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Identification of an enhancer-like element in the 5' flanking region of the rat apolipoprotein A-I gene

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ABSTRACT

In order to study the expression of the apolipoprotein (apo) A-I gene, we have isolated and characterized the structural gene encoding rat apo A-I. The 5' flanking sequence of the apo A-I gene was placed upstream of the coding sequence of the bacterial chloramphenicol acetyl transferase (CAT) gene, such that the expression of CAT activity in cultured cells is under the control of the promoter and regulatory sequences of the rat apo A-I gene. By transient transfection, nucleotide deletion and substitution methods, it was demonstrated that the nucleotide sequences between -464 and -148 upstream from the start of transcription of the rat apo A-I gene are required for the expression of this gene in Hep G2 cells and that these sequences function with an enhancer-like activity.

Apolipoprotein (apo) A-I is a major constituent of plasma high density lipoproteins (HDL) and a cofactor of plasma lecithin cholesterol acyltransferase (1). The levels of plasma HDL and apo A-I are negatively correlated with the incidence of coronary heart disease (2,3), so an increased level of plasma apo A-I is desirable.

Apo A-I is synthesized primarily in the liver and intestine (4,5). Plasma apo A-I and HDL levels can be increased by several pharmacological agents, such as ethanol (6) and phenobarbital (7). Recent studies have shown that phenobarbital, estrogen, insulin and dexamethasone increase levels of apo A-I mRNA in rat liver and cultured HepG2 cells (8-10). Thus an understanding of the mechanism by which the apo A-I gene is regulated becomes crucial.

In order to identify cis-acting regulatory elements in the 5' flanking region of apo A-I gene that are required for expression, we have isolated the rat apo A-I structural gene. The 5' flanking region of the apo A-I gene which contains the promoter and regulatory sequences was placed upstream of the coding sequence of the bacterial chloramphenicol acetyltransferase (CAT) gene, such that the expression of CAT activity is under the control of apo A-I promoter and regulatory sequences. This chimeric gene and its related

deletion and insertion constructs were introduced into HepG2 cells, which synthesize and secrete apo A-I under normal conditions (11). The results show that the 5' flanking region of the rat apo A-I gene contains sequences that are required for the expression of this gene and that these sequences function as an enhancer.

MATERIALS AND METHODS

Identification of Rat Apo A-I Genomic Clone

Approximately 10^6 plaques from a rat EcoRI and Hae III genomic library (12) were screened with a rat apo A-I cDNA (8) with procedures described by Rothkopf et al. (13). A clone, λAI-517, was identified and found to contain the complete rat apo A-I structural gene.

Construction of Plasmids

pSVO-CAT (ATCC #37153), a recombinant plasmid that contains the coding sequence for bacterial CAT gene, was digested with Hind III and the ends were filled in with dNTP as catalyzed by the large fragment of DNA polymerase I. A 2.1 Kb DNA fragment, which contains the apo A-I 5' flanking region was removed from λAI-517 by digestion with EcoRI and Sac I and was blunt-ended with the large fragment of DNA polymerase I and mung bean nuclease. These two blunt-end DNA fragments were ligated with T4 DNA ligase and the resulting construct is designated as pAICAT-5. Deletion mutants were prepared from pAICAT-5 by using restriction endonucleases. The deletion and insertion mutants were verified by a series of restriction enzyme digestions.

Cell Growth, DNA Transfection and Assay for CAT Activity

HepG2 cells were maintained in minimum essential medium containing 100 U/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) fetal calf serum, and were grown at 37°C with 7% CO_2.

Cells were seeded at 2.5×10^5 per 9 cm^2 dish and transfected with plasmids (10 μg) by calcium phosphate precipitation and glycerol shock methods (14). After 4 hours, the cells were washed twice with culture medium and maintained in culture. After 2 days the cells were collected, cell extracts were prepared and CAT activities were determined as described by Gorman (14). Each reaction mixture (200 μl) contained 25 to 100 μg protein, 0.44 mM acetyl CoA, 3.34 nmole [^{14}C]chloramphenicol (~1 x 10^5 cpm/nmole) in 0.2 M Tris buffer (pH 7.5). The samples were incubated at 37°C for 40 minutes. The incubation conditions were determined empirically such that all assays were at linear phase with respect to time and concentration of protein. At the end of the incubation, the reaction mixture was extracted with equal volumes of ice-cold

ethyl acetate. [^{14}C]acetyl chloramphenicol and unreacted [^{14}C] chloramphenicol were separated by thin layer chromatography, visualized by autoradiography, scrapped from plates and quantitated by liquid scintillation spectrometry. Protein was determined by the method of Lowry, et al. (15).

Primer Extension Assays

A 17 base oligonucleotide, 5'-TTCTTCATTTTAGCTTC-3' complementary to CAT mRNA (14) was synthesized and used for extension studies to determine the transcription initiation site of pAICAT-5. This primer was 5'-end labeled with ^{32}P, annealed to total RNA prepared from Hep G2 cells (16) which has been transfected with pAICAT-5 and extended in the presence of reverse transcriptase according to the methods described in (16). The resulting transcripts were analyzed on 8% polyacrylamide gels in the presence of 8M urea.

RESULTS

The nucleotide sequence of the cloned rat apo A-I gene, λAI-517, was determined and except for a few substitutions, the sequence of λAI-517 was identical to the sequences of the rat apo A-I gene reported by Haddad et al. (17). Primer extension studies show that there are two transcription initiation sites for the rat apo A-I gene (unpublished data and 17). For convenience, the position of the first transcription initiation site is designated as +1 (17).

A 2.1 Kb, EcoRI-SacI fragment which contains part of the first exon and the upstream sequences of the rat apo A-I gene (for detailed map see reference 17) was isolated from λAI-517 and ligated in the correct 5'--3' orientation into the Hind III site of pSVO-CAT, which contains the coding sequence of the CAT gene. The resulting plasmid was designated pAICAT-5. When pAICAT-5 was transfected into cultured Hep G2 cells, CAT activity was detected from the cell extracts after 48 hours.

To ensure that the expression of CAT gene in pAICAT-5 transfected Hep G2 cells is using the rat apo A-I promoter, the primer extension study was conducted. The results are shown in Fig. 1. It shows that there are two primer extension products and that they are 56 and 60 nucleotides in length. Previous studies of Haddad et al (17) have shown that these are two transcription initiation sites in the rat apo A-I gene. Taken together, it is established that the expression of CAT gene in pAICAT-5 transfected Hep G2 cells is using the rat apo A-I promoter.

In order to identify the DNA sequences that control the expression of apo A-I gene, nucleotide sequence deletion experiments were carried out by

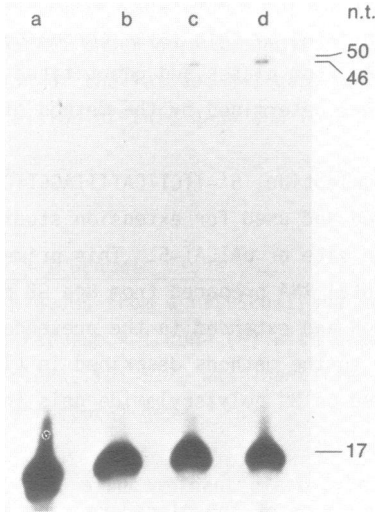

Figure 1. Primer extension analysis of the transcription intitiation sites of pAICAT-5. A 17 base nucleotide primer was annealed with total RNA from Hep G2 cells transfected with pAICAT-5 and extended with reverse transcriptase. The extension products were analyzed on 8% polyacrylamide gels. The amounts of total RNA used were 0 (lane a), 25 μg (lane b), 50 μg (lane c) and 75 μg (lane d). The nucleotide length (n.t.) of the primer (17 nucleotides) and the extended products (56 and 60 nucleotides) are indicated.

using restriction enzyme digestion and ligation methods. The structure of the regenerated apo A-I promoter-CAT chimeric genes and the relative levels of expression of these chimeric genes in transiently transfected Hep G2 cells are shown in Figure 2.

These data show that sequential deletion of the 5' flanking sequences of the rat apo A-I gene resulted in decreases of the promoter activities in Hep G2 cells. The DNA sequences between positions -464 and -148 are of particular interest, because removal of these sequences (pAICAT-4 and pAICAT-28) results in a complete loss of the promoter activity in Hep G2 cells.

In order to examine if the DNA sequences located between -464 and -148 of the rat apo A-I gene function like an enhancer, these sequences were isolated and placed at the positions of -2320 or -1470 of pAICAT-4 or at the Bam HI site which is located at nucleotide position +1651 (14) and is outside of the CAT structural gene of pAICAT-6 (Fig. 3). The resulting constructs were transiently transfected into Hep G2 cells. The CAT activities were determined

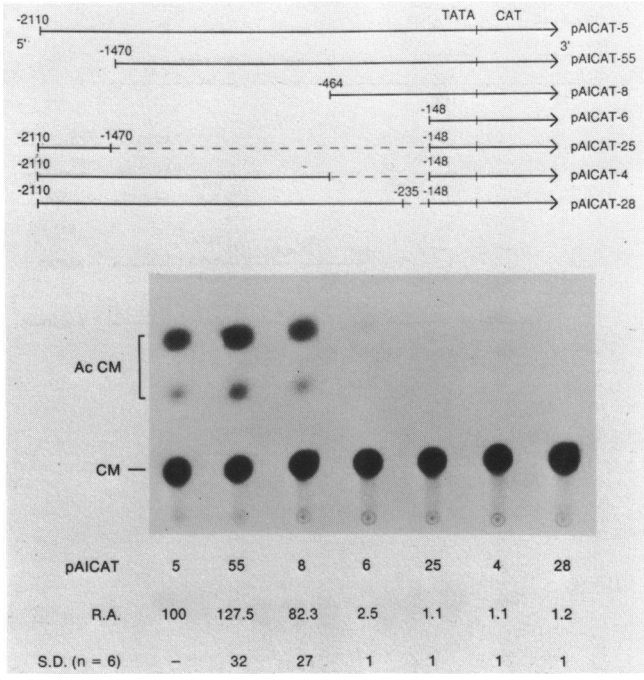

Figure 2. Structure of apo A-I promoter-CAT chimeric genes (top) and expression CAT activities in Hep G2 cells transfected with these chimeric genes (bottom). All deletion constructs were generated from pAICAT-5 after it had been digested with appropriate restriction enzymes and ligated with T4DNA ligases. The amount of cellular protein in each assay was 50 µg. The relative activity (R.A.) are expressed as percentages of the CAT activity observed with the pAICAT-5 which is set as 100%. Each value shows the mean ± standard deviation (S.D.) of six separate experiments. The autoradiogram shows the conversion of [^{14}C] chloramphenicol (CM) to its acetylated products (AcCM).

from these cell extracts and shown in Fig. 3. The results show that the DNA sequences located between positions -464 and -148 of the rat apo A-I gene increase the expression of CAT gene in Hep G2 cells when they are placed in either the upstream sequences of pAICAT-4 or the downstream sequences of pAICAT-6 and that these DNA sequences function in both the correct (pAICAT-62) and the reversed (pAICAT-63) orientations.

Furthermore, a plasmid, pAICAT-1, which contains two repeated sequences of this DNA fragment at position -148 (Fig. 3) showed an increased expression of CAT gene in Hep G2 cells in relation to pAICAT-8 which contains only one copy of this fragment (Fig. 3). These results demonstrated that the DNA

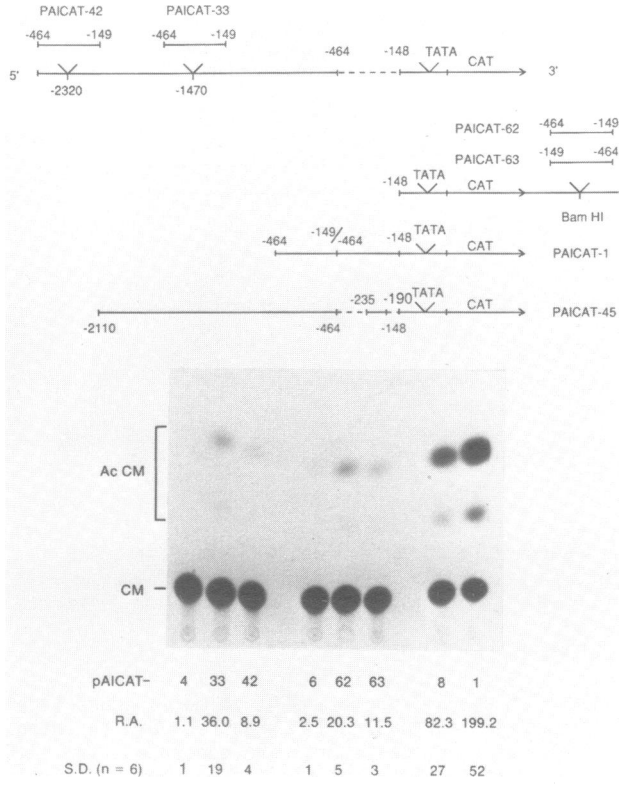

Figure 3. Structure of apo A-I promoter-CAT chimeric genes (top) and expression of CAT activities in Hep G2 cells transfected with these genes (bottom). Plasmids pAICAT-42 and pAICAT-33 were generated by removal of the DNA sequence between -464 and -149 from pAICAT-5 and placed them at positions -2320 or -1470, respectively. Plasmids pAICAT-62 and pAICAT-63 were generated by insertion of the DNA sequence of -464 to -149 into the Bam Hl site of pAICAT-6 with correct or inversed orientation, respectively. Plasmid pAICAT-45 was constructed by deletion of the -464 to -149 DNA sequence from pAICAT-5 and replacing them with the -235 to -190 sequence. The amount of cellular protein in each assay was 50 µg. The relative activities (R.A.) represent the percentages of the CAT activity observed with the pAICAT-5 which is set as 100%. Each value represents the mean ± standard deviation (S.D.) of six separate experiments.

sequences located between positions -464 and -148 in relation to the transcription initiation site of the rat apo A-I gene have enhancer-like characteristics.

Data presented in Fig. 3 also showed that the DNA sequences located between positions -235 and -148 of the rat apo A-I gene are required for the

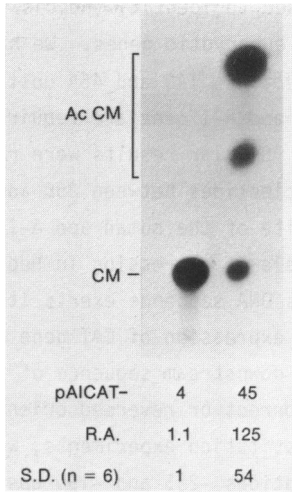

Figure 4. Expression of CAT activities in cultured Hep G2 cells transfected with pAICAT-4 and pAICAT-45. The structure of pAICAT-45 is shown in Fig. 5. The amounts of cellular protein used were 50 μg and 75 μg for pAICAT-4 and pAICAT-45, respectively. The relative activities (R.A.) represent the percentages of the CAT activity observed with the pAICAT-5 gene which is set as 100%.

expression of this gene in Hep G2 cells. In order to further identify the DNA sequences which are required for the expression of the rat apo A-I gene in Hep G2 cells, a plasmid, pAICAT-45 (Fig. 3) was constructed by inserting a DNA fragment which consisted of the sequences between positions -235 and -190 of the rat apo A-I gene into the positions of -464 and -148 of pAICAT-4. pAICAT-45 was transiently transfected into Hep G2 cells. The CAT activity were determined from these cell extracts and shown in Fig. 4. It shows that the DNA sequence between positions -235 and -190 of the rat apo A-I gene is essential for the expression of this gene. However, the possibility that additional enhancer activity located in the -190 to -148 is not excluded.

DISCUSSION

The expression of eukaryotic genes is usually regulated by the 5' flanking sequences of the genes. In this study we have examined the DNA sequences that are required for the expression of the apo A-I gene in Hep G2 cells by placing the 5' flanking sequences of the rat apo A-I gene in front of the CAT structural gene so that the expression of the CAT gene is under the control of the regulatory and promoter elements of the rat apo A-I gene. This

system has been commonly used to identify the cis-acting elements which regulate the expression of eukaryotic genes. We have demonstrated that the DNA sequences between nucleotides 149 and 464 upstream from the transcription initiation site of the rat apo A-I gene are required for the expression of this gene in Hep G2 cells. Similar results were reported by Sastry et al. (18). They showed that nucleotides between 256 and 41 upstream from the transcription initiation site of the human apo A-I gene is necessary and sufficient for maximal levels of expression in Hep G2 cells. In this study we also demonstrated that this DNA sequence exerts its function by acting like an enhancer. It enhanced the expression of CAT gene when they were placed at either the upstream or the downstream sequence of the CAT gene. They also functioned at either the correct or reversed orientation.

By deletion and substitution experiments, we have identified that the DNA sequence between nucleotides -235 and -190 upstream are sufficient to direct the expression of this gene in Hep G2 cells. Using gel mobility retardation, exonuclease III and DNase I footprinting assays, we have identified nuclear proteins isolated from Hep G2 cells bind this DNA fragment (unpublished results). So the expression of the rat apo A-I gene is regulated by the nuclear protein(s) which activate the transcription of this gene by binding to the nucleotides between 235 and 190 upstream from the transcription initiation site of the gene. Identification of this regulatory nuclear protein(s) will gain further insight to the regulation of the expression of this gene.

Recently, Shelley and Baralle (19) reported that the sequences between 912 and 653 base pairs upstream from the transcription initiation site of apo A-II have enhancer-like properties and is involved in the tissue-specific expression of the gene. We have observed that the expression of pAICAT-6 in mouse L cells, which do not synthesize apo A-I under normal conditions, is about 50-fold higher than that of pAICAT-5, pAICAT-62 and pAICAT-63 (data not shown). Thus the sequences upstream from -148 of the rat apo A-I gene also direct the tissue-specific expression of this gene. Because apo A-I and apo A-II are thought to be derived from a common ancestor (20), it is likely that all the common regulatory elements of these genes are conserved during evolution. In this regard, the regulation of the expression of the apo A-I gene should be in accord with that of the apo II gene. It remains to be determined if the upstream elements of apo A-I and apo A-II genes bind to the same nuclear regulatory protein(s).

Since the levels of plasma apo A-I are negatively correlated with the

incidence of coronary heart disease, an understanding of the regulation of the synthesis of apo A-I at the molecular level is important. Data presented in this manuscript demonstrate that the promoter activity of the apo A-I gene is regulated by both cis- and trans-acting elements. Identification of these elements will help delineate the mechanism of regulation of the apo A-I gene and should provide a rational approach to modulate plasma levels of apo A-I and HDL.

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Abbreviations: apo, apolipoproteins; CAT, Chloramphenicol acetyl transferase; HDL, high density lipoproteins.

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