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Regulated expression of the human acetylated low density lipoprotein receptor gene and isolation of promoter sequences

(transcription factor AP-1/macrophages/atherosclerosis/phorbol 12-myristate 13-acetate/retinoic acid)

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ABSTRACT **The acetylated low density lipoprotein (AcLDL) receptor is expressed on tissue macrophages after their differentiation from monocyte precursors and has been proposed to play a role in the generation of foam cells in atherosclerotic lesions. In the present studies, THP-1 human monocytic leukemia cells were used to investigate mechanisms responsible for expression of the AcLDL receptor gene after treatment with phorbol 12-myristate 13-acetate (TPA). TPA-dependent accumulation of AcLDL receptor mRNA was not detected until after a lag phase of 12 hr and was blocked by concurrent treatment with cycloheximide. In addition, the TPA-dependent induction of AcLDL receptor activity and mRNA levels was inhibited by retinoic acid and dexamethasone treatment. Isolation and sequence analysis of the promoter regions for the human and bovine AcLDL receptor genes indicated high sequence similarity. Binding sites for AP-1 proteins or other known transcription factors were not conserved between the two species, suggesting that novel factors are required for AcLDL receptor expression.**

The acetylated low density lipoprotein receptor (AcLDL R) is a scavenger receptor that is expressed on most, if not all macrophages. Scavenger receptors were initially identified by their ability to bind and internalize AcLDL, a chemically modified form of LDL that is not recognized by receptors for native LDL (1, 2). In addition to AcLDL, scavenger receptors also bind and degrade oxidized LDL, which appears to be present in atherosclerotic lesions (3, 4). Unlike the expression of LDL receptors, the expression of scavenger receptors is not down-regulated by high levels of intracellular cholesterol (1). These observations have led to the hypothesis that scavenger receptor-mediated uptake of modified LDL is a critical event in the development of foam cells of early atherosclerotic lesions.

High AcLDL R activity is observed exclusively in macrophages, although low activities have been observed in phorbol ester-treated smooth muscle cells and other cell types (5–7). Because AcLDL R activity is low in circulating monocytes, the development of high levels of expression in macrophages is linked to general developmental events involved in the differentiation of these cells. Specific physiologic regulators of AcLDL R gene expression are poorly defined, but they may include macrophage colony-stimulating factor (M-CSF) and platelet secretory products (5, 8). In THP-1 cells and primary monocytes, treatment with phorbol 12-myristate 13-acetate (TPA, for 12-*O*-tetradecanoylphorbol 13-acetate) promotes the differentiation of macrophage-like cells and induces expression of the AcLDL receptor. The effects of TPA on THP-1 differentiation and AcLDL R expression can be blocked by using a highly selective inhibitor of protein kinase C (9). It is therefore likely that at least some of the actions of substances that promote macrophage differentiation *in vivo* are mediated by the protein kinase C system.

The isolation of AcLDL R cDNAs has made it possible to investigate the molecular mechanisms controlling developmental regulation of this gene (10–12). In the present studies, we used the THP-1 human monocytic leukemia cell line to study activation of AcLDL R gene expression in response to treatment with TPA. In addition, we have isolated the bovine and human AcLDL R promoter regions and determined their transcriptional start sites.¶

MATERIALS AND METHODS

Tissue Culture. THP-1 cells (American Type Culture Collection) were cultured in RPMI medium 1640 supplemented with heat-inactivated 10% fetal calf serum (Gemini), 10 mM Hepes at pH 7.5, and 50 μM 2-mercaptoethanol. TPA (Sigma) was used at 0.2 μM. Cycloheximide was added at 10 μg/ml. All-*trans*-retinoic acid (Sigma) and dexamethasone (Sigma) were used at 1.0 μM and 0.1 μM, respectively. Degradation of AcLDL as a measure of AcLDL R expression was assayed according to published methods (13).

RNA Analysis. Total RNA was isolated by using guanidium thiocyanate procedures (14, 15). Human AcLDL R (hAcLDL R) mRNA was detected by RNase protection methods, using a [^{32}P]UTP-labeled antisense RNA probe representing the 281-base pair (bp) region from codon 247 to codon 339 (14). This probe was derived from a partial hAcLDL R cDNA clone generated by the polymerase chain reaction (PCR) using two degenerate oligonucleotides, 5′-GCAGCCATG-GTNYTNAARACNAARAAYTG-3′ and 5′-CCACAAGCT-TYTCNCCYTTYTGNCCYTTYTG-3′ (N = any nucleotide, Y = C or T, and R = G or A), corresponding to nucleotides +213 to +247 and +1022 to +992 in the hAcLDL R cDNA sequence (12). The resulting 794-bp cDNA fragment, extending from codon 76 to codon 339, was subcloned in the Bluescript SK II plasmid (Stratagene) and sequenced.

RNase protection analysis of the 5′ end of hAcLDL R mRNAs utilized an hAcLDL R antisense RNA probe that contained the sequences −87 to +53 from exon 1 and 17 bp from exon 2. This antisense probe was produced by using the longest 5′ hAcLDL R cDNA clone obtained by anchored PCR cloning methods (see below). Additionally, RNase

Abbreviations: LDL, low density lipoprotein; AcLDL, acetylated LDL; AcLDL R, AcLDL receptor; hAcLDL R and bAcLDL R, human and bovine AcLDL receptors; TPA, phorbol 12-myristate 13-acetate (12-*O*-tetradecanoylphorbol 13-acetate).

¶The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M93188 for bovine and M93189 for human).

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protection analysis of THP-1 RNA was conducted by using RNA probes prepared from hAcLDL R genomic clones encompassing the putative transcription start sites. The 5′ end of bovine AcLDL R (bAcLDL R) mRNAs was determined by using a probe corresponding to the region from −242 to +48 of the transcriptional start site.

Genomic Cloning and Sequencing. Genomic clones encoding the AcLDL R gene were isolated from human and bovine EMBL 3 genomic libraries (Clontech). The bovine genomic library was screened by using a PCR-generated probe corresponding to nucleotides −12 to +368 of the bAcLDL R cDNA sequence (10). The human genomic library was screened by using the previously described hAcLDL R cDNA probe and an hAcLDL R-specific oligonucleotide referred to as A1 (5′-CCTAAAGAAAGCAGCACTGATTTATCCACT-3′), which represented the 5′ end of the known hAcLDL R cDNA. Positive clones were plaque purified and genomic fragments were subcloned in the Bluescript SK II plasmid. Sequencing was performed with double-stranded templates (16). Sequence data were collected from a minimum of two overlapping runs derived from each DNA strand and analyzed by the IntelliGenetics gene analysis program.

Cloning the 5′ End of hAcLDL R cDNA. An anchored PCR strategy was used to amplify the 5′ end of hAcLDL R cDNAs derived from TPA-treated THP-1 mRNA and modified to contain a poly(G) tract at the 5′ end (17). The first amplification utilized the hAcLDL R A8 oligonucleotide (5′-GCAGCTATCAGTGTCCTCCTGTTGATTGTG-3′, representing nucleotides +51 to +22 of the hAcLDL R cDNA sequence) and previously described oligonucleotides that contained a poly(C) tract and polylinker sequences (18). A second amplification was performed with the polylinker oligonucleotide and another hAcLDL R-specific oligonucleotide (5′-GAAAGTCGACCCACTGCTCCATACTTCTTT-3′). Amplified products were ligated into the Bluescript SK plasmid and multiple clones were sequenced.

Primer Extension. Total RNA (50 μg) isolated from bovine lung tissue and TPA-treated or untreated THP-1 cells was hybridized overnight with 500,000 cpm of ^{32}P-labeled oligonucleotides (specific activity 10^6 cpm/ng) according to published methods (14). Bovine lung RNA was primed with either the bAcLDL R A8 (5′-ACAGCTGTCAGTGTCCTCTTGCTGATCAGG-3)′ or the bAcLDL R A1 (5′-GTCCTAAAGACGGCAGCACCGATT-3′) oligonucleotide, corresponding to nucleotides +51 to +22 and −10 to −33 of the bovine cDNA sequence (10). RNA from TPA-treated and control THP-1 cells was analyzed by using the hAcLDL R A8 and A1 oligonucleotides. The exact locations of transcription initiation for both human and bovine genes were determined by comparing the sizes of the primer extension bands with a sequencing reaction ladder.

Measuring Newly Transcribed RNA. Nuclei were isolated from THP-1 cells cultured for selected time intervals in the presence of 0.2 μM TPA. Each nuclear run-off reaction used 40 × 10^6 nuclei and 100 μCi (3.7 MBq) of [^{32}P]UTP as described (19). hAcLDL R transcripts were detected by using the described hAcLDL R cDNA plasmid and another plasmid containing a 2-kilobase (kb) *Xba* I genomic fragment, which represented hAcLDL R gene sequences from −696 to +53 and 1.3 kb of the first intron. Plasmids containing cDNA sequences encoding portions of the human LDL and β-actin genes were employed to measure newly transcribed mRNA species for their respective genes.

RESULTS

TPA-Mediated Induction of AcLDL R in THP-1 Cells. In agreement with previous studies (20, 21), AcLDL R activity was low in untreated THP-1 cells and was first noted to increase at 24 hr after TPA treatment (Fig. 1*A*). Activity continued to rise for up to 72 hr. No AcLDL R mRNA was detected in untreated THP-1 cells (Fig. 1*C*). AcLDL R mRNA transcripts were initially observed after 12 hr of TPA treatment, and further accumulation of these mRNA transcripts was observed during the next 48 hr. The TPA-dependent induction of AcLDL R mRNA was blocked by the concurrent addition of cycloheximide, demonstrating that new protein synthesis was required for this effect.

Recent studies of TPA-dependent signal transduction have demonstrated that glucocorticoids and retinoic acid can antagonize some of the effects of TPA on patterns of gene expression (22, 23). This antagonism appears to reflect interactions between glucocorticoid or retinoic acid receptors and AP-1 transcription factors. To assess whether TPA-dependent induction of the AcLDL R gene may involve AP-1 proteins, the effects of retinoic acid and the glucocorticoid dexamethasone on this process were examined. As shown in Fig. 1 *B* and *D*, retinoic acid or dexamethasone treatment alone had little effect on AcLDL R activity or mRNA level but significantly inhibited TPA-dependent expression.

Isolation of b- and hAcLDL R Promoter Sequences. Initial characterization of 20 independent bovine genomic clones indicated that the bAcLDL R gene locus spans more than 33 kb. The longest clone, containing the extreme 5′ end of the bAcLDL R gene, included 7 kb of 5′ flanking sequence. Exon 1 consists entirely of noncoding sequence. Exon 2, which is separated from exon 1 by a 9-kb intron, contains the site of translation initiation (Fig. 2*A*).

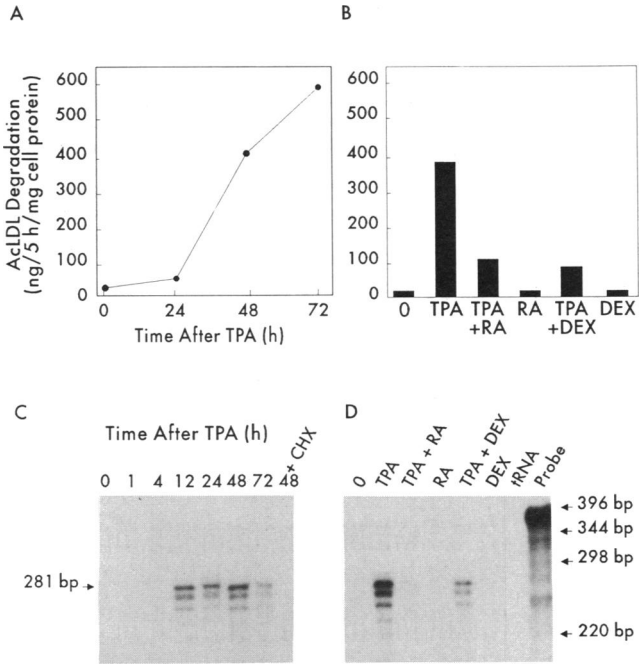

FIG. 1. AcLDL R expression in THP-1 cells after TPA treatment. (*A*) Degradation of ^{125}I-labeled AcLDL measured in THP-1 cells as a function of time (hr) after treatment with 0.2 μM TPA. (*B*) AcLDL R activity was measured 48 hr after treatment with indicated agents used at the following concentrations: TPA, 0.1 μM; retinoic acid (RA), 1.0 μM; and dexamethasone (DEX), 0.1 μM. Each bar represents the mean of duplicate determinations. Results are representative of three independent experiments. (*C*) Levels of AcLDL R mRNA were detected by RNase protection in total RNA (20 μg) isolated from THP-1 cells treated with 0.2 μM TPA for increasing time intervals (hr). Cycloheximide (CHX) was added concurrently with TPA. (*D*) Effects of RA and DEX on the TPA-dependent accumulation of AcLDL R mRNA. Treatment conditions were the same as described for *B*. The full-length protected fragment of 281 bp is indicated by an arrow in *C*.

Initial characterization of human genomic clones led to the isolation of a 15-kb insert encompassing 12 kb of 5′ flanking sequence, exon 1, and a portion of the first intron (Fig. 2*A*). Another nonoverlapping 16-kb clone contained the 3′ end of the first intron and exon 2 sequences. The first intron in the hAcLDL R gene is greater than 5 kb in length and maps to the same splice junction as in the bovine gene. A high degree of sequence conservation (74% identity) is observed within 300 bp from the transcription start sites (Fig. 2*D*). Analysis of the human and bovine AcLDL R promoter sequences for putative binding sites of known transcription factors by using the Signal Scan program (24) failed to identify consensus binding sites that were conserved in both the human and bovine promoters. A consensus AP-1 binding site was observed at −1242 (TGAGTCA) in the hAcLDL R upstream region, and near-consensus AP-1 binding sites were observed at positions −199 (TGACTTCT) and −1216 (TGAGTAA) in the bovine and human promoters, respectively. Consensus binding sites for the myeloid-specific transcription factor PU.1 were observed at −161 in the bovine gene and at −1642 in the human gene. Intriguingly, a 9-bp motif of sequence AAAGATTTT was observed just upstream of each of the two major transcriptional start sites of the human promoter, and it was highly conserved in the bovine promoter sequence (Fig. 2*D*).

Sites of Transcription Initiation. Three populations of clones with different lengths of 5′ noncoding cDNA sequence were identified by using anchored PCR cloning methods (Fig. 2*D*). These data suggested transcription started at two major

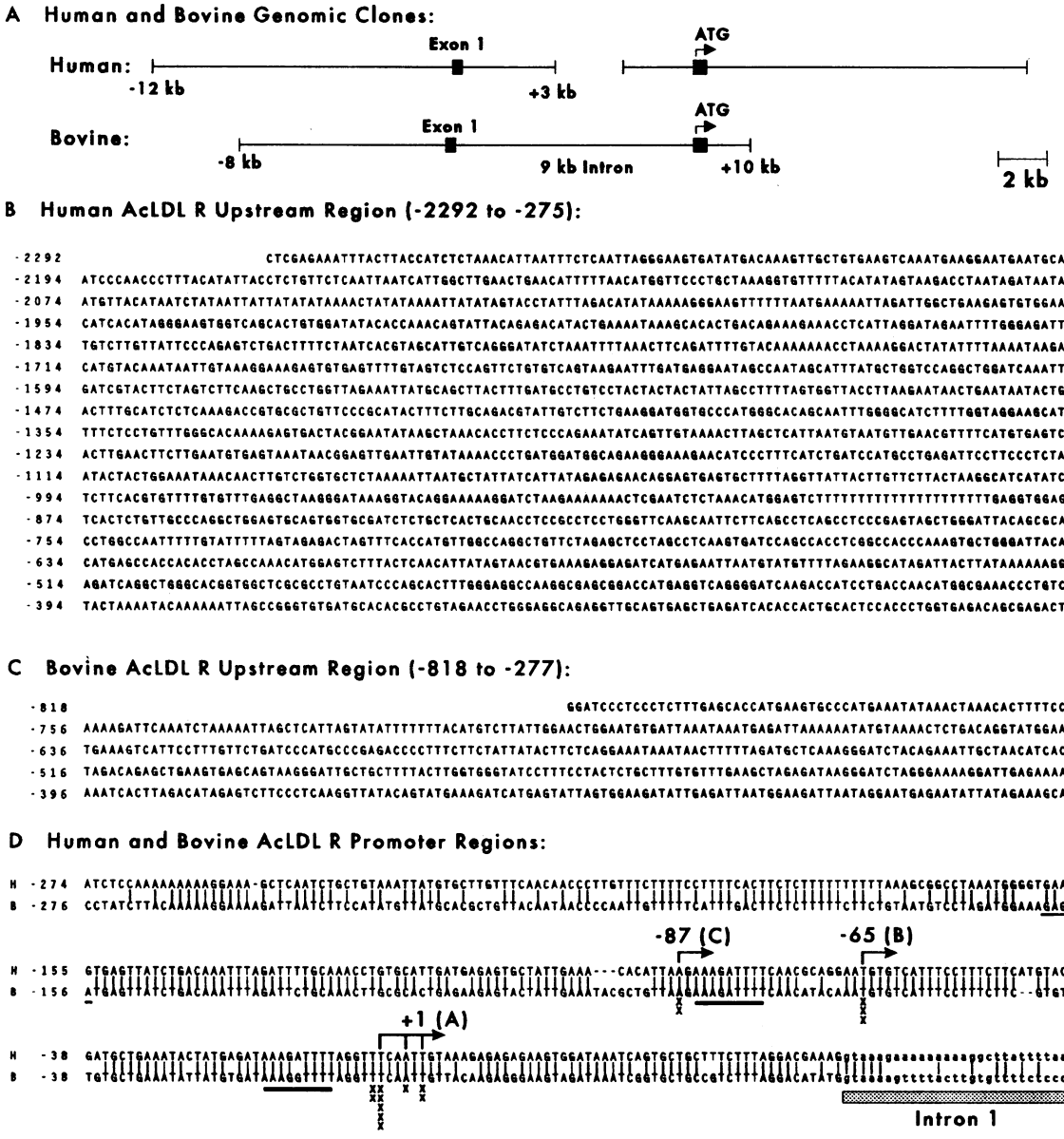

FIG. 2. Organization and sequence of the 5′ end of the AcLDL R gene. (*A*) Schematic representation of inserts isolated from human and bovine genomic clones. Exon 2 contains the ATG translational start site. (*B*) Nucleotide sequence for the upstream region of the hAcLDL R gene. (*C*) Nucleotide sequence for upstream region of the bAcLDL R gene. (*D*) The most proximal sequences of human and bovine AcLDL R promoter regions were aligned to indicate areas of high sequence conservation. The nucleotide numbering scheme was determined by using +1 to denote the major position of transcriptional initiation. In the case of multiple transcription start sites (A, B, and C) for the hAcLDL R gene, +1 was assigned to the A nucleotide located 53 bp upstream from the intron junction. The same corresponding nucleotide in the bovine promoter sequences was identified as the major transcription start site in the bAcLDL R gene. Arrows indicate sites of transcription initiation. The 5′ ends of individual hAcLDL R cDNA clones that were isolated by the anchored PCR cloning strategy are designated by Xs. Bold underlines denote a repeated motif that occurs just upstream of the major transcriptional start sites. A thin underline denotes a consensus PU.1 binding site present in the bovine promoter.

and one minor initiation site, referred to as A, B, and C respectively, and predicted exon 1 sizes of 53, 118, or 140 bp. The start site A actually represents a cluster of start sites that are close together. To verify that the 5′ ends of the isolated hAcLDL R cDNA clones represented the 5′ ends of hAcLDL R mRNA transcripts, primer extension studies were performed with the hAcLDL R A8 oligonucleotide. Major primer extension products of 108 bp (A) and 173 bp (B) sizes were uniquely represented in RNA isolated from TPA-treated THP-1 cells as compared with untreated cells (Fig. 3*A*). A third primer extension product of 195 bp (C) represented a minor transcription initiation site that is found in less than 10% of AcLDL R mRNA transcripts. The other bands in the primer extension reaction likely represent nonspecific priming of other RNA transcripts, since they were also present in untreated THP-1 RNA. The human A1 oligonucleotide, located 61 bp closer to the 5′ end of hAcLDL R transcripts, produced primer extension products with sizes of 47 and 112 bp (data not shown) and therefore predicted the same major start sites obtained with the hAcLDL R A8 oligonucleotide. The transcription initiation start sites A and B were further confirmed by RNase protection studies. The hAcLDL R cDNA clone that contained the longest 5′ noncoding sequence served as a template to generate the antisense RNA probe. Protected RNA fragments with the sizes corresponding to start sites A and B were identified only in RNA from TPA-treated THP-1 cells (Fig. 3*B*). Results obtained with two additional probes generated from hAcLDL R genomic fragments also predicted the same transcription initiation sites (data not shown).

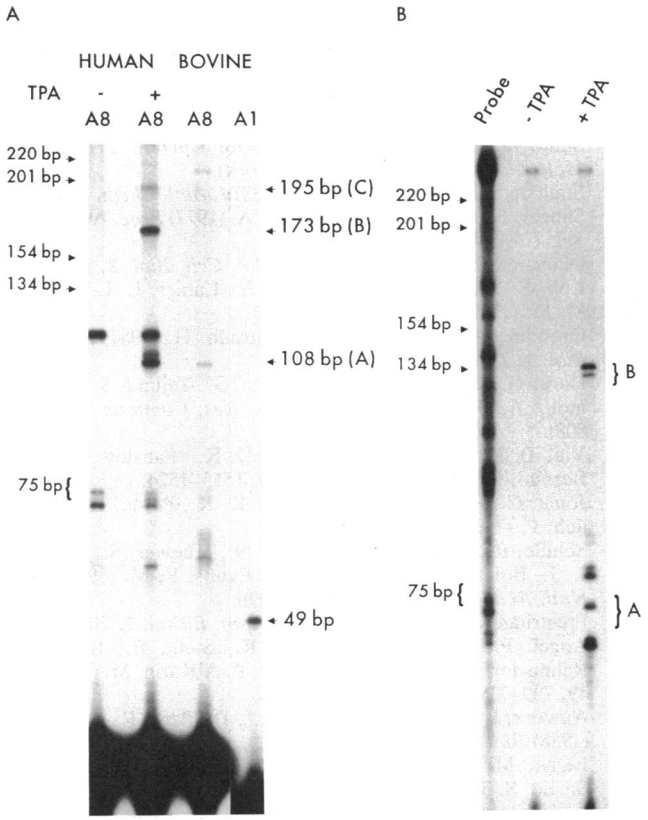

FIG. 3. Determination of transcription initiation sites for human and bovine AcLDL R mRNA. (*A*) Three A8 oligonucleotide-primed products, with sizes corresponding to the transcription initiation sites A, B, and C (Fig. 2*D*), were observed in RNA isolated from TPA-treated THP-1 cells (lane 2). Primer extension results with the bovine A8 and A1 oligonucleotides predicted a transcriptional start site in bovine lung RNA that is analogous to the A start site described for the human AcLDL R gene (lanes 3 and 4). (*B*) RNase protection analysis. An hAcLDL R RNA probe was derived from the cDNA clone that contained the most 5′ hAcLDL R cDNA sequence. The sizes of protected fragments obtained with THP-1 RNA isolated from TPA-treated cells agree with the locations of major transcription start sites A and B (lane 3).

When bovine lung RNA was used, the major primer extension product generated from the bAcLDL R A8 primer was 108 bp long (Fig. 3*A*). This start site corresponded to the primer extension product (A) observed for the human gene in TPA-treated THP-1 cells. The bAcLDL R A1 primer, which is located 59 bp closer to the site of transcription initiation, produced a primer extension band that measured approximately 49 bp when compared with a sequence ladder. The 53-bp size of exon 1 for the AcLDL R mRNA in bovine lung RNA was also verified by RNase protection (data not shown).

Nuclear Run-Off. The effects of TPA on transcriptional activation of the AcLDL R gene were directly examined in nuclear run-off assays. By using both cDNA and genomic sequences as hybridization targets, transcription of the AcLDL R gene was easily detected in untreated and TPA-treated THP-1 cells (Fig. 4). Densitometric scanning of autoradiograms indicated that TPA maximally stimulated transcription approximately 6-fold at 16 hr after treatment. Similar kinetics of induction were observed with target plasmids containing either genomic sequences corresponding to the 5′ end of the gene or cDNA sequences corresponding to a region of the gene located more than 12 kb downstream. In comparison, the rate of transcription from the β-actin gene remained relatively constant after TPA treatment. The finding of a measurable basal rate of AcLDL R gene transcription without being able to detect any mature hAcLDL R mRNAs in untreated cells implies that additional posttranscriptional mechanisms may also regulate the stability of AcLDL R mRNA.

DISCUSSION

The AcLDL R represents a particularly useful marker for the study of macrophage development because of its preferential and perhaps exclusive expression in macrophages. Treatment of THP-1 cells with TPA has been demonstrated to induce expression of the AcLDL R gene by a protein kinase C-dependent mechanism (9). Activation of the protein kinase C system by TPA is known to stimulate the activities of several classes of transcription factors, including AP-1 proteins, leading to rapid increases in the rates of transcription of immediate target genes such as c-*fos* and lipoprotein lipase (25, 26). In contrast, TPA-dependent accumulation of AcLDL R mRNA in THP-1 cells occurred after a lag phase

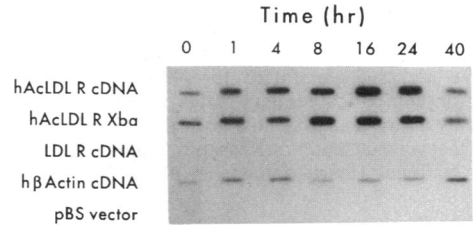

FIG. 4. Effects of TPA on the transcription rate of specific genes in THP-1 cells. Newly transcribed AcLDL R mRNA was detected by using both a plasmid containing hAcLDL R cDNA sequences (hAcLDL R cDNA) and another plasmid, termed hAcLDL R Xba, which contained exon 1 sequences plus 1.3 kb of the first intron. Plasmids containing cDNA sequences encoding portions of the human LDL receptor (LDL R cDNA) and β-actin (hβActin cDNA) genes were used to measure new transcription from their respective genes. The parent plasmid (pBS vector) controlled for nonspecific hybridization of newly transcribed RNAs.

and required new protein synthesis. These observations distinguish the regulation of the AcLDL R gene from the regulation of immediate/early response genes. Promoters of early response genes, such as c-*fos* or collagenase, contain TPA-response elements that bind activated AP-1 proteins and thereby enhance their transcription rates (e.g., see refs. 22 and 25). Although potential TPA-response elements were identified in the human and bovine promoters, these elements do not support TPA-dependent transcription in transient transfection studies (K.S.M. and C.K.G., unpublished data).

Although AP-1 transcription factors may not play an immediate role in trans-activation of the AcLDL R gene, one line of evidence suggests an indirect mode of action. Retinoic acid and dexamethasone were both found to inhibit the TPA-dependent accumulation of AcLDL R mRNA. Antagonism of the actions of TPA by retinoic acid and dexamethasone has previously been shown to involve interactions between the receptors for retinoic acid and glucocorticoids and the c-*jun* product, which is a major component of AP-1 (22, 23). These findings and the cycloheximide sensitivity of TPA-regulated AcLDL R mRNA levels suggest an indirect role of AP-1 factors in the activation of the AcLDL R gene, possibly by directing the synthesis of intermediate regulatory proteins. Regardless of the mechanisms involved, these observations suggest that pharmacologic doses of glucocorticoids or retinoic acid may alter AcLDL R expression by macrophages *in vivo*.

An intriguing aspect of the AcLDL R promoter sequence is the absence of a TATA motif, which is usually observed in genes that are expressed in a cell type-specific pattern. However, the AcLDL R promoter also differs significantly from so called "housekeeping" genes that lack a TATA box. The promoters of housekeeping genes tend to be G+C-rich, contain one or more binding sites for the transcription factor SP-1, and initiate transcription from many sites that may be widely spaced. In contrast, the AcLDL R promoter is A+T-rich and lacks SP-1 binding sites, and the majority of transcripts initiate from two closely spaced start sites. A small number of developmentally regulated genes have been identified that share some of these characteristics. Included in this class are *Drosophila* homeotic genes [e.g., Ultrabithorax (27)] and genes that are regulated during immunodifferentiation [e.g., the terminal deoxynucleotidyltransferase (28) and the T-cell receptor β-chain genes (29)]. These observations suggest that developmental regulation of the AcLDL R gene may involve basal transcription factors that are distinct from those that recognize the TATA motif. The identification and characterization of the factors regulating AcLDL R gene expression are likely to provide insights into the more general mechanisms that regulate development of monocytes and macrophages from bone marrow precursor cells.

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