We have isolated the human apolipoprotein (apo) A-IV gene from a cosmid library and determined its complete nucleotide sequence. The gene contains three exons of 162, 127, and 1180 nucleotides separated by two introns of 357 and 777 nucleotides. A sequence polymorphism has been identified in the 5' non-coding portion of the third exon. The human apoA-IV gene lacks an intron in the area encoding the 5' nontranslated region of its mRNA, which distinguishes it from all the other human apolipoprotein genes whose sequences are known. Comparison matrix analysis of the human apoA-IV gene sequence revealed evidence for an ancestral 11-nucleotide repeat unit that spans the third exon. These repeated sequences are much more highly conserved than those present in either rat apoA-IV or in any other human apolipoprotein. Optimal alignment of the 5' flanking regions of the rat and human apoA-IV genes disclosed multiple deletions in the rat sequence as well as a highly conserved region of 90 nucleotides (90% sequence identity) located within 170 nucleotides of the start site of transcription. The 5' flanking regions of the human and rat apoA-IV genes were ligated to the bacterial chloramphenicol acetyltransferase gene, then transfected into different cultured cells. The apoA-IV gene sequences elicited preferential expression of chloramphenicol acetyltransferase activity when introduced into intestinally derived Caco-2 cells and liver-derived Hep-G2 cells, consistent with the tissue specificity of the native gene. Analysis of deletion mutants of the human apoA-IV 5' flanking region indicated that regions from -293 to -233 and from -127 to -60 upstream of the transcription start site contain sequences required for maximum gene expression. These findings on the structure and expression of rat and human apoA-IV should prove useful in studying the control of the apoA-IV gene.
of the apoA-IV gene to investigate its cell-specific expression as well as the functional domains that determine promoter activity.

EXPERIMENTAL PROCEDURES

Screening of Human Genomic Library—Five hundred thousand clones in a human cosmid genomic library (kindly provided by Dr. Chris Lau, University of California, San Francisco) were screened with a 32P-labeled human apoA-IV cDNA probe using the conditions described in Ref. 1. Three positive recombinants, with an average insert length of 34 kilobases, were identified. These DNAs were digested with a variety of restriction endonucleases, and Southern blots were prepared (15). When the 32P-labeled human apoA-IV cDNA was used to probe these blots, it was apparent that all three cosmids had inserts that contained the complete apoA-IV gene, and that the restriction maps of the apoA-IV gene in each insert were identical (data not shown). Therefore, a single cosmid, pHAIVG52, was employed for all subsequent studies.

Restriction fragments derived from this cosmid DNA were subcloned in bacteriophage M13 mp18 and mp19 prior to subsequent nucleotide sequence analysis by the dideoxy chain termination method (16). Initially, the sequences of both ends of these fragments were defined using the universal M13 primer. The resultant partial nucleotide sequences were subsequently used for designing synthetic oligonucleotides for further sequence analysis. This strategy was followed for both strands of the human apoA-IV gene. All oligodeoxynucleotides were produced by an Applied Biosystems (Foster City, CA) Model 380A synthesizer.

Primer Extensions—To determine the initiation site for transcription, a 27-nucleotide primer was synthesized that was complementary to a region beginning 79 nucleotides upstream from the initiator methionine codon of apoA-IV mRNA (1). The 27-mer was labeled at its 5' end using polynucleotide kinase and was subsequently hybridized to 2.5 μg of poly(A)-containing RNA from human small intestine. After hybridization, 5 mM dNTPs, 5 mM MgCl2, 40 mM dithiothreitol, and 50 mM Tris at pH 8.3. The solution was incubated for 30 min at 60 °C and then for 15 min at 42 °C. After hybridization, 2 μl of 5 mM dATP, dGTP, dCTP, and dTTP, as well as 21 units of reverse transcriptase, were added to the reaction. The mixture was incubated at 60 °C and then for 15 min at 42 °C. After hybridization, the mixture was incubated for an additional 30 min at 42 °C. After ethanol precipitation, the resultant partial sequencing reaction mixture was analyzed on an 8% polyacrylamide sequencing gel (17).

Computer-assisted Comparative Sequence Analyses—All computations were carried out on a MicroVAX II computer (Digital Equipment Corp.), using version 4.2 of the VMS operating system. Protein and nucleic acid comparison matrices were generated using the CSPSEQ84 program (obtained from Dr. Andrew McLachlan, Laboratory of Molecular Biology, Medical Research Council, Cambridge, United Kingdom). The NUCALN and PRTALN programs (17) were used to compute optimal alignments of nucleic acid and protein sequences, respectively. The FASTN program (18) was used to search the Genetic Sequence Data Bank (GenBank™).

Construction and Characterization of Deletion Mutants—XbaI/SacI (-893 to 26) and BglII/XmnI (-800 to 21) restriction fragments were isolated from the human and rat apoA-IV genes. These fragments were ligated into the polylinker region of the plasmid pLS1 (pLS1 is modified pTE19 without thymidine kinase promoter). Deletions in the 5' portions of the human apoA-IV sequences were introduced either by restriction endonuclease digestion or by digestion with Bal-31 and DNA polymerase I. The digested fragments were ligated to BglII linkers and inserted into the BglII/SacI site of pLS1, adjacent to the bacterial chloramphenicol acetyltransferase (CAT) gene (19). These constructions were introduced into different cell lines and examined for chloramphenicol acetyltransferase activity. A calcium phosphate co-precipitate containing 10 μg of DNA was added to different cultured mammalian cell lines. Cells were collected 48 h after addition of the DNA, and extracts were prepared by freezing and then heat shock at 60 °C (20). The reaction mixture (20) contained Tris (pH 7.8), 140 mM; acetyl coenzyme A, 0.44 mM; [3H]chloramphenicol (40–60 mCi/mmol, New England Nuclear), 0.2 mM; and cell extract (60 μg of total protein). The reactions were allowed to proceed for up to 60 min. Samples were extracted with 1 ml of ethyl acetate. The solution was dried down, and the residue was resuspended in 10 μl of ethyl acetate and analyzed by ascending thin-layer chromatography using chloroform/methanol (95/5, v/v). The chromatograms were subjected to autoradiography. Chloramphenicol acetyltransferase activity was quantitated by counting scraped regions of the chromatograms in a liquid scintillation spectrometer. All chloramphenicol acetyltransferase activity measurements were normalized for the differences in the transfection efficiency between cells by co-transfecting the cells with plasmid containing the β-galactosidase gene under the control of Rous sarcoma virus promoter and measuring the total β-galactosidase activity in cell protein extracts as described (21).

RESULTS AND DISCUSSION

Nucleotide Sequence Analysis—The strategy used to determine the complete nucleotide sequence of the human apoA-IV gene is shown in Fig. 1. A comparison of this sequence with the previously determined nucleotide sequence of human apoA-IV cDNA (1, 10) disclosed that the gene contains three

![Fig. 1. Restriction endonuclease map and nucleotide sequencing strategy for the human apoA-IV gene. The apoA-IV gene and its flanking regions are presented on the top line. Exon locations are indicated by the boxes located just below the line. Restriction endonuclease sites used for subcloning and sequence analyses are indicated by vertical lines. The solid lines below represent reactions primed with the universal M13 primer. The dashed lines indicate where synthetic oligodeoxynucleotides complementary to portions of the apoA-IV gene sequence were used. The lines with open boxes indicate that the sequence was determined from a second genomic clone, pHAIVG51.](image-url)

Nucleotides x 10⁻²
The complete nucleotide sequence of the human apoA-IV gene and flanking regions. The start site of transcription, indicated by the arrow (1), was determined by primer extension analysis (see "Experimental Procedures"). The TATA box is denoted by a single asterisk, was determined by primer extension analysis (see "Experimental Procedures"). The TATA box is denoted by a single asterisk, was determined by primer extension analysis (see "Experimental Procedures"). The TATA box is denoted by a single asterisk, was determined by primer extension analysis (see "Experimental Procedures"). The TATA box is denoted by a single asterisk, was determined by primer extension analysis (see "Experimental Procedures"). The TATA box is denoted by a single asterisk, was determined by primer extension analysis (see "Experimental Procedures"). The TATA box is denoted by a single asterisk, was determined by primer extension analysis (see "Experimental Procedures"). The TATA box is denoted by a single asterisk, was determined by primer extension analysis (see "Experimental Procedures"). The TATA box is denoted by a single asterisk, was determined by primer extension analysis (see "Experimental Procedures"). The TATA box is denoted by a single asterisk, was determined by primer extension analysis (see "Experimental Procedures"). The TATA box is denoted by a single asterisk, was determined by primer extension analysis (see "Experimental Procedures"). The TATA box is denoted by a single asterisk, was determined by primer extension analysis (see "Experimental Procedures"). The TATA box is denoted by a single asterisk, was determined by primer extension analysis (see "Experimental Procedures"). The TATA box is denoted by a single asterisk, was determined by primer extension analysis (see "Experimental Procedures"). The TATA box is denoted by a single asterisk, was determined by primer extension analysis (see "Experimental Procedures"). The TATA box is denoted by a single asterisk, was determined by primer extension analysis (see "Experimental Procedures"). The TATA box is denoted by a single asterisk, was determined by primer extension analysis (see "Experimental Procedures"). The TATA box is denoted by a single asterisk, was determined by primer extension analysis (see "Experimental Procedures"). The TATA box is denoted by a single asterisk, was determined by primer extension analysis (see "Experimental Procedures"). The TATA box is denoted by a single asterisk, was determined by primer extension analysis (see "Experimental Procedures"). The TATA box is denoted by a single asterisk, was determined by primer extension analysis (see "Experimental Procedures"). The TATA box is denoted by a single asterisk, was determined by primer extension analysis (see "Experimental Procedures"). The TATA box is denoted by a single asterisk, was determined by primer extension analysis (see "Experimental Procedures"). The TATA box is denoted by a single asterisk, was determined by primer extension analysis (see "Experimental Procedures"). The TATA box is denoted by a single asterisk, was determined by primer extension analysis (see "Experimental Procedures"). The TATA box is denoted by a single asterisk, was determined by primer extension analysis (see "Experimental Procedures").
Fig. 3. The structure of the human apoA-IV gene. A, the structures of the human and rat apoA-IV genes are compared. The exons are indicated by solid bars, and the introns by open bars. The numbers above the exons and introns indicate their length in nucleotides. Amino acid codon positions that contain intron-exon junctions are indicated. B, the structure of the human apoA-IV gene is compared with that of two closely linked human apolipoprotein genes. Exons are indicated by solid bars, with their lengths in nucleotides indicated by numbers below the bars. Intron lengths are indicated by numbers below the lines that indicate their locations. Amino acid codons that contain introns are indicated above the lines. Overall gene lengths are indicated at the right end of the lines. The lengths of the human apolipoprotein genes are taken from the following references: 25, A-I; and 24, C-III.

rupts both genes at precisely the same place: within the glycine codon corresponding to position -4 of the signal peptide (Fig. 3A). Alignment of the two signal peptides disclosed a sequence identity of 81%. Both the human and rat apoA-IV genes lack an intron within the 5' noncoding region of the corresponding mRNA, making them unique among apolipoprotein genes (see Ref. 12). In all other defined mammalian apolipoprotein gene sequences (12), an intron is located about 20 nucleotides upstream from the mRNA translation start site. The lack of this intron in both apoA-IV genes raises the possibility that it was deleted during the postulated duplication event(s) (13) that gave rise to the apoA-IV and apoA-I genes. Because of this common ancestor, the two introns in the human apoA-IV gene are located in positions similar to those in other

Fig. 4. Intrasequence comparison matrix analyses of human apoA-IV. A, a self-comparison of the human apoA-IV gene nucleotide sequence is shown. A span length of 11 nucleotides was used. The unitary matrix (31) was used to score comparisons between spans of nucleotides. Only spans reaching or exceeding a score that had a probability of occurring by chance alone of less than 1 in 10 were plotted. Spans exceeding the predetermined threshold were plotted with a point that indicates only the center of the aligned span. B and C, a self-comparison of the amino acid sequence of preapoA-IV using a span length of 23 residues is shown. The PAM250 mutation data matrix (31) was used to score comparisons between spans. A threshold was selected for plotting such that in infinite random sequences with the same amino acid composition as this protein, the probability of achieving the threshold score was less than 1 in 600 (B) or 1 in 100 (C).
known major apolipoprotein genes. To illustrate this observation, the structure of the human apoA-IV gene is compared with that of the apoC-III (24) and apoA-I (25) genes (Fig. 3B) to which it is linked closely (1, 26) on chromosome 11 (27). This similarity in structure is consistent with the notion that these apolipoprotein genes evolved from a common ancestral gene through a series of gene duplications, deletions, and chromosome translocations (11–13).

The second intron of the human apoA-IV gene interrupts the codon for Asn-39 in the mature protein-coding portion of the gene. Thus, the second exon specifies the last 4 residues of the signal peptide and the first 39 residues of the mature plasma protein. The amino-terminal oligopeptide domains encoded by the second exons of the human and rat genes exhibit a higher degree of sequence identity (80%) than those encoded by their third exons (61%). The first and second introns in the human gene, respectively, 80 and 104 nucleotides larger than those in the rat gene (see Fig. 3A). The human apoA-IV gene sequence was compared with the primate, rodent, and mammalian DNA libraries contained in GenBank™. No Alu family sequences were found in any region of this gene or its defined flanking sequences. Such sequences have been previously noted in the human apoE gene sequence (28) and in the human apoC-II gene sequence (29).

Comparison of the human apoA-IV exonic sequences with the nucleotide sequence of human apoA-IV cDNA (1) revealed only one difference. The portion of the third exon that specifies the 3' untranslated region of apoA-IV mRNA contained four tandem repetitions of the sequence TGTC (beginning at nucleotide position 2494, Fig. 2), whereas this sequence was repeated only three times in the cDNA sequence. The gene sequence was unambiguous, having been confirmed on both strands. In addition, a second apoA-IV gene clone (pHAIVG51, Ref. 1) from the same genomic library was examined and found to have the same nucleotide sequence in this region. The cDNA sequence was also unambiguous, since it was confirmed on both strands of three independent recombinants (1). Therefore, it is unlikely that this discrepancy represents a cloning artifact. Furthermore, the nucleotide sequence that we observed for the potentially polymorphic site in the gene agrees with the corresponding sequence of an apoA-IV mRNA that was determined independently for a different individual (10). Because the genomic and cDNA libraries were prepared from different individuals, the sequence variation probably represents a naturally occurring polymorphism.

Repeated Sequences in the Human ApoA-IV Gene—The human apoA-IV gene sequence was compared against itself using the comparison matrix algorithm of McLachlan (30) and a span length of 11 nucleotides (Fig. 4A). In this analysis, similarities between nucleotide spans of the chosen length are evaluated by determining similarity scores for each span (30, 31). The scores above a statistically significant threshold are plotted as a single point representing the center of the span. Because only the center of a span is plotted, the comparison matrix program requires that the span length be an odd integer. It is apparent in Fig. 4A that the third exon of this gene contains multiple repeats, indicated by the cluster of short diagonals offset from the main diagonal. A similar distribution was obtained when longer span lengths (23, 45, and 67 residues) were analyzed and when the threshold scores for plotting were raised (data not shown). Measurements of the displacements of these shorter diagonals from each other and from the main diagonal indicated that an 11-nucleotide repeat was present in this exon of the apoA-IV gene. These repeats cannot be detected in the second exon.

Analyses of repeated sequences in rat apoA-IV by our group (13, 32) and others (12) have used amino acid sequences because they permit a more sensitive analysis. This extra sensitivity is helpful because Luo et al. (12) have recently shown that the rate of nucleotide substitution among apolipoprotein genes is higher than the average rate observed in other mammalian genes. This difference may reflect the fact that selective pressures act only to conserve a specific pattern of generic lipophilic and hydrophilic side chains as opposed to the conservation of a specific sequence of amino acids. The
Fig. 5. Alignment of the 5' non-transcribed regions of the human, rat, and mouse apoA-IV genes. Optimal alignments were generated using NUCLAN (17), a k-tuple of 3, a window size of 20, and a gap penalty of 7. Dashed lines, indicating hypothetical deletions, were placed in the sequences to achieve maximum homology. The numbers indicate the nucleotide positions of the human sequence upstream from the start of transcription. A colon between the rat and human sequences indicates an identity between their nucleotides. A colon between the mouse and rat sequences indicates a nucleotide position that is identical in mouse, rat, and human genes. A period between the mouse and rat sequences indicates an identity between nucleotides of only these two genes.

The relative sequence conservation among repeat units within the human and rat apoA-IV polypeptides was indicated by the probability distribution of comparison span scores observed in the self-comparison matrix analysis (Table 1). A comparison score equals the sum of similarity scores (31) for amino acid pairs contained within the span. The frequency of an observed score was compared with the expected frequency of that score in randomly shuffled sequences having the same amino acid composition as authentic apoA-IV. The repeated sequences in human apoA-IV achieved higher overall comparison scores than the corresponding sequences in rat apoA-IV. For example, there were 34 spans in the human sequence that had comparison scores greater than 285, whereas this was the highest score achieved for spans in the rat apoA-IV intrasequence comparison matrix. The ratio of observed/expected scores for the highest scoring spans in rat apoA-IV (i.e. the four spans with scores of 295) was about 10-fold lower than the ratio for spans with the same score in human apoA-IV. These data indicate that the repeat units in human apoA-IV are much more highly conserved with respect to one another than the repeat units present in the rat protein.

The self-comparison matrix statistics for human and rat apoA-IV were compared with the previously published analysis of human apoA-I and apoE (13). The degree of sequence
Fig. 6. Stimulation of chloramphenicol acetyltransferase
gene transcription by the human apoA-IV promoter. A 919-
nucleotide fragment, Xba/I/Sacl, including 893 nucleotides of the 5'
flanking DNA of the human genes and 26 nucleotides of the first
exon, was ligated into the polylinker region of the pLSI vector at
the 5' end of the chloramphenolic acetyltransferase gene coding sequence. Plasmids containing deletion in the
served region present in the apoA-IV gene of humans and rats was
developed as described under Experimental Procedures. The constructs were
introduced into various cell lines, and transient expression of chloramphenolic acetyltransferase activity was measured (see "Experimental Procedures"). The data are expressed as a percentage of activity
found in the human intestine-derived cell line Caco-2. The Rous sarcoma virus (RSV)-β-galactosidase vector was used as an internal
control for the comparison of the activities of chloramphenolic acetyltransferase vector products as described (37). The actual expres-
sion of Rous sarcoma virus-directed β-galactosidase in different cells
as compared with that of Caco-2 cells is shown.

### TABLE I

| Cell culture | Description | ApoA-IV | RSV-β-galactosidase |
|--------------|-------------|---------|---------------------|
| Caco-2       | Human colonic carcinoma | 100 | 100 |
| Hep-G2       | Human hepatoma | 112 | 129 |
| HeLa         | Human epithelioid carcinoma | 12 | 107 |
| BaGl         | Human fibroblast | 7 | 106 |
| U937         | Human monocyte-like | 5 | 1 |
| L            | Mouse fibroblast-like | 11 | 127 |
| J774.1       | Mouse macrophage-like | 12 | 134 |

### TABLE III

| Tissue            | Relative levels | % |
|-------------------|-----------------|---|
| Small intestine   | 100             | 100 |
| Liver             | 12              | 2 |
| Large intestine   | 1               | 2 |
| Testis            | 1               | 1 |
| Spleen            | 1               | 1 |
| Pancreas          | 1               | 1 |
| Kidney            | 1               | 1 |
| Lung              | 2               | 1 |
| Stomach           | 1               | 1 |
| Brain             | 1               | 1 |
| Adrenal           | 1               | 1 |
| Heart             | 1               | 1 |

The expression of apoA-IV gene in vivo was performed using aliquots of total cellular RNA taken from the listed tissues, applied
to nitrocellulose filters, and examined by hybridization and autoradiograms as described previously (1).
favor this intermolecular reaction over one involving apoA-IV and the surfaces of human plasma lipoproteins (36).

Analysis of the 5' Flanking Region of the ApoA-IV Gene—Previously, we have used RNA blot hybridization to compare the expression of the apoA-IV gene in a variety of rat, mouse, and human tissues (1). One obvious difference between rodents and primates is that apoA-IV mRNA is abundant in rat liver but not in the livers of the other two species. It was of interest, therefore, to compare the 5' nontranscribed regions of the human and rat genes using the following two approaches. Alignment of the rat and human 5' flanking region (Fig. 5) revealed a typical TATA box beginning at comparable positions in both genes. Two other features shown in the alignment are particularly noteworthy. First, a region extending from nucleotide -77 to -167 in the human gene exhibits striking sequence similarity (90% identity) to its rat homolog. This high level of extended sequence conservation in gene 5' flanking regions between species is unusual. A further comparison to the promoter region of the mouse apoA-IV gene (37) reveals the same striking homology in this domain. Second, the human gene contains a large insertion that spans positions -346 to -999, which is surrounded by regions of 49% homology in the flanking 500 nucleotides. One hypothesis arising from this comparison is that these various conserved domains and/or the multiple insertions and deletions could be important in determining the observed patterns of apoA-IV tissue-specific expression in rodents and primates.

Therefore, we initiated a series of in vitro studies to identify functionally important domains in the 5' flanking region of the orthologous human and rat apoA-IV genes. DNA sequences containing the 5' flanking region of the human and rat apoA-IV genes (i.e., 893 and 800 nucleotides, respectively, upstream from the start of transcription) were fused to the 5' end of the CAT gene (Fig. 6). These recombinants were introduced into different cell lines, and the transient expression of the CAT gene, mediated by the promoter activity of the apoA-IV gene fragments, was measured (Table II). To assess potential differences in cell transfection efficiency, all cells were transfected with a plasmid containing the Escherichia coli β-galactosidase gene linked to the Rous sarcoma virus promoter (38). The β-galactosidase activity in the various cells examined did not vary by more than 30% (0.5-fold) from the activity in Caco-2 cells (Table II).

The results in Table II show that the 5' flanking regions of the rat and human apoA-IV genes exhibit the greatest promoter activity in cultured cells derived from tissues that have been shown previously (1, 39) to express the gene (Table III). Both the rat and human apoA-IV gene fragments were at least 10-fold more active in the human hepatoma cell line Hep-G2 and the intestinally derived cell line Caco-2 than in the other cells tested. These data are consistent with in vivo results in the rat (39), which show that the liver and intestine are the only organs that express significant levels of apoA-IV. The human apoA-IV promoter was expressed about 20% more efficiently than the rat apoA-IV promoter in the Caco-2 cells (data not shown). The finding that the human apoA-IV gene promoter elements function in Hep-G2 cells was not unexpected because apoA-IV mRNA was detected in these cells at a relative level of 15% of that of the small intestine (data not shown). However, the normal human liver has only a very low level of apoA-IV mRNA. These differences in the efficiency of human apoA-IV promoter element expression in normal and neoplastic hepatoma cells may reflect a number of physiological differences.

To define further the location of functionally significant sequences in the 5' flanking domain of the human apoA-IV gene, additional mapping studies were performed. Fig. 6 shows that deletions of nucleotides -983 to -293 resulted in no significant change in apoA-IV promoter activity in Hep-G2 cells. However, deletion of the region from -293 to -233 resulted in a 50% reduction in the promoter activity. Deletion of the region from -127 to -60 resulted in a dramatic reduction in the promoter activity. This encompasses most of the regions of the orthologous rat and human apoA-IV genes that show exceptionally high sequence conservation (Fig. 5). These data therefore support the hypothesis that this domain represents a region of functional importance for efficient gene expression.

The loss of chloramphenicol acetyltransferase activity in transfected human hepatoma cells associated with progressive deletion of the 5' flanking region of the apoA-IV gene suggests that specific control elements are located within 300 base pairs of the transcription start site and may be part of a positive regulatory system that modulates expression of this human apolipoprotein gene. Although our data are consistent with the notion that sequences affecting cell-specific expression are situated within 300 nucleotides of the transcription initiation site, further mapping studies employing experimental strategies similar to those described here will be required to delineate their location in the apoA-IV gene.

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