Expansion and Molecular Evolution of the Interferon-Induced 2′–5′ Oligoadenylate Synthetase Gene Family

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The mammalian 2′–5′ oligoadenylate synthetases (2′–5′OASs) are enzymes that are crucial in the interferon-induced antiviral response. They catalyze the polymerization of ATP into 2′–5′-linked oligoadenylates which activate a constitutively expressed latent endonuclease, RNaseL, to block viral replication at the level of mRNA degradation. A molecular evolutionary analysis of available OAS sequences suggests that the vertebrate genes are members of a multigene family with its roots in the early history of tetrapods. The modern mammalian 2′–5′OAS genes underwent successive gene duplication events resulting in three size classes of enzymes, containing one, two, or three homologous domains. Expansion of the OAS gene family occurred by whole-gene duplications to increase gene content and by domain couplings to produce the multidomain genes. Evolutionary analyses show that the 2′–5′OAS genes in rodents underwent gene duplications as recently as 11 MYA and predict the existence of additional undiscovered OAS genes in mammals.

Introduction

Interferons (IFNs) function as antiviral cytokines in both mammals and birds. They interfere with viral replication by inducing several effector proteins, including those that block viral protein synthesis. The 2′–5′ oligoadenylate synthetases (2′–5′OASs) form one such set of enzymes (reviewed in Lengyel 1982; Sen and Lengyel 1992; Rebouillat et al. 1999). When activated by double-stranded RNA, 2′–5′OAS catalyzes the polymerization of ATP into 2′–5′-linked oligoadenylates, $pppA(2'p5'A)_n$ ($1 \leq n \leq 30$), which bind to and activate a latent endonuclease, RNaseL (Kerr and Brown 1978; Floyd-Smith, Slattery, and Lengyel 1981; Zhou, Hassel, and Silverman 1993). RNaseL activation is short-lived because $pppA(2'p5'A)_n$ is rapidly degraded by cellular phosphodiesterases and 2′–5′ exoribonucleases (Schmidt et al. 1979; Schröder et al. 1980). Because double-stranded RNA is frequently produced during viral infections, activation of 2′–5′OAS prevents viral replication by degrading mRNA (Rice et al. 1985; Chebath et al. 1987; Kumar et al. 1988). Although 2′–5′OAS activity is highest in virus-infected cells, these enzymes may also function to regulate the stability of RNAs that control normal cellular processes such as cell division, differentiation, and apoptosis (Kumar et al. 1994; Diaz-Guerra, Rivas, and Esteban 1997; Salzberg et al. 1997; Yu and Floyd-Smith 1997).

The 2′–5′OAS–RNaseL system has been most extensively characterized for primates (human) and rodents (mouse and rat). There are three 2′–5′OAS genes in humans, encoding small (p40/p46), medium (p69/71), and large (p100) isoforms which are found on human chromosomal segment 12q24.1 (Hovnanian et al. 1998). Alternative splicing of transcripts from individual genes generate the p40 and p46 isoforms, as well as the p69 and p71 isoforms (Benech et al. 1985; Marié and Hovanessian 1992). Alignment of the putative translation products has identified a homologous sequence of about 350 amino acid residues that is present as a single domain in p40, two domains in p69, and three domains in p100 (Benech et al. 1985; Marié and Hovanessian 1992; Rebouillat et al. 1999). Functional interaction between domains is likely, since the p40 isoform is only active as a tetramer and the p69 isoform functions as a dimer (Ghosh et al. 1997; Sarkar et al. 1999*a*). Another gene (OASL) encoding a protein with significant sequence similarity to the three 2′–5′OAS genes (p40, p69, and p100), but lacking 2′–5′OAS activity, has been mapped to human chromosome segment 12q24.2 (Hartmann et al. 1998; Rebouillat, Marie, and Hovanessian 1998; Hovnanian et al. 1999).

In mice, several isoforms of 2′–5′OAS have been identified which correspond to the small, medium, and large size classes of the human enzymes (Hovanessian 1991; Rebouillat et al. 1999). A related sequence p54OASL has also been identified in mice. 2′–5′OAS activity has been detected in rats, pigs, marmots, and rabbits (Hartmann et al. 1998), among other mammals.

Birds, like mammals, have an interferon-inducible antiviral system, which includes a 2′–5′OAS-RNaseL system. A single chicken 2′–5′OAS gene has been identified which shows sequence similarity with mammalian 2′–5′OAS genes (Hartmann et al. 1998; Yamamoto et al. 1998). Components of the 2′–5′OAS system have also been detected in reptiles and amphibians (Cayley et al. 1982). The 2′–5′OAS-RNaseL system has not been extensively characterized for nonvertebrates. However, high levels of 2′–5′OAS activity have been reported for a marine sponge (*Geodia cydonium*) (Kuusksalu et al. 1995, 1998). Protein binding to the effector oligonucleotide, as well as its function in the antiviral host defense system, has not been established for nonvertebrates.

The recent accumulation of 2′–5′OAS sequences from mammals and birds provides us with an opportunity to understand the evolution and diversification of 2′–5′OAS genes. Furthermore, a partial sequence of human chromosomal fragment 12q24.1 (PAC RPCI1-71H24) containing known human OAS genes is now

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available; hence, the genomic structure and phylogenetic history of the human 2′–5′OAS locus can now be determined. In this paper, we employed a molecular evolutionary approach for this purpose.

Materials and Methods

A 162,346-bp human genomic PAC clone (PAC RPCI1-71H24) was scanned for putative open reading frames (ORFs) using the ORF-Finder software (T. Tatusov and R. Tatusov, NCBI). ORFs were tested for significant sequence similarity to all nonredundant GenBank CDS translations + PDB + SwissProt + PIR + PRF using BLASTP (Altschul et al. 1990). Nucleotide sequences of all ORFs showing significant sequence similarity to the known 2′–5′OAS cDNA sequences were then aligned using CLUSTAL W (Higgins, Thompson, and Gibson 1996).

All published 2′–5′OAS polypeptide sequences were retrieved from GenBank, and all very short and redundant sequences were removed from further analysis. Two cDNA sequences (accession numbers M63849 and M63850) were 99% identical to human 2′–5′OAS sequences and were excluded from further analysis because their origins were not known with certainty (Rebouillat et al. 1999). A list of GenBank/EMBL accession numbers and references are given in table 1. A protein sequence alignment was constructed for these sequences using CLUSTAL W, and the alignments were manually corrected. We used the slow-and-accurate alignment option in CLUSTAL W, along with a BLOSUM30 amino acid substitution matrix. Penalties for alignment gap creation and gap extension were set at 10 and 0, respectively. The former is recommended by default in CLUSTAL W, and the latter was chosen because higher gap extension penalties produced poor alignments due to the presence of multiple evolutionarily related domains within genes. Final evolutionary analyses were conducted on an alignment consisting of protein sequence of all homologous OAS domains (fig. 4). This alignment was constructed using the default options in CLUSTAL W.

Phylogenetic analyses were conducted using the neighbor-joining (NJ) method (Saitou and Nei 1987), as implemented in the *MEGA* program (Kumar, Tamura, and Nei 1993). Because the sequences were distantly related, we used *p*-distances for constructing the phylogenetic trees. For a pair of sequences, the *p*-distance is simply the proportion of sites that contain different residues between the given sequences. Reliability of the NJ trees was examined by the bootstrap test (Felsenstein 1985). PAUP* was used for maximum-parsimony (MP) analyses (Swofford 1998) for conducting 100 replications of the heuristic search with the tree-bisection-and-reconnection branch-swapping algorithm on the initial trees obtained by random sequence addition order. For both MP and NJ trees, 1,000 bootstrap replicates were generated for the bootstrap tests. The branching pattern obtained using the NJ tree with Poisson correction distance and the MP methods were different from the *p*-distance–based NJ (NJ-*p*) tree. In all of these cases, the statistical confidence for the inferred branching pattern for the NJ-*p* trees was higher than that for the NJ trees with Poisson correction distance and the MP trees. Therefore, only the NJ-*p* trees have been presented and considered in this paper with the indicated robustness of the inferred branching patterns obtained using the bootstrap test (bootstrap confidence levels [BCLs] (Felsenstein 1985; Kumar, Tamura, and Nei 1994).

To place a temporal perspective on the evolution and diversification of the OAS gene family, we estimated the divergence time for different branching points in the inferred phylogeny using the molecular clock concept (Zuckerkandl 1987). A Poisson model of amino acid substitution was used to correct for multiple substitutions, and the equalities of evolutionary rates in sister lineages were tested using the two-cluster relative-rate tests (Takezaki, Rzhetsky, and Nei 1995), as implemented in the PHYLTEST program (Kumar 1995).

Table 1
Sequences Used in the Study

Species and Genes	No. of Domains	Accession No.	References[a]
Chordata			
Mammals			
Homo sapiens (*Hsa*)			
Hsa-p40	1	D00068	1
Hsa-p42	1	X04371	2
Hsa-oasE	1	X02874	3, 4
Hsa-PAC	6	AC004551	5
Hsa-p69	2	M87434	6
Hsa-OASL	1	AJ225089	7
Hsa-oas2	2	NM_002535	6
Sus scrofa (*Ssc*)			
Ssc-p42	1	AJ225090	7
Rattus norvegicus (*Rno*)			
Rno-oas	1	Z18877	8
Rno-oas2	1	AF068268	9
Mus musculus (*Mmu*)			
Mmu-L1	1	X55982	10
Mmu-L2	1	X58077	10
Mmu-L	1	X04958	11
Mmu-OASL	1	AF068835	12
Marmota monax (*Mmo*)			
Mmo-oas	1	AF082498	13
Bird			
Gallus gallus (*Gga*)			
Gga-oasA	1	AB002585	14
Gga-oasB	1	AB002586	14
Porifera			
Geodia cydonium (*Gcy*)			
Gcy	1	Y18497	15

[a] 1 = Shiojiri et al. (1986); 2 = Wathelet et al. (1986); 3 = Merlin et al. (1983); 4 = Benech et al. (1985); 5 = Muzny (1998); 6 = Marié and Hovanessian (1992); 7 = Hartmann et al. (1998); 8 = Truve et al. (1993); 9 = Shimizu (1998); 10 = Rutherford et al. (1991); 11 = Ichii et al. (1986); 12 = Heufler (1998); 13 = Zhou, Hu, and Seeger (1998); 14 = Yamamoto et al. (1998); 15 = Wiens et al. (1999).

Results

Genomic Structure of the Human OAS Locus

The ORF-Finder predicted 936 potential ORFs in the human PAC clone RPCI1-71H24. BLASTP analysis of these ORFs and the exploration of the related literature yielded 33 exons matching the known OAS sequences (fig. 1*A*). The most distal region containing six

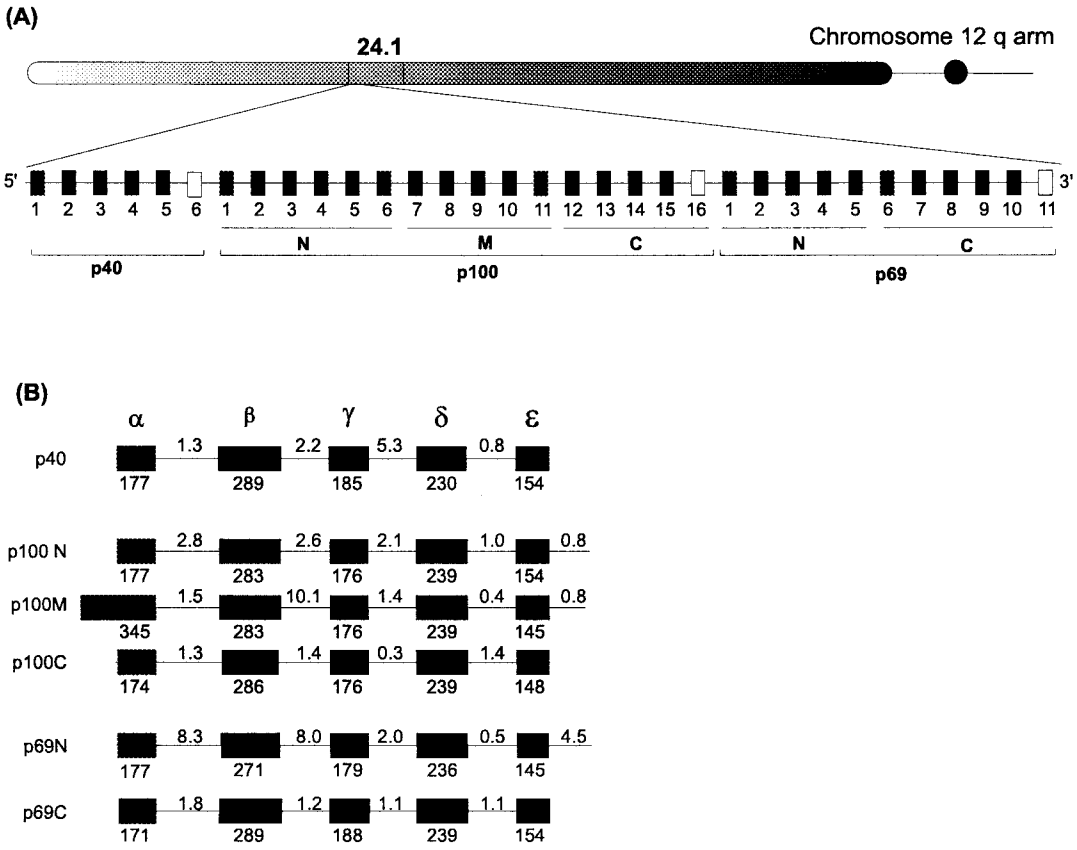

FIG. 1.—*A,* Map of human chromosome 12, showing the genomic location of the OAS genes found on human PAC clone 12q24.1. Exons are indicated with rectangular boxes and are numbered for each gene. The orientation of the PAC clone was established based on the tentative relative position of the p40 and p69 STS identified in the human genome sequencing projects. *B,* Exon-intron structure of the OAS genes in more detail. Exons are drawn to scale, with their lengths given in nucleotides. Intron lengths are given in kilobases and are not drawn to scale.

exons matching p40 (Benech et al. 1985) is 13-kb long. This segment is within 19 kb of a 37-kb region containing 15 novel exons that can be aligned to form three domains (N, amino; M, middle; C, carboxyl) for the p100 OAS cDNA (see Rebouillat et al. 1999). The intron separating domains N and M and that separating M and C are 0.8 kb each. The proximal region contains 11 exons spanning 32 kb that match two domains (N and C) of the cDNA sequence for p69. A 4.5-kb intron is found between these two domains. The p100 and p69 genes are separated by a 4-kb intergenic region. Therefore, the three OAS genes occupy 103 kb of the 163-kb genomic fragment. Table 2 shows exon and intron lengths of human p40, p100, and p69 genes. In total, the OAS protein-coding sequences account for a small fraction of the genomic fragment occupied by this gene cluster. Since no other putative genes were found within the chromosomal region containing p40, p100, and p69 genes, these genes appear to form an uninterrupted cluster on human chromosome 12 (fig. 1*A*).

Table 2
Genomic Structure of the OAS Locus Containing Human p40, p100, and p69 Genes

	p40	p100-N	p100-M	p100-C	p69-N	p69-C
Exon α	177 (0)	177 (0)	345 (0)	174 (0)	177 (0)	171 (0)
Intron I	1,316	2,862	1,487	1,335	8,252	1,831
Exon β	289 (1)	283 (1)	283 (1)	286 (1)	271 (1)	289 (1)
Intron II	2,226	2,623	10,095	1,388	7,987	1,190
Exon γ	185 (0)	176 (0)	176 (0)	176 (0)	179 (0)	188 (2)
Intron III	5,273	2,091	1,417	342	2,045	1,104
Exon δ	230 (2)	239 (2)	239 (2)	239 (2)	236 (2)	239 (2)
Intron IV	808	964	410	1,433	510	1,143
Exon ϵ	154 (0)	154 (0)	145 (0)	148 (0)	145 (0)	154 (0)
Intron V	1,786	759	777	5,296	4,521	1,133
Exon η	162 (0)			12 (0)		108 (0)

NOTE.—Sequence lengths are given as numbers of nucleotides, and intron phases are shown in parentheses.

Phylogenetic Analysis of the Human PAC Clone RPCI1-71H24

An alignment of 33 exons was constructed to identify homologous exons using CLUSTAL W. The NJ-*p* tree identified five well-defined classes of exons (α, β, γ, δ, and ε). For the last alternatively spliced exons (exons η), two out of three showed sequence similarity to genes of the ubiquitin family, and one was too short to be analyzed (table 2). Bootstrap analysis of the phylogeny of the remaining 30 exons shows the monophyly of the five exon groups to be statistically supported (89% $\leq$ BCL $\leq$ 100%; fig. 2). When the relative positions of most closely related exons are considered along with the exon homology in figure 2, it is clear that there are six ordered sets of five core exons each. These ordered sets

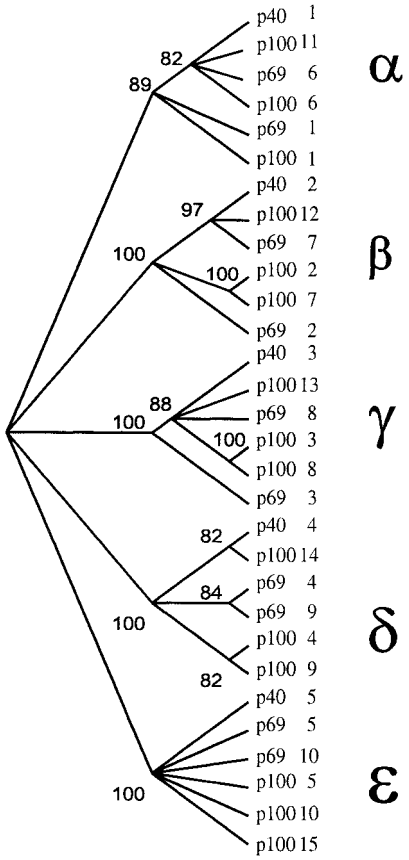

FIG. 2.—A neighbor-joining (NJ) tree of the 2′–5′ oligoadenylate synthetase exons identified in human PAC clone RPCI1-H74. The NJ tree was inferred using the *p*-distances between nucleotide sequences. Branches with bootstrap values less than 80% were collapsed to construct the condensed tree. Numbers after the exons refer to their relative positions within respective genes given in figure 1*A*.

define the three human 2′–5′OAS genes: the one-domain p40 gene, the two-domain p69 gene (p69N and p69C), and the three-domain p100 gene (p100N, p100M, and p100C) (fig. 1). Exons of a related sequence, OASL (Hartmann et al. 1998; Rebouillat, Marie, and Hovanessian 1998), can be similarly aligned, and its first five exons show close sequence similarities to the α, β, γ, δ, and ε exons, respectively, of the three OAS genes. The final OASL exon shows sequence similarity to ubiquitin. The OASL gene has been mapped to an adjacent site on human chromosome 12q24.2 (Hovnanian et al. 1999), but the genomic sequence of that region is not yet available. Thus, it is unclear whether the OASL forms an uninterrupted cluster with p40, p100, and p69 genes, which reside on 12q24.1 (fig. 1*A*).

Table 2 and figure 1*B* clearly show that the exon lengths within groups are more similar than those between groups. Exon lengths always differ by multiple of 3 nt within groups, indicating insertion/deletion of complete codons. This is expected because of strong purifying selection against frameshift mutations. In the α group, the first exon in domain p100M is almost twice the size of other α-type exons (fig. 1*B*). Exon nucleotide sequence alignment shows that the 3′ half of the p100M-α exon is homologous to other α exons. In order to identify the evolutionary origin of the 5′ half, we split this exon into 3′ and 5′ regions and constructed an alignment of all exons. This alignment was then used to construct an NJ-*p* tree. The 5′ region of p100M-α does not cluster significantly with any other OAS exon, which rules out its origin by duplication of an existing OAS exon. Since the 5′ region is contiguous with the 3′ half on the genomic sequence, it appears that a single change in the splice site resulted in the recruitment of an upstream nucleotide position as a splice site to lengthen the p100M-α exon.

The exon-intron boundaries for the α-I, β-II, γ-III, δ-IV, and ε-V junctions are in intron phases 0, 1, 0, 2, and 0, respectively (table 2). If an intron interrupts the coding sequence between first and second codon positions, then the intron is said to be in phase 1. Phase 2 introns interrupt the codon between the second and third positions, and phase 0 introns are found between codons. Identity of intron phases among different domains indicates that the genomic structures of human 2′–5′OAS genes have remained unchanged since their origin, with the exception of the splice site mutation in p100M. Conservation of the genomic sequence around the exon-intron boundaries is further reflected in the preservation of nucleotide sequences around the homologous exon-intron and intron-exon boundaries (fig. 3), as inferred using the Schneider and Stephens (1990) and Gorodkin et al. (1997) methods.

Phylogenetic Analysis of Human 2′–5′OAS Domains and Other Homologous Sequences

A protein sequence alignment (fig. 4) consisting of six domains comprising the human 2′–5′OAS genes and all other representative protein sequences was used to construct the NJ-*p* tree shown in figure 5. In this analysis, sites containing missing data or alignment gaps were removed in a pairwise-deletion fashion (Kumar, Tamura, and Nei 1994). Use of the complete-deletion option produced an identical topology, except for *Ssc*-p42 clustering as a sister group to *Hsa*-p40 group rather than to *Mmo*-oas. The evolutionary relationships of the human 2′–5′OAS domains show that (1) p40, p100C, and p69C group together (BCL = 91%); (2) the p100C and p69C domains are more closely related to each other than either is to p40 (BCL = 93%); and (3) p100N and p100M are each other's closest relatives (BCL = 99%).

In figure 5, the four allelic p40 human sequences (*Hsa*-PAC-p40, *Hsa*-p40, *Hsa*-p42, and *Hsa*-oasE) cluster tightly. The pig *Ssc*-p42 and the rodent *Mmo*-oas sequences cluster with these four human sequences to form subgroup C-I (fig. 5) with high bootstrap support (BCL = 100%). The close relationship of the artiodactyl (*Ssc*-p42) sequence to the rodent sequence (*Mmo*-oas), rather than to human sequences, is not statistically supported. It is likely to have occurred because the *Mmo*-oas sequence available is less than half the length of the *Ssc*-p42 and *Hsa*-p40 sequences (as mentioned above, use of the complete-deletion option led to clustering of *Ssc*-p42 with the human p40 genes).

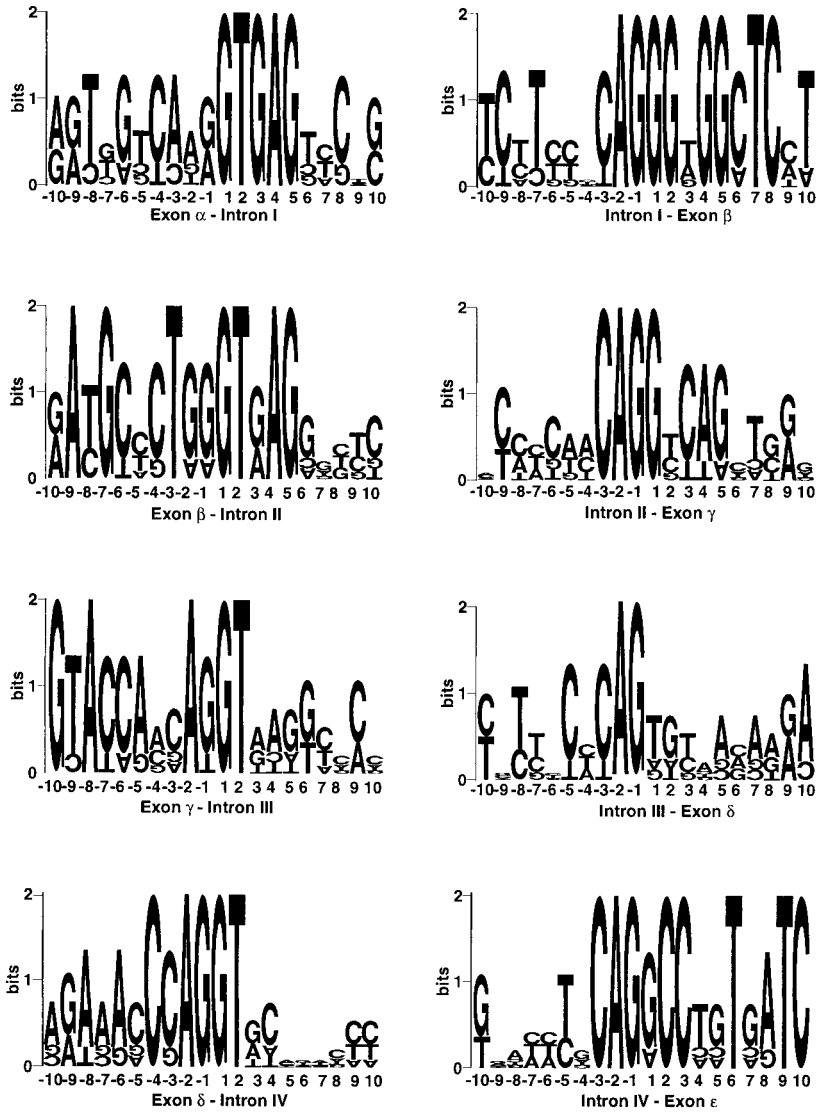

FIG. 3.—The sequence logo graph showing the consensus sequences at the homologous exon-intron and intron-exon junctions of the OAS genes in the human PAC clone. These graphs were drawn using the Sequence logos method of Schneider and Stephens (1990) and Gorodkin et al. (1997). Type I plain sequence logos with heights based on the proportional frequencies of nucleotides are shown. Symbols are displayed upsidedown when a nucleotide appears with less than expected frequency. Negative and positive numbers denote nucleotide distances to the left and to the right of the junction, respectively.

Five rodent sequences comprise subgroup C-II, which shares a most recent common ancestor with subgroup C-I (BCL = 100%). These five rodent sequences are further subdivided into groups C-IIa (*Rno*-oas2, *Mmu*-L1) and C-IIb (*Rno*-oas, *Mmu*-L2, *Mmu*-L). Groups C-I and C-II diverged before the primate-rodent split; this divergence was followed by the emergence of subgroups C-IIa and C-IIb in the common ancestor of murid rodents (mouse and rat). Within *Mus,* the presence of two distinct p40-like genes (*Mmu*-L2 and *Mmu*-L) shows recent evolution of additional genes. Rutherford (1991) noted that *Mmu*-L1 and *Mmu*-L2 are linked. In light of the close relationship of the *Mmu*-L1, *Mmu*-L2, and *Mmu*-L sequences (fig. 5), it is likely that all three genes form a linked unit. Therefore, p40 genes compose a multigene family within rodents.

The mammalian p40 genes are closely related to the p100C and p69C domains (BCL = 91%). Together, they compose the supergroup C. Human and mouse OASL proteins, lacking 2′–5′OAS activity, show a statistically supported cluster (BCL = 98%) and group with the known bird OAS (*Gga*-oas) sequence (BCL = 67%; group D). In an attempt to determine the root of this tree to identify the most ancient group(s), we used the marine sponge sequence (*Gcy*) in phylogenetic analysis. However, the root could not be placed reliably in that phylogenetic analysis, because sequence similarity between *Gcy* and all other sequences is less than 20% (table 3). Therefore, the status of the marine sponge sequence as a member of the OAS gene family is uncertain at this time, and thus it is not included in figure 5.

Discussion

The human genome contains 15 times as many genes as yeast (Goffeau et al. 1996) and 5 times as many genes as *Caenorhaditis elegans* (The *C. elegans* Sequencing Consortium 1998). Much of this increase in gene content is due to gene duplications through a variety of mechanisms, including chromosome, genome, and individual gene duplications, and the recruitment of preexisting modules to form new composite genes (Ohno 1970; Nei 1987; Li 1997). The gene family containing the vertebrate OAS genes is one gene family which has expanded by gene duplications, domain coupling to form multidomain genes, block duplications to produce copies of multiple genes at the same time, and domain duplications within genes, as discussed below.

Evolution by Gene Duplication

We begin with the evolution-by-gene-duplication scenario for p40, p100C, and p69C, which show the highest sequence similarity and form a statistically supported group to the exclusion of all other domains (fig. 5). Because p40, p69C, and p100C do not occupy adjacent positions in the human genome (fig. 1*A*), an explanation of their origins by recent tandem duplications would require multiple gains and losses of domains. Instead, they appear to have shared a common ancestor in the earliest stages of the OAS gene family expansion. This is illustrated in a parsimonious gene duplication scenario given in figure 6, which is based on the phylogeny in figure 5 and the relative positions of OAS genes on the human genome.

In this scenario, the first gene duplication produced a pair of genes that later gave rise to seven domains comprising the OASL, p40, p100, and p69 genes. In the absence of a reliable rooting point, the first duplication could have occurred either prior to or after the bird-mammal divergence 310 MYA. In the first case, the *Gga*-oas and mammalian OASL genes are related by speciation. In the second case, they are paralogous genes, as they are related by a gene duplication event in the common evolutionary history. In either case, the ancestral gene (fig. 6) duplicated to produce direct ancestors of the OASL and group C genes.

The next event in the history of OAS gene family evolution was a chromosomal segment duplication. Two of these genes were the ancestors of the modern human p59 and p40 genes, and the other pair underwent domain coupling to form a two-domain gene (p69). The two-domain gene, reported only in mammals, duplicated again to give rise to a total of three genes (p40, p69, and p100). Subsequently, an internal domain duplication converted the two-domain p100 gene into a three-domain gene. Due to extensive heterogeneity of evolutionary rates among lineages in this gene, the timing of this duplication event cannot be established unless homologous sequences are known from other mammals. However, this internal domain duplication appears to have occurred much later than the initial origin of the p100 gene, as indicated by the length of the ancestral branch for p100M and p100N. In this regard, it is interesting to note that the 64–65-cM segment of mouse chromosome 5 shares an ordered two-gene conserved synteny with human chromosomal segment 12q24.1–12q24.3 (Mouse Genome Database 1999). In this case, two loci, *Tbx*5 and *Tcf*1, in mouse chromosome 5, are also found in human chromosome 12 at positions 12q24.1 and 12q24.3, respectively. Therefore, the human OASL, p40, p100, and p69 2′–5′OAS genes are flanked by the two loci in an apparent conserved synteny. This suggests that the current genomic structure of the human OAS genes was established in the earliest known history of placental mammals.

In rodents, there are multiple p40-like genes (groups C-IIa and C-IIb) with high sequence similarity to the human and other rodent p40 genes (group C-I). The first gene duplication that led to the emergence of the ancestor of group C-II genes occurred in the common ancestor of primates and rodents. This was followed by a gene duplication in the ancestor of murid rodents (rats and mice). Because groups C-I and C-IIb show similar rates of evolution, we estimated the divergence time for the split of C-IIa and C-IIb in a lineage-specific manner by using only the C-IIb lineage length to date the divergence of C-IIa and C-IIb. In this case, the length of the C-IIb lineage from the common ancestor of C-I and C-IIb is 0.2132, and that from the common ancestor of C-IIa and C-IIb is 0.0866. Assuming a divergence time of 110 MYA for humans and mice (Kumar and Hedges 1998), the rate of evolution is computed to be 1.9×10^{-3} substitutions/Myr. This suggests the presence of one gene duplication $\sim 65 \pm 11$ MYA to produce subgroups IIa and IIb, followed by a gene duplication within subgroup C-IIb in mice 11 ± 4 MYA. Therefore, the 2′–5′OAS gene family in rodent genomes has expanded recently to include multiple small 2′–5′OAS isoforms.

The *Hsa*-OASL and *Mmu*-OASL genes form a sister group to the bird *Gga*-oas sequence. Based on the evolutionary relationships, we would expect *Hsa*-OASL and *Mmu*-OASL to be orthologous. However, the evolutionary distance observed between these two sequences is much larger than that expected for two orthologous sequences between these mammals if *Gga*-oas is an outgroup. In fact, if we conservatively assume that the branch connection between the two OASL genes with the bird sequence is the oldest splitting point, then the assumption of a molecular clock indicates a minimum divergence time of 276 MYA for *Hsa*-OASL and *Mmu*-OASL, which predates their species divergence by 160 Myp. This suggests either that the mammalian OASL genes have been evolving two times as fast as bird sequences or that the OASL genes are paralogous.

Gene Content in Vertebrate Genomes

Based on the evolution by gene duplication scenario in figure 6, a number of predictions can be made about the gene contents in genomes of different vertebrates (table 4). Within vertebrates, the OAS gene activity is induced by IFN in all mammals tested, and chicken IFN strongly induces 2′–5′OAS in cultured cells. RNaseL activity is also detected in chicken cells,

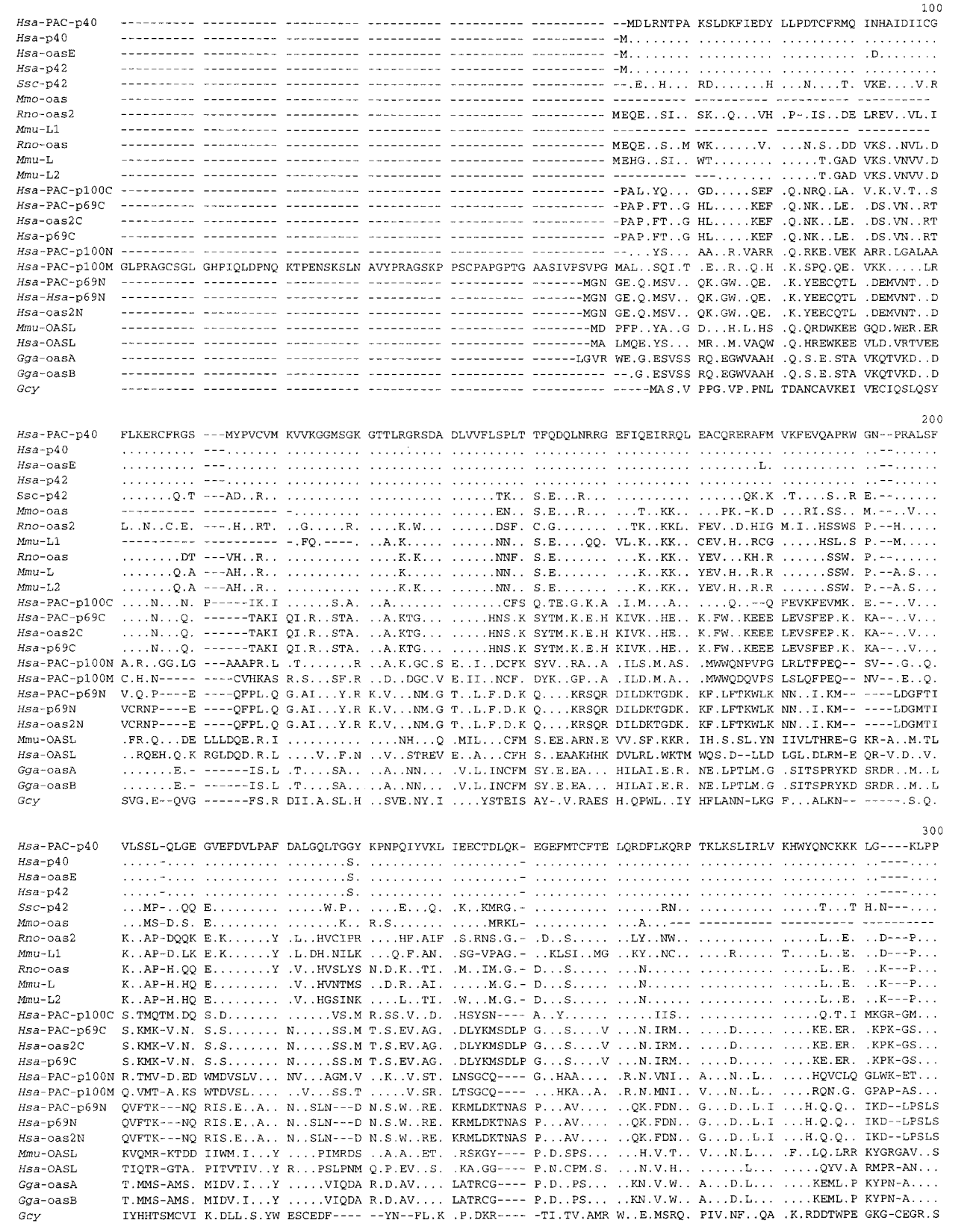

FIG. 4.—A representative alignment of the OAS sequences and domains. Exons are separated by vertical lines are based on the genomic structure of the OAS genes in humans. Identity with the first sequence is shown using dots, and dashes are used to indicate alignment gaps.

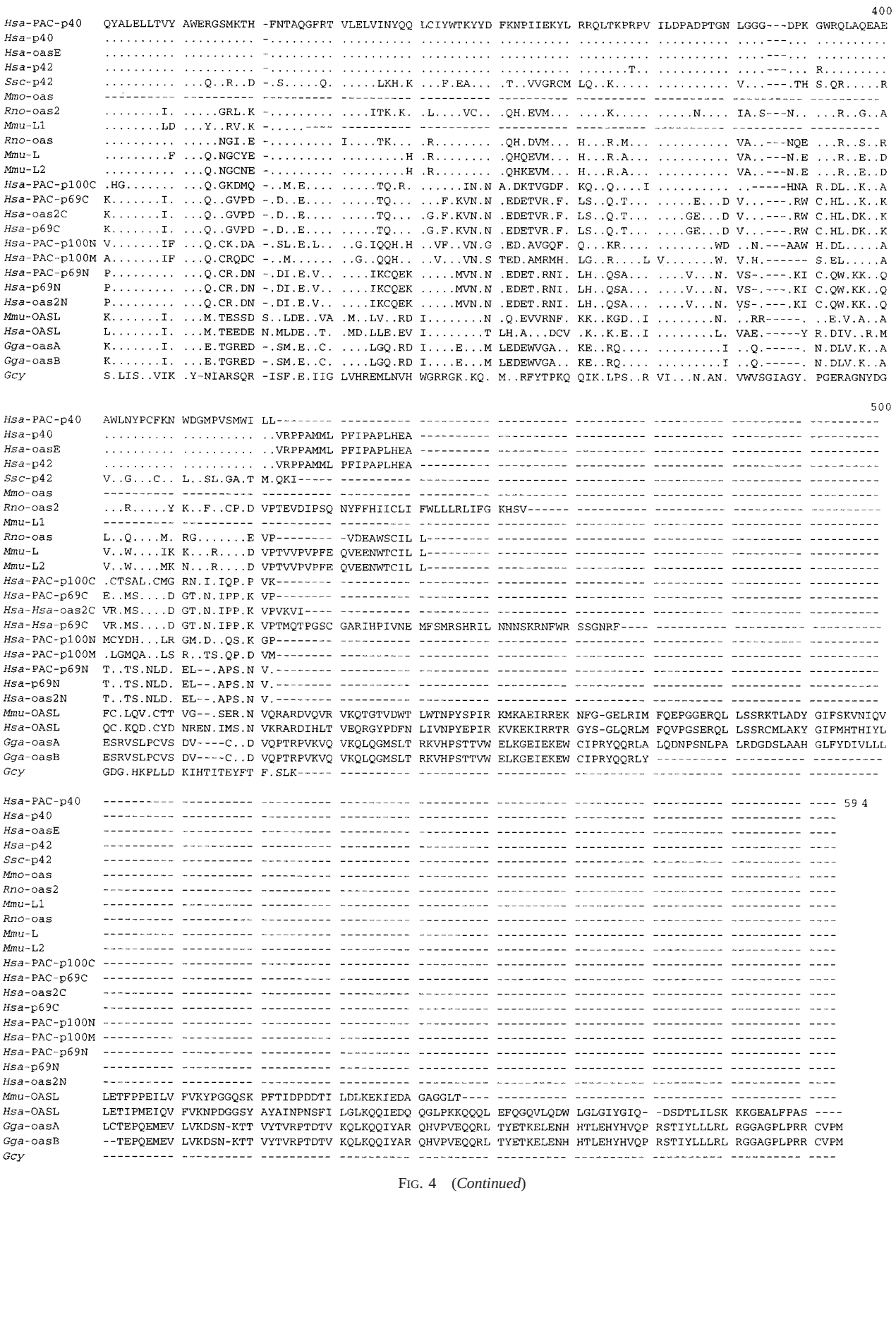

FIG. 4 (*Continued*)

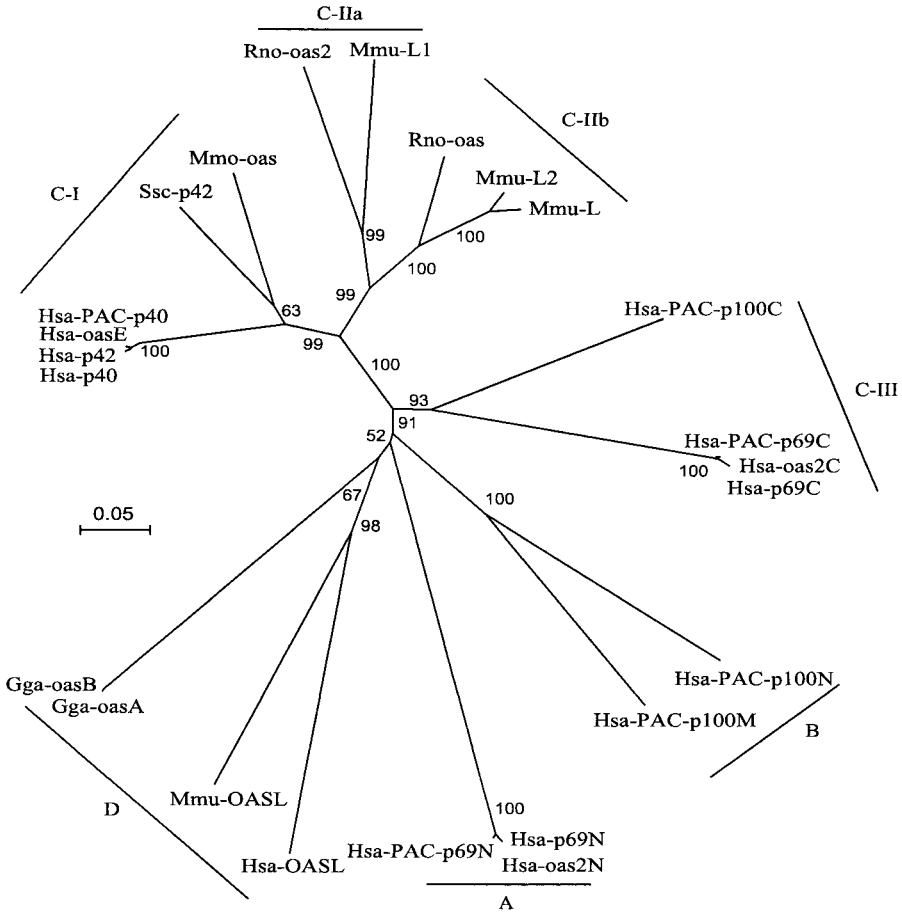

FIG. 5.—A neighbor-joining (NJ) tree of the OAS and OAS-like sequences. Protein sequence alignment was used to compute a pairwise matrix of *p*-distances for constructing the NJ tree. Bootstrap confidence values given are based on 1,000 bootstrap replications.

in which it appears to be involved in mediation of an antiviral response (West and Ball 1982; Sokawa and Sokawa 1986; Hartmann et al. 1998; Yamamoto et al. 1998). 2′–5′OAS and pppA(2′p5′A)$_n$-binding activities have also been detected in three reptilian species, *Agama caudospinosa*, *Gekko gekko*, and *Mabuya brevicollis*, suggesting that they may also have this inducible antiviral system (Cayley et al. 1982). Therefore, the most recent common ancestor of birds and mammals may have already contained an OAS gene with 2′–5′OAS and pppA(2′p5′A)$_n$-binding activities. While the amphibians contain low levels of pppA(2′p5′A)$_n$-binding activity, the 2′–5′OAS activity has not been detected (Cayley et al. 1982) to date. Neither 2′–5′OAS nor pppA(2′p5′A)$_n$-binding activity has been detected in fish (*Salmo trutta*). Therefore, the evolution of the 2′–5′OAS host-defense activity appears to be specific to tetrapods, and the earliest gene duplication events do not seem to have occurred prior to the amphibian and amniote divergence 350 MYA (Benton 1993; Kumar and Hedges 1998).

Table 3
Proportion of Amino Acids Different Between OAS Domains Found on PAC RPCI1-7H24, the Human OASL Gene, and the Marine Sponge (Gcy) Sequence

Genes/ Domains	p40	p100C	p69C	p69N	p100M	OASL	Gcy
p69C	0.45						
p100C ...	0.40	0.39					
p69N	0.57	0.55	0.58				
p100N ...	0.55	0.53	0.51	0.62			
p100M ...	0.51	0.54	0.49	0.63	0.39		
OASL ...	0.56	0.64	0.57	0.66	0.59	0.64	
Gcy	0.78	0.81	0.81	0.83	0.92	0.81	0.83

Clearly, multiple gene duplications have produced the modern OAS genes involved in innate immunity in placentals. The three different size classes of OAS genes found in placentals differ in their subcellular locations, induction parameters, and enzymatic characteristics (Marié et al. 1997; Yu and Floyd-Smith 1997; Floyd-Smith, Wang, and Sen 1999; Rebouillat et al. 1999; Sarkar et al. 1999*b*; Yu and Floyd-Smith 1999). This suggests that multiple isoforms of these enzymes underwent duplication followed by diversification of function, which presumably would enable the cell to more effectively respond to diverse viral pathogens. The p69 OAS is myristalated and localized to membranes, whereas the p40 isoform is distributed throughout the cytoplasm and the p100 isoform is associated with ribosomes (Chebath et al. 1987; Hovanessian et al. 1988; Hovanessian 1991). Induction of p40 and p100 is protein synthesis–indepen-

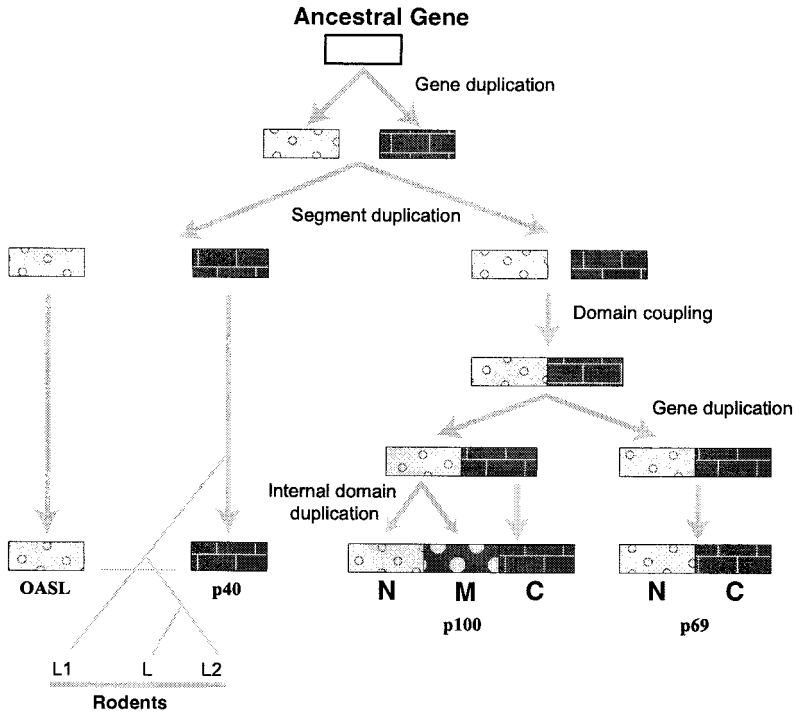

FIG. 6.—One possible scenario for the expansion of the OAS gene family by gene duplication, domain coupling, and chromosomal segment duplication.

dent, whereas p69 induction occurs in two phases: an initial protein synthesis–independent phase followed by a more prolonged protein synthesis–dependent phase (Yu and Floyd-Smith 1999). Relative induction of each isoform is variably dependent on cell type, cell growth rates, and protein kinase C activation, suggesting divergence among these similar enzymes in mediating a cellular antiviral response (Marié et al. 1997; Yu and Floyd-Smith 1997; Floyd-Smith, Wang, and Sen 1999; Yu and Floyd-Smith 1999). Activation of the p100 isoform is maximal at 1 μg/ml dsRNA, while the induction of p69 requires 100 μg/ml dsRNA (Marié et al. 1997). The three isoforms also differ in the lengths of 2′–5′ oligomers produced: the p69 isoform produces 2′–5′ oligonucleotides up to 30 residues, whereas the small and large isoforms preferentially produce dimers and trimers (Marié et al. 1997; Rebouillat et al. 1999; Sarkar et al. 1999*b*).

Table 4
Predicted Gene Contents in Some Vertebrate Genomes

Species	p40-like	p69-like	p100-like	p59-like
Primates	2 (1)	1 (1)	1 (1)	1 (1)
Rodents				
Rat	3 (2)	1 (0)	1 (0)	1 (0)
Mouse	4 (4)	1 (0)	1 (0)	1 (1)
Hamster	2 (0)	1 (0)	1 (0)	1 (0)
Birds	1 (0)	0	0	1 (1)
Reptiles	1 (0)	0	0	1 (0)
Amphibians	1[a] (0)	0	0	1[a] (0)

NOTE.—Numbers of genes for which sequence data are already available are given in parentheses.

[a] Assuming that the mammalian OASL and avian OAS genes are related by a speciation event.

The OAS genes contain several highly conserved subregions. These include a glycine-rich region, GGS(S/T)(G/A)(K/R)/GT, an adjacent DAD motif, and an FDVLP motif within the β exons of the small isoforms and the β exons of the C-terminal domains of p69 and p100 (group C in fig. 5). The glycine-rich region resembles an ATP/GTP-binding motif (Hartmann et al. 1998; Rebouillat et al. 1999). Molecular modeling analysis suggests that these C-terminal domains form a αβ-βαβββ structure corresponding to the three-dimensional crystal structures of several DNA and RNA polymerases (Sarkar et al. 1999*b*). In DNA polβ, three aspartate residues corresponding to aspartates within the DAD and FDVLP motifs of OAS form the active site of the enzyme. Mutation of any of these aspartates to alanine inactivates OAS, suggesting that they are essential for catalytic activity (Sarkar et al. 1999*b*). Similar protein folding of this region to DNA polβ suggests that the OAS enzymes may have evolved from an ancestral polymerase.

Interestingly, the DAD motif in group C domains is also found in a distantly related Gga-*oas* sequence, which also has OAS activity. In contrast, the OASL genes have lost this signature, as have the N and M domains (groups A and B in fig. 5). Therefore, domains in groups A and B may have lost the catalytic activity after gene/domain duplications. The small isoform (group C), which presumably evolved first, is active only as a tetramer (Ghosh et al. 1997), where the p69 isoform is a dimer (Marié, Rebouillat, and Hovanessian 1999; Sarkar et al. 1999*b*) and the p100 isoform is active as a monomer (Rebouillat et al. 1999).

Given that the tetrameric protein needs only one domain to retain catalytic activity, the additional do-

mains did not need to retain the conserved DAD motif (Sarkar et al. 1999*b*). This suggests that domain duplication and linking into a single transcription unit might have evolved to increase the efficiency of function enzyme production, rather than substantially altering the gene function by specific mutational changes in the protein sequence. The apparent recent divergence of multiple rodent genes that are not shared among human genes suggests that the evolution of the OAS locus may be an ongoing process in which mammals continue to develop new defensive strategies against viral pathogens.

Expansion of the OAS gene family points to the concurrent evolution of specific and innate immunity in early mammalian history. This component of the antiviral system appears to have evolved only in tetrapods, and, once evolved, it became established in these genomes. The antiviral activity for this system can be demonstrated in many highly divergent organisms. For instance, plants are not known to have a 2′–5′OAS-RNaseL system (Cayley et al. 1982; Mitra et al. 1996); however, the addition of pppA(2′p5′A)$_n$ to tobacco (*Nicotiana glutinosa*) confers resistance to tobacco mosaic viral infection (Devash et al. 1984), and the expression of mammalian 2′–5′OAS in transgenic potato plants correlates with resistance to potato virus X (Truve et al. 1993). In this regard, the fact that these genes appear to have undergone gene duplications recently, at least in placentals mammals, may further suggest that stronger antiviral response confers selective advantage.

Thus, the OAS genes involved in the antiviral response originated early in the ancestors of the modern tetrapods and underwent expansion in mammals. This expansion led to the evolution of genes with OAS activity, which is required in the innate immunity response known to exist *uniquely* in vertebrates. The expansion of the immunoglobulin and the major histocompatibility complex (MHC) genes in response to selective pressures is well known. Proliferation of the OAS gene family for facilitating an antiviral response could have occurred as a mechanism for increasing the overall magnitude of induction in response to interferons or other inducers. Alternatively, the ancestral and the duplicated genes could have evolved and diversified to provide for distinct mechanisms of induction and/or distinct enzymatic properties. In the present case, the adaptive nature of the antiviral host-defense and the existence of the cellular machinery to evolve a new developmental pathway appear to have led to the generation of a dynamic gene family.

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