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Differentiation-Dependent Expression of a Human Carboxylesterase in Monocytic Cells and Transcription Factor Binding to the Promoter

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Carboxylesterases play an important role in defense and clearance mechanisms of the monocyte/macrophage system. During the differentiation process of cells from the monocytic cell line THP-1 we observed a transient transcriptional upregulation of a human carboxylesterase analyzed by means of Northern blots. In PMA-treated THP-1 cells we could detect three major transcription initiation sites as revealed by Nuclease Protection Assay carried out with two overlapping antisense RNA probes. We have recently cloned the carboxylesterase upstream sequence and showed its basal promoter activity in CHO cells. Using electrophoretic mobility shift analysis we demonstrated that the promoter region spanning base pairs −1 to −275, which contains several putative binding sites for transcription factors, is bound by nuclear factors Sp1 and IRBP but not by C/EBPs. Taken together these data indicate that carboxylesterase gene transcription in THP-1 cells starts at multiple initiation sites and that Sp1 and IRBP may be critical factors for modulating the differentiation-dependent transcription of this human carboxylesterase gene.

Monocytes/Macrophages are active phagocytic cells that play an important role in the immune system (1). During monocyte differentiation, pluripotent stem cells become comitted to myeloid precursor cells, which differentiate into monocytes that enter the circulation (2). The process of monocytic differentiation involves the gain and loss of several functions, e.g. enzymatic activity, which are accompanied by a complex pattern of gene regulation and expression.

Mammalian carboxylesterases (E.C. 3.1.1.1), which constitute a family of isoenzymes (3) are involved in several physiological processes, such as cholesterol homeostasis (4) and detoxification mechanisms (5). These enzymes are responsible for the hydrolysis of ester, thioester, and amide bonds of a series of xenobiotics (6). Although the expression of carboxylesterase genes is not restricted to macrophages, these enzymes posess major functions in the monocyte/macrophage defense system. Thus, a 60-kDa serine esterase which is released by human alveolar macrophages was shown to be capable of initiating and modulating inflammatory processes (7). Information on molecular mechanisms of transcriptional regulation of carboxylesterases is very limited, mainly due to the lack of analysis of promoter elements and transcription factors. Recently, we have reported the cloning of a complete human carboxylesterase gene including the promoter region and demonstrated that the first 195 bp proximal to the transcription start site exhibit basal promoter activity in CHO cells as determined by luciferase reporter assay (8). Within this region, putative binding sites for the transcription factors Sp1 (9) and CAAT enhancer binding protein (C/EBP) (10) were found. In the present study, we describe the transcriptional regulation of this gene in the monocytic leukemia cell line THP-1 after PMA-induction, the identification of alternative transcription start sites and binding of transcription factors Sp1 and IRBP to their coressponding *cis*-acting elements in the carboxylesterase promoter.

MATERIALS AND METHODS

Cell culture. THP-1 cells were obtained from the American Type Culture Collection, Rockville, MD, and were cultured in RPMI 1640 (Sigma) with 10% fetal bovine serum (Gibco BRL), 2 mM L-glutamine in a 5% CO_2 incubator. THP-1 cells were induced to differentiate by

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addition of 160 nM phorbol 12-myristate-13-acetate (PMA). Upon addition of PMA, THP-1 cells, which are propagated in suspension, stop dividing, become adherent, and differentiate to a macrophage-like state.

Preparation of RNA and Northern blot analysis. Total cellular RNA was isolated from THP-1 cells stimulated with PMA for the indicated times by guanidium isothiocyanate lysis and CsCl centrifugation (11). The RNA isolated was quantitated spectrophotometrically and 10 μg samples were separated on a 1.2% agarose-formaldehyde gel and transferred to a nylon membrane (Schleicher & Schüll). Probes were radiolabeled with [α-^{32}P]dCTP (Amersham) using the Oligolabeling kit from Pharmacia, and were allowed to anneal bound RNA using a hybridization solution recommended by the manufacturer of the membrane. Final washes prior to exposure to film were usually 5% SDS, 1× SSC at 60° C.

Ribonuclease protection assay. The plasmid constructs used to make riboprobes were prepared by the cloning of DNA fragments into a pGem4Z vector (Promega). DNA fragments were amplified from genomic λ-clones containing the carboxylesterase gene described previously (8) by polymerase chain reaction (PCR) performed with the Expand-PCR system (Boehringer). Oligonucleotides to amplify probe PA1 were, P1 (5′GCGGGGATCCGGCGCCAGGGCTGGACAG3′) and P3 (5′CCCCGAATTCGGGAAGCAGAGAGAGTGG3′), and to generate PA2 were, P2 (5′GCTTTTCGGATCCCTCCCAATTAGAGGATT3′) and P3. To use these plasmids for the generation of [^{32}P] riboprobes, plasmids were linearized with the appropriate restriction enzymes. Riboprobes were prepared by *in vitro* transcription of the plasmid constructs with [α-^{32}P]CTP and SP6 RNA polymerase with the SP6 Riboprobe kit (Promega). The labeled transcripts were treated with DNase I and purified with Sephadex G50 columns (Boehringer). Nuclease protection assays were performed with the RNase Protection kit (Boehringer). 5-20 μg of total RNA from untreated or PMA-induced THP-1 cells were hybridized overnight at 50° C with the [^{32}P]-labeled riboprobes. After the incubation of hybridized samples with RNase A and RNase T1, the protected fragments were analyzed on a 6% polyacrylamide gel containing 8M urea. The gel was fixed, dried, and exposed to film. The molecular weight markers used were MspI digested 5′end-[^{32}P]-labeled DNA fragments from pBR322 (NEB) and a sequencing reaction.

Electrophoretic mobility shift assays (EMSA). Nuclear extracts were prepared from THP-1 cells as previously described (12). The concentrations of nuclear proteins were determined with Bio Rad protein-assay reagent. EMSA (13) were performed with 10 μg of nuclear extracts and 5′end[^{32}P]labeled double-stranded oligonucleotides, which were assembled in the following reaction mixture: 50 mM HEPES, pH 7.9, 6 mM $MgCl_2$, 50 mM KCl, 5 mM DTT, 100 μg/ml BSA, 0.01% NP40, and 10 mg/ml poly(dI-dC). After incubation for 20 minutes at room temperature, aliquots were loaded on a 8% polyacrylamide gel. In competition experiments nuclear extracts were preincubated with a 50-fold molar excess of competitor for 10 minutes prior to addition of the radiolabeled probe. Oligonucleotides used in EMSA are listed in table 1.

RESULTS AND DISCUSSION

PMA-mediated induction of carboxylesterase mRNA in THP-1 cells. Since it has been shown that a carboxylesterase can be released by alveolar macrophages (7), we attempted to clarify the mechanisms controlling the expression of this enzyme. To investigate the expression of the carboxylesterase at the RNA level in differentiating macrophages, we have studied cells from the monocytic cell line THP-1, that may be induced to differentiate toward a macrophage-like state by culture with phorbol ester. Results from other laboratories (14) indicate that THP-1 cells may serve as model system for the study of gene expression during monocyte to macrophage differentiation. RNA was prepared at different times after the cells were treated with PMA and sequentially hybridized with the carboxylesterase cDNA and a GAPDH-probe (Fig.1). No carboxylesterase mRNA was detected in unstimulated cells, however, the expression was strongly upregulated after PMA treatment for 3 hours followed by a continuous decrease of mRNA in cells stimulated for longer periods. This shows, that the amount of carboxylesterase mRNA is modulated by the differentiation state of the THP-1 cell. To determine whether this regulation occurs in native monocytes as well as the THP-1 cell line, we analyzed mRNA levels for the carboxylesterase in total RNA from human peripheral blood monocytes and from monocyte-derived macrophages after 7 days in culture (data not shown). Similarly, the carboxylesterase message was not detectable in monocytes but in

TABLE 1

Oligonucleotides Used in Electrophoretic Mobility Shift Assay (EMSA)

Name	Sequence	Location[1]	Size
Sp1 (1)	GCAGGGCGGTAACTCTGGGCGGGGCTGGGCG	−37 to −7	(31)
Sp1 (2)	GGCGCCCAGCCCCGCCCAGAGTTACCGCCCT	−37 to −7	(31)
Sp1 con. (1)	ATTCGATCGGGGCGGGGCGAGC		(22)
Sp1 con. (2)	GCTCGCCCCGCCCCGATCGAAT		(22)
C/EBP (1)	GATCTCTCCCAATTAGAGGATTAGGCAATTGGCAG	−73 to −39	(35)
C/EBP (2)	CGCTGCCAATTGCCTAATCCTCTAATTGGGAGAGA	−73 to −39	(35)
E-BOX (1)	TTGGGGGCCGTCACAGTGCACTGAGGTTAGAGTCCTGCAAG	−275 to −235	(41)
E-BOX (2)	CTTGCAGGACTCTAACCTAAGTGCACTGTGACGGCCCCCAA	−275 to −235	(41)

[1] The sequence of oligonucleotides used in EMSA and their location within the carboxylesterase promoter are shown. Position +1 refers to the transcription initiation site previously identified by 5′-RACE-PCR (8). The size of each oligonucleotide in base pairs is given as numbers in brackets. Double-stranded oligonucleotides (DO) were obtained by mixing equal molar amounts of single-stranded oligonucleotides 1 and 2, respectively.

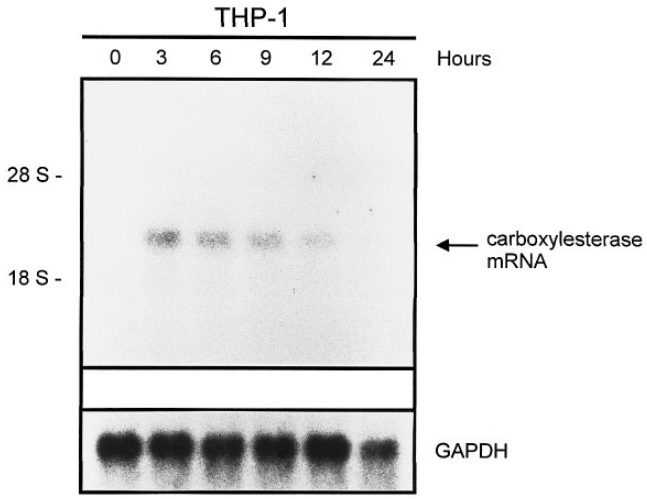

FIG. 1. Northern blot analysis of carboxylesterase mRNA. Total RNAs were isolated from THP-1 cells cultured in the presence of 160 nM PMA for 0, 3, 6, 9, 12, and 24 hours. Each lane was loaded with 10 μg of total RNA. The blot was sequentially hybridized with a [^{32}P]-labeled carboxylesterase cDNA and a GAPDH-probe to verify equivalent RNA loading. PMA, phorbol 12-myristate 13-acetate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

differentiated macrophages. Since the activation of the protein kinase C system is known to be a common mechanism to mediate phorbol ester induced expression of several immediate-early genes such as c-fos and lipoprotein lipase (15, 16), the obtained results reveal an involvement of PKC in activating carboxylesterase transcription.

Mapping of transcription start sites. Because the sequence data from the 5′region of the carboxylesterase gene showed no apparent TATA box or transcription initiator (Inr) sequences, ribonuclease protection assays were performed to determine the transcriptional start site(s) of the carboxylesterase message. Two overlapping genomic fragments PA1 and PA2 spanning from −19 to +119 and −81 to +119 (8), respectively, were PCR-amplified and cloned into the plasmid vector pGem4Z (Promega). Antisense RNA probes were generated using SP6 RNA Polymerase. The probes were hybridized to different amounts of RNA from THP-1 cells stimulated with PMA for 3 hours or yeast tRNA at 50°C overnight and were subjected to ribonuclease protection analysis. Protected fragments of 119, 106, 87, and 84 nucleotides were detected with both probes PA1 and PA2 (Fig. 2). Since all protected fragments generated with probe PA1 are also found with probe PA2 and since no additional bands occur with probe PA2, further upstream initiation sites can be excluded. This demonstrates that in addition to the unique transcription initiation site detected previously by RACE (8), three alternative start sites downstream from this point are being used. When comparing the amounts of protected RNA fragments we conclude that transcription mainly occurs from the newly identified start sites. These data are in agreement with many other TATA-less (G+C)-rich promoters which routinely initiate transcription at multiple start sites, although initiation at a single site has been documented in a minority of cases (17, 18).

Transcription factor binding to the carboxylesterase promoter. Double-stranded oligonucleotides were synthesized corresponding to nucleotides −37 to −7 (DO-Sp1), −73 to −39 (DO-C/EBP), and −275 to −235 (DO-E-BOX) in the (G+C)-rich region of the carboxylesterase promoter. Nuclear extracts were prepared from THP-1 cells treated with PMA for 3 hours and electrophoretic mobility shift assays (EMSA) were performed as described. Duplex oligonucleotide DO-Sp1, which contains two consensus binding sites for transcription factor Sp1, binds a nuclear protein in THP-1 cell extracts as well as purified human Sp1 protein (Fig. 3A).

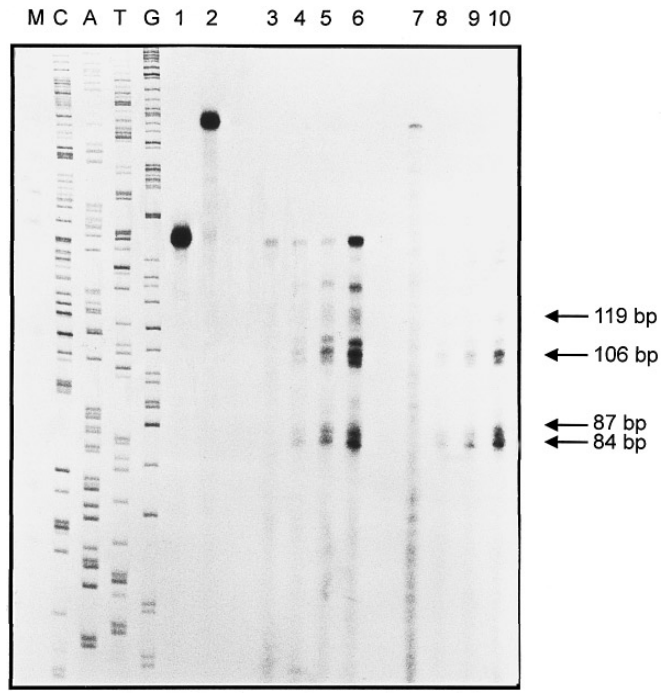

FIG. 2. Localization of transcription start sites. The autoradiograph from a ribonuclease protection assay using two overlapping antisense RNA probes PA1 and PA2 spanning from −19 to +119 and −81 to +119, respectively, is shown. The [^{32}P]-RNA probes were allowed to hybridize to total RNA from THP-1 cells stimulated with 160 nM PMA for 3 hours, and digested with RNase A and RNase T1. Lane M contains a labeled dephosphorylated MspI digested pBR322 marker. Lanes C, A, T, G show a sequencing ladder. Lanes 1 and 2 contain the undigested probes PA1 and PA2, respectively. Yeast tRNA was used as negative control in the protection experiments (lanes 3, 7). Lanes 4-6 are hybridizations of probe PA1 with 5, 10, and 20 μg of THP-1 RNA. Lanes 8-10 show hybridization products of probe PA2 with the same quantities of RNA. The protected fragments of 119 bp, 106 bp, 87 bp, and 84 bp are indicated.

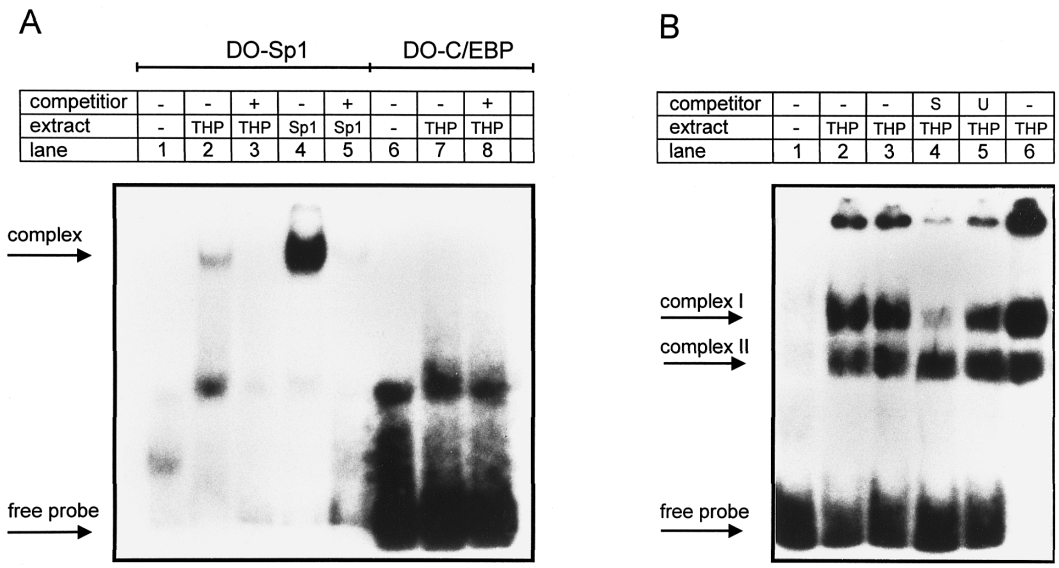

FIG. 3. Gel mobility shift analysis (EMSA). (A) Binding of DO-Sp1 oligonucleotide by Sp1 transcrition factor. Competition experiments with double-stranded oligonucleotides DO-Sp1 (lanes 1-5) and DO-C/EBP (lanes 6-8) are shown. 10 μg of nuclear extracts (7) from PMA-induced THP-1 cells (lanes 2, 3, 7, 8) or purified human Sp1 protein (Promega) (lanes 4, 5) were used in EMSA. Lanes 1 and 6 show negative controls with no nuclear extract. Using 10,000 cpm of probe DO-Sp1 (Table 1), a specific DNA-protein complex (indicated by an arrow) could be detected with THP-1 extract as well as with Sp1 protein. Addition of a 50-fold molar excess of unlabeled competitor oligonucleotides DO-Sp1 (lane 3) and DO-Sp1 con. (lane 5) resulted in a complete inhibition of complex formation. Lanes 6-8 show the results obtained with oligonucleotide DO-C/EBP. In this experiment, no bandshift with THP-1 cell extract could be observed. An intrinsic interaction of the probe, that is also present in the negative control, very likely presents a non-specific complex. In order to achieve adequate separation of bands, the gel was electrophoresed sufficiently long such that unbound probe was run off the bottom. (B) Binding of DO-E-BOX by IRBP. Competition experiments using oligonucleotide DO-E-BOX (Table 1) encompassing the IR (inverted repeat) element from the junB promoter (20) as a probe. Lane 1 represents a negative control without nuclear extract. Two protein-DNA complexes were detected (I and II), of which complex I was inhibited by pre-incubation of the nuclear extract with a 50-fold molar excess of unlabeled DO-E-BOX oligonucleotide (lane 4; s, specific competition). Competition with an excess of poly dI-dC resultet neither in an inhibition of complex I nor of complex II formation (lane 5; u, unspecific competition). Lane 6 demonstrates complete binding of probe using excess (20 μg) of nuclear extract, as shown by the absence of free probe.

Both bandshifts have the same molecular weight, indicating that the nuclear factor isolated from the THP-1 cells is identical with Sp1. This is an interesting finding because the zinc-finger protein Sp1 can mediate tissue specific expression as well as differentiation dependent expression (19). In contrast to many other transcriptional activators that interact with the TATA box factor TFIID, SP1 can act independently. The specificity of the formed complexes was confirmed using an excess of unlabeled oligonucleotide and a consensus oligonucleotide (Table 1) as competitors. When using DO-C/EBP duplex oligonucleotide, which contains CAAT boxes, no bandshift could be detected with THP-1 nuclear extracts (Fig. 3A). This is evidence that the putative CAAT boxes in the carboxylesterase promoter are not functional in vivo and that no transcription factors interacting with this sequence are expressed in THP-1 cells. Thus, C/EBPs are not capable of mediating the differentiation-dependent expression of the carboxylesterase.

The inverted repeat AGTGCACT was previously shown to be a mediator of PMA-induced transcription from the junB promoter (20). Since this *cis*-acting element was found in the carboxylesterase upstream region and the transcription is regulated by PMA, we attempted to identify specific DNA-protein interactions between the DO-E-BOX oligonucleotide (Table 1) and nuclear extracts from THP-1 cells. As shown in Fig. 3B, two complexes were observed. Complex I was specifically competed with unlabeled E-BOX oligonucleotide. In contrary, complex II-formation was not inhibited by specific as well as non-specific competitor, indicating that this second complex is formed by an unspecific binding protein.

The previous study (8) and the experiments presented here demonstrate that at least part of the differentiation-dependent regulation of the carboxylesterase promoter involves the 275 base pairs upstream of the transcription start site. Our results suggest that the putative binding sites for transcription factor C/EBP exert no effect on the transcription mechanismn in THP-1 cells. In contrast, the transcription factor Sp1 and the E-BOX binding protein (IRBP, inverted repeat binding protein) could interact to mediate the differen-

tiation-induced expression of the carboxylesterase gene in THP-1 cells.

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