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## Evolution and organization of the fibrinogen locus on chromosome 4: Gene duplication accompanied by transposition and inversion

(multigene families/gene evolution/somatic cell hybrids/*in situ* hybridization)

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**ABSTRACT** Human fibrinogen cDNA probes for the  $\alpha$ -,  $\beta$ -, and  $\gamma$ -polypeptide chains have been used to isolate the corresponding genes from human genomic libraries. There is a single copy of each gene. Restriction endonuclease analysis of isolated genomic clones and human genomic DNA indicates that the human  $\alpha$ -,  $\beta$ -, and  $\gamma$ -fibrinogen genes are closely linked in a 50-kilobase region of a single human chromosome: the  $\alpha$ -gene in the middle flanked by the  $\beta$ -gene on one side and the  $\gamma$ -gene on the other. The  $\alpha$ - and  $\gamma$ -chain genes are oriented in tandem and transcribed toward the  $\beta$ -chain gene. The  $\beta$ -chain gene is transcribed from the opposite DNA strand toward the  $\gamma$ - and  $\alpha$ -chain genes. The three genes have been localized to the distal third of the long arm of chromosome 4, bands q23–q32, by *in situ* hybridization with fibrinogen cDNAs and by examination of DNA from multiple rodent–human somatic cell hybrids. Alternative explanations for the present arrangement of the three fibrinogen genes involve either a three-step mechanism with inversion of the  $\alpha/\gamma$ -region or a two-step mechanism involving remote transposition and inversion. The second more simple mechanism has a precedent in the origin of repeated regions of the fibrinogen and immunoglobulin genes.

The major clotting factor, fibrinogen, is a complex protein of 340,000  $M_r$  composed of paired groups of three nonidentical polypeptides designated the  $\alpha$ -,  $\beta$ -, and  $\gamma$ -chains (1, 2). Significant homologies among amino acid (1, 2) and cDNA (3–6) sequences for the human  $\alpha$ -,  $\beta$ -, and  $\gamma$ -fibrinogen chains indicate that the genes encoding these polypeptides have arisen by duplication and subsequent evolutionary divergence of an ancestral gene. Data from a number of multigene families, including  $\alpha$ - and  $\beta$ -globins (7), immunoglobulin heavy (8) and light (9–11) chains, cardiac myosin heavy chains (12),  $\alpha$ -interferons (13), histones (14), and a variety of insect genes (15–18), illustrate that such duplications are often local, leading to clustering of related genes in a small region of a single chromosome. In fact, the  $\alpha$ - and  $\gamma$ -fibrinogen genes have been shown to be molecularly linked in the rat (19). In these studies we have used human fibrinogen  $\alpha$ -,  $\beta$ -, and  $\gamma$ -chain cDNA probes to isolate each gene in a single copy from human genomic libraries and show that the genes are closely linked in a small region on the long arm of chromosome 4.

### MATERIALS AND METHODS

**Materials.** Partial *Hae* III–*Alu* I and *Eco*RI human genomic libraries constructed in the bacteriophage vector Charon 4A were kindly provided by Terry Robbins (National Cancer

Institute), Philip Leder (Harvard University), and Edward J. Benz, Jr. (Yale University). Cloned genomic probes for human  $\alpha$ -fetoprotein were graciously provided by Shirley Tilghman and Rob Krumlauf (Fox Chase Institute for Cancer Research). Human fibrinogen cDNAs have been described (3).

Hybrid human–mouse and human–hamster cell lines were generated by fusing human fibroblasts with rodent hypoxanthine phosphoribosyltransferase-negative or thymidine kinase-negative fibroblasts as described (20, 21); description of each group of hybrid cell lines used is given in the table or figure legends. Restriction endonucleases were purchased from Bethesda Research Laboratories and New England Biolabs; DNase I and DNA polymerase were from Boehringer Mannheim. Unlabeled deoxynucleotide triphosphates were purchased from P-L Biochemicals; deoxynucleotide [ $\alpha$ - $^{32}$ P] triphosphates was purchased from Amersham.

**Genomic Library Screening and Mapping.** The human genomic libraries were screened according to Benton and Davis (22) by using  $^{32}$ P-labeled, nick-translated ( $>10^8$  dpm/ $\mu$ g) cDNAs for the  $\alpha$ -,  $\beta$ -, and  $\gamma$ -chains of human fibrinogen (3). DNA was prepared from isolated bacteriophage clones hybridizing with these probes according to Maniatis *et al.* (23). DNAs were digested with restriction endonucleases according to the supplier's instructions and analyzed on agarose and polyacrylamide gels.

**Chromosomal Assignment of Fibrinogen Genes.** Cell pellets from rodent–human somatic cell hybrids were prepared simultaneously for DNA isolation (24) and isoenzyme analyses. Human fibrinogen cDNAs were hybridized to nitrocellulose filters containing *Eco*RI-digested, size-fractionated cellular DNAs (25). The human chromosomes present in each hybrid cell line were determined from starch gel electrophoretic analyses (26, 27) of the isoenzyme markers that have been assigned previously to each of the human chromosomes (28).

For *in situ* hybridization studies, human metaphase and prometaphase chromosomes were prepared from methotrexate-synchronized peripheral blood cultures (29). All slide preparations were aged 1 week before use. Human fibrinogen cDNAs were nick-translated according to Lai *et al.* (30) to a specific activity of  $\approx 10^7$  cpm/ $\mu$ g using [ $^3$ H]dATP (60 Ci/mmol; 1 Ci = 37 GBq) and [ $^3$ H]dTTP (97 Ci/mmol). *In situ* hybridizations were performed according to Harper and Saunders (31) at 37°C for 12–14 hr with probe concentrations of 0.25–1.0 ng/ml in 0.3 M NaCl/0.03 M sodium citrate, 50% formamide with 10% dextran sulfate, and a 500-fold excess of carrier DNA. The slides were rinsed three times in 50% formamide/0.3 M NaCl/0.03 M sodium citrate and five

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Abbreviation: kb, kilobase(s).

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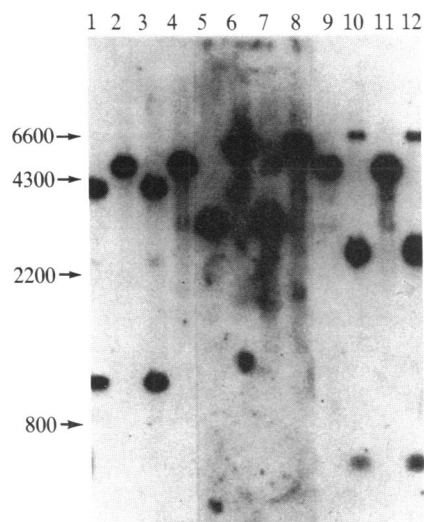


FIG. 1. Human genomic DNA probed with fibrinogen cDNAs. Human leukocyte DNA from two individuals was digested with restriction endonucleases *Hind*III (lanes 1, 3, 5, 7, 9, 11) or *Eco*RI (lanes 2, 4, 6, 8, 10, 12). Ten micrograms was run per lane on a 0.7% agarose gel, transferred to nitrocellulose, and hybridized to human  $\gamma$ - (lanes 1-4),  $\beta$ - (lanes 5-8), and  $\alpha$ - (lanes 9-12) fibrinogen cDNAs. DNA from one is in lanes 1, 2, 5, 6, 9, and 10. DNA from a second individual is in lanes 3, 4, 7, 8, 11, and 12. Molecular sizes are given at the side. The cDNAs were 2275, 855, and 736 base pairs for the  $\alpha$ -,  $\beta$ -, and  $\gamma$ -chains, respectively.

times in 0.3 M NaCl/0.03 M sodium citrate at 39°C, dehydrated, and coated with Kodak NTB2 emulsion (diluted 1:1 with water). After 5-30 days at 4°C in light-tight boxes with Drierite, the slides were developed and chromosomes G-banded as described by Chandler and Yunis (32). Grains were scored only if they touched at least one chromatid.

**RESULTS**

**Isolation of Single-Copy Fibrinogen Genes.** Three human genomic libraries were screened initially with human fibrino-

gen  $\alpha$ -,  $\beta$ -, and  $\gamma$ -cDNAs. Of  $\approx 2 \times 10^6$  plaques screened 5, 11, and 24 hybridized with the  $\alpha$ -,  $\beta$ -, and  $\gamma$ -cDNAs, respectively. Several pieces of evidence indicate that each fibrinogen gene exists in a single copy in the human genome. A variety of restriction endonucleases produced a simple hybridization pattern often with a single band on Southern transfers of genomic DNA probed with the fibrinogen cDNAs (Fig. 1). Moreover, the restriction endonuclease maps of each genomic isolate that hybridized with a single cDNA could be overlapped with all others hybridizing with the probe (see Fig. 2). In the absence of a very recent gene duplication, these data indicate that each isolate comes from a single genomic locus. Finally, each restriction fragment from leukocyte DNA that hybridized to an individual fibrinogen cDNA on Southern blots could be accounted for by an identically sized restriction fragment from the genomic clones that also hybridized with that cDNA (Figs. 1 and 2).

**Molecular Linkage of Human  $\alpha$ -,  $\beta$ -, and  $\gamma$ -Fibrinogen Genes.** Restriction endonuclease maps of the genomic isolates for individual fibrinogen genes could be overlapped, indicating that the three single-copy genes were directly adjacent *in vivo* (Fig. 2). The  $\alpha$ -chain gene is located in the center of the cluster with the  $\gamma$ -chain gene  $\approx 10$  kilobases (kb) away to one side and the  $\beta$ -chain gene  $\approx 13$  kb to the other. There was 3 kb of overlap between isolates  $\gamma_3$  and  $\alpha_1$ , as judged by identically placed sites for four restriction enzymes (all enzymes not indicated in Fig. 2). In addition, a nonrepetitive 450-base-pair *Hind*III fragment isolated from the most distal portion of clone  $\gamma_3$  in the predicted region of overlap with clone  $\alpha_1$  hybridized strongly with the expected region in  $\alpha_1$ . A much larger overlap of roughly 12 kb was seen between clones  $\alpha_2$  and  $\beta_1$ . When 4.8- and 4.3-kb *Hind*III fragments from these regions of apparent identity were subcloned independently from  $\alpha_2$  and  $\beta_1$  and compared with the five base recognition enzymes *Ava* II and *Hinf*I, an identical array of fragments was seen.

**Transcriptional Orientation of the Linked Fibrinogen Genes.** The transcriptional orientation of each human fibrinogen gene was determined by comparing hybridization patterns of the genomic isolates with full-length and 3' fragments from the  $\alpha$ - and  $\beta$ -cDNAs and by nucleotide sequenc-

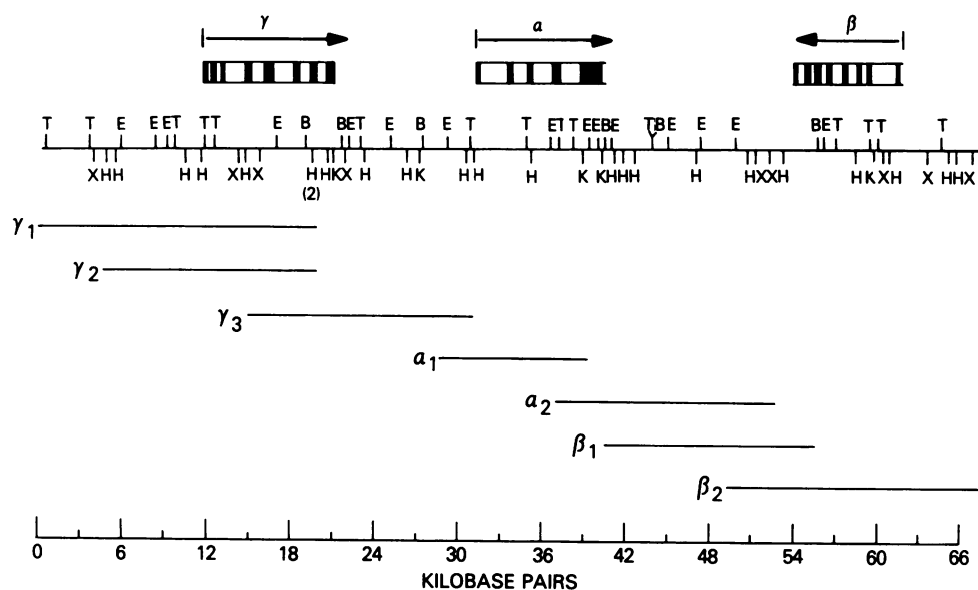


FIG. 2. Organization of the human fibrinogen gene locus. Restriction endonuclease maps are shown of representative overlapping genomic isolates that hybridized with human  $\alpha$ -,  $\beta$ -, and  $\gamma$ -fibrinogen cDNAs (3). Restriction endonucleases: E = *Eco*RI, B = *Bam*HI, T = *Sst* I, H = *Hind*III, X = *Xba* I, K = *Kpn* I. The estimated length of each gene and exon-intron positions are depicted above each gene. These lengths were determined for the  $\alpha$ -chain gene based on hybridization with the nearly full-length cDNA and comparison to the rat  $\alpha$ -gene sequence (33), for the  $\beta$ -chain gene based on cDNA hybridization and the results of Chung *et al.* (5), and for the  $\gamma$ -chain gene based on cDNA hybridization and nucleotide sequence studies (34, 35).

ing for the  $\gamma$ - and  $\beta$ -chain genes (34, 35). The  $\gamma$ - and  $\alpha$ -chain genes are transcribed in the same direction toward the  $\beta$ -chain gene. In contrast, the  $\beta$ -chain gene is transcribed in the opposite direction toward the  $\alpha$ - and  $\gamma$ -chain genes (Fig. 2).

**Localization of Human Fibrinogen Genes on Chromosome 4.** The three human genes were mapped to chromosome 4 by analysis of a series of human–mouse and human–hamster somatic cell hybrid lines. Each cell line's human chromosomal composition was characterized by isoenzyme analysis. DNA from each was digested with *EcoRI* and analyzed on Southern blots with fibrinogen cDNAs using the characteristic 2500-, 6000-, and 5000-base-pair bands as respective markers for the  $\alpha$ -,  $\beta$ -, and  $\gamma$ -chain genes (e.g., Fig. 1). Preliminary experiments on 32 such lines showed that the three genes segregated together (data not shown). These and subsequent analyses of DNAs from 35 additional hybrid lines using the  $\alpha$ -cDNA probe alone showed no discordancy with human chromosome 4 (Table 1). A typical experiment in Fig. 3 compares hybridization using the  $\alpha$ -cDNA probe with chromosomal composition by isoenzyme analysis; only chromosome 4 showed a match.

*In situ* hybridization of fibrinogen cDNAs to human metaphase chromosomes further localized each of the three genes approximately two-thirds of the way down the long arm of chromosome 4, bands q23–q32. Fig. 4 displays the localization of silver grains on particular human chromosomes. An average of 1.6 silver grains was observed in the 71 metaphases analyzed. Grains were located on 4q in 45% of the metaphases. Of the grains on chromosome 4, 73% were located in bands q23–q32 for a total of 23% of all grains counted. This represented an 11.3-fold increase over background counts or normal distribution along other chromosomes.

## DISCUSSION

It is clear from these studies that the genes encoding the  $\alpha$ -,  $\beta$ -, and  $\gamma$ -chain polypeptides of human fibrinogen occur as single copies closely linked in a roughly 50-kb region of chromosome 4, bands q23–q32. The chromosomal assignment agrees with previous reports for the  $\gamma$ -chain gene by family studies (36) and a report by Henry *et al.* using cDNA clones and somatic cell hybrids (37). The arrangement of all genes

Table 1. Segregation of human  $\alpha$ -fibrinogen with human chromosome 4

Series	Total hybrids	$\alpha$ -Fibrinogen positive	% discordancy	
			Chromosome 4	Other chromosomes
A	16	6	0	$\geq 25^a$
B	34	18	0	$\geq 26^b$
C	17	10	0	$\geq 24$

Human  $\alpha$ -fibrinogen cDNA was hybridized to nitrocellulose filters containing *EcoRI*-digested, size-fractionated cellular DNA from a number of hybrid cell lines. Series A represents hybrids isolated after fusing human fibroblasts containing a reciprocal X:14 chromosome translocation (GM0073) with hypoxanthine phosphoribosyltransferase-negative Chinese hamster fibroblasts as described (21). Series B represents subclones of one human–hamster and three human–mouse hybrid cell lines. Series C is described in the legend to Fig. 3. Independent segregation of a human chromosome and human  $\alpha$ -fibrinogen represents discordancy. Detection of hybridizing bands of the size expected from genomic clones (see Fig. 1) correlated with the presence or absence of human chromosome 4 in all hybrids when compared to the isoenzyme profile (26, 27); the presence of chromosome 4 was corroborated in these lines by positive hybridization with cloned genomic probes to  $\alpha$ -fetoprotein. The minimal discordancy between segregation of all other human chromosomes and  $\alpha$ -fibrinogen is shown with the following exceptions: a, 13% discordancy with chromosome 17; b, single discordancy (3%) with chromosome 3.

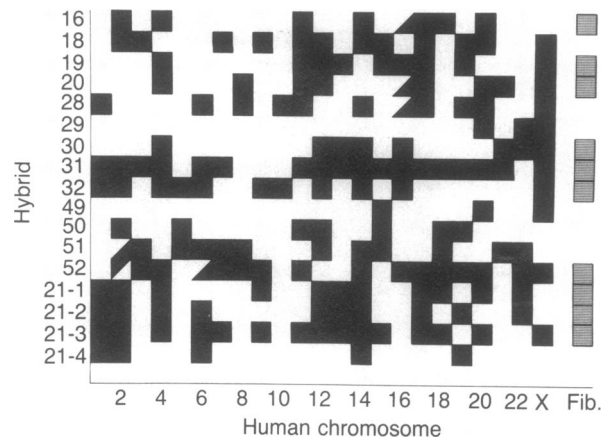


Fig. 3. Distribution of the human fibrinogen gene and chromosomal composition in human–rodent hybrid cell lines. Individual hybrid cell lines are represented on the ordinate and specific human chromosomes are represented on the abscissa. Solid boxes indicate the presence of a particular human chromosome in a hybrid line and open boxes indicate absence of the chromosome. The presence of human  $\alpha$ -fibrinogen (Fib.) sequences is shown by stippled boxes. The symbols ▽ and ▲ indicate the presence of markers for the short or long arm of a chromosome, respectively. Lines 16, 18–20, and 28 are human–mouse primary hybrid cell lines described previously (20). Lines 29–52 were isolated after fusing well-characterized human fibroblasts containing a reciprocal 2:6 chromosome translocation (GM2658) with Chinese hamster fibroblasts. Lines 21-1 to 21-4 are four subclones of a human–mouse hybrid line.

encoding multichain protein in a small region of a single chromosome is somewhat unusual in eukaryotic systems. The genes that form such proteins are generally found on separate chromosomes, as exemplified by globins (7), immunoglobulins (8–11), and thyrotropin (38).

The tight clustering of the three genes is reminiscent of that seen in a number of eukaryotic gene families that have arisen by duplication and divergence from an ancestral gene (7–18). It is clear from amino acid and nucleotide sequence homologies among the three fibrinogen genes (1–6) as well as similarities of exon–intron domains at the 5' ends of the genes (33) that the  $\alpha$ -,  $\beta$ -, and  $\gamma$ -fibrinogen genes have evolved in this fashion. An ancestral gene duplicated to form the  $\alpha$ -chain gene and a pre $\beta$ - $\gamma$ -gene  $\approx 1$  billion years ago. The pre $\beta$ - $\gamma$ -gene then duplicated somewhat more than 500 million years ago to yield individual  $\beta$ - and  $\gamma$ -genes (1, 33).

Given the fact that the  $\beta$ - and  $\gamma$ -chain genes are products of a more recent duplication than the  $\alpha$ -chain gene, several aspects of fibrinogen gene organization are unexpected. First, the presence of the  $\alpha$ -gene between the more closely related  $\beta$ - and  $\gamma$ -genes rather than at one end of the cluster is not predicted by simple duplication or unequal crossing over at meiosis. Second, the opposite transcriptional orientation of the  $\beta$ - and  $\alpha$ -genes is again not predicted by simple duplication or unequal crossing over. Tandem orientation of each gene in the same direction, head to tail, is the rule for most mammalian multigene families (7–14); however, it seems likely that other gene families with divergently oriented transcriptional units will be described, given the example of fibrinogen and the presence of a number of divergently oriented genes in prokaryotes (39) and insects (15–18).

Alternative mechanisms explaining the arrangement and transcriptional orientation of the human fibrinogen genes are presented in Fig. 5. The more conventional and more complex model involves the three steps shown in Fig. 5A. The first two steps are gene duplications, and the third step involves inversion of the  $\alpha/\gamma$ -regions. An alternative simpler mechanism involving only two steps is shown in Fig. 5B and is suggested by the presence within fibrinogen and immuno-

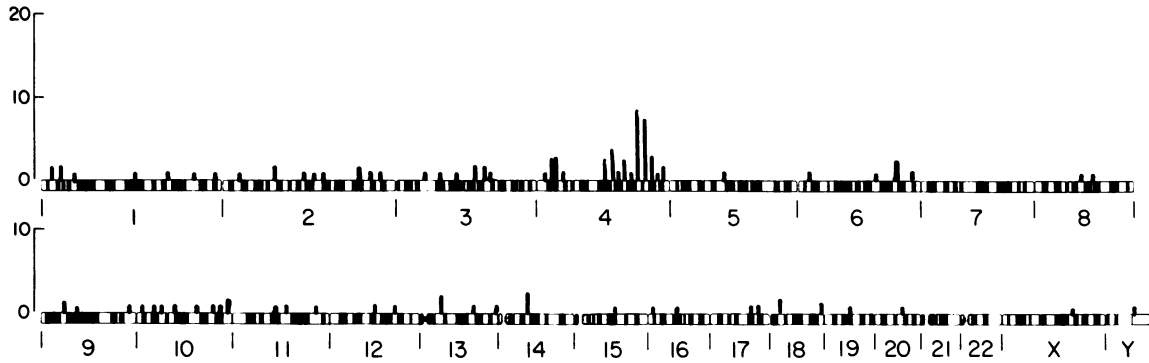


FIG. 4. Chromosomal localization of the human fibrinogen genes by *in situ* hybridization. Distribution of grains counted in 71 metaphases hybridized with human fibrinogen cDNA; 23% of all grains were located in bands 4q23-4q32.

globulin genes of small duplications flanked by single-copy inverted repeats (34). These structures appear to have arisen about 10-20 million years ago since a similar degree of homology exists between the two arms of the inverted repeat and between the duplicated sequence and its origin. These previously described duplications suggest a mechanism for the organization of the fibrinogen genes involving, first, gene duplication to produce the  $\alpha$ - and  $\beta/\gamma$ -precursors and, second, duplication of the  $\beta/\gamma$ -precursor accompanied by remote transposition and inversion. The lack of inverted repeats in the published fibrinogen gene sequence (33, 34) is easily explained since the arms of the inverted repeat evolve under no selective pressure and hence would have disappeared in the 500-1000 million years since the second duplication.

Since the  $\alpha$ -,  $\beta$ -, and  $\gamma$ -chains are present in fibrinogens spanning a wide variety of species from lampreys to primates, differences in the organization of the fibrinogen complex in distantly related species will be of interest. Note that the model presented in Fig. 5A predicts the existence of spe-

cies with all three genes in the same transcriptional orientation. The linkage and orientation of  $\alpha$ - and  $\gamma$ -genes in rats are the same as in humans (19), although examination of 8-10 kb at the 3' end of both the  $\beta$ - and  $\alpha$ -genes and 15-20 kb at the 5' ends of both the  $\beta$ - and  $\gamma$ -genes has not yet shown linkage of the  $\beta$ -gene.

The mRNAs for the three fibrinogen genes are coordinately regulated (40) at the level of transcription (unpublished data). The close chromosomal linkage of the three fibrinogen genes reported in this paper suggests that regional chromosomal influences and/or the homologous regions at the 5' ends of each fibrinogen gene (41) could play a role in coordinating the activity of these three genes. However, the opposing transcriptional orientation of the three genes proves that the coordinate expression of the fibrinogen genes is not related to the production of a single nuclear transcript encompassing all three genes.

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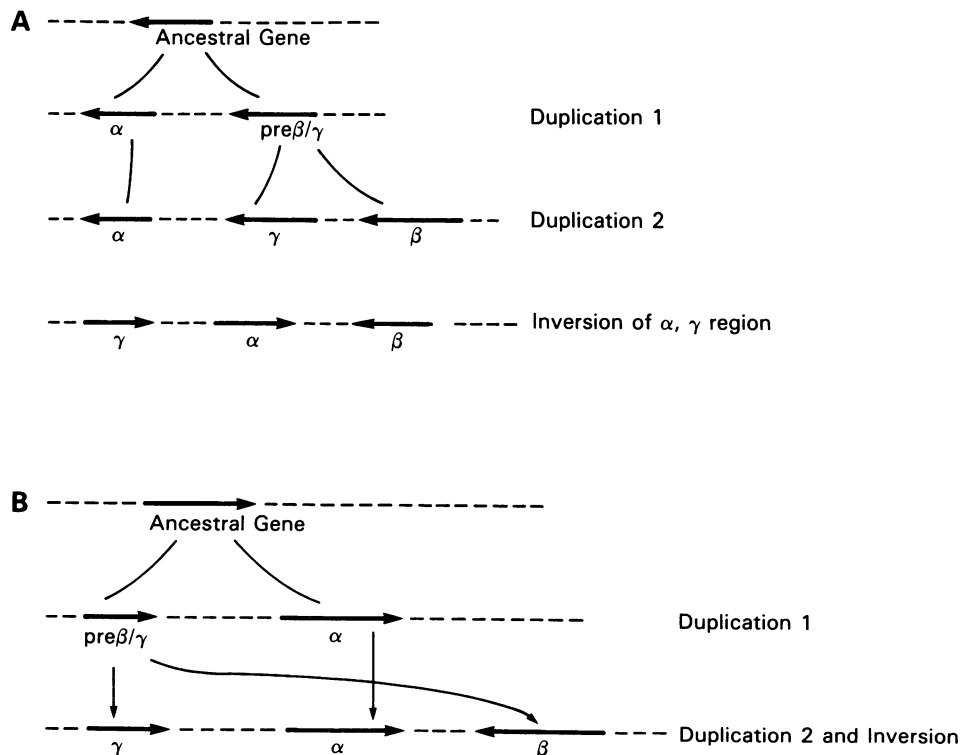


FIG. 5. Alternative mechanisms for the present arrangement of the fibrinogen genes. Note that the first mechanism postulates the existence of species that do not have the inverted  $\beta$ -chain.

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