The complete nucleotide sequence of mouse immunoglobulin γ2a gene and evolution of heavy chain genes: further evidence for intervening sequence-mediated domain transfer

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ABSTRACT

We have determined the complete nucleotide sequence (1990 base pairs) of mouse immunoglobulin γ2a gene, and compared it with the sequences of other γ subclass genes so far sequenced, i.e. γ1 and γ2b genes. Divergence of the nucleotide sequences between a compared pair of the γ genes varies extensively among different segments of the gene. For example, comparison of the γ2a and γ2b genes has revealed a remarkable homology in a long continuous segment (about 900 bases) that covers from the 3' portion of the first intervening sequence to the third intervening sequence. However, there is no particular segment of the γ gene that is conserved universally among the three γ genes. These findings suggest that, during their evolution, segments of the γ genes had been scrambled between different subclass genes through recombinations within intervening sequences, thus providing further evidence for the intervening sequence-mediated domain transfer hypothesis. We have discussed several possible phylogenic trees which can explain the difference of divergence in various segments of the γ genes.

INTRODUCTION

Gene duplication has been proposed to be a driving force for gene evolution in higher organisms (29). Recent studies on the cloned genes have clearly demontrated examples of gene duplication in various multi-gene families such as α and β globin, ovalbumin, amylase, immunoglobulin heavy (H) chain, interferon, chorion and vitellogenin genes (13, 17-19, 21, 26, 31, 33, 40). In case of immunoglobulin H chain genes, both variable (V) and constant (C) region genes have been derived by duplication of respective ancestral genes, evolving a family of V_H genes and eight classes and subclasses of C_H genes in mouse. Similar V_H genes, which are probably derived by more recent duplication, are shown to be clustered (16, Kataoka et al., in preparation). Molecular cloning experiments of mouse C_H genes (20, 27, 33) have demonstrated that organization of mouse C_H gene is 5'-V_H genes-(unknown distance)-J_H-(6.5 kb)-C_μ-(4.5 kb)-C_δ-(unknown distance)-$C_{\gamma 3}$-(34 kb)-$C_{\gamma 1}$-(21 kb)-$C_{\gamma 2b}$-(15 kb)-$C_{\gamma 2a}$-(14.5 kb)-C_ε-(12.5 kb)-C_α-3'. The results also indicate that more related genes such as γ subclass genes (1, 7-9, 30, 41) are clustered.

Cloning and nucleotide sequence determination of mouse immunoglobulin C_H genes (2, 6, 12, 14, 15, 32, 39, 42) have clearly demonstrated that all the C_H genes are interrupted by intervening sequences (IVSs) at the junction of the domains and the hinge region, which constitute the functional and structural units of the H chain protein. These studies indicate that the IVS was introduced into C_H genes probably during duplication of ancestral DNA segment encoding a domain to establish a multi-domain structure of the prototype C_H gene (6, 14, 32).

Comparison of the nucleotide sequences of the γ1 and γ2b genes has revealed a remarkable homology region containing the entire CH1 coding region and about the 5' half of the IVS located at the 3' side of the CH1 domain (23). These results suggest that, during the γ1 and γ2b gene evolution, a double unequal crossing-over event has taken place in the IVSs, resulting in the transfer of the DNA segment coding for the CH1 domain. Amino acid sequence comparison of the γ1, γ2a and γ2b chains also suggested a similar domain transfer between the γ2a and γ2b genes. We have proposed that such an IVS-mediated domain transfer event may have played a significant role in the evolution of many other eukaryote genes. IVS-mediated domain transfer was recently found in globin genes as well (Miyata, Nishida and Hayashida, in preparation; 35).

To test the above possibility we have determined the complete nucleotide sequence of the mouse γ2a gene and compared it with those of other immunoglobulin genes, i.e. γ1 (12), γ2b (42) and μ (15) genes. These results provide further evidence for IVS-mediated domain transfer among these γ subclass genes.

MATERIALS AND METHODS

Preparation of DNA and Nucleotide Sequence Determination

Ch·M·Igγ2a-9 (33) was digested with Eco RI and ligated with pBR322 DNA which had been digested with Eco RI and treated with bacterial alkaline phosphatase. The ligated DNA was then used to transform an E.coli strain LE392 in a P3 facility. Transformants which contain the structural sequence of the γ2a gene were screened using a nick-translated γ2b gene fragment as probe (28). Plasmid DNA was prepared as described (3). DNA sequencing was carried out according to the Maxam and Gilbert (22).

Alignment of Nucleotide Sequence and Calculation of Divergence

Nucleotide sequences of three γ subclass genes were aligned using a computer program (23). Alignment was done first between the γ2a and γ2b genes which are the most homologous pair among the three genes, and then the γ1 gene

sequence was aligned to the paired sequences. The extent of the nucleotide sequence divergence was calculated for each functional or structural unit as described (23-25). Divergences or sequence difference is defined as the number of sites which differ between aligned sequences relative to the total number of sites compared. The divergence of the noncoding region (Kn) was calculated by comparison of every nucleotide of the aligned sequences. Since lengths of noncoding regions vary considerably, gaps are introduced to maximize homology. Given no routine procedures in treating gaps, it might be appropriate to estimate the Kn value by two different approaches: in Method I gaps are excluded from comparison and in Method II a gap is considered as a substitution, Nucleotide positions consisting of more than 10 consecutive gaps are excluded from comparion in both methods. Obviously Methods I and II would give values correspoding to the lower and upper bounds, respectively, of the true Kn value. Averages of values obtained by the two methods were used in the present study. Divergence at amino acid-substituting sites (Ka) and at synonymous sites (Ks) are evaluated separately for coding regions according to the methods described previously (24).

Previously, we compared several mammalian gene sequences (25) and showed that the evolutionary rate at the synonymous sites (Vs) is 5.1×10^{-9} per site per year in average and is approximately constant among different genes within relatively a short period of time. We have also proposed that this property is suitable for a molecular clock to determine the evolutionary relationships and branching order of closely related duplicated genes. The time since divergence of duplicated genes (T) is estimated by the equation $T = Ks^c/2Vs$ where $Ks^c = -(3/4)\ln [\, 1 - (4/3)Ks \,]$. The estimation of the divergence time is accurate only for the recent events. We have set the upper limit of the meaningful value arbitrarily at 75 million years, which is the approximate divergence time between primates and rodents (25).

Materials

γ-^{32}P-ATP (spec. act. >5000Ci/mmole) was purchased from New England Nuclear (Boston, Massachusetts) and from Radiochemical centre (Amersham, U.K.). Sources of restriction endonucleases and other enzymes are as described previously (14).

RESULTS

Sequencing Strategy

Mouse immunoglobulin γ2a gene clone was previously isolated from a library

of embryonic mouse DNA by screening with a ^{32}P-labeled mouse γ2b gene as probe (33). The 3' Eco RI fragment (5.2 kb) of the clone called Ch·M·Igγ2a-9 was shown to contain the structural sequence and subcloned into the plasmid vector pBR322 to obtain a large quantity of DNA.

The detailed restriction enzyme cleavage map was constructed by a conventional procedure. The ranges and restriction sites used to determine the DNA sequence of the γ2a gene is shown in Figure 1. The nucleotide sequence was determined by the chemical modification method of Maxam and Gilbert (22). The entire 1990 base pairs (bp) of DNA sequence determined includes, in addition to the coding sequences and IVSs, the 5' region (248 bp) flanking the CH1 domain and the 3' region (119 bp) flanking the poly(A) addition site. The nucleotide sequences of the coding regions and the 3' untranslated region agree completely with those determined for the γ2a cDNA clone (34). The amino acid sequence determined for the γ2a chain protein of MOPC 173 (9) differs at 25 positions from that predicted from the nucleotide sequences. The coincidence of the nucleotide sequences determined independently in two different laboratories suggest that these differences are not due to errors in nucleic acid sequencing.

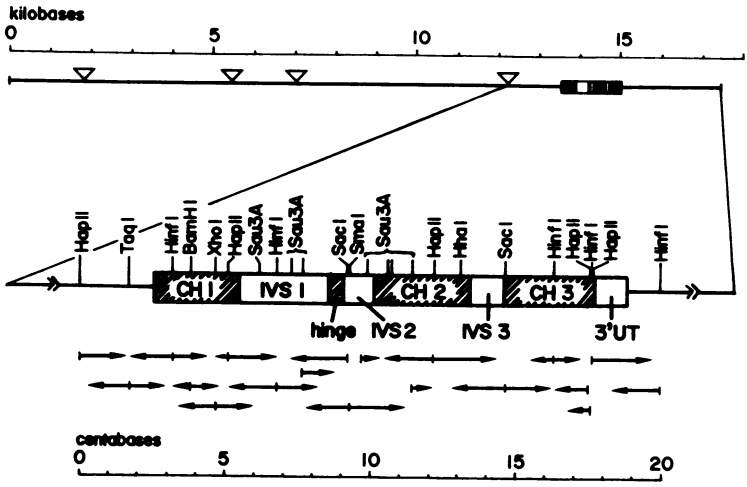

Figure 1. Diagram of Restriction Endonuclease Cleavage Sites of the γ2a Gene and Strategy for Sequencing

The top line represents the insert of the phage clone Ch4A·Ig·M·γ2a-9 (33). The 5.2 kb Eco RI fragment subcloned into pBR322 is magnified below. Wide rectangles indicate the structural gene. The direction and range of the sequences read are indicated by horizontal arrows. (IVS) intervening sequence; (UT) untranslated sequence; (▽) Eco RI site.

Sequence and General Organization of the γ2a Gene

The complete nucleotide sequence of the γ2a gene is shown in Figure 2. The sequence of the γ2a gene reveals that functional units of protein domains and the hinge region are separated by IVSs as shown in all the immunoglobulin heavy chain genes thus far examined including mouse γ1 (12), γ2b (14, 39, 42), μ (2, 11, 15), α (6) and δ (20) genes and also human μ gene (37). The lengths of homologous domains and IVSs are similar among γ subclass genes as shown in Table 1.

Figure 2. Nucleotide Sequence of the Mouse γ2a Gene

The nucleotide sequence of the strand corresponding to the mRNA is displayed 5' to 3' on the bottom row. The amino acid predicted by the nucleotide sequence is shown above the coding sequence. (Term) refers to the termination codon UGA. [poly(A)] indicated the site of poly(A) addition (34). Amino acids are expressed by a one letter code as follows: (A) alanine; (C) cysteine; (D) aspartic acid; (E) glutamic acid; (F) phenylalanine; (G) glycine; (H) histidine; (I) isoleucine; (K) lysine; (L) leucine; (M) methionine; (N) asparagine; (P) proline; (Q) glutamine; (R) arginine; (S) serine; (T) threonine; (V) valine; (W) tryptophan; (Y) tyrosine.

Table 1. Lengths of Domains and IVSs of γ Subclass Genes

Segments of Gene	γ1	γ2a (base pairs)	γ2b
CH1	291	291	291
IVS1	356	310	316
Hinge	39	48	66
IVS2	98	107	107
CH2	321	330	330
IVS3	121	112	112
CH3	321	321	321
3' UT	93	103	103

Data of the γ1 and γ2b genes are taken from Honjo et al. (12) and Yamawaki-Kataoka et al. (42), respectively. The lengths of the 3' untranslated sequence of the γ2b and γ2a genes were determined by Tucker et al. (38) and Sikorav et al. (34), respectively. UT, untranslated sequence.

Comparison of the Nucleotide Sequences of the γ1, γ2b and γ2a Genes

We have compared the nucleotide sequences of three γ subclass genes, γ1, γ2b and γ2a genes to see whether or not the divergence of the sequences varies among different segments of the genes. Insomuch as the γ2a and γ2b genes are similar to each other, the two genes were first aligned to maximize homology. Then, the γ1 gene sequence was aligned to the matched sequences using a computer program described previously (23). The aligned sequences are shown in Figure 3. The divergence of the coding region was calculated separately for synonymous (Ks) and amino acid-substituting (Ka) sites as shown in Table 2. It should be noted that nucleotide divergence at the synonymous sites of the coding regions is free from the selective constraint operating at the protein level. The divergence of the noncoding region (Kn) was calculated by comparing every nucleotide position.

The divergence of nucleotide sequences at amino acid-substituting sites is roughly consistent with that evaluated by the comparison of the amino acid sequences (23). The nucleotide sequences coding for the CH1 and CH2 domains are more conserved than that for the CH3 domain between the γ2a and γ2b genes, while the sequence coding for the CH1 domain is more conserved than those for the CH2 and CH3 domains between the γ2a and γ1 genes.

The nucleotide divergence at the synonymous sites of the coding regions and that in the noncoding regions were plotted against segments of the γ genes in Figure 4. When the γ2a and γ2b genes are compared, the segments of compared sequences are classified into three groups on the basis of the extent of

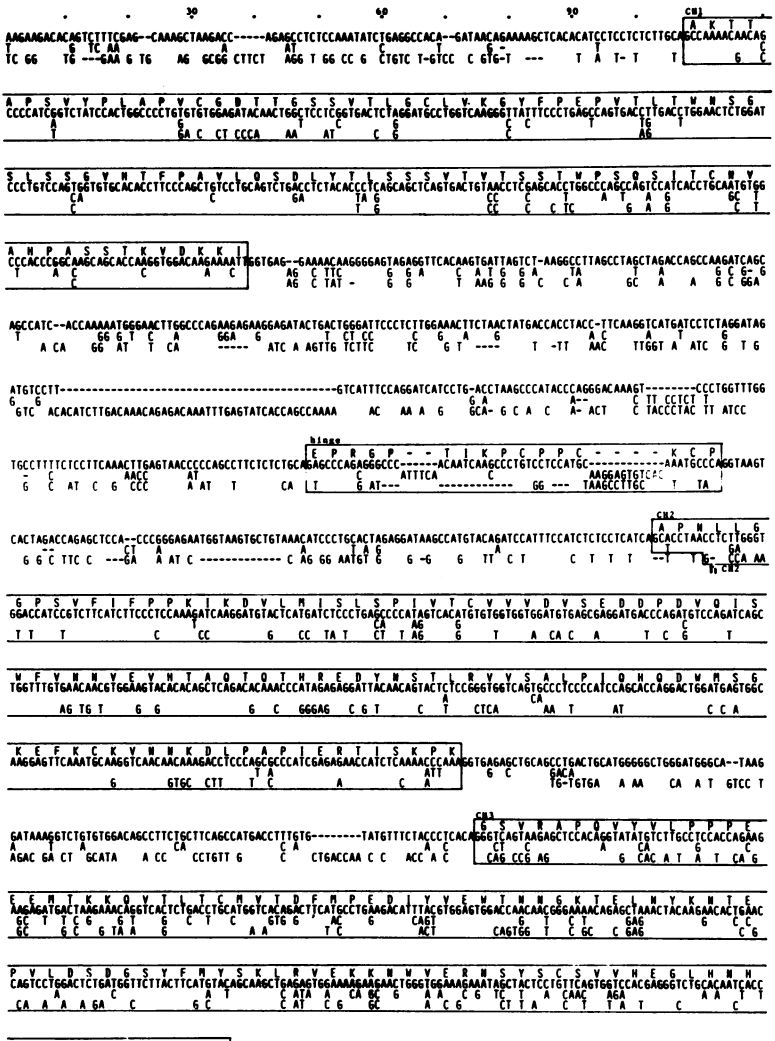

Figure 3. Aligned Nucleotide Sequences of the τ1, τ2b and τ2a Genes

The nucleotide sequences of the τ2a, τ2b and τ1 genes are aligned at the top, middle and bottom rows, respectively. Only the nucleotides which differ from those of the τ2a gene are shown for the τ1 and τ2b genes. Dashes indicate deletion inserted to maximize homology. The amino acid sequence of the τ2a gene is shown above the coding regions which are also boxed. Amino acids are expressed by a one letter code as described in the legend of Figure 2.

Table 2. Comparison of Nucleotide Sequences of the γ1, γ2a and γ2b Genes

Segment of Gene	Nucleotide Position[a]	Divergence (per site) γ2a vs γ2b		γ2a vs γ1	
		Ka	Ks or Kn	Ka	Ks or Kn
5' Flanking	1-107		0.139		0.527
CH1	108-398	0.069	0.291	0.084	0.236
IVS1 5' portion	399-513		0.269		0.306
3' portion	514-767		0.182		0.447
Hinge	768-833	0.054	0.0	0.269	0.563
IVS2	834-942 (951)[b]		0.084		0.394
CH2	943-1272 (952)[b]	0.053	0.104	0.178	0.493
IVS3	1273-1394		0.134		0.458
CH3	1395-1715	0.228	0.356	0.225	0.524
3' Flanking 5' portion	1716-1775		0.140		0.439
3' portion	1776-1880		0.076		0.241

a) nucleotide position shown in Figure 3. b) position for the γ1 gene. Ka, divergence at amino acid substituting sites; Ks, divergence at synonymous sites. Kn, divergence at noncoding regions.

divergence. The 5' flanking region, the 3' portion of IVS 1, the hinge region, IVS 2, the CH2 domain, IVS 3, and the 3' flanking region are the most conserved segments. The CH3 domain is the least conserved segment. The CH1 domain and the 5' portion of IVS 1 fall inbetween. When the γ2a and γ1 genes are compared, it is obvious that the CH1 domain and the 5' portion of IVS 1 in addition to the region surrounding the poly (A) addition site are distinctly more conserved than the other segments. The overall divergence profile obtained by the comparison of the γ2a and γ1 genes is similar to that of the γ1 and γ2b gene.

We have divied the γ gene into six segments based on the extent of divergence (Figure 4). Segment A is the 5' flanking region. Segment B includes the CH1 domain and the 5' portion of IVS 1. Segment C comprises the region between the 3' portion of IVS 1 and IVS 3. Segments D, E and F include the CH3 domain, the 3' untranslated sequence and 3' flanking region, respectively.

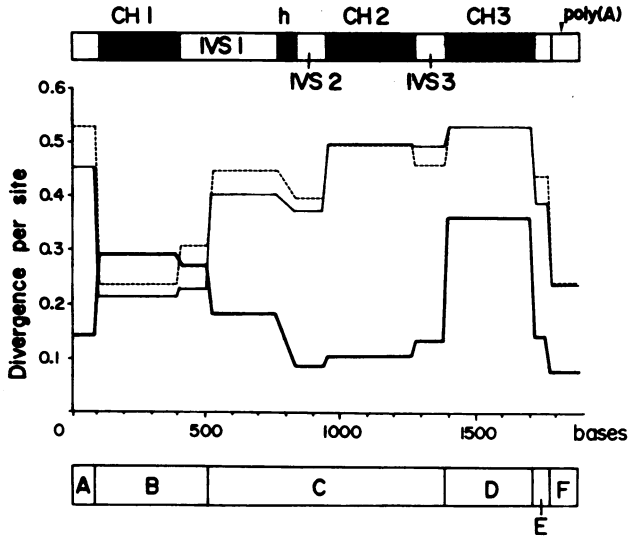

Figure 4. Nucleotide Divergence of Various Segments of the τ Genes

Divergence values (Ks and Kn) shown in Table 2 are plotted across the segment of the τ gene. Values for comparison between τ1 and τ2b genes are taken from the previous data (23) except for slight changes in noncoding regions due to re-alignment. For coding regions, divergence at the synonymous sites (Ks) alone was shown. The τ gene was divided into segments A, B, C, D, E and F depending on the extent of divergence as indicated at the bottom. Divergence values are shown by a wide line (τ2a-τ2b), a narrow line (τ2b-τ1) and a broken line (τ2a-τ1).

DISCUSSION

IVS-Mediated Domain Transfer

In most mammalian genes so far examined, amino acid-substituting sites of coding regions and the 3'portions of 3' untranslated regions show strong sequence conservation, whereas IVSs and the 5' portions of 3' untranslated regions are almost comparable to or slightly lower in divergence than synonymous sites of coding regions, the rapidly evolving sites (25). Thus, it is expected that, if no recombination events had occured between the τ genes or there are no additional constraints, the Kn value is approximately the same as the Ks value. The divergence profiles obtained by the comparison of three pairs of the τ genes (Figure 4) clearly show that there are no specific segments conserved among all the τ genes except for the 3' flanking region which is obviously under the functional constraint to conserve the nucleotide sequence surrounding the poly(A) addition site. The extent of homology is almost constant among dif-

ferent domains when the amino acid sequence of a human γ chain was compared with those of mouse γ chains (23). Therefore, it is difficult to explain varied divergence among different segments of the γ gene by selective constraint. We have proposed that the homology region might have been generated by the exchange of the DNA segment due to the relatively recent recombination events between γ subclass genes (23). The present results are also explained by unequal crossing-over at the boundary of each segment shown in Figure 4.

The recombination required for such domain exchange is a double unequal crossing-over event. Since the sequence divergence change drastically at the middle of IVS 1, one site of the unequal crossing-over seems to be located within IVS 1. The precise crossing-over point was estimated by comparison of every 50 base segment of IVS 1 along the gene sequence (Figure 5). Assuming that recombination took place at the drifting point of divergence, the crossing-over point between the γ2a and γ2b gene seems to be located at around 160 bp (= 110 bp at the starting point of the 50 bp segment) 3' to the end of the CH1 domain or position 558 of Figure 3. The crossing-over points for the other two pairs, γ1-γ2b and γ1-γ2a genes is located at around 115 bp (= 65 bp at the starting point of the 50 bp segment) 3' to the end of the CH1 domain or position 513 of Figure 3.

Judging from marked shifts in divergence at the junction of the domains and IVSs (or flanking sequences), it seems likely that other unequal crossing-over occured at points close to the boundary of the IVSs (or flanking sequences) and the domains although difficult to pin point (Figure 4). In general, the recombination within the coding region is difficult to accomplish fruitfully because it is often accompanied by deleterious changes to the structural sequences which include deletion, addition and frameshift. On the other hand, recombination within IVS is more efficient and safer means to exchange a segment (domain) of the gene. It is obvious that deletions and/or additions are rather frequent in IVSs which do not code for any protein (see Figure 3). These consideration lead us to a tentative conclusion that other crossing-over events took place at the boundary of domains and IVSs. Dunnick, Rabbitts and Milstein (5) have reported that a variant (IF2) of the γ1 chain-producing myeloma MOPC21 contains an expressed γ1 gene that has lost the CH1 domain by the recombination at the middle (position 721) of IVS 1.

It has been proposed that IVS is a trait of the flanking sequences that have been incorporated into the gene by duplication of domains or by linking different prototype genes during evolution of eukaryote genes (4, 10, 12, 14, 32). IVS may have played not only a passive role as a foreign segment for-

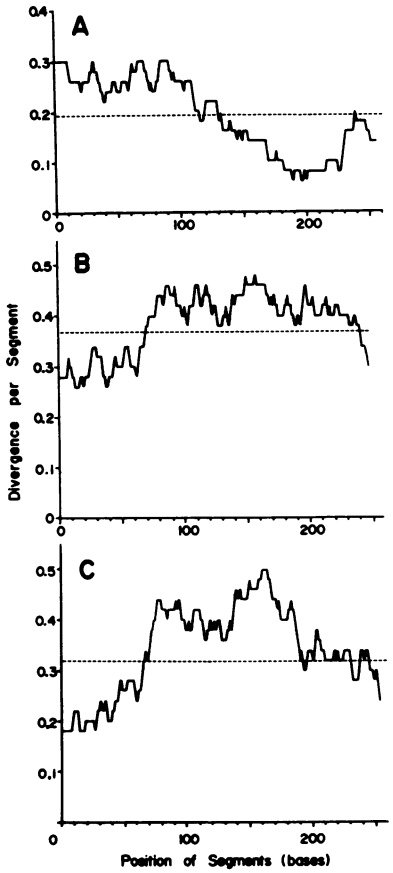

Figure 5. Comparison of Nucleotide Sequences of IVS 1 among γ1, γ2b and γ2a Genes

Homologous 50 bp long segments of IVS1 were compared between (A) γ2a and γ2b, (B) γ2a and γ1 and (C) γ1 and γ2b genes. The every 50 bp segment is used for comparison. Values are plotted against the nucleotide where the 50 bp segment starts. Dashed horizontal lines indicate average values.

tuitously incorporated into the gene but also a positive role as a mediator of domain exchange between related genes.

Evolution of Mouse γ Genes

Since the nucleotide substitution rate at the synonymous site is almost constant within relatively a short period of time regardless of the type of the mammalian genes (25), we have evaluated the time since devergence of each segment of the γ gene as summarized in Table 3. Although many other complicated

Table 3. Divergence Time of Segments of the γ Gene

Genes Compared	Time Since Divergence of Each Segment (10^6 years) B	C	D
γ2a and γ2b	36	11	≤75
γ1 and γ2a	28	≥75	≥75
γ1 and γ2b	25	≥75	≥75

Segments B, C,and D are indicated in Figure 4. Using average of the Ks values of the coding regions involved in each segment, divergence time was estimated as described in Mateials and Methods. Since the value larger than 75 million years is not accurate, the calculated value is not shown. Considering the extent of divergence (Kn) shown in Table 2, the divergence time of segments A and E may be approximately identical to that of segment C between the γ2a and γ2b genes. Similarly, the divergence times of segments A and E may be the same as those of segments C and D for the other combinations.

phylogenic trees can be drawn, we will describe relatively simple examples of the evolutionary relationship among γ genes based on the two alternative assumption. The first model assumes that the γ2a and γ2b genes were separated from each other by duplication at a relatively early stage (around 75 million years ago) or as early as the segment D (the CH3 domain) began to diverge (Figure 6A). Then, the segment B (the CH1 domain and the 5' portion of IVS1) was

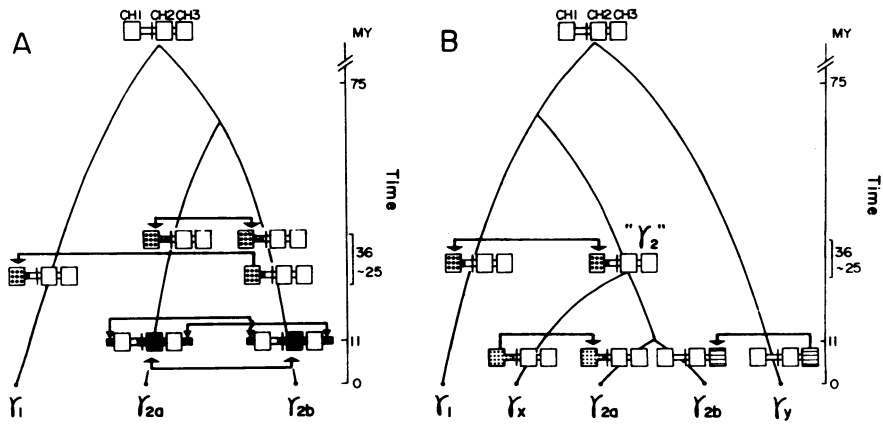

Figure 6. Evolutionary Relationship of Mouse γ Genes

Two possible phylogenic trees are shown. Horizontal lines with arrows indivate exchange or transfer of segments by recombination. Squares and narrower bars indicate domains and IVS, respectivly. See text for explanation of each pathway.

exchanged between the γ2a and γ2b genes at about 25-36 million years ago, immediately followed by the second double unequal crossing-over event to transfer the segment B from the γ2b gene to the γ1 gene. At a later stage (about 11 million years ago), the segment C was exchanged by another double unequal crossing-over event between the γ2a and γ2b genes. At about the same time, two crossing-over events took place, one at the boundary of the segments A and B and the other at the boundary of the segments D and E.

Alternatively, one can assume that the γ2a and γ2b genes were separated at a relatively recent stage (about 11 million years ago) or as recently as the segment C began to diverge (Figure 6B). After the separation of the γ2a and γ2b genes, the segments B and D were transfered from other diverged γ or related genes. We have postulated two genes γ_x and γ_y as the donors of the segments B and D, respectively. The γ_x gene, the donor of the segment B was separated from the prototype "γ2" gene at about 36 million years ago and the γ_y, the donor of the segment D arose probably more than 75 million years ago. The γ1 and "γ2" genes were separated at around 75 million years ago. Then, the segment B seems to have been exchanged between the γ1 and "γ2" genes at around 25-36 million years ago. It is possible that γ_x gene is the γ3 gene, which is homologous to the other γ genes with the decreasing order of γ2a, γ2b and γ1.(41). The segment D could heve been transfered from the γ_y gene either to the γ2a or γ2b gene. We have recently found several pseudo γ genes around the γ3 gene during molecular cloning of the whole chromosomal region containing C_H genes (Takahashi, Shimizu, Kataoka and Honjo, in preparation). One of these pseudo γ genes can be a candidate for the putative γ_y gene. It is also conceivable that the γ_y gene is one of the other C_H genes such as the γ3, ε and α genes whose sequences are not yet determined.

We prefer the latter possibility because it is simpler. Obviously, there are many other possible phylogenic trees which can explain the differential divergence of the γ gene segments. In any case, it is clear that the γ subclass genes have undergone several recombinational events within IVS resulting in the exchange of the segments of the genes.

Shift of the Splicing Site May Have Created the Hinge Region

Since primitive vertibrates posess only IgM-type immunoglobulin and higher vertibrates have IgG-type (and IgA-type) as well as IgM-type immunoglobulin, it is reasonable to assume that the γ gene has evolved from the μ gene which contains the four domains and no hinge region. When the γ1 and γ2b genes were compared with μ gene, the CH2 domain of the γ gene is more homologous to CH3 domain (about 45%) than to the CH2 domain (about 40%) of the μ gene (15). Likewise, the

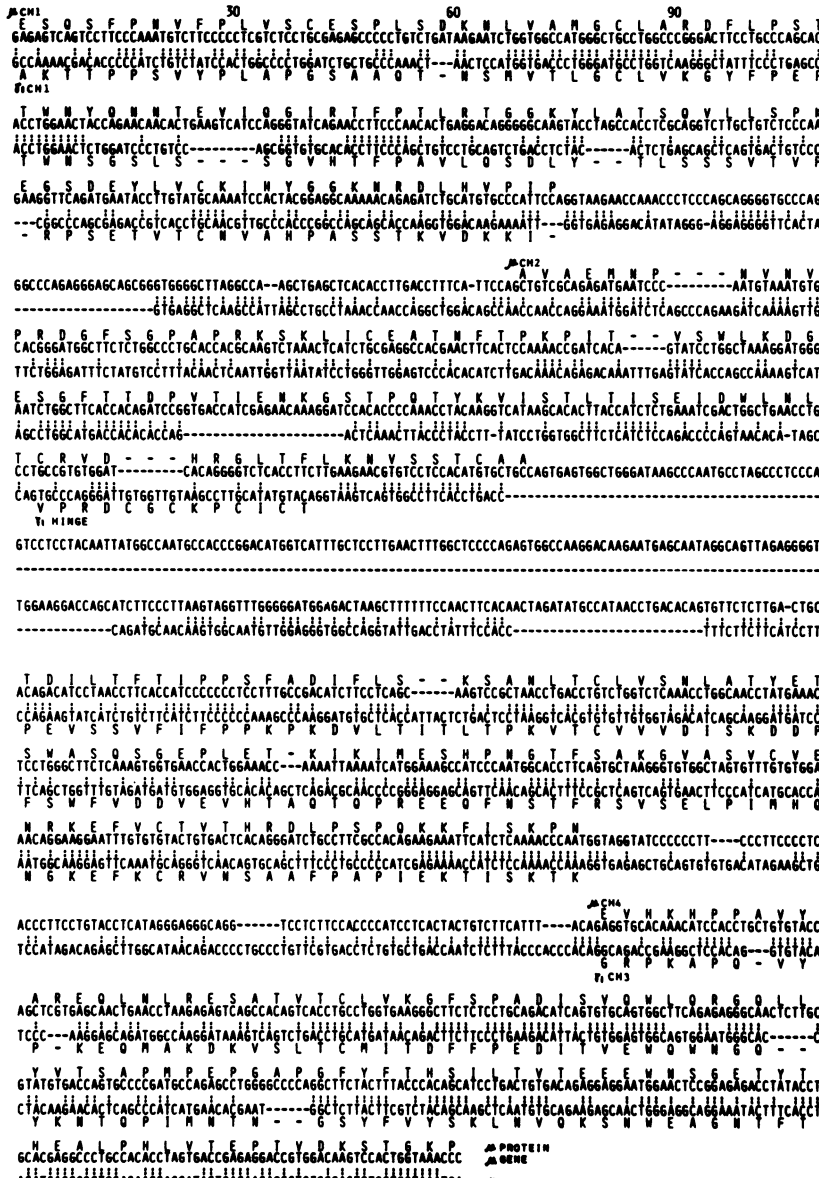

Figure 7. Aligned Nucleotide Sequences of Mouse γ1 and μ Genes

The sequence of the μ gene is shown above that of the γ1 gene. Homologous nucleotides are linked by dots. Dashes indicate deletions introduced to maximize homology. Amino acids are shown by a one letter code at the coding regions.

CH3 domain of the γ gene is more similar to the CH4 domain (about 50%) than to the CH3 domain (about 40%) of the μ gene. The results suggest that the hinge region of the γ gene might have been derived from the CH2 domain of the μ gene.

We have aligned the nucleotide sequences of the γ1 and μ genes with a computer program which is useful to align such diverged pair of the sequences. As shown in Figure 7, the hinge region is aligned to the 3' end of the CH2 domain of the μ gene. Since the IVS 1 of the γ1 gene can be paired with the CH2 domain of the μ gene, it is likely that the hinge region may have been created by shifting the acceptor site of the splicing from the 5' end of the CH2 domain to the 3' side. A similar conclusion was drawn by Tucker et al. (39) who compared the nucleotide sequence of the hinge region of the γ2b gene with those of the domains of the same gene. It is interesting that the lengths of the hinge regions vary rather extensive among γ genes while lengths of other domains remain constant.

Difference of Nucleotide Sequences of γ2b Genes

The complete nucleotide sequence of the γ2b gene was published from two laboratories (39, 42). Two sequences match very well except for positions 1-44 (Figure 3) in the 5' flanking region where two sequences diverge more than 25 percents. The most striking difference is the presence of different restriction sites; the Bgl II site (AGATCT) of Wisconsin group is replaced by the Hinf I site (AGAATCT) of our group at position 42-48.

We have reexamined our sequence and also tested whether or not the Bgl II site is present in the 5' flanking region of the γ2b gene by Southern (1976) blot experiments. Our data clearly indicate digestion with Bgl II produces a 5.3 kb fragment that hybridizes with the structural sequence of the γ2b gene. The sizes of the fragment expected from restriction maps of Wisconsin and our groups are 3.1 and 5.3 kb, respectively. The presence of two γ2b genes is unlikely because molecular cloning of the C_H gene region demonstrated the order 5'-γ3-(34 kb)-γ1-(21 kb)-γ2b-(15 kb)-γ2a-(14.5 kb)-ε-(12.5 kb)-α-3' (33). Although both groups used BALB/c mice we cannot exclude the possibility of the polymorphism of the γ2b gene.

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