide primers corresponding to nts 25 to 48 (5' to 3') and 330 to 307 (3' to 5') of published sequence[13]; isolate was confirmed by dideoxy sequencing (United States Biochemicals).

Characterization of primate ECP and EDN genes. Non-human primate sequences were isolated by PCR using gene specific primer pairs (EDN: nts 46 to 69 (5' to 3') and 528 to 505 (3' to 5'), ECP: nts 55 to 78 (5' to 3') and 550 to 527 (3' to 5'), as per published cDNA sequences[6,7]) and genomic DNA (BIOS) as templates. PCR products were subcloned into pBluescript (Stratagene) for dideoxy sequencing (USB). Sequence analysis (as well as GenBank access and polypeptide analysis as described below) was performed with the assistance of algorithms within the Wisconsin Genetics Computer Group Program on-line at the National Institutes of Health. There was no evidence of aberrant priming/hybrid product formation in any of the isolated sequence pairs.

The primate ribonuclease sequences described in this work have been assigned GenBank accession numbers as follows: chEDN, U24102; gEDN, U24100; oEDN, U24104; mmEDN, U24099; chECP, U24103; gECP, U24097; oECP, U24101; mcECP, U24098; mcEDN, U24096.

Analysis of primate coding sequences. Calculations shown in Table 2 were derived from sequences reported to GenBank (12/94 version), except for SRY, where calculations were taken directly from ref. 20. Nonsynonymous substitutions per nonsynonymous site (Nd/N = Ka) and synonymous substitutions per synonymous site (Sd/S = Ks) were calculated from open reading frames of mature proteins (without signal or pro-sequences). Sequences were selected on the basis of (a) the availability of at least one other orthologous non-human primate sequence, and (b) the presence of a complete open reading frame for analysis. Proteins whose function involves the generation of significant intra-species and individual diversity (such as variable immunoglobulin domains, T-cell receptors) were not included in this analysis. The value T (years since species divergence) was determined from consensus values for species divergence reported by Sibley and Ahlquist[19]; 2T represents the total years of divergent evolution[8], which includes 12 million years (my) for human/chimpanzee, 16 my for human/gorilla, 26 my for human/orangutan, 55 my for Old World monkey/human, and 100 my for New World monkey/human.

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