Transcriptional regulation of lysosomal acid lipase in differentiating monocytes is mediated by transcription factors Sp1 and AP-2

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Abstract **Human lysosomal acid lipase (LAL) is a hydrolase required for the cleavage of cholesteryl esters and triglycerides derived from plasma lipoproteins. It is shown here that during monocyte to macrophage differentiation, the expression of LAL-mRNA is induced. This induction is dependent on protein kinase C activity and protein synthesis. The cell type-specific increase in LAL expression is further investigated in the THP-1 cell line with respect to transcriptional regulation. The human monocytic leukemia cell line THP-1 differentiates into macrophage-like cells when treated with phorbol esters. In order to determine the *cis*-acting elements necessary for both basal and phorbol 12-myristate-13 acetate (PMA)-enhanced promoter activity, we performed deletion analysis and reporter gene assays. A PMA responsive element has been identified between −182 bp and −107 bp upstream of the major transcription start site. Gel mobility shift assays demonstrated that binding of Sp1 and AP-2 to the LAL promoter is increased by PMA in THP-1 cells. Co-transfections with expression plasmids for Sp1 and AP-2 further emphasized the important role of these transcription factors in both basal and PMA-enhanced LAL expression. Our data suggest that differentiation dependent increase of lysosomal acid lipase (LAL) expression in THP-1 cells is mediated by a concerted action of Sp1 and AP-2.—**Ries, S., C. Büchler, T. Langmann, P. Fehringer, C. Aslanidis, and G. Schmitz. **Transcriptional regulation of lysosomal acid lipase in differentiating monocytes is mediated by transcription factors Sp1 and AP-2. *J. Lipid Res.* 1998.** 39: **2125–2134.**

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Human lysosomal acid lipase (LAL; E.C. 3.1.1.13) catalyzes the hydrolysis of both cholesteryl esters and triglycerides that are delivered to lysosomes by low density lipoprotein (LDL) receptor-mediated endocytosis (1). Free cholesterol is released from lysosomes, which leads to activation of acyl-CoA:cholesterol acyltransferase (ACAT) and to down-regulation of LDL receptor gene expression and HMG-CoA reductase activity. Therefore, LAL contributes to the homeostatic control of plasma lipoprotein levels and to the prevention of cellular lipid overload in liver, spleen, macrophages, and smooth muscle cells of the arterial wall (2). LAL activity is significantly reduced in mononuclear cells from patients suffering from premature coronary heart disease and familial hypercholesterolemia (3, 4). The role of LAL in the pathogenesis of atherosclerosis is also emphasized by the existence of an autosomal recessive disease, namely cholesteryl ester storage disease (CESD) (5). In this disorder LAL activity is reduced due to mutations within the coding region of the LAL gene. In contrast, patients with a complete lack of LAL activity (Wolman disease) die within the first year of life (6–8). The LAL gene is approximately 36 kb long, consists of 10 exons, and is transcribed as a 2.4 kb long mRNA (9–11).

Mature macrophages extend their lysosomal compartment required for intracellular degradation processes, and therefore enhanced expression of lysosomal enzymes is expected (12). To investigate LAL expression in differentiating monocytes/macrophages, we have analyzed both peripheral blood monocytes and the human promonocytic leukemia cell line THP-1 (13). The human leukemia cell line THP-1 may be induced toward a macrophage like state by culture with phorbol esters; this is characterized by adherence, increased phagocytic activity, and the induction of lysozyme and non-specific esterase activities (14). THP-1 cells thus provide a useful in vitro model to investigate biological properties of macrophages. Recently, many studies have focused on the alteration of gene expression

Abbreviations: LAL, lysosomal acid lipase; M-CSF, macrophage-colony stimulating factor; PMA, phorbol 12-myristate-13-acetate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SR-A, scavenger receptor type A; EGR, early growth response; PKC, protein kinase C; TSS, transcriptional start site; TBP, TATA-box binding protein.

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that occurs along with the maturation of macrophages. For example, expression of scavenger receptor type A, vacuolar H^+-ATPase, and several lysosomal enzymes like cathepsin L are induced during differentiation (15–17). We have examined LAL expression during monocyte to macrophage differentiation and provide evidence for both transcriptional and post-transcriptional regulation. As the putative promoter region of the LAL gene has been cloned (10, 11), the 5′ flanking region was amplified by PCR and promoter elements responsible for transcriptional control were characterized. A genomic fragment 182 bp upstream of the major transcription start site possesses full promoter activity, that is inducible up to 3.5-fold in THP-1 cells cultured in the presence of PMA. Binding of transcription factors to specific promoter elements has been demonstrated by gel mobility shift assays. Our data clearly indicate that transcriptional induction of LAL during monocyte to macrophage differentiation is mediated by a concerted action of transcription factors Sp1 and AP-2.

MATERIALS AND METHODS

Reagents

Phorbol 12-myristate-13-acetate (PMA), the protein kinase C inhibitors, staurosporine and H7 (1-[5-isoquinolonesulfonyl]-2-methylpiperazine dihydrochloride), actinomycin D, chloroquine, DEAE-dextran, and cycloheximide were purchased from Sigma (St. Louis, MO). Human recombinant M-CSF was purchased from Genzyme (Cambridge, MA).

Cell culture

THP-1 cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (Gibco BRL, Eggenstein), 100 U penicillin per ml, and 100 μg of streptomycin per ml in a humidified atmosphere in 10% CO_2 at 37°C. To induce monocyte to macrophage differentiation, THP-1 cells were cultured in the presence of 160 nm PMA. Peripheral blood monocytes were isolated from normolipidemic volunteers by leukapheresis and subsequent counterflow centrifugation (18). Fractions containing >90% CD14 positive cells were pooled and cultured for 7 days on plastic petri dishes in macrophage SFM medium (Gibco BRL, Eggenstein) supplemented with 50 ng/ml human recombinant M-CSF.

RNA isolation and Northern blot analyses

Cells were washed twice with PBS (Gibco BRL, Eggenstein) and total RNA was extracted by the isothiocyanate/cesium chloride ultracentrifugation method as described elsewhere (19).

Ten μg of total cellular RNA was separated on 1.2% agarose gels containing 6% formaldehyde, transferred to nylon membranes (GeneScreen Plus, DuPont, Bad Homburg), and UV-crosslinked. LAL specific transcripts were detected by hybridization using a 1.2 kb cDNA fragment generated by PCR with LAC1/LAC2 primers radiolabeled using the Oligolabeling kit (Pharmacia, Freiburg), and hybridized to the membranes. The primer sequences for LAC1 and LAC2 were 5′-CCCGGCAGGA CAGCTCCAGA-3′ and 5′-GTGTGACACAGCTCAAGTCCAGC TT-3′, respectively. Hybridization and wash conditions were as recommended by the manufacturer (DuPont, Boston, MA). Subsequently, the membranes were exposed to Kodak AR X-Omat films at −70°C with intensifying screens.

Ribonuclease protection assay

An LAL specific cDNA probe was generated by PCR using primers LACup 5′-CAAGGATCCACGATTACCAGATCATCTCA TTAAG-3′ and LACdown 5′-TAGAATTCTGGCTCCAGTGTAA CATGTTTTGC-3′ and cloned into a pGEM4Z vector (Promega, Madison, WI). To generate radiolabeled cRNA probes, the plasmid was linearized and transcribed in vitro using α[^{32}P]CTP and SP6 RNA polymerase. The ribonuclease protection assay was performed with the RNase protection kit (Boehringer, Mannheim). To determine the number of transcriptional start sites, two overlapping genomic fragments PA1 and PA2, spanning from −219 to +59 and −109 to +59 relative to the major transcription start site, were PCR amplified and cloned into the plasmid vector pGEM4Z. The P1 fragment was amplified using the primers SR1-up 5′-CCGGGATCCAGCCTGCGAGGCGGAGGACG-3′ and SR-down 5′-GGAGAATTCCTGCCGGGCCGCTGTCTCGAG-3′. The probe PA2 was generated using SR2-up 5′CCTGGATCCGAG GCACTTCCCGGTGGCTG-3′ in combination with SR-down.

Nuclear run-on analyses

Nuclear run-on assays were performed using nuclei from resting THP-1 cells and THP-1 cells cultured in the presence of 160 nm PMA for 24 h as previously described (20). For each nuclear run-on experiment, 2×10^7 nuclei were used. The LAC1/LAC2-amplified 1.2 kb LAL cDNA fragment and cDNA fragments of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and scavenger receptor type A (SR-A) were subcloned into the pUC18 plasmid vector (Pharmacia, Freiburg). Five μg of these plasmids was linearized, alkaline denatured, and transferred to nylon membranes (Schleicher & Schüll, Dassel), using the pUC18 vector as negative control. Hybridization and washing of the membranes were carried out as described by Srivastava, Cable, and Bonkovsky (20).

Immunoblotting

Cells were first homogenized by sonication and subjected to SDS-PAGE in a Mini Protean II electrophoresis chamber (Bio-Rad, Hercules, CA) (21). The proteins were then transferred onto the PVDF FluoroTrans™ membrane (Pall, Dreieich) using the Trans-Blot® semi-dry transfer cell (Bio-Rad). Blocking was done overnight at 4°C with 5% non-fatty dried milk, followed by a 1-h incubation at room temperature with polyclonal rabbit antiserum against recombinant LAL from *E. coli* (dilution 1:1000 in blocking buffer). Detection of the anti-LAL antibodies was performed by a 1-h incubation with a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Dianova, Hamburg). Unbound antibody was removed by extensive washing after each incubation step. The bound antibodies were visualized by chemiluminescence with the ECL™ Western blotting detection reagent (Amersham, Braunschweig), and protein concentration was determined by the method of Smith et al. (22).

Preparation of THP-1 nuclear extracts

Nuclear extracts from resting and PMA-induced THP-1 cells were prepared as described previously (23). THP-1 cells (5×10^7) were used in each experiment. Nuclear extracts were aliquoted and stored at −80°C prior to use. The protein concentration of each nuclear extract has been determined by the method of Smith et al.(22).

Construction of LAL gene promoter/luciferase chimeras

The various 5′ regions of the LAL gene were generated by means of PCR using specific primers for each construct and were cloned into the promoterless pGL3-basic luciferase vector (Promega, Madison, WI). Primers for pLAL1 were Pro 2586 5′-TTTGGTACCCCC ACATGCTCTCTGTCAGGCGCAGAAG-3′ and Pro3005i 5′-ACA

GATCTCTGGAGCTGTCCTGCCGGGCCGCT G-3′; for pLAL2, Pro2730 5′-GCCGGTACCGCGGAGGACGGGCTCCATCTCTT AG-3′ and Pro3005i; for pLAL3, Pro2740 5′-GGGCTAGCGAA ACGCCTACGGCGCATGCTCTAT-3′ and Pro3005i; for pLAL4, Pro2750 5′-GGGCTAGCGGGGTCAACTGGGGGGCTG GCAAGC-3′ and Pro3005i; for pLAL5, Pro2780 5′-GGGCTAGCGGCAG CGCTGGTCTGGGGCGGAGTCT-3′ and Pro3005i; for pLAL6, Pro2830 5′-CGGGTACCCCGAGGCACTTCCCGGTGG CTGGC-3′ and Pro3005i; for pLAL7, Pro2805 5′-GGGCTAGCTGCTCT GATTGGCTGAACAAATAGTC-3′ and Pro3005i.

The PCR conditions were described previously (7). The PCR products were purified by Qiaex (Qiagen, Hilden), digested with *Bgl*II and *Kpn*I (pLAL1, 2, and 7) or *Bgl*II and *Nhe*I (pLAL3, 4, 5, and 6) and cloned into the pGL3-basic luciferase vector. All LAL/luciferase fusion constructs had a common 3′ terminus of the LAL gene located at nucleotide +67, with the fused promoter fragment varying in length from 149 bp to 419 bp. The LAL gene promoter/luciferase constructs were sequenced prior to use.

Transient transfection of THP-1 cells and luciferase assays

For each experiment 5×10^7 THP-1 cells were transfected with 10 μg reporter plasmid DNA by the DEAE-dextran method as previously described (24). Two μg of pSVβ-galactosidase plasmid (Promega, Madison, WI) was included in all assays and luciferase activities were normalized to β-galactosidase activities. In co-transfection experiments, 100 ng of a transcription factor expression vector or pUC18 plasmid (negative control) was added. To increase transfection efficiency, cells were incubated with 100 μm chloroquine. After removal of chloroquine, cells were resuspended in 30 ml complete RPMI 1640 medium, divided into two 75-cm^2 culture flasks, and incubated for 24 h.

PMA (160 nm) was added to one flask 24 h after transfection. Forty-eight h after transfection cells were harvested and luciferase activity was measured in a Lumat LB9501 (Berthold, Munich) according to the instructions of the manufacturer (Promega, Madison, WI). β-Gal activity was evaluated in a standard color reaction to detect differences in transfection efficiency (25).

Gel mobility shift assays

An equivalent of 30,000 cpm of labeled double-stranded oligonucleotide probe containing the desired promoter sequence was incubated with 10 μg of nuclear extract in a buffer containing 50 mm HEPES/HCl, pH 7.9, 6 mm $MgCl_2$, 50 mm KCl, 5 mm dithiothreitol, 100 μg/ml BSA, 0.01% NP40, and 2 μg poly(dI-dC) (Pharmacia, Freiburg) at room temperature for 20 min. Supershift analyses were carried out using antibodies against Sp1 and AP-2 (Santa Cruz Biotechnology, Santa Cruz, CA). For Sp1, a rabbit polyclonal antibody against residues 436–454 was used (catalog number sc-59x) and AP-2 binding was examined using a rabbit polyclonal antibody against residues 420–437 (catalog number sc-184x). In competition experiments nuclear extracts were preincubated with a 50-fold molar excess of competitor for 10 min prior to addition of the radiolabeled probe. DNA–protein complexes were resolved by electrophoresis through 8% polyacrylamide gel and visualized by autoradiography.

RESULTS

Characterization of LAL expression in monocytic cells

To study the mechanisms regulating differentiation dependent lysosomal acid lipase (LAL) expression in monocytes, we performed Northern blot analyses on total cellular RNA isolated from peripheral blood monocytes and monocyte-derived macrophages. **Figure 1A** shows a Northern blot hybridized with a 1.2 kb long cDNA fragment generated with the LAL primers LAC1 and LAC2. Steady-state levels of LAL mRNA increase during differentiation. Quantification of LAL mRNA was achieved with a ribonuclease protection assay using an RNA probe containing an LAL-specific sequence located +720 to +963 downstream of the major transcription start site. We allowed the probe to hybridize to 10 μg of total cellular RNA from monocytes and monocyte-derived macrophages cultivated in the presence of 50 ng/ml human recombinant M-CSF for 7 days, or to a negative control (yeast t-RNA). Isolation and measurement of the protected RNA fragments after gel electrophoresis revealed a strong increase in LAL mRNA induction up to 12.4-fold after 7 days cultivation (numbers in boxes below Fig. 1).

To determine whether this regulation also occurs in the leukemia cell line THP-1, we analyzed the LAL mRNA expression pattern in THP-1 cells differentiated with PMA by Northern blots. As can be seen in Fig. 1B, steady state levels of LAL mRNA increased after PMA treatment.

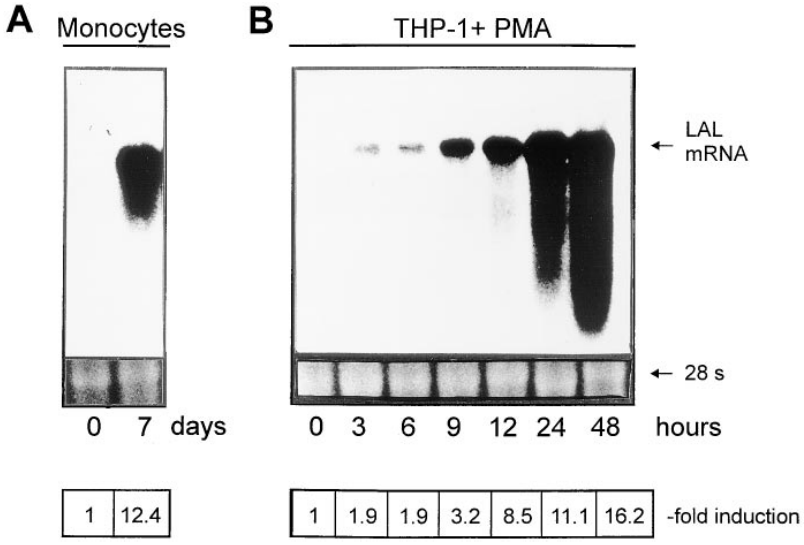


Fig. 1. Northern blot analysis of LAL mRNA during differentiation of monocytes to macrophages. Total cellular RNA was isolated from (A) human blood monocytes (day 0) and monocyte-derived macrophages (day 7), or (B) THP-1 cells cultured in the presence of 160 nm PMA for the indicated time. Each lane was loaded with 10 μg of total cellular RNA. Northern hybridization was carried out using the [^{32}P]-labeled 1.2 kb LAL cDNA fragment generated with LAC1 and LAC2. The induction factor of LAL mRNA levels is depicted at the bottom. The ethidium bromide stained agarose gel showing the 28S rRNA confirms equal RNA loading.

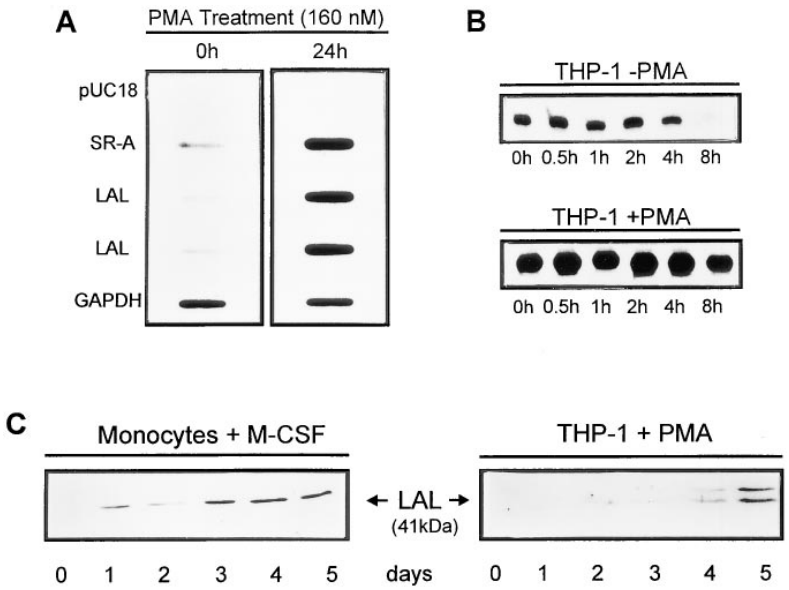


Fig. 2. Comparison of transcriptional and post-transcriptional LAL mRNA induction. A: Nuclear run-on analysis of LAL mRNA. Nuclei were isolated from resting THP-1 cells (0 h) and cells cultured in the presence of PMA (24 h). Nascent transcripts were labeled with $\alpha[^{32}P]$UTP and hybridized to cDNA fragments for scavenger receptor type A, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), pUC18, and the LAL 1.2 kb cDNA probe generated with primers LAC1 and LAC2, immobilized on nylon membranes. B: Analysis of LAL mRNA stability in undifferentiated and differentiated THP-1 cells. Cells were grown in the absence or presence of PMA for 24 h. After the addition of actinomycin D (8 μg/ml) to either differentiated (lower panel) or undifferentiated cells (upper panel), total cellular RNA was isolated at the times indicated. RNA samples were then analyzed by RNase protection assay. C: Western blot analysis of total cellular LAL protein in differentiating monocytes (left panel) and THP-1 cells (right panel). Cellular extracts were prepared from freshly isolated blood monocytes and monocyte-derived macrophages that were cultured on plastic petri dishes for various times in the presence of 50 ng/ml M-CSF and from THP-1 cells incubated with 160 nm PMA. Proteins (10 μg/lane) were separated on a 12% SDS-PAGE and immunoblotted against a polyclonal LAL antiserum.

Lysosomal acid lipase is regulated both at transcriptional and post-transcriptional levels

To examine the relative contribution of transcriptional and post-transcriptional processes responsible for the higher LAL mRNA levels, rates of synthesis and degradation of LAL mRNA were determined in nuclear run-on and actinomycin D experiments. Nascent transcripts from untreated and PMA-treated (24 h) THP-1 cells were labeled with $\alpha[^{32}P]$ UTP and hybridized to an LAL-specific cDNA fragment, a scavenger receptor type A cDNA plasmid, a GAPDH cDNA plasmid, and a pUC 18 vector. Scavenger receptor type A is known to be activated transcriptionally in THP-1 cells immediately upon monocytic differentiation, which is induced by PMA, and was thus used as a positive control (15). The LAL transcript was barely detectable in resting THP-1 cells and increased substantially 24 h after PMA treatment (**Fig. 2A**). Densitometric scanning of autoradiograms indicated that transcrip-

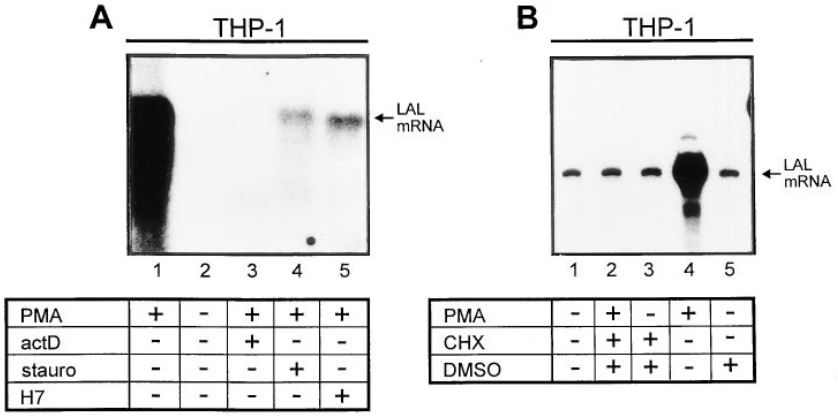


Fig. 3. Induction of LAL mRNA depends on PKC activity and new protein synthesis. A: Northern analysis demonstrating the effect of PKC inhibitors on PMA-induced LAL expression in THP-1 cells. Lane 1, THP-1 cells cultured in the presence of 160 nm PMA; lane 2, THP-1 cells cultured in the absence of PMA; lane 3, same as lane 1 but in the presence of actinomycin D (8 μg/ml); lane 4, same as lane 1 but in the presence of 100 nm staurosporine; lane 5, same as lane 1 but in the presence of 100 μm H7. B: Ribonuclease protection assay demonstrating the effect of cycloheximide on PMA induced LAL expression. Lane 1 contains RNA from uninduced THP-1 cells; lane 2, THP-1 cells preincubated with 10 μg/ml cycloheximide prior to stimulation with 160 nm PMA for 14 h; lane 3, THP-1 cells cultured with 10 μg/ml cycloheximide for 14 h; lane 4, THP-1 cells cultured in the presence of 160 nm PMA for 14 h; lane 5, THP-1 cells incubated with DMSO (solvent).

tion was induced approximately 4.9-fold in THP-1 cells treated with PMA for 24 h. In comparison, the rate of transcription from the GAPDH gene remained relatively constant after PMA incubation. Because a basal LAL mRNA expression is detectable in THP-1 cells, the observed 4.9-fold increase after PMA stimulation might also be due to altered mRNA stability. For a number of other genes, PMA has been shown to enhance gene expression by induction of transcriptional activity as well as enhancement of the stability of the corresponding mature mRNA (26, 27).

To evaluate potential post-transcriptional control of LAL mRNA expression, actinomycin D (8 μg/ml) was added to undifferentiated or PMA-exposed THP-1 cells. RNA extracted at different times from undifferentiated and differentiated THP-1 cells after actinomycin D treatment were subjected to RNase protection analysis. As can be seen in Fig. 2B, LAL mRNA from undifferentiated THP-1 cells was eliminated more rapidly when transcription was inhibited by actinomycin D (40% of control after 4 h). In PMA-differentiated THP-1 cells, the relative amount of LAL mRNA was unaffected up to 8 h. These data suggest that the LAL mRNA is degraded faster in resting THP-1 cells than in THP-1 cells cultured in the presence of PMA. Immunoblots from peripheral blood monocytes and THP-1 cells clearly demonstrate that more LAL protein is synthesized as a result of higher LAL mRNA levels (Fig. 2C). These data indicate that PMA-enhanced LAL expression occurs both on transcriptional and post-transcriptional levels in THP-1 cells.

PMA-induced LAL mRNA up-regulation depends on protein kinase C (PKC) activation and new protein synthesis

Inhibitors of the PKC pathway were used in conjunction with PMA to characterize the signal transduction pathways involved in the regulation of LAL gene expression. As can be seen in **Fig. 3A**, the PKC inhibitors, staurosporine and H7, caused a remarkable decrease of LAL mRNA in PMA-stimulated THP-1 cells, while the GAPDH mRNA was not affected (data not shown). Incubation of THP-1 cells with PMA and cycloheximide completely abolished up-regulation of LAL mRNA, while cycloheximide alone did not induce LAL mRNA (Fig. 3B).

Taken together, these results indicate that the transcriptional induction of the LAL gene during PMA-induced monocytic differentiation is dependent upon activation of protein kinase C and requires new protein synthesis.

Mapping of LAL transcriptional start sites

5′ RACE-PCR analysis indicated two different transcription start points for the LAL gene (data not shown). In addition, the DNA sequence of the putative promoter region is (G + C)-rich and is deficient of a TATA-box. The exact position of the multiple LAL mRNA start sites was determined using two antisense RNA probes (PA1, PA2) derived from a genomic clone. These probes were hybridized to different amounts of total cellular RNA from resting and PMA-treated THP-1 cells or a negative control. Two strong signals (TSS A, 59 bp; TSS B, 62 bp) and two weak signals (TSS C and TSS D) have been generated with either probe (**Fig. 4**) indicating major and minor transcription start points. Such a preference is typical for TATA-less and (G + C)-rich promoters (28). As all protected fragments generated with the shorter PA2 probe are also generated with the longer PA1 probe, further upstream initiation sites can be excluded. Transcriptional activity from the two major initiation sites is markedly up-regulated in THP-1 cells after incubation in PMA.

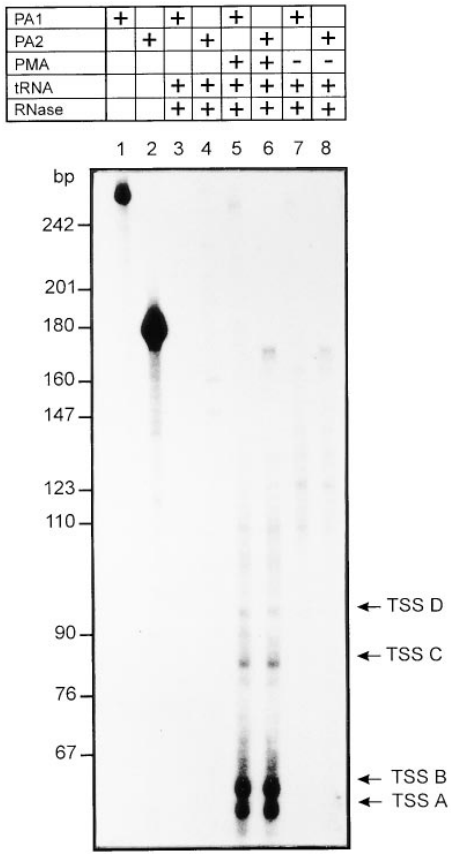
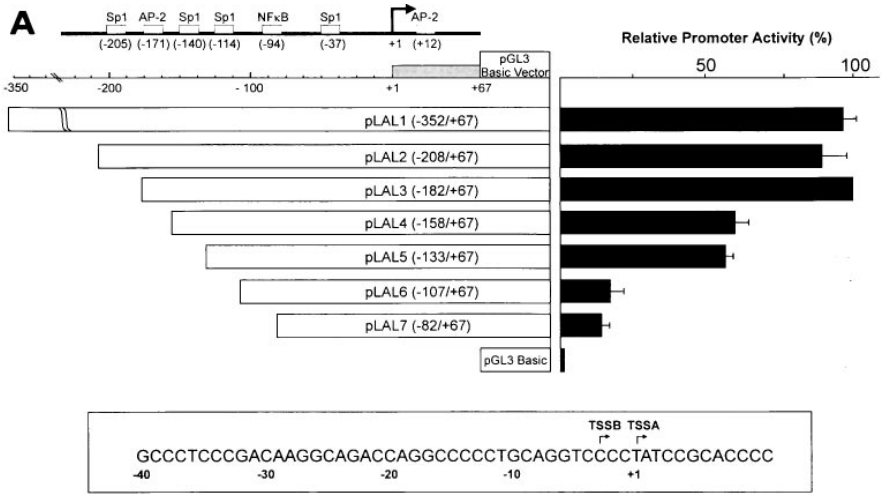


Fig. 4. Identification of the LAL transcription start sites by RNase protection assay. The autoradiograph from a ribonuclease protection assay using two overlapping RNA probes, PA1 and PA2 spanning from nucleotides −219 to +59 and −109 to +59, respectively, is shown. Lanes 1 and 2 contain the undigested probes, yeast-tRNA was used as negative control (lanes 3 and 4). Ten μg of total RNA from untreated THP-1 cells (lane 7) or 24-h incubated THP-1 cells (lane 5) were hybridized to probe PA1. Lanes 6 and 8 contain the same as lanes 5 and 7 hybridized to probe PA2. The protected fragments of 59 bp, 62 bp, 85 bp, and 95 bp are indicated by arrows. The use of total RNA from PMA-stimulated THP-1 cells is indicated by a plus (+); minus (−) refers to total RNA from non-PMA-stimulated cells.

5′-Deletion analysis of the LAL promoter

To identify sequences important for promoter activity, seven LAL promoter plasmids were constructed by fusing DNA fragments, containing sequential 5′ truncations of the putative LAL promoter to the firefly luciferase re-



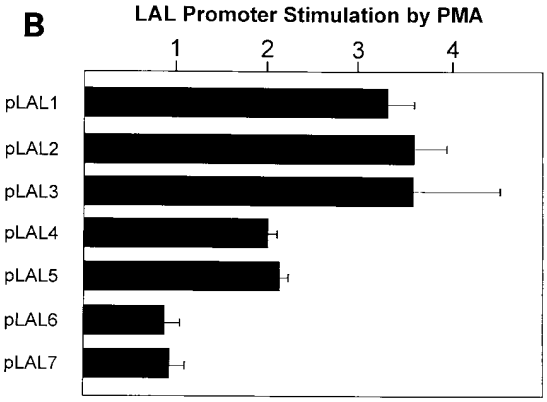


Fig. 5. Deletion analysis of 5′ flanking region of LAL gene by transient transfection experiments. A: Basal promoter activity in THP-1 cells. THP-1 cells were transfected with 10 μg of each reporter gene construct and 2 μg of pSVβ-gal plasmid and cultured for 48 h. Aliquots of cell extracts were assayed for luciferase and β-galactosidase activity. Assays were performed in triplicate and results were normalized in respect to the β-galactosidase expression. The data are expressed as relative luciferase activity/β-galactosidase activity ± SE from 5 independent experiments with the pLAL3 construct being set to a value of 100. Putative *cis*-acting consensus sequences are denoted by boxes and numbers indicate their position relative to the major transcriptional start site A. DNA sequence at the transcriptional start site of the LAL gene is depicted at the bottom. B: Effect of PMA on promoter activity of different LAL promoter constructs in THP-1 cells**.** THP-1 cells were transfected with 10 μg of the various LAL–luciferase constructs and 2 μg pSVβ-gal plasmid for 24 h. Luciferase and β-galactosidase activity was measured in cellular extracts. The normalized luciferase activity of each construct in the absence of PMA was taken as 100%, and the PMA stimulation factor was determined. The data represent the average of 5 independent experiments of triplicate measurements.

porter gene. The deletions, as shown in **Fig. 5A**, range from −352 bp to −82 bp in regard to the major transcriptional start site A. Transcriptional activity of the LAL promoter was quantified by measuring luciferase activity expressed by transiently transfected THP-1 cells (Fig. 5A, B). While the three promoter truncations pLAL1, pLAL2, and pLAL3 result in similar activities, the larger truncation in pLAL4 and pLAL5 revealed only 60% activity and promoter activity is nearly abolished in the shortest promoter constructs pLAL6 and pLAL7. In the THP-1 cell line, all but the latter two constructs retained PMA-induced promoter activity (2.2 to 3.6-fold, Fig. 5B). These values correlate well with the increase in transcription rates found in the nuclear run-on analyses (Fig. 2A).

Co-transfection with Sp1 and AP-2 expression vectors

The DNA region exhibiting PMA-inducible promoter activity has been localized between nucleotides −107 and −182. Several putative *cis*-acting consensus sequences are present within this LAL promoter region. One AP-2 site is located at position −179 to −171 and AP-2 sites are known to be phorbol ester-inducible. Furthermore, there are two consensus binding sites for the transcription factor Sp1 at positions −146 to −140 and −120 to −114. In order to elucidate the role of these putative binding sites, we co-transfected THP-1 cells with the LAL promoter construct pLAL3 and expression vectors for Sp1 (pPacSp1) or AP-2α (pCMXAP2α), respectively. As shown in **Fig. 6**, expression of Sp1 or AP-2 caused a significant increase in LAL promoter activity (2.7-fold, and 1.8-fold, respectively), demonstrating the involvement of both transcription factors in PMA-induced up-regulation of LAL mRNA in THP-1 cells. Simultaneous transfection of THP-1 cells with Sp1 and AP-2 resulted in a 3.5-fold increase of luciferase activity. These results suggest that both transcription factors act in concert to modulate LAL promoter activity.

Transcription factor binding to the PMA-inducible LAL promoter region

To verify whether Sp1 and AP-2 are indeed components of the binding complex responsible for the inducibility by PMA, we performed gel shift analyses with crude nuclear extracts prepared from resting or PMA-treated THP-1 cells. Three double-stranded oligonucleotides, GSA1 (−218/−168), GSA2 (−167/−128), and GSA3 (−127/

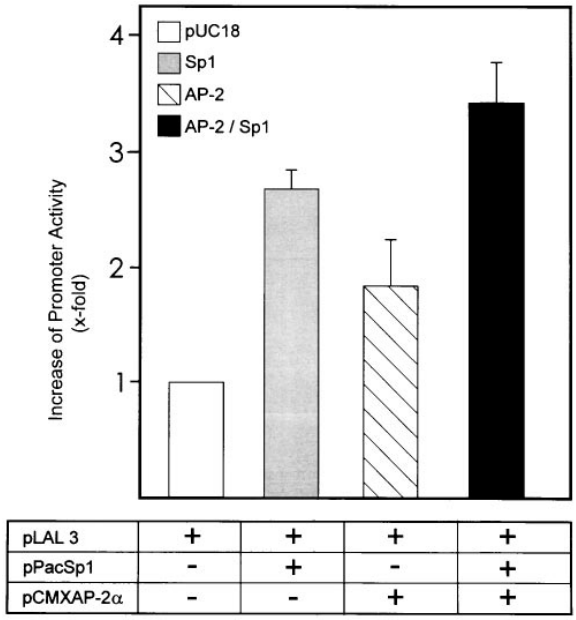


Fig. 6. Co-transfection with transcription factor expression plasmids. THP-1 cells were cotransfected with 10 μg of pLAL3 promoter construct and 100 ng of transcription factor expression vectors for Sp1 (pPacSp1), AP2α (pCMXAP2α), or a combination of Sp1 and AP-2. Cell lysates were used for measurement of luciferase activity. The data represent the average of 3 independent experiments with triplicate measurements.

−88), were radiolabeled and used as probes in gel mobility shift experiments.

GSA1 bound to one protein complex in THP-1 cell extracts (**Fig. 7A**). Using nuclear extracts from PMA-induced THP-1 cells, a marked increase of this complex I was observed. Additionally, a second protein complex (II) appeared. Complex I has been identified as the Sp1 gene product based on interaction with anti-Sp1 antibodies (Fig. 7A, lane 3). When anti-AP-2 antibodies were included in the binding reaction, the complex II was retarded, indicating that it is formed by the interaction of GSA1 with transcription factor AP-2 (lane 4). Binding of Sp1 and AP-2 to oligonucleotide GSA1 can be disrupted by preincubation of the THP-1 cellular extract with unlabeled GSA1 oligonucleotide in a 50-fold molar excess (data not shown). Antibodies against transcription factor PU.1 caused no specific supershift of the complex pattern.

Incubation of probes GSA2 and GSA3 with nuclear extracts from THP-1 cells resulted in the formation of one major protein complex (Fig. 7B, C). The intensity of this complex (I) is markedly increased when nuclear extracts from PMA-treated THP-1 cells are used. Antibodies specific to Sp1 transcription factor incubated with the reaction mixture resulted in a decrease in the intensity of complex I and the appearance of a new complex with high molecular weight. These data indicate that Sp1 binds to both oligonucleotides GSA2 and GSA3. Preincubation of nuclear extracts with antibodies against PU.1 did not lead to a change in the complex pattern using GSA3 as a probe. Addition of antibodies against PU.1 to the reaction mixture with GSA2 resulted in a faster migrating complex of unknown origin. Antibodies against AP-2 did not change the complex pattern with GSA2 and GSA3. Specificity of the Sp1 or AP-2 interaction with the respective DNA sequences was demonstrated by competition experiments using a 50-fold molar excess of non-labeled double-stranded oligonucleotides (data not shown).

DISCUSSION

In the present study, we characterized the transcriptional regulation of the LAL gene in response to PMA. As a cellular model we used the human monocytic leukemia cell line THP-1 which upon specific stimulation can differentiate to a macrophage like state thus mimicking monocyte / macrophage differentiation (13, 14).

We have demonstrated that phorbol esters, which are known to activate protein kinase C and stimulate monocytic differentiation (13), do increase the level of lysosomal acid lipase mRNA transcripts. The increase in LAL mRNA resulted in higher amounts of LAL enzyme within the cells. Although the up-regulation of the LAL mRNA is mostly due to strong activation of the promoter, additional post-transcriptional mechanisms like mRNA stability appear to contribute to differentiation-dependent increase in LAL mRNA.

The LAL promoter region is (G + C)-rich and lacks a classical TATA-box. Therefore, the identification of multiple transcription start points was not surprising (28). However, unlike certain other TATA-less housekeeping promoters, the LAL promoter is regulated in a differentiation-dependent manner. Using deletion derivatives of the promoter region the minimal PMA-inducible promoter sequence has been identified between nucleotides −107 and −182. Within this 76-bp promoter region one AP-2-like consensus sequence is present. The activity of the transcription factor AP-2 is subject to distinct regulatory mechanisms. Phorbol esters and signals that elevate cyclic AMP concentration have been found to induce AP-2 activity without affecting AP-2 mRNA and protein expression (29). Additionally, an alternatively spliced AP-2 gene product may negatively regulate AP-2 activity (30).

In addition to the AP-2 site, we found two Sp1 binding sites within the PMA-responsive region of the LAL gene promoter. Sp1 has been shown to interact with the TATA-box binding protein (TBP)-associated protein TAF 110 and, in cooperation with another TBP-associated protein, TAF 250, activate transcription (31, 32). Therefore, Sp1 is thought to be important in the regulation of many TATA-less promoters and may function to recruit or stabilize the TFIID initiation complex via a “tethering factor” (33, 34). Even though Sp1 is ubiquitously expressed, its role in cell-specific and differentiation-dependent regulation processes has been implicated (35–37). In particular, its involvement in the TATA-less promoters of the myeloid specific integrin CD11b (36), the monocyte specific gene CD14 (37), and the neural specific acetylcholine receptor α_3 gene (38) have been analyzed.

We have demonstrated that these two transcription fac-

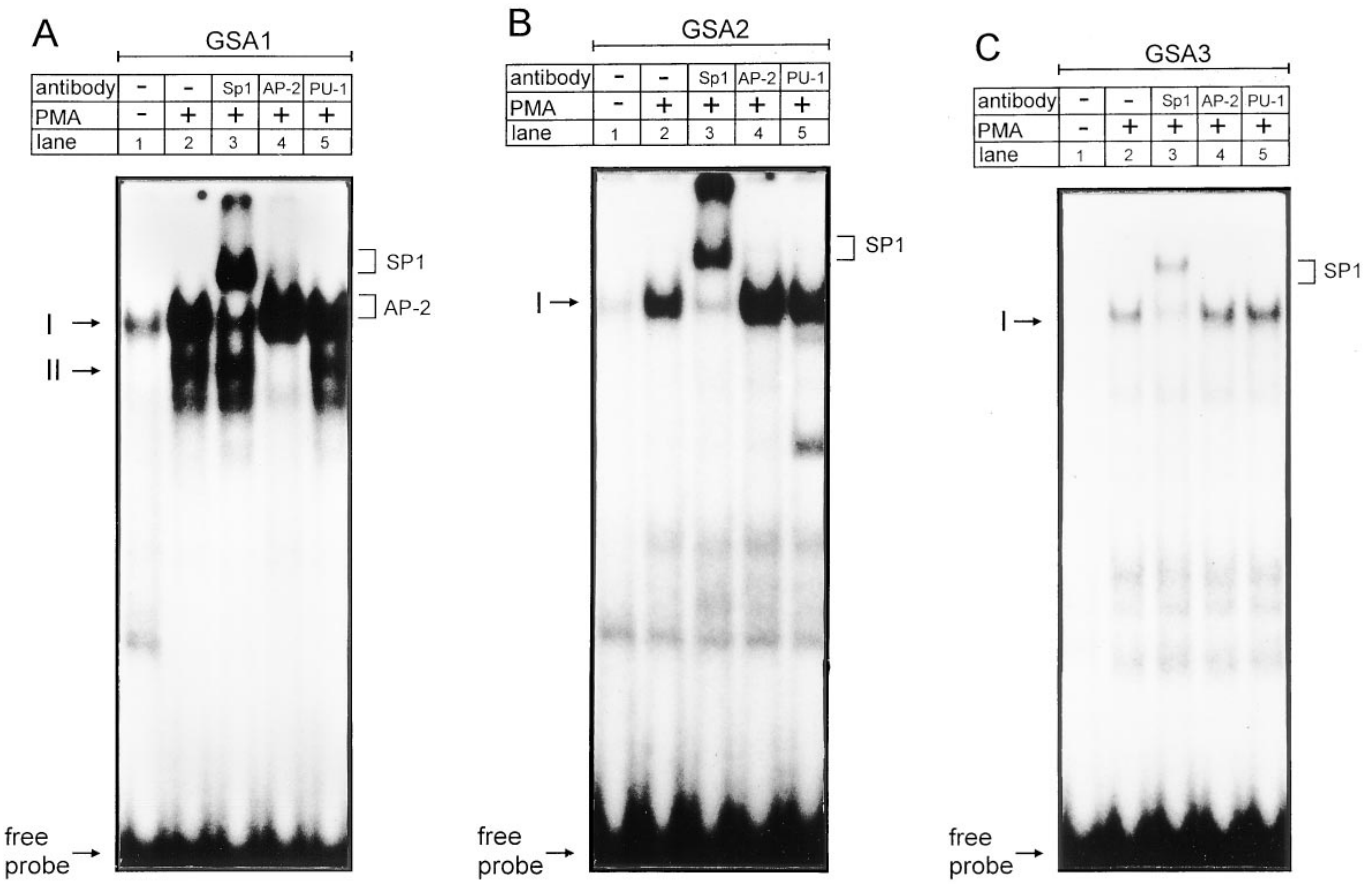


Fig. 7. Gel mobility shift assays with nuclear extracts from THP-1 cells and oligonucleotides corresponding to the PMA-responsive element in the LAL promoter. A: GSA1 (−218/168); lane 1, extract from uninduced THP-1 cells; lane 2, PMA extract; lane 3, PMA extract plus Sp1-antibodies; lane 4, PMA extract plus AP-2-antibodies; lane 5, PMA extract plus PU.1-antibodies. B: GSA2 (−167/−128); lane 1, extract from uninduced THP-1 cells; lane 2, PMA extract; lane 3, PMA extract plus Sp1-antibodies; lane 4, PMA extract plus AP-2-antibodies; lane 5, PMA extract plus PU.1-antibodies. C: GSA3 (−127/−88); lane 1, extract from uninduced THP-1 cells; lane 2, PMA extract; lane 3, PMA extract plus Sp1-antibodies; lane 4, PMA extract plus AP-2-antibodies; lane 5, PMA extract plus PU.1-antibodies. Brackets to the right indicate the specific supershifted complexes. Arrows to the left indicate complexes in induced nuclear extracts.

tors, AP-2 and Sp1, play an important role in phorbol ester-mediated expression of the LAL gene. Deletion of the AP-2 site (−171) decreased both basal and PMA-enhanced promoter activity by 40%. Elimination of the Sp1 binding site (−140) did not further suppress promoter function, whereas deletion of the second Sp1 site (−114) resulted in an almost complete elimination of both basal and PMA-induced promoter activity.

In order to provide evidence for the involvement of the Sp1 and AP-2 sites in LAL gene transcription, we analyzed LAL promoter activity in THP-1 cells after co-transfection with Sp1 and AP-2 expression plasmids and an LAL promoter/luciferase construct. Co-transfection of Sp1 with the shortest fully inducible LAL promoter construct pLAL3 resulted in a 2.7-fold augmentation of promoter activity. Co-transfection of AP-2 expression vector also increased promoter activity of the pLAL3 (−182/+67) construct, but to a lesser extent (1.8-fold). Simultaneous transfection of THP-1 cells with Sp1 and AP-2 led to a 3.5-fold increase of promoter activity. These experiments indicate that Sp1 and AP-2 act in concert to enhance LAL transcription.

Using gel mobility shift assays, we have shown that PMA treatment of THP-1 cells led to an increased binding of several nuclear factors to labeled oligonucleotides derived from the PMA-responsive LAL promoter region. Binding activities interacting with this region responsible for PMA inducibility have been characterized as transcription factors Sp1 and AP-2 by experiments using specific antibodies. In contrast to AP-2, little is known about a possible role of Sp1 in PMA-mediated transcriptional regulation. In a recent study, Biggs, Kudlow, and Kraft (39) have shown that Sp1 is implicated in the PMA-induced expression of the WAF1/CIP1-gene in U937 cells. They demonstrated that a reporter plasmid containing multiple Sp1 sites upstream of a TATA-box is induced by PMA in the U937 cell line. D'Angelo et al. (40) reported that thromboxane receptor gene expression in K562 cells is enhanced by PMA due to increased Sp1 protein levels and Sp1 binding to a C-rich element. To prove whether Sp1 protein levels increase during PMA treatment of THP-1 cells, we carried out immunoblots of THP-1 nuclear extracts and found that the amount of Sp1 protein was significantly higher in PMA-induced THP-1 cells compared

to resting THP-1 cells (data not shown). These data and our results demonstrating the necessity of protein synthesis for PMA-enhanced LAL gene expression suggest that induction is partially mediated by an increased Sp1 synthesis and binding to the promoter in a differentiation-dependent manner.

In recent studies there is evidence that Sp1 expression itself is regulated. Thus Sp1 levels decrease during myogenesis, which contributes to the repression of GLUT1 gene transcription (41). In addition, Sp1 binding alone is not sufficient for maximal PMA enhancement of LAL gene expression. However, the response of the LAL gene to PMA may involve interactions of Sp1 and AP-2. It has previously been reported that Sp1 interacts with other transcription factors, like Egr-1 (PDGF-expression), AP-2 (acetylcholine receptor α_3 gene expression), or YY1 (38, 42, 43). Therefore, it is conceivable that under some circumstances Sp1 can act as a transcriptional enhancer or modulator independent of its action in transcriptional initiation. Phosphorylation and glycosylation of Sp1 in various cell types may also contribute to variable modes of regulation of promoter activity (44, 45).

In conclusion, the results of our study indicate: *1*) induction of LAL gene expression in the macrophage cell line THP-1 depends on protein kinase C activity and new protein synthesis; *2*) transcription factors Sp1 and AP-2 may act in concert to enhance transcription of the LAL gene after PMA treatment; and *3*) LAL activity is partly regulated by post-transcriptional mechanisms. Furthermore, our data suggest that far from being a ubiquitously expressed "housekeeping" transcription factor, Sp1 is a dynamically regulated enhancer playing a critical regulatory role for numerous genes. In the future, it will be interesting to see whether potential Sp1 binding sites in other monocytic genes also contribute to specific regulation during the monocyte/macrophage differentiation process.

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