

# DNA inversion within the apolipoproteins AI/CIII/AIV-encoding gene cluster of certain patients with premature atherosclerosis

(apolipoprotein gene fusion/deficiency of apolipoproteins AI/CIII/chromosome 11/recombination)

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**ABSTRACT** The genes coding for apolipoproteins (apo) AI, CIII, and AIV, designated *APOA1*, *APOC3*, and *APOA4*, respectively, are closely linked and tandemly organized in the long arm of the human chromosome 11. A DNA rearrangement involving the genes encoding apoAI and apoCIII in certain patients with premature atherosclerosis has been associated with deficiency of both apoAI and apoCIII in the plasma of these patients. Structural characterization of the genes for apoAI and apoCIII in one of these patients indicates that this rearrangement consists of a DNA inversion containing portions of the 3' ends of the apoAI and apoCIII genes, including the DNA region between these genes. The breakpoints of this DNA inversion are located within the fourth exon of the apoAI gene and the first intron of the apoCIII gene. Thus, this DNA inversion results in reciprocal fusion of the apoAI and apoCIII gene transcriptional units. Expression of these gene fusions in cultured mammalian cells results in stable mRNA transcripts with sequences representing fusions of the apoAI and apoCIII mRNAs. These results indicate that absence of transcripts with correct apoAI and apoCIII mRNA sequences causes apoAI and apoCIII deficiency in the plasma of these patients and suggest that these apolipoproteins are involved in cholesterol homeostasis and protection against premature atherosclerosis.

Noncovalent association of lipids with certain plasma proteins (apolipoproteins) results in formation of lipid-protein complexes (lipoproteins), the major carriers of lipids in the plasma. The concentration of high density lipoprotein (HDL) in the plasma is inversely correlated with the risk for coronary artery disease (1). HDL promotes cholesterol efflux from cells (reverse cholesterol transport) and by this process may protect against atherosclerosis (2). The major protein constituent of HDL is apolipoprotein AI (apoAI) derived from the liver and from the surface of lymph triglyceride-rich lipoprotein particles (chylomicrons) during hydrolysis of these particles upon their entry in the circulation (reviewed in ref. 3). In addition, apoAI activates lecithin:cholesterol acyltransferase, an enzyme thought to play a key role in reverse cholesterol transport (4, 5). A less prominent protein constituent of HDL is apolipoprotein CIII (apoCIII), which is actively exchanged between HDL and triglyceride-rich lipoprotein particles and appears to inhibit both the hydrolysis of these particles by the enzyme lipoprotein lipase and the apolipoprotein E (apoE)-mediated uptake of remnants of these particles by the liver (3).

The genes coding for apoAI, apoCIII, and another apolipoprotein, apolipoprotein AIV (apoAIV), are closely linked and tandemly organized in the long arm of human chromosome 11 (6) and are designated *APOA1*, *APOC3*, and *APOA4*, respectively. It has been shown recently that the organization of these genes in the rat is remarkably similar to that in

humans, and it was proposed that these genes are similarly organized in the genomes of all mammals (7).

Previous studies indicated that an inherited DNA rearrangement involving the genes for apoAI and apoCIII but not the gene for apoAIV in certain patients with premature atherosclerosis is associated with deficiency of apoAI, apoCIII, and HDL in the plasma of these patients (8-10). This report shows that the DNA rearrangement in the genome of one of these patients is due to inversion of a 6.0-kilobase (kb) DNA segment containing portions of the 3' sequences in the genes for apoAI and apoCIII including the DNA region between these genes. Because of the close physical linkage and convergent transcription of these genes in the normal human genome (6), this DNA inversion results in reciprocal fusion of portions of 5' apoAI with 3' apoCIII and 5' apoCIII with 3' apoAI gene transcriptional units. Expression of these gene fusions in cultured mammalian cells results in mRNAs with sequences representing fusions of the apoAI and apoCIII mRNAs. These results indicate that absence of transcripts with correct apoAI and apoCIII mRNA sequences causes deficiency of apoAI and apoCIII in these patients and suggest that these apolipoproteins are involved in HDL metabolism and protection against premature atherosclerosis.

## MATERIALS AND METHODS

**Genomic Libraries, Hybridization Probes, Restriction Mapping, and Heteroduplex Mapping Analyses.** Human genomic DNA libraries in the cloning vector EMBL3 were constructed by using peripheral blood lymphocyte DNA (11, 12). Screening of these libraries, purification of positive clones, and preparation of clone DNA were carried out as described (12). Hybridization probes were prepared using cloned DNA fragments as detailed (13). DNA restriction mapping was carried out by digestion with various restriction enzymes (New England Biolabs), electrophoresis in 1% agarose gels, transfer onto nitrocellulose filters, and hybridization with various DNA probes (14). Heteroduplex mapping was carried out by the formamide-spreading technique (15).

**Nucleotide-Sequencing Determinations.** DNA sequencing was carried out by subcloning relevant DNA fragments in bacteriophage M13 vectors (16) and using the resulting single-stranded DNA templates for nucleotide sequencing by the dideoxy-chain-termination method (17).

**Plasmid Constructions, Transfection of Gene Constructs into Mammalian Cells, RNA Hybridization, and S1-Nuclease and Primer-Extension Analyses.** An  $\approx$ 250-base-pair (bp) *HincII*-*Pvu* II DNA fragment containing the 72-bp and 21-bp repeats of the simian virus 40 (SV40) enhancer but not the "TATA box" of the early promoter of this virus was ligated by using

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Abbreviations: apoAI, apoCIII, and apoAIV, apolipoproteins AI, CIII, and AIV; HDL, high density lipoprotein; SV40, simian virus 40.

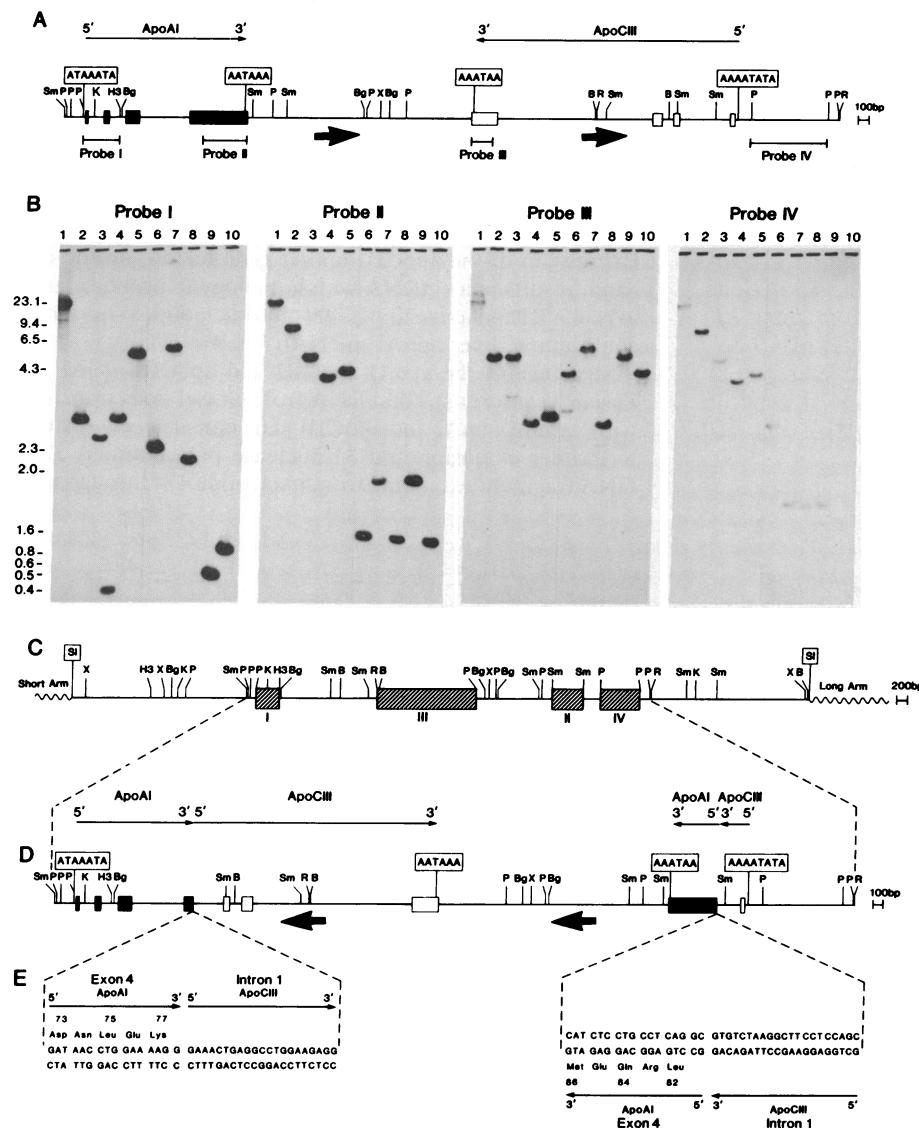
*Bgl* II linkers into the *Bam*HI site of the plasmid pUC9 (18). The resulting vector (pUC9-SV) was selected so that the 72-bp repeats of the enhancer are proximal to the *Pst* I site in the polylinker region of pUC9. DNA fragments containing the normal genes for apoA-I and apoC-III and those derived from the apoAI/apoCIII-deficient patient were isolated by *Pst* I digestion of relevant genomic clones and subcloned in the *Pst* I site of pUC9-SV. The resulting constructs were selected so that the TATA box in these genes is proximal to the 72-bp repeats. These plasmids were purified by alkaline lysis (19), chromatography in Sepharose 4B (Sigma) columns, extraction with phenol, and precipitation with ethanol and were used to transfect cultured monkey kidney COS-1 cells by the calcium phosphate technique (20, 21). Forty-eight hours after transfection, these cells were harvested and used to prepare total RNA (22). This RNA was used for blotting and hybridization and for S1-nuclease and primer-extension analyses (23–25).

## RESULTS

**Inversion of apoAI and apoCIII Gene Sequences in the Genome of a Patient with apoAI-apoCIII Deficiency and Premature Atherosclerosis.** A genomic DNA library was constructed with DNA extracted from peripheral blood from one of the apoAI/apoCIII-deficient patients and screened with an apoAI gene probe (6). One of the 11

identified positive clones ( $\lambda$ B7) was mapped with restriction enzymes (Fig. 1C) and hybridized with apoAI and apoCIII gene probes. The relative location of these probes (probes I–IV) with respect to the normal genes for apoAI and apoCIII is shown in Fig. 1A. The resulting hybridization patterns (Fig. 1B) are identical to those obtained by similar analysis of DNA from blood taken from this patient (data not shown; ref. 9). These results show that the arrangement of DNA segments with hybridization homology to these probes in  $\lambda$ B7 is I–III–II–IV (Fig. 1C), while that in the normal human genome is I–II–III–IV (Fig. 1A). Therefore, a DNA segment containing sequences homologous to probes II and III is inverted in the genome of the apoAI/apoCIII-deficient patient. Comparison of the restriction map of  $\lambda$ B7 (Fig. 1C) with that of the normal human genome (Fig. 1A) indicates that this DNA inversion is 6.0 kb long and that it contains portions of the 3' sequences in the apoAI and apoCIII genes including the DNA region between these genes. This comparison also shows that the organization of the remaining 5' portions, promoter, and 5' flanking regions of these genes is not affected by this DNA inversion.

**The DNA Inversion Is Confined Within the apoAI–apoCIII Gene Region.** A clone ( $\lambda$ J5) containing the apoAI and apoCIII genes in their normal arrangement (see Fig. 1A) was isolated from a genomic library constructed with DNA from blood taken from a normal (with regard to coronary artery disease) donor. This clone contains 8.5 kb and 2.5 kb of the 5' flanking



**FIG. 1.** (A) Restriction map of the normal genes encoding apoAI and apoCIII. Direction of transcription (arrows), TATA boxes and polyadenylation signals (boxed), and exons in the apoAI (filled boxes) and apoCIII (open boxes) genes are indicated. The scale of the map is shown by a 100-bp bar. Restriction sites: X, *Xba* I; H3, *Hind*III; Bg, *Bgl* II; K, *Kpn* I; P, *Pst* I; Sm, *Sma* I; and R, *Eco*RI. DNA fragments used for probes are indicated by lines below the map. *Alu* I repetitive DNA elements (27) are shown by thick arrows. (B) Clone  $\lambda$ B7 DNA was digested with the enzymes (abbreviated as above plus S1 for *Sal* I) S1, S1/Bg, Bg/K, Bg/R, X/R, Sm, P, P/Sm, P/H3, and Sm/H3, electrophoresed in gel lanes 1–10, respectively, blotted, and hybridized with probes I, II, III, and IV. The resulting autoradiograms are shown. The migration of DNA size markers is indicated by numbers along the side of the autoradiograms. (C) Restriction map of clone  $\lambda$ B7. The arms of the cloning vector (EMBL3) are shown by wavy lines. Restriction sites are abbreviated as above. Sites at the junctions of the vector and recombinant DNA are boxed. The scale of the map is indicated by the 200-bp bar. DNA segments with hybridization homology to probes I, II, III, and IV are indicated by hatched boxes. (D) Restriction map of the apoAI–apoCIII gene region in clone  $\lambda$ B7. All symbols and notations are as in A. (E) Nucleotide sequences at the junctions of the DNA inversion in clone  $\lambda$ B7.

regions of the apoAI and apoCIII genes, respectively (data not shown). The clone  $\lambda$ B7 contains 6.5 kb and 5.0 kb of the 5' flanking regions of the apoAI and apoCIII genes, respectively (Fig. 1C). The DNA inserts in these clones have the same orientation with respect to the arms of the cloning vector. Comparison of the structure of these clones by heteroduplex mapping shows that, with the exception of the 6.0-kb DNA inversion in clone  $\lambda$ B7, the remaining sequences are very similar between these clones (Fig. 2). These results indicate that the DNA inversion is confined within the apoAI-apoCIII gene region of these patients.

**The DNA Inversion Results in Reciprocal Fusion of Portions of the apoAI and apoCIII Genes.** The sequences at the junctions of the DNA inversion in clone  $\lambda$ B7 were determined (Fig. 1E). The results show that as a consequence of this DNA inversion, the 3' end of a portion of the apoAI gene exon 4 (26) is linked to the 5' end of a portion of the apoCIII gene intron 1 (27), while the 3' end of a portion of the apoCIII gene intron 1 is linked to the 5' end of a portion of the apoAI gene exon 4 (Fig. 1D). The sequences flanking the junctions of this DNA inversion were aligned and compared with the corresponding sequences in the normal human genome. This comparison shows that 9 nucleotides in the apoAI gene exon 4 (AGACAGAGG) and 21 nucleotides in the apoCIII gene intron 1 (CCAGTCCTACCCAGACAGG) have been deleted in clone  $\lambda$ B7 (Fig. 3). Therefore, this DNA inversion results in fusion of nucleotide 1 in codon 78 of the message translated into the mature apoAI amino acid sequence with nucleotide 305 in the apoCIII gene intron 1 (apoAI-apoCIII fusion) and fusion of nucleotide 283 in the apoCIII gene intron 1 with nucleotide 2 in codon 81 of the message translated into the mature apoAI amino acid sequence (apoCIII-apoAI fusion) (Fig. 3). It also should be noted that translation of the apoAI-apoCIII fusion, according to the frame of apoAI, results in a termination codon (boxed TGA) located two codons 3' to codon 77 in apoAI (Fig. 3).

**Expression of the apoAI-apoCIII and apoCIII-apoAI Gene Fusions Results in Stable mRNA Transcripts.** *Pst* I DNA fragments of 2.2 kb and 4.5 kb containing the apoAI and apoCIII genes, respectively, were isolated from clone  $\lambda$ J5 (see Fig. 1A) and subcloned in the plasmid vector pUC9-SV. Similarly, *Pst* I DNA fragments of 5.5 kb and 1.4 kb containing the apoAI-apoCIII and apoCIII-apoAI gene fusions, respectively, were isolated from clone  $\lambda$ B7 (Fig. 1D)

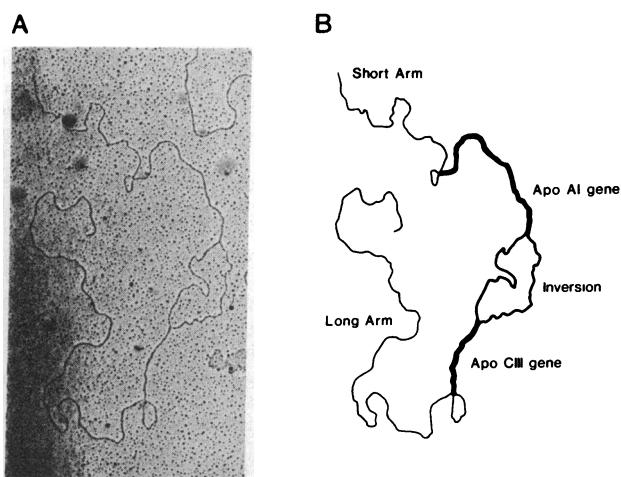


FIG. 2. (A) Electron micrograph of the heteroduplex formed between clones  $\lambda$ B7 and  $\lambda$ J5. (B) Drawing of the heteroduplex in A showing similarities (thick lines) and differences (thin lines) of the apoAI-apoCIII gene region of the recombinant DNA inserts in clones  $\lambda$ B7 and  $\lambda$ J5. The junctions between the arms of the cloning vector (EMBL3) and the DNA inserts in these clones are the indicated loops.

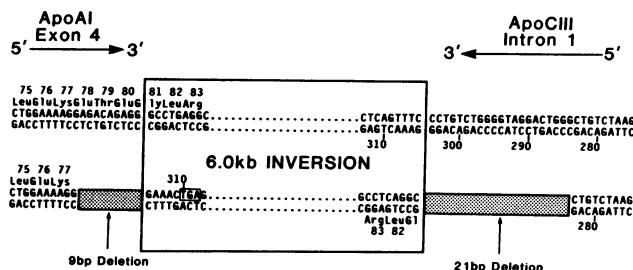


FIG. 3. Alignment of nucleotide sequences at the junctions of the DNA inversion in clone  $\lambda$ B7 (lower sequence) with the corresponding sequences in the normal human genome (upper sequence). Direction of transcription is indicated by arrows. The amino acid sequence encoded by the portion of apoAI gene exon 4 shown is numbered according to the first amino acid in the mature plasma apoAI. A termination codon in frame with the apoAI translation frame is indicated by a boxed TGA. The nucleotide sequence in the portion of apoCIII gene intron 1 shown is numbered according to the first nucleotide at the 5' end of this intron in the normal human apoCIII gene (27). The DNA segment that is inverted in clone  $\lambda$ B7 and the corresponding segment in the normal human genome are boxed. The nucleotide sequences deleted in clone  $\lambda$ B7 are indicated by stippled boxes.

and subcloned in pUC9-SV. The resulting gene constructs were transfected into monkey kidney COS-1 cells, and total RNA from these cells was studied by blotting and hybridization with probes containing the last exon of the apoAI (probe II) and apoCIII (probe III) genes (Fig. 1A). The resulting autoradiograms show that expression of the apoAI and apoCIII gene constructs results in mRNAs with sizes very similar to the corresponding mRNAs in adult human liver (Fig. 4). In contrast, expression of the apoAI-apoCIII gene construct results in a mRNA with homology to probe III but not to probe II and a size that is 400–500 nucleotides larger than adult human liver apoCIII mRNA (Fig. 4). Expression of the apoCIII-apoAI gene fusion, on the other hand, results in an mRNA with homology to probe II but not to probe III and a size that is 400–500 nucleotides shorter than adult human liver apoAI mRNA (Fig. 4).

**Structure of the apoAI-apoCIII and apoCIII-apoAI Gene Fusion Transcripts.** Total RNA from COS-1 cells transfected with various apoAI and apoCIII gene constructs was studied by primer extension and S1-nuclease protection. A 121-bp DNA fragment extending from nucleotide 474 through nucle-

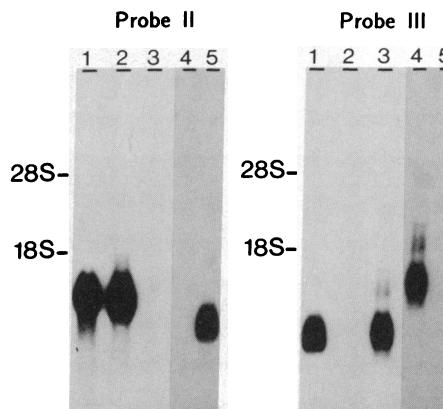


FIG. 4. Total RNA (20  $\mu$ g) prepared from adult human liver (lane 1) and COS-1 cells transfected with apoAI (lane 2), apoCIII (lane 3), apoAI-apoCIII (lane 4), and apoCIII-apoAI (lane 5) gene constructs were blotted and hybridized with probes containing the last exon of the apoAI (probe II) and apoCIII (probe III) genes (see Fig. 1A). The resulting autoradiograms are shown. The relative electrophoretic migration of 28S and 18S rRNAs is indicated.

otide 594 in the apoAI gene exon 3 and a 276-bp cDNA fragment spanning 56 bp of exon 3, the entire exons 2 (63 bp) and 1 (17 bp), the TATA box and 29 bp of the 5' flanking region in the apoAI gene (including 23 G-C residues), and 59 bp of plasmid pBR322 sequences (26) were used as primer-extension and S1-nuclease-protection probes, respectively. The results show that the sizes of nucleotide fragments obtained with RNA from the apoAI-apoCIII fusion are similar to those obtained with RNA from either the apoAI gene construct or adult human liver (Fig. 5A and B). In addition, S1-nuclease-protection analysis using as probe a 1260-bp cDNA fragment spanning 265 bp of exon 4, the entire exons 3 (124 bp) and 2 (67 bp), part of exon 1 (16 bp) in the apoCIII gene, and 789 bp of pBR322 sequences shows that the size of the protection fragment obtained with RNA from the apoAI-apoCIII fusion is very similar to that obtained with RNA from either the apoCIII gene construct or adult human liver (Fig. 5C). However, S1-nuclease analysis using as probe a 2000-bp *Sma* I DNA fragment spanning 22 bp of the apoCIII gene exon 2, 321 bp of the apoCIII gene intron 1, the entire apoAI gene region in the apoAI-apoCIII fusion, and 250 bp of SV40 sequences indicates that only RNA from the apoAI-apoCIII fusion protects a 450-nucleotide-long fragment (Fig. 5D). These results indicate that the transcription start site of the apoAI-apoCIII fusion mRNA is very similar to that of the normal apoAI mRNA and that the apoAI gene introns 1, 2, and 3 and apoCIII gene introns 2 and 3, present in the primary transcript of this fusion, are spliced similarly to the corresponding introns in the normal apoAI and apoCIII genes (Fig. 5F). These results also show that neither the portion of apoAI exon 4 nor the portion of apoCIII gene intron 1 is spliced from the primary transcript of this gene fusion (Fig. 5F). The

structure of the apoCIII-apoAI fusion mRNA was characterized by S1-nuclease mapping using as probe a 325-bp DNA fragment that spans 95 bp of the apoAI exon 4 and 228 bp of apoCIII intron 1 (Fig. 5E). The results show that this RNA protects a 123-nucleotide-long probe fragment (Fig. 5E). Since the sizes of the apoCIII gene exon 1 and apoAI gene exon 4 in this fusion are 35 bp and 545 bp, respectively, and the size of its mRNA is 500–600 nucleotides long (Fig. 4), it can be concluded that the start site of this mRNA is similar to that of the normal apoCIII mRNA and that the portion of apoCIII gene intron 1 present in this fusion is spliced via a cryptic 3' splice site (TTCTTCAGGC) located 256 nucleotides 3' to the 5' splice junction of the apoCIII gene intron 1 (Fig. 5G; ref. 27).

## DISCUSSION

Reciprocal recombination events leading to DNA inversions are essential for the assembly of functional immunoglobulin and T-cell receptor genes in eukaryotes (28, 29). The DNA inversion within the apoAI/apoCIII-encoding gene cluster of the apoAI/apoCIII-deficient patients and a similar inversion within the  $\beta$ -globin gene cluster of certain patients with  $\delta$ , $\beta$ -thalassemia (30) raise the possibility that eukaryotic cells have a generalized recombination mechanism for DNA rearrangements and that, under certain structural and/or functional constraints, this mechanism may give rise to DNA inversions such as those found in the genomes of these patients. The presence of *Alu* I repetitive DNA elements at similar distances from the breakpoints of the DNA inversions in both the apoAI-apoCIII (Fig. 1D; ref. 27) and the  $\beta$ -globin (30) gene clusters may indicate the involvement of these DNA elements

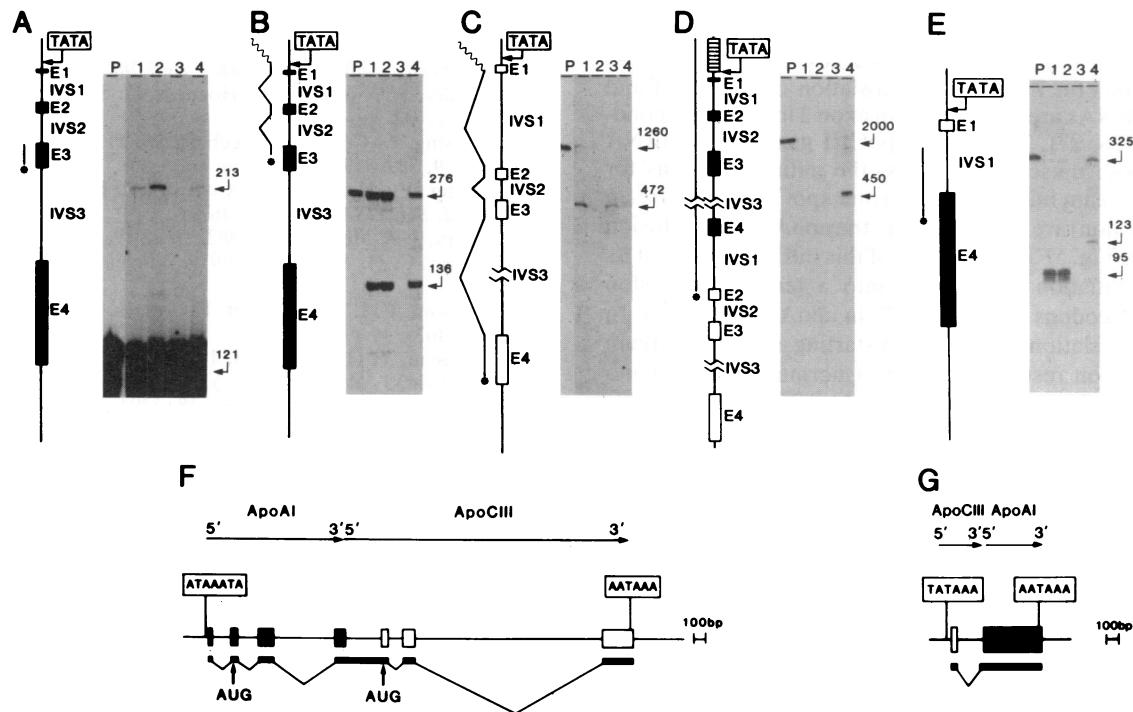


Fig. 5. Total RNA (20  $\mu$ g) isolated from adult human liver (lane 1) and COS-1 cells transfected with the apoAI (lane 2), apoCIII (lane 3), apoAI-apoCIII (lane 4 in A, B, C, and D), and apoCIII-apoAI (lane 4 in E) gene constructs were used for primer-extension (A) and S1-nuclease-protection (B, C, D, and E) analyses. The apoAI and apoCIII gene sequences within the primer-extension and S1-nuclease-mapping probes (vertical lines with a filled circle at one of their ends) are indicated by the vertical maps of the apoAI (A and B) and apoCIII (C) genes and the apoAI-apoCIII (D) and apoCIII-apoAI (E) gene fusions. TATA boxes (boxed), exons (E), and introns (IVS) of these genes are indicated as in Fig. 1A. Plasmid pBR322 (wavy lines) and the SV40 enhancer (striped box) DNA sequences as well as the radioactively labeled end (filled circle) of each of these probes are indicated. The resulting autoradiograms are shown. The size (in nucleotides) of each probe (lane P) and the primer-extension or S1-nuclease-protection products obtained using these probes are indicated by numbers along the right sides of the autoradiograms. (F) Splicing pattern of the apoAI-apoCIII gene fusion primary transcript. The location of the natural initiation codons (AUG) of the apoAI and apoCIII genes are indicated. (G) Splicing pattern of the apoCIII-apoAI gene fusion primary transcript.

in stabilization of DNA stem-loop structures necessary for the recombination events that led to these inversions. *Alu I* repetitive DNA elements have also been implicated in the recombination events that led to two different DNA deletions within the low density lipoprotein receptor gene of certain patients with familial hypercholesterolemia (31).

Because of the close physical linkage and convergent transcription of the apoAI and apoCIII genes in the normal human genome (6), the DNA inversion in the genome of the apoAI/apoCIII-deficient patients results in reciprocal fusion of portions of the apoAI and apoCIII gene transcriptional units. These gene fusions are expressed into stable mRNAs with sequences representing fusions of the normal apoAI and apoCIII mRNAs. Specifically, the apoAI-apoCIII gene fusion mRNA contains the apoAI gene exons 1, 2, and 3 and part of exon 4 as well as the apoCIII gene exons 2, 3, and 4. In addition, this mRNA contains the entire portion of apoCIII gene intron 1 present in this gene fusion. Therefore, it is interesting that the presence of the apoCIII intron 1 3' splice site and several GT dinucleotides 5' to this site are not sufficient to generate a cryptic 5' donor splice site. These results contrast those obtained with a  $\beta$ -thalassemia mutation in which inactivation of a 5' donor site leads to activation of several cryptic 5' splice sites (32). Thus, although events at the 3' splice site may be important for the events leading to cleavage at the 5' splice site (33), additional factors (such as the pre-mRNA secondary structure) may also be required for precise definition of cleavage at the 5' splice site. The apoCIII-apoAI gene fusion mRNA, on the other hand, contains the apoCIII gene exon 1 and the 3' portion of the apoAI gene exon 4. Thus, the portion of apoCIII intron 1 present in this gene fusion is spliced via a cryptic 3' splice site located within the apoCIII intron 1. This finding suggests that, in agreement with previous reports (33), the normal branch-point sequence for lariat formation is not essential for pre-mRNA splicing in higher eukaryotes.

The codons for initiation of translation of the apoAI and apoCIII mRNAs are located within exon 2 in the corresponding genes (26, 27). The apoAI-apoCIII gene fusion mRNA contains both these exons. Thus, the initiation codons for apoAI (upstream initiation codon) and apoCIII (downstream initiation codon) are both present in the apoAI-apoCIII fusion mRNA (see Fig. 5F). Translation of this mRNA starting at its upstream initiation codon runs into a termination codon located two codons 3' to codon 77 in apoAI (see Fig. 3). In contrast, translation of this mRNA starting at its downstream initiation codon results in the intact normal apoCIII amino acid sequence. However, the apoAI/apoCIII-deficient patients lack apoCIII in their plasma. It is possible that apoCIII is being synthesized *in vivo*, but disturbance(s) of the lipoprotein metabolism due to the absence of apoAI results in hypercatabolism of this protein. Alternatively, initiation of translation of this mRNA may occur only at its upstream initiation codon. However, it should be pointed out that the presence of a termination codon in frame with the upstream initiation codon may result in translation starting from the downstream initiation codon (34). On the other hand, the apoCIII-apoAI gene fusion mRNA does not contain any of the natural initiation codons for apoAI and apoCIII. However, the first AUG proximal to the 5' end of this mRNA coincides with codon 86 in apoAI mRNA (26). This AUG is located within a nucleotide sequence context (AGGAG-AUGA) that may allow its utilization by ribosomes (34) as an initiation codon. Thus, translation of this mRNA would result in a protein containing the 86th to 243rd amino acid portion of apoAI. Therefore, this protein would contain the amphipathic amino acid repeats in apoAI (26) but not its signal

peptide and may be accumulated and/or degraded intracellularly.

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