MOLECULAR AND CELLULAR BIOLOGY, Oct. 1992, p. 4622–4633
0270-7306/92/104622-12$02.00/0

Vol. 12, No. 10

Characterization of the Human Lipoprotein Lipase (LPL) Promoter: Evidence of Two *cis*-Regulatory Regions, LP-α and LP-β, of Importance for the Differentiation-Linked Induction of the LPL Gene during Adipogenesis

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Received 12 March 1992/Returned for modification 17 April 1992/Accepted 21 July 1992

When preadipocytes differentiate into adipocytes, several differentiation-linked genes are activated. Lipoprotein lipase (LPL) is one of the first genes induced during this process. To investigate early events in adipocyte development, we have focused on the transcriptional activation of the LPL gene. For this purpose, we have cloned and fused different parts of intragenic and flanking sequences with a chloramphenicol acetyltransferase reporter gene. Transient transfection experiments and DNase I hypersensitivity assays indicate that several positive as well as negative elements contribute to transcriptional regulation of the LPL gene. When reporter gene constructs were stably introduced into preadipocytes, we were able to monitor and compare the activation patterns of different promoter deletion mutants at selected time points representing the process of adipocyte development. We could delimit two *cis*-regulatory elements important for gradual activation of the LPL gene during adipocyte development in vitro. These elements, LP-α (−702 to −666) and LP-β (−468 to −430), contain a striking similarity to a consensus sequence known to bind the transcription factors HNF-3 and fork head. Results of gel mobility shift assays and DNase I and exonuclease III in vitro protection assays indicate that factors with DNA-binding properties similar to those of the HNF-3/fork head family of transcription factors are present in adipocytes and interact with LP-α and LP-β. We also demonstrate that LP-α and LP-β were both capable of conferring a differentiation-linked expression pattern to a heterolog promoter, thus mimicking the expression of the endogenous LPL gene during adipocyte differentiation. These findings indicate that interactions with LP-α and LP-β could be a part of a differentiation switch governing induction of the LPL gene during adipocyte differentiation.

3T3-F442A preadipocytes differentiate with high frequency into adipocytes (21). This process is characterized by a transcriptional activation of several genes together with morphological changes. This in vitro system has been shown to closely resemble fat cell development in vivo (22). The transformation of a fibroblastlike preadipocyte into a fat-laden adipocyte is initiated when rapidly dividing preadipocytes reach confluence. At this early phase of adipocyte development, the lipoprotein lipase (LPL) gene is transcriptionally activated, whereas exceedingly low levels of LPL activity and LPL-specific mRNA are present in preconfluent preadipocytes (1, 8, 46). During adipocyte differentiation, there is a gradual increase of LPL-specific mRNA that reaches a plateau level when the cell has matured into an adipocyte (35). As a consequence of LPL enzyme action, free fatty acids are released. In the adipocyte, these are taken up by the cell, reestrified, and stored as lipid droplets in the cytoplasm. This latter process is catalyzed by the action of adipocyte-specific proteins. The glycerol-3-phosphate dehydrogenase (11) and adipocyte P2 (37) genes are both members of this group of genes that are induced during terminal adipocyte differentiation. Transcriptional activation of CCAAT/enhancer-binding protein α (C/EBPα) is a crucial component of terminal adipocyte development; several adipocyte-specific genes have been shown to be transactivated by C/EBPα (6, 25, 28). Growth arrest and ability to take up lipid have also been shown to be influenced by C/EBPα (35, 40). In a recent publication, Cao et al. (4) have characterized new isoforms of C/EBP, C/EBPβ and C/EBPδ, which are induced at an early stage of adipocyte development. In contrast, C/EBPα is activated during the terminal phase of adipocyte conversion.

In an earlier work, we studied terminal adipocyte differentiation (35). In an effort to analyze factors important for the immediate-early phase of fat cell development, we have isolated and fused 5′- and 3′-flanking, as well as intragenic, sequences derived from the human LPL gene with a chloramphenicol acetyltransferase (CAT) reporter gene. This was done with the aim of identifying *cis*-regulatory elements and *trans*-acting factors important for the differentiation-linked activation of the LPL gene. Stable and transient transfection assays delineate two such regions, LP-α (−702 to −666) and LP-β (−468 to −430). We demonstrate that these two regions interact with a set of closely related DNA-binding proteins that are enriched during adipogenesis. Some of these factors are shown to have DNA-binding properties similar to those of the HNF-3/fork head (30, 42) family of transcription factors. To assess the functional importance of these elements, chimeric constructs were made and stably introduced into preadipocytes that were allowed to differentiate into adipocytes. We demonstrated that LP-α and LP-β were both capable of conferring a differentiation-linked expression pattern to a heterologous promoter, thus mimicking the activation pattern of the LPL gene during adipocyte differentiation.

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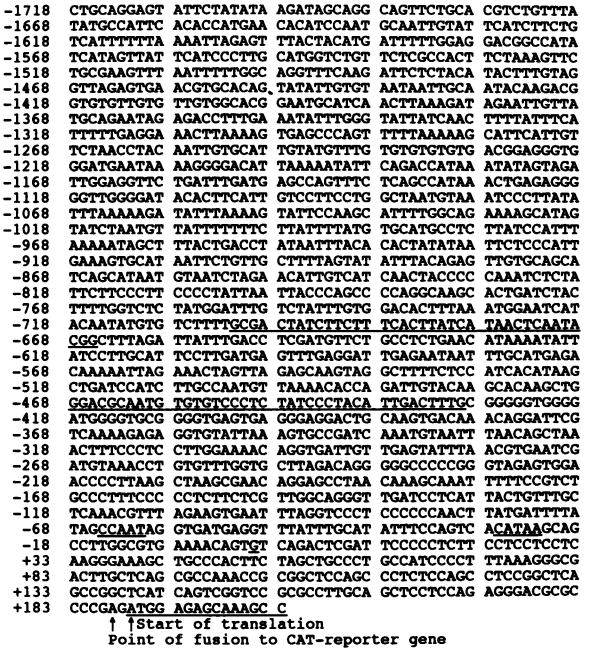

FIG. 1. Sequence of the 5'-flanking region and part of exon I of the human LPL gene. *cis*-regulatory regions LP-α (−702 to −666) and LP-β (−468 to −430) are underlined. CCAAT and TATA motifs are present at −65 and −27, respectively. The start of transcription at +1 is as determined by Deeb and Peng (10) and Kirchgessner et al. (29).

MATERIALS AND METHODS

Cell culture. 3T3-F442A cells (21) were maintained in Dulbecco's modified Eagle's medium, and THP-1 cells (39) were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 100 IU of penicillin per ml, and 100 μg of streptomycin (Gibco Ltd., Paisley, Scotland) per ml. 3T3-F442A cells received 5 μg of insulin (Sigma, St. Louis, Mo.) per ml in the medium after confluence of growth; THP-1 cells were induced to express LPL by addition of phorbol 12-myristate 13-acetate (PMA; Sigma) to a final concentration of 1.6×10^{-7} M. Cells were cultured at 37°C in 5% CO_2 and humidified air.

Cloning and plasmid constructs. An oligonucleotide corresponding to the 48 5'-most bases in the human LPL cDNA clone described by Wion et al. (45) was used to screen a human genomic λEMBL 3 library (Clontech Inc., Palo Alto, Calif.). The oligonucleotide was phosphorylated with [γ-^{32}P]ATP (Amersham International, Amersham, United Kingdom), using T4 polynucleotide kinase (Boehringer, Mannheim, Germany). Screening and hybridization were carried out under standard conditions as outlined by Maniatis et al. (31). Various LPL-derived 5'-flanking sequences were fused upstream of a 1.63-kb cassette containing the CAT-coding region and simian virus 40 splice and polyadenylation signals derived from pSVEcat (5, 24) and were cloned into plasmid pTZ18R (Pharmacia, Uppsala, Sweden), thus placing the LPL 5'-flanking DNA and/or sequences derived from exon I immediately upstream of the CAT-coding cassette (Fig. 1 and 2). When intragenic and 3'-flanking sequences were included in the constructs, they were cloned at the very 3' border of the CAT cassette. To abolish background activity due to spurious transcripts initiated in plasmid sequences, an upstream mouse sequence (UMS) was included and placed above the LPL promoter. The UMS has the functional properties of a transcription terminator (32, 47). For generating stable transfectants, we used plasmid pXTI, in which a selectable neomycin resistance (Neo^r) gene is under a viral long terminal repeat derived from Moloney murine leukemia virus (Stratagene Inc., La Jolla, Calif.). Nucleotide sequences were determined by the dideoxy-chain termination method (36).

Transient and stable transfections. 3T3-F442A preadipocytes were transfected by using Lipofectin (16). Plasmid DNA was diluted in 1.5 ml of Opti-MEM (Gibco Ltd.) and mixed with 1.5 ml of Opti-MEM containing 20 μg of Lipofectin. Preconfluent monolayers of preadipocytes in 60-mm-diameter dishes were washed twice with serum-free medium before addition of the plasmid-lipofectin mix. After ~18 h, 3 ml of Dulbecco's modified Eagle's medium with 10% fetal calf serum was added and incubation continued. 3T3-F442A cells that had been confluent for 8 days and exhibited a clear adipocyte morphology with lipid droplets present in the cytoplasm were used as adipocytes. These cells were transfected by electroporation. Plasmid DNA was added in 50 μl of Opti-MEM to 0.45 ml of cell suspension, mixed, and placed in a sterile 1-ml disposable cuvette (Kartell, Milan, Italy). Electroporation was performed with a BTX Transfector-300 (BTX Inc., San Diego, Calif.) with a 3.5-mm electrode gap and with settings of 220 V and 2,200 μF. Cells were kept in the cuvette for 60 s after discharge and then transferred to a tissue culture dish. Medium was changed after ~18 h. To compensate for differences in transfection efficiency, a β-galactosidase-encoding construct, pRSV-βgal (12), was cotransfected together with CAT constructs. Stable transfectants were generated by electroporation as previously described (35); briefly, the settings specified above were used to transfect various CAT constructs together with plasmid pXTI encoding Neo^r. Medium was changed after ~18 h, at which time point 1 mg of G418 sulfate (Gibco Ltd.) per ml was added to the medium (38). After 8 to 12 days, resistant foci appeared; at this time point, all cells had died in a control experiment (transfected only with plasmid pUC), and the G418 concentration was lowered to 0.8 mg/ml.

CAT and β-galactosidase assays. Cells were harvested 48 to 72 h after transfection, and enzyme extracts were made by three freeze-thaw cycles. One aliquot (50 μl of ~150 μl) was removed for the β-galactosidase assay (33), and the rest was treated for 15 min at 65°C. Extracts (20 to 50 μl) were used in a diffusion CAT assay as described by Neuman et al. (34). [^{14}C]butyryl coenzyme A (100 nCi per assay; NEN) was used; the reactions were allowed to proceed at room temperature while the samples were counted in a liquid scintillation counter at constant time intervals. Counts per minute versus time was plotted for each sample, and CAT activity was calculated from the slope of the curve within its linear range. The values (counts per minute per hour per picomole of plasmid) were normalized against β-galactosidase activities to compensate for variations in transfection efficiency. For stable transfectants, CAT activities were compared with that derived from cells harvested at day −1; these values were normalized for differences in protein concentrations by the method of Whitaker and Granum (44).

DNase I hypersensitivity assay. Cells were suspended in a hypotonic lysis buffer (10 mM *N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES; pH 8.0], 10 mM KCl, 1.5 mM $MgCl_2$) and passed through a 25-gauge needle five times, nuclei were pelleted, and the supernatant was removed. Aliquots were then resuspended in a DNase I reaction buffer (60 mM KCl, 15 mM NaCl, 0.5 mM dithiothreitol [DTT], 0.5

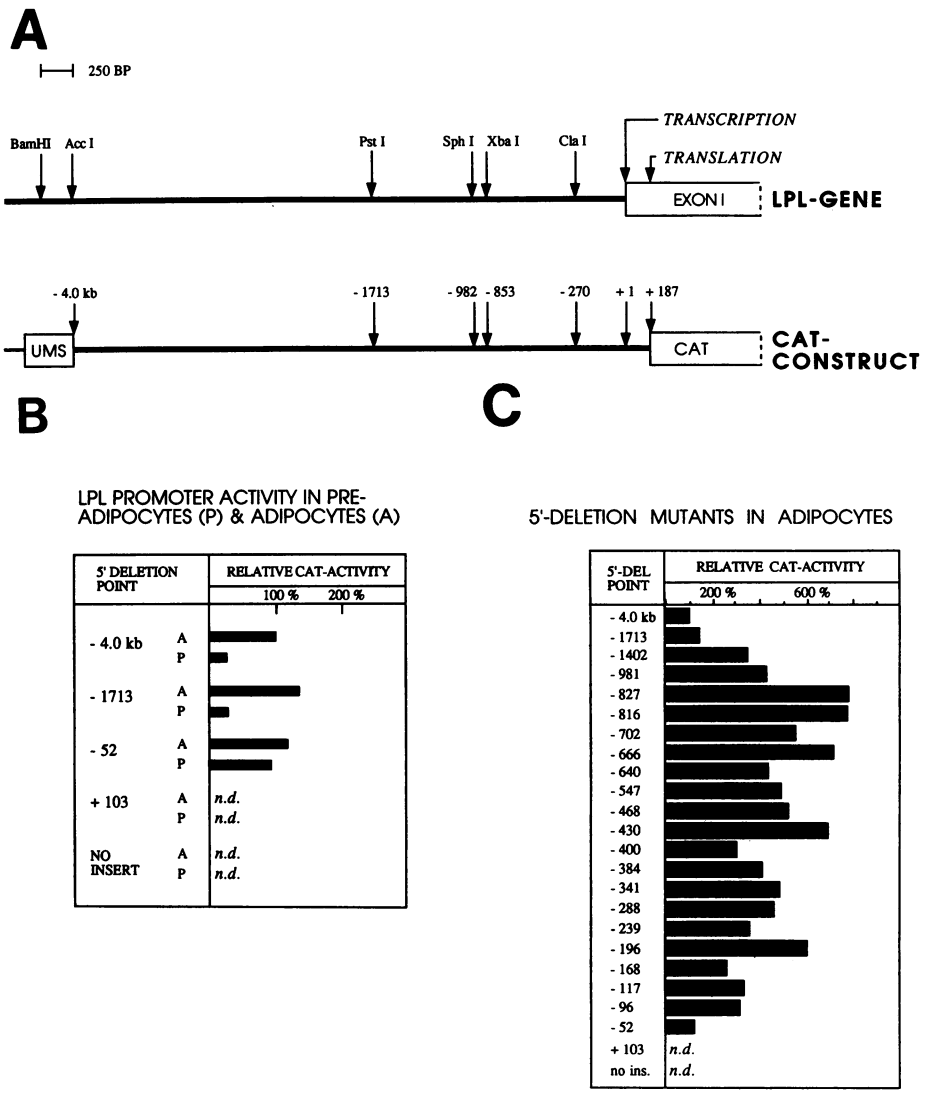

FIG. 2. (A) Comparison of a part of the LPL gene with the 5'-flanking region and CAT construct −4 kb. Restriction enzyme sites in the LPL map are indicated in the CAT construct relative the transcription start. The position of the UMS (47) is indicated. (B) LPL promoter activity after transient transfections (for details, see Materials and Methods) in adipocytes (A) and preadipocytes (P). The CAT activity of construct −4 kb in adipocytes is set to 100%. *n.d.*, not detected in comparison with nontransfected cells. (C) Activity of 5'-deletion mutants transiently transfected into adipocytes. The CAT activity of construct −4 kb is set to 100%. All transfections in panels B and C are means of at least three independent experiments. The largest variation between three such normalized values (see Materials and Methods) was ±24% (standard deviation) of the calculated mean value.

mM phenylmethylsulfonyl fluoride, 5 mM $MgCl_2$, 0.1 mM EGTA, 15 mM Tris-HCl [pH 7.6]). Nuclei were incubated on ice for 10 min with various concentrations of DNase I (Sigma); reactions were terminated by the addition of one-half reaction volume of a stop solution (10% sodium dodecyl sulfate [SDS], 0.4 M EDTA, 2 mg of proteinase K [Boehringer] per ml). After precipitation, the DNA was digested with appropriate restriction enzymes. Southern blots were prepared and probed with corresponding cDNA probes (Fig. 3). Probes were radiolabeled with [α-^{32}P]dCTP (15, 18).

Nuclear extracts and gel mobility shift assay. Nuclei were isolated by pelleting through a sucrose cushion as described by Gorski et al. (20) and extracted with a buffer containing 0.42 M KCl. After sedimentation of the chromatin and other insoluble material by centrifugation, the supernatant was collected and frozen in liquid nitrogen. For gel mobility shift assays, we used double-stranded oligonucleotides radiolabeled either at the 5' end by phosphorylation with [γ-^{32}P] ATP (Amersham), using T4 polynucleotide kinase (Boehringer), or by a 3' fill-in reaction with [α-^{32}P]dATP and/or [α-^{32}P]dCTP, using Klenow polymerase. The protein-DNA binding reaction was carried out in a volume of 20 μl containing 20 mM Tris · HCl (pH 7.6), 75 mM NaCl, 0.1 mM EGTA, 1 mM DTT, 5% glycerol, and 1.5 μg of poly(dI-dC) (Pharmacia). Approximately 40,000 Cerenkov cpm (0.3 to 1 ng of probe) was added to each reaction, which proceeded at room temperature for 20 min after protein extracts had been added. In some experiments, an antiserum was added to the binding reaction as described by Lai et al. (30). Competitions were performed by inclusion of a ~200-fold molar excess of unlabeled double-stranded oligonucleotide, as specified in the figure legends; the nonspecific competitor used (5'-

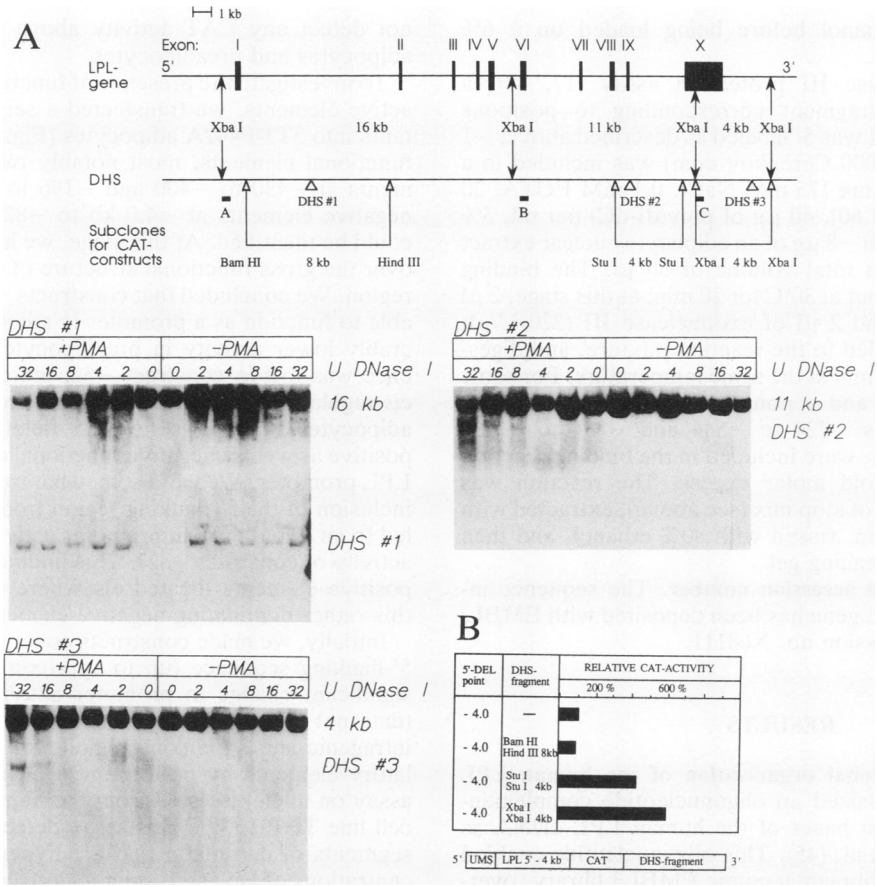

FIG. 3. (A) Schematic representation of the LPL gene (10, 29) and the four *Xba*I sites that divided the gene into 16-, 11-, and 4-kb fragments. DNase I-treated nuclei derived from THP-1 cells (39) treated or not treated with PMA, as indicated, were digested to completion with *Xba*I and used for Southern blots (see Materials and Methods). cDNA probes A, B, and C, corresponding to the 5′ end of each *Xba*I fragment, were used. Each *Xba*I fragment contains DHS 1, 2, or 3. DHS 2 and 3 are present only in PMA-treated cells that express LPL-specific mRNA as described by Auwerx et al. (3). A separate experiment on the same batch of nuclei confirmed this finding; the positions of DHS 1, 2, and 3 have been confirmed by use of cDNA probes corresponding to the 3′ end of the *Xba*I fragments (data not shown). (B) Transient transfection assay. Three genomic fragments each harboring a DNase I-hypersensitive site were cloned immediately adjacent to the 3′ end of the CAT gene of construct −4 kb. These constructs were then transiently transfected into adipocytes, with the activity of construct −4 kb without a DNase I-hypersensitive site set to 100%. Results in panel B are means of three independent experiments. The largest variation between three such normalized values (see Materials and Methods) was ±31% (standard deviation) of the calculated means.

GCGCTGGGCAAAGGTCACCTGCTGACC-3′) lacks any known sequence similarity to the LPL promoter region. The reaction mixture was then electrophoresed through a native 5% polyacrylamide gel containing 0.25× TBE (2.2 mM Tris borate, 2.2 mM boric acid, 0.5 mM EDTA) and 3% glycerol in order to separate DNA-protein complexes from unbound probe.

Protein separation. Protein extracts were thawed and diluted in a buffer containing 20 mM HEPES (pH 7.8), 0.5 mM EDTA, 17% glycerol, 1 mM DTT, antipain (2.5 μg/ml), leupeptin (2.5 μg/ml), chymostatin (2.5 μg/ml), pepstatin (2.5 μg/ml), and 1 mM phenylmethylsulfonyl fluoride (Sigma). Approximately 25 mg of total nuclear extract was applied to a 12-ml heparin-Sepharose 6B column (Pharmacia) at 4°C. Proteins were eluted with a linear KCl gradient (0.1 to 1.0 M) and collected in 105 fractions of 0.5 ml. Protein concentrations were determined according to Whitaker and Granum (44).

DNase I and exonuclease III in vitro protection assays. The DNase I protection assay was performed essentially as described by Jones et al. (27) with an end-labeled restriction enzyme fragment corresponding to positions −724 to −544 in Fig. 1. Approximately 1 ng of this fragment (~40,000 Cerenkov cpm) was added to a binding reaction with a final volume of 50 μl in the presence of various amounts of adipocyte extract or 5 μg of bovine serum albumin (BSA) [20 mM Tris · HCl (pH 7.8), 75 mM KCl, 0.5 mM EDTA, 1 mM DTT, 2% polyvinyl alcohol, 5% glycerol, 30 μg of poly(dI-dC) per ml] and incubated for 15 min on ice and then for 2 min at room temperature. In some experiments, unlabeled LP-α oligonucleotide corresponding to positions −702 to −666 in Fig. 1 was included in the binding reaction as a competitor in a ~100-fold molar excess. Binding reaction mixtures were then diluted with equal volumes of a mixture containing 5 mM $CaCl_2$ and 10 mM $MgCl_2$ and digested for 60 s at room temperature with various amounts of DNase I (Worthington Inc., Freehold, N.J.) to achieve partial digestion of the probe. The DNase I digestion was terminated by the addition of 100 μl of stop mix (20 mM EDTA, 1% SDS, 0.2 M NaCl, and 325 μg of yeast total RNA per ml), extracted with phenol and chloroform, precipitated, and

rinsed with 80% ethanol before being loaded on a 6% sequencing gel.

For the exonuclease III protection assay (17, 48), a restriction enzyme fragment corresponding to positions −854 to −544 in Fig. 1 was 5′ labeled as described above; ~1 ng of this probe (40,000 Cerenkov cpm) was included in a binding reaction mixture [75 mM NaCl, 0.1 mM EGTA, 20 mM Tris · HCl (pH 7.60), 40 μg of poly(dI-dC) per ml, 5% glycerol] together with ~8 μg of an adipocyte nuclear extract or 5 μg of BSA in a total volume of 50 μl. The binding reaction was carried out at 30°C for 20 min; at this stage, 2 μl of 137 mM $MgCl_2$ and 2 μl of exonuclease III (220 U/μl; Boehringer) were added to the reaction mixture, and digestion proceeded for 20 min at the same temperature. For some reactions, a specific and a nonspecific competitor, corresponding to positions −724 to −544 and −854 to −723, respectively, in Fig. 1, were included in the binding reaction mixture in a ~100-fold molar excess. The reaction was terminated with 50 μl of stop mix (see above), extracted with phenol and chloroform, rinsed with 80% ethanol, and then loaded on a 6% sequencing gel.

Nucleotide sequence accession number. The sequence information for the LPL gene has been deposited with EMBL/GenBank under accession no. X68111.

RESULTS

Cloning and functional organization of the human LPL promoter. We synthesized an oligonucleotide complementary to the 48 5′-most bases of the human LPL cDNA as described by Wion et al. (45). This oligonucleotide enabled us to isolate, from a human genomic EMBL3 library, overlapping λ clones covering the entire human LPL gene as well as shorter segments of 5′- and 3′-flanking regions. When we compared the sequence derived from our first λ clone (Fig. 1) with those of Deeb and Peng (10) and Kirchgessner et al. (29), we found a perfect match up to the point where those sequences start, −480 and −730, respectively. In Fig. 1, we have also marked the major start site of transcription as described by the same authors. As shown in Fig. 1 and 2A, we fused a 4.2-kb fragment derived from 5′-flanking sequence and a part of exon I to a CAT reporter gene (19). Immediately upstream of the LPL-derived sequence, we added a UMS (32, 47) which has the functional properties of a transcription terminator, thus abolishing background activity due to spurious transcripts initiated in flanking plasmid sequence. When the UMS was added to a naked CAT gene or construct +103 deleted of the LPL transcription start site, no difference in CAT activity was detected in comparison with untransfected cells (data not shown).

On the basis of construct −4 kb (Fig. 2A), we made a number of 5′-deletion mutants that were transiently transfected into adipocytes and preadipocytes derived from the cell line 3T3-F442A (for details, see Materials and Methods). In Fig. 2B, we set the CAT activity of construct −4 kb in adipocytes to 100%. It is interesting to note that the activities of constructs −4 kb and −1718 are about one-fourth as great in preadipocytes as in adipocytes. In construct −52, the difference in CAT activity is no longer present. Thus, the tissue distribution of CAT activity is similar to that of the expression pattern of the endogenous LPL gene in constructs −4 kb and −1718, whereas construct −52 does not show any clear difference in CAT activity between adipocytes and preadipocytes. In the case of construct +103 and a construct without any LPL-derived sequence, we could not detect any CAT activity above that of untransfected adipocytes and preadipocytes.

To investigate the presence of functional, transcriptionally active elements, we transfected a series of 5′-deletion mutants into 3T3-F442A adipocytes (Fig. 2C). Several possible functional elements, most notably two major positive elements at −430 to −400 and −196 to −168 and two major negative elements at −4.0 kb to −827 and −239 to −196, could be identified. At this stage, we had a general overview over the gross functional structure of the human 5′-flanking region. We concluded that constructs −4 kb and −1718 were able to function as a promoter in adipocytes, with a considerably lower activity in preadipocytes. This tissue preference was lost in construct −52, indicating the presence of *cis*-regulatory elements important for LPL expression in adipocytes. A longer series of 5′-deletions identified several positive as well as negative functional elements in the human LPL promoter. We were somewhat troubled by the fact that inclusion of the 5′-flanking region from base −827 to kb −4 led to an 8-fold drop in promoter activity compared with the activity of construct −827. This finding motivated us to seek positive elements located elsewhere that could counteract this rather dominating negative element.

Initially, we made constructs containing larger portions of 5′-flanking sequence (up to approximately −8 kb), but no significant change in promoter activity could be detected (data not shown). We then tried another approach to scan intragenic and 3′-flanking regions for the presence of regulatory elements by performing a DNase I hypersensitivity assay on nuclei isolated from the human monocyte-derived cell line THP-1 (39). This assay detects structurally altered segments of chromatin that are hypersensitive to low concentrations of DNase I; such regions have been shown to be involved in the transcriptional regulation of genes (23). THP-1 turned out to be especially suitable for this purpose, since this cell line expresses LPL only after it has been stimulated to differentiate into a macrophagelike cell type, by use of the phorbol ester PMA (3). Thus, we were able to identify DNase I-hypersensitive regions present only in PMA-treated, LPL-expressing cells. As shown in Fig. 3A, three DNase I-hypersensitive regions, DHS 1, 2, and 3, were characterized. DHS 1 was present in both PMS-treated and untreated cells, whereas DHS 2 and 3 were detected only in PMS-treated cells. To investigate whether these DNase I-hypersensitive sites contained regulatory elements important for the transcriptional regulation of LPL in 3T3-F442A adipocytes, the corresponding genomic fragments were isolated and ligated into the −4 kb promoter construct downstream of the CAT gene as outlined in Fig. 3B, an approach adopted from Arnow et al. (2). These constructs were then transiently transfected into adipocytes. Two of these constructs, those containing DHS 2 and 3, were able to overcome the negative element(s) present in the construct −4 kb at kb −4 to base −827. These experiments show the presence of several positive as well as negative elements present not only in the 5′-flanking region but also in intragenic and 3′-flanking regions. Hence, a strong negative element at kb −4 to base −827 can be overruled by positive elements present within the gene and in the 3′-flanking region.

Stable transfectants pinpoint two *cis*-regulatory regions important for differentiation-linked expression of the LPL gene. Having gained an overall idea of the functional properties of the human LPL promoter, we stably introduced a series of 5′-deletion mutants into preadipocytes, which, after a selection procedure, were allowed to develop into adipocytes (for details, see Materials and Methods). This was

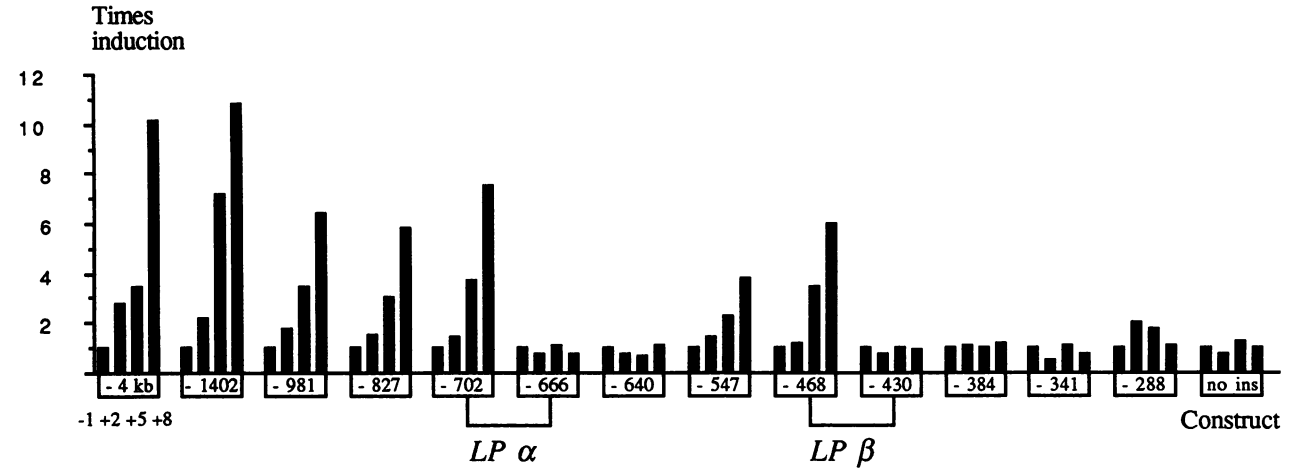

FIG. 4. Transfection assay. Preadipocytes (3T3-F442A) were stably transfected with some of the constructs depicted in Fig. 2C. Stably transfected cells were harvested at different time points (days) before (−) and after (+) confluence. Total RNAs from cells at day −1 and +8 were used for a Northern (RNA) blot assay and were shown to contain LPL-specific mRNA at day +8 and to lack this transcript at day −1 (data not shown); cells at day +8 also showed all morphological characteristics of an adipocyte. The results are expressed as times of induction relative to day −1; thus, CAT activity for day −1 was set to 1.0. This experiment was performed three times and assayed in duplicate, with identical results pinpointing the same breakpoints of the induction pattern, LP-α and LP-β. These results have also been normalized for differences in protein concentrations (44).

done with the aim of identifying *cis*-regulatory regions important for induction of the LPL gene during adipogenesis (1, 35). Encouraged by the results demonstrated in Fig. 2B, indicating the presence of *cis* elements at positions −1718 to −52 of importance for tissue-specific expression, we cotransfected CAT constructs with plasmid pXTI carrying a Neo^r marker gene (see Materials and Methods) at a molar ratio of 20:1. During the selection procedure, no decrease in the number of resistant foci was observed when the CAT constructs were included in comparison with plasmid pUC or control plasmid pXTI (data not shown) during the selection procedure (see Materials and Methods). Approximately 300 resistant foci were pooled in each experiment to compensate for differences in integration site and copy number (9). Figure 4 shows the result of a representative experiment; this experiment was performed three times, and each experiment was assayed in duplicate, with the same result pinpointing the same breakpoints of induction at −702 to −666 and −468 to −432. The Neo^R cells were cultured for various periods before and after the cells reached confluence (Fig. 4), and protein extracts were prepared for measurements of CAT activity. The time points were chosen to reflect the process of adipocyte differentiation, from a preconfluent preadipocyte at day −1 to a completely matured, fat-laden adipocyte at day +8 (Fig. 4). The morphological changes during fat cell development have previously been correlated to activation of several genes, e.g., the LPL gene (35). As shown in Fig. 4, there is a gradual increase in CAT activity during adipocyte development, mimicking the endogenous LPL expression during adipocyte differentiation as described by Samuelsson et al. (35). Constructs −4 kb to −702 show this gradual induction pattern during adipocyte development, whereas there seems to be a breakpoint with regard to this induction pattern located at positions −702 to −666, as demonstrated by construct −666. Constructs −666 and −640 do not show any induction of CAT activity during adipocyte development. In the case of constructs −547 and −468, there is a gradual reconstitution of the induction pattern. A new breakpoint is present at positions −468 to −430 which is similar to that located at positions −702 to −666. Further downstream, no reconstitution of the induction pattern could be demonstrated, nor did the construct void of LPL sequence show any time-dependent induction. This experiment with stable transfectants pinpoints two regions, LP-α (−702 to −666) and LP-β (−468 to −430; Fig. 1), that seem to be important for the regulation of LPL during adipocyte conversion of 3T3-F442A cells.

Transcription factors that are enriched during adipogenesis and with similarities in DNA-binding properties interact with LP-α and LP-β. A reasonable explanation for the functional properties of LP-α and LP-β would be that one or several distinct DNA-binding proteins, mediating the time-dependent induction pattern (Fig. 4), interact with DNA-binding motifs on LP-α and LP-β. To investigate this possibility, we made two double-stranded oligonucleotides identical to LP-α and LP-β, spanning the regions from −702 to −666 and from −468 to −430 (Fig. 1). These oligonucleotides were radiolabeled and used in a gel mobility shift assay (see Materials and Methods). Figure 5 shows that both LP-α and LP-β probes specifically interact with nuclear proteins derived from 3T3-F442A adipocytes. By itself, unlabeled LP-α and LP-β could compete for the nuclear proteins bound to both radiolabeled LP-α and LP-β. In contrast, a nonspecific double-stranded oligonucleotide competitor (see Materials and Methods), with no known sequence homology to the LPL gene or its flanking regions, did not compete with either LP-α or LP-β interactions (Fig. 5). Thus, nuclear proteins with similar DNA-binding properties seem to interact with both LP-α and LP-β. To further characterize the LP-α and LP-β interactions, nuclear extracts from adipocytes or preadipocytes were passed over a heparin-Sepharose column (see Materials and Methods). Approximately the same total amount of protein extract (~25 mg) from each cell type was used. There is a striking difference in signal strength when adipocyte and preadipocyte extracts are compared, indicating that during differentiation of preadipocytes into adipocytes, there is an increase of nuclear proteins that interact with LP-α and LP-β (Fig. 6). These results demonstrate that

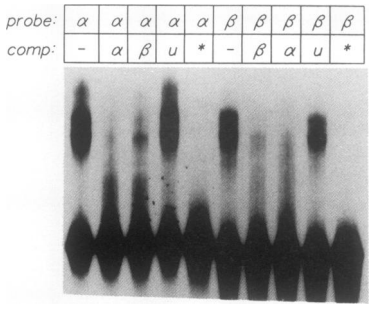

FIG. 5. Gel mobility shift experiment with LP-α (α) and LP-β (β) as probes and competitors, used with an adipocyte extract. A nonspecific double-stranded oligonucleotide (see Materials and Methods) was also used for competition experiments (u). −, absence of competitor; *, absence of competitor and protein extract (for details, see Materials and Methods).

TTR-S	-96	TATTGACTTAG	-106	11/11
TTR-W	-140	TATTTGTGTAG	-130	8/11
α1-AT	-376	TATTGACTTTG	-366	11/11
α1-AT	-195	CATTGATTTAG	-185	11/11
AFP	-6630	TATTGACTTTG	-6620	11/11
AFP	-5029	TGTTGACTTTT	-5019	10/11
LP-α	-669	TATTGAGTTAT	-679	10/11
LP-β	-440	CATTGACTTTG	-430	11/11
CONSENSUS:		TATTGACTTAG		
		C T TT		

FIG. 7. Sequence comparison of six HNF-3 binding sites (7) with LP-α and LP-β. TTR-S and TTR-W, the strong and weak HNF-3 sites in the transthyretin promoter; α1-AT, such sites in the α1-antitrypsin promoter; AFP, HNF-3 sites in the α-fetoprotein promoter. Positions relative the start of transcription are indicated. Degree of similarity to the consensus sequence is shown at the right.

LP-α and LP-β interact with a set of common transcription factors that are enriched during adipogenesis and with similarities in DNA-binding properties as well as band pattern in a gel mobility shift assay.

Factors similar in DNA-binding properties to the HNF-3/fork head family of transcription factors interact with LP-α and LP-β. To explain the fact that the DNA-protein interactions with LP-α and LP-β demonstrated such a clear resemblance with regard to DNA-binding specificity, we sought the existence of sequence similarities and known DNA-binding motifs present in LP-α and LP-β. The most striking motif that we could find on both LP-α and LP-β turned out to be very similar to the consensus sequence reported to bind members of the HNF-3 family of transcription factors (7) (Fig. 7). LP-α is the only sequence in Fig. 7 that displays a perfect symmetry centered around position −674. In promoter regions known to contain HNF-3 binding sites,

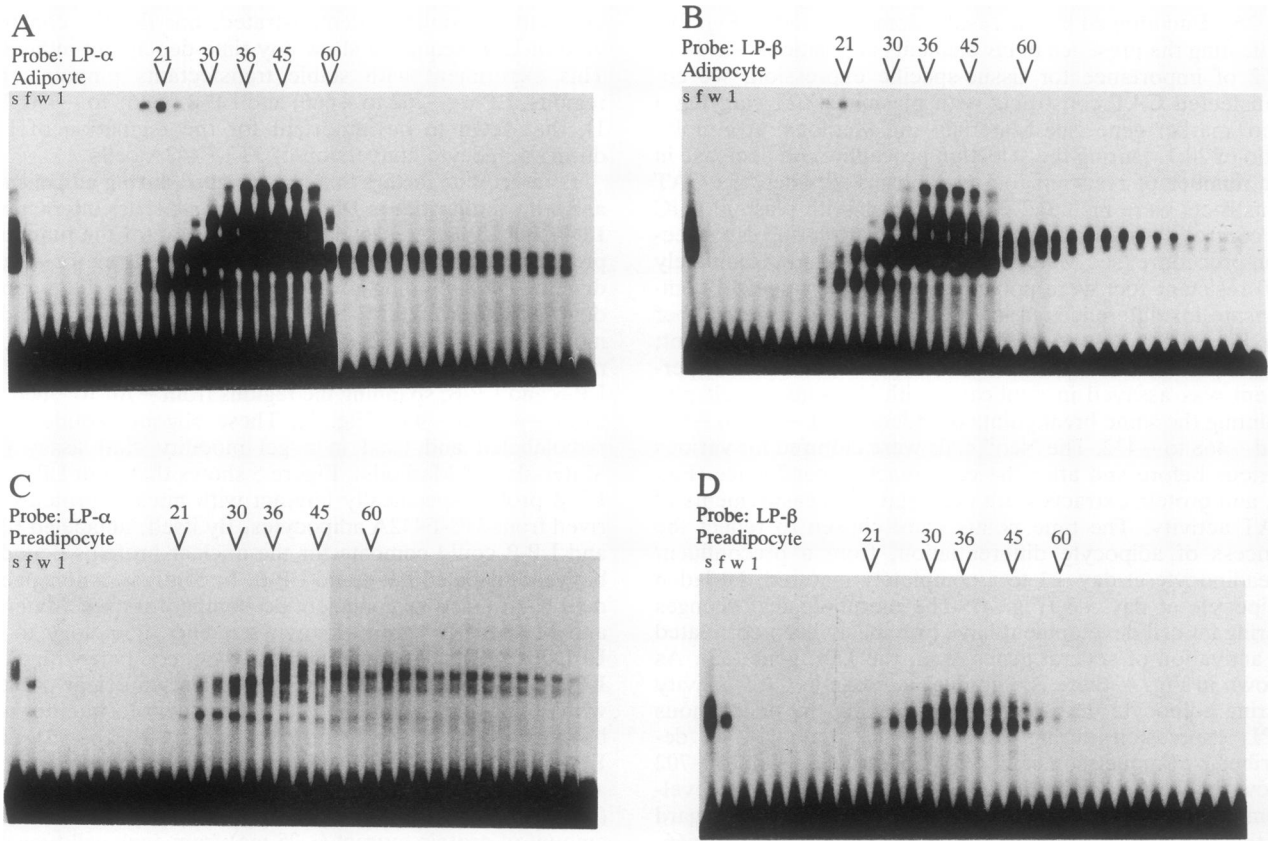

FIG. 6. Gel shifts performed on fractions collected from a heparin-Sepharose column as described in Materials and Methods. Each third fraction is included, with the exception that each fifth fraction is included between fractions 45 and 60. s, protein extract before it was loaded on the column; f, flowthrough; w, gel shift performed with a sample collected after the column had been washed with several column volumes of buffer containing 100 mM KCl. The column was then eluted with a linear salt gradient (0.1 to 1.0 M KCl). Probes and extracts used are indicated in each panel. The same amount of protein extract (approximately 25 mg) was used in each experiment.

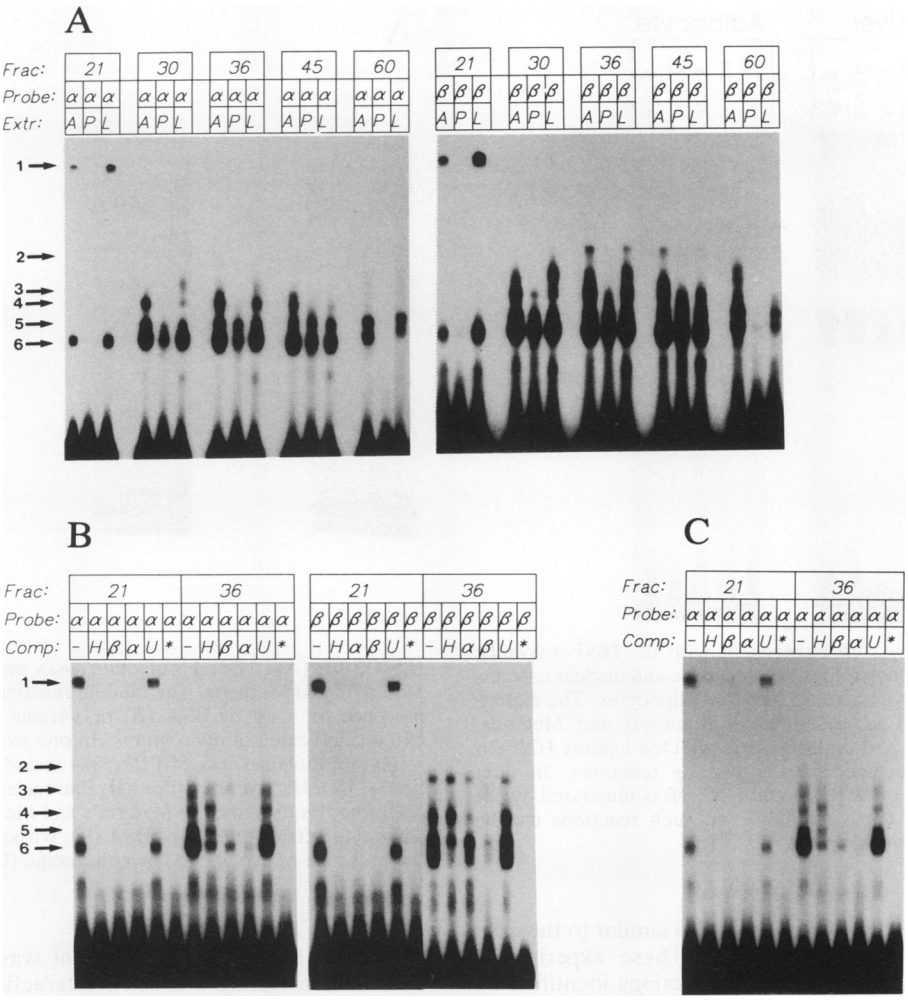

FIG. 8. (A) Comparison of gel shift patterns from fractions 21, 30, 36, 45, and 60, derived from the experiments shown in Fig. 6 and 9. LP-α (α) and LP-β (β) were used as probes with fractions derived from adipocytes (A), preadipocytes (P), and mouse liver (L). (B) Competition experiment with fractions 21 and 36, derived from an adipocyte extract. LP-α (α) and LP-β (β) were used as probes and competitors. −, absence of competitor; *, absence of competitor and extract; U, presence of a nonspecific competitor. (C) Experiment identical to that in panel B except that fractions derived from mouse liver were used.

typically two such sites have been identified, as indicated in Fig. 7 (7). HNF-3 is a family of transcription factors expressed mainly in the liver and, to a lesser degree, in the lung (30). To investigate whether a nuclear extract of hepatic origin would interact with LP-α and LP-β, approximately 25 mg of nuclear extract derived from mouse liver was passed over a heparin-Sepharose column. This separation revealed a gel shift pattern in liver extract, for both LP-α and LP-β (data not shown), similar to that found with adipocyte extract (Fig. 6A and B).

Figure 8A compares the various DNA-binding proteins in nuclear extracts from adipocytes (3T3-F442A), preadipocytes (3T3-F442A), and mouse liver. Fractions 21, 30, 36, and 45 from each chromatographed extract (Fig. 6) were assayed in parallel, facilitating a detailed comparison. As can be seen in Fig. 8A, bands 1 and 6 are present in fraction 21 of adipocyte and liver extracts but totally absent in a preadipocyte extract. In fractions 30, 36, and 45, bands 5 and 6 are present in all three extracts, whereas bands 2, 3, and 4 seem to be clearly more abundant in adipocyte and liver extracts. In fraction 60, bands 4 and 5 are present in adipocyte and liver extracts; in a preadipocyte extract, no signal can be observed. In a gel mobility shift assay, no clear difference in band pattern can be demonstrated between adipocyte and liver extracts. It should also be noted that the LP-β probe identifies band 2 more distinctly than does the LP-α probe. In Fig. 8B, fractions 21 and 36 from an adipocyte extract were selected for a competition experiment (see Materials and Methods). As can be seen, bands 1 and 6 in fraction 21 could be effectively competed for by a double-stranded oligonucleotide identical to the one used by Lai et al. (30) to purify HNF-3α. LP-α and LP-β could also compete for these interactions, whereas a nonspecific double-stranded oligonucleotide could not. In fraction 36, it is clear that LP-α is the only competitor able to totally compete for all interactions when LP-α is used as a probe. The same is true for LP-β when LP-β is the probe (Fig. 8B). The HNF-3 oligonucleotide can clearly compete for bands 6 and 5; with LP-β as a probe, there is complete competition for band 6. Bands 2, 3, and 4 do not seem to be as sensitive to competition with the HNF-3 oligonucleotide. Figure 8C, in which fractions 21 and 36 derived from liver extract were used, demonstrates not only that LP-α can interact with factors present in a liver extract but also that these interac-

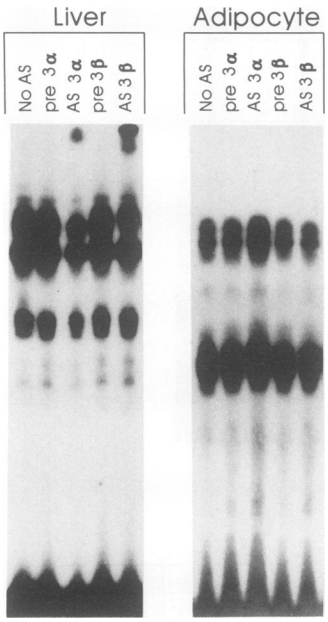

FIG. 9. Gel mobility shift experiment with the HNF-3-specific double-stranded oligonucleotide (30) as a probe and nuclear extracts derived from mouse livers and 3T3-F442A adipocytes. The experiment was carried out as described in Materials and Methods. Preimmune sera (pre) and antisera (AS) directed against HNF-3α and HNF-3β were included in the binding reactions. In liver extracts, the presence of HNF-3α and HNF-3β is illustrated by the supershifts formed in these reactions; no such reactions can be detected in the adipocyte extract.

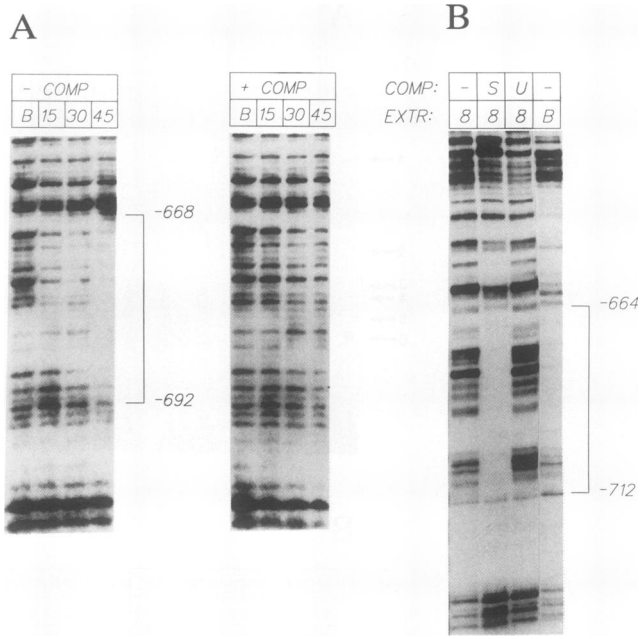

FIG. 10. (A) DNase I protection assay performed as described in Materials and Methods. The binding reaction was performed in the presence of 5 μg of BSA (B) or various amounts of adipocyte extract, indicated in micrograms. In one experiment (+ COMP), a ~100-fold molar excess of LP-α was included in the binding reaction. − COMP, no competitor. (B) Exonuclease III protection assay performed as described in Materials and Methods, using 8 μg (8) of adipocyte extract or 5 μg of BSA (B), without competitor (−) or in the presence of specific (S) or nonspecific (U) competitor.

tions can be competed for in a way that is similar to the case with an adipocyte extract (Fig. 8B). These experiments support the idea that some of the interactions identified by LP-α and LP-β are similar in DNA-binding specificity to the HNF-3 family of transcription factors. We also used as a probe the double-stranded HNF-3 oligonucleotide, which is known to bind both HNF-3α and HNF-3β (30), in gel mobility shift assays together with nuclear extracts derived from mouse liver and 3T3-F442A adipocytes. When antisera specific to HNF-3α and -β were included in these reactions as described by Lai et al. (30), we could demonstrate the absence of HNF-3α and -β in adipocyte extracts, whereas the antisera produced supershifts indicating the presence of these factors in liver extracts (30) (Fig. 9). Also, LP-α and LP-β interact with factors present in liver in a way that shares many resemblances with interactions that are present when an adipocyte extract is used (Fig. 8A). These results indicate that factors with no immunological identity to HNF-3α and -β but similar in DNA-binding specificity to the HNF-3 family interact with LP-α and LP-β in adipocytes. It should also be pointed out that some of the interactions cannot be competed for by the HNF-3 probe as a competitor, indicating that proteins with other DNA-binding specificities also interact with LP-α and LP-β.

To determine the exact location of the DNA-protein interaction, we used an end-labeled fragment from the LPL 5′-flanking region containing LP-α, corresponding to −724 to −544 in Fig. 1, as a probe in an in vitro DNase I protection assay (27) (Fig. 10A). With an extract derived from adipocytes, a fragment encompassing −692 to −668 was protected. When a ~100-fold molar excess of oligonucleotide LP-α was added to the binding reaction prior to DNase I treatment, the protected fragment was no longer present, thus demonstrating a specific interaction. In Fig. 10B, we used an exonuclease III in vitro protection assay (18, 48) with a fragment corresponding to positions −854 to −544. In this assay, a somewhat larger stretch of DNA is shown to contain several exonuclease stop bands, present between positions −712 and −664 (Fig. 1). A specific competitor in a ~100-fold molar excess can completely extinguish these stop bands, whereas a nonspecific competitor lacks this capability. When these results are compared with those of Fig. 7, it is clear that the HNF-3 consensus binding sequence is a part of the two protected fragments. However, the interactions extend in the 5′ direction to −692 and −712, respectively, demonstrating a similarity to the DNase I protection experiment reported by Costa et al. (7) in which affinity-purified HNF-3 protected a fragment of 29 nucleotides in the α1-antitrypsin promoter. Here the consensus sequence is located at the extreme 3′ border of the protected region, very similar to the results shown in Fig. 10A. Despite several attempts, we have not been able to demonstrate any interactions with protein extracts derived from preadipocytes (data not shown).

The LP-α and LP-β *cis*-regulatory regions confer a differentiation-dependent induction pattern to a heterolog promoter during adipogenesis. We have shown that LP-α and LP-β represent two breakpoints with regard to a differentiation-linked expression pattern during adipocyte differentiation in vitro. It has also been demonstrated that a group of DNA-binding proteins very similar with regard to band pattern in gel mobility shift assays and DNA-binding specificity interact with LP-α and LP-β. Some of these factors are similar in

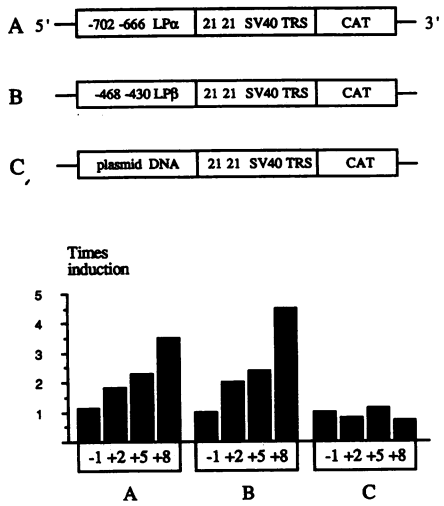

FIG. 11. Stable transfections performed as described in Materials and Methods with chimeric constructs containing LP-α (A), LP-β (B), or plasmid DNA (C) fused to a simian virus 40 (SV40) promoter lacking the enhancer but containing the transcription start site (TRS) and the 21-bp repetitions. The experiment was performed three times and assayed in duplicate, with identical results illustrating a stepwise induction pattern in constructs A and B but not in construct C.

DNA-binding specificity to the HNF-3 family of transcription factors (7, 30), whereas others clearly show different specificities. To further investigate the functional properties of LP-α and LP-β, we made three chimeric constructs in which we fused LP-α, LP-β, and unrelated plasmid DNA upstream of an enhancerless simian virus 40 early promoter containing the transcription start and the 21-bp repetitions. These constructs were then stably transfected into preadipocytes. After a selection procedure, the cells were allowed to differentiate into adipocytes (see Materials and Methods). Figure 11 shows that LP-α and LP-β are capable of conferring the LPL induction pattern to a heterologous promoter. The level of induction is ~4-fold in each construct, compared with constructs −4 kb and −1402 (Fig. 4), which are induced ~10-fold, indicating that other *cis*-regulatory elements also are involved.

DISCUSSION

To study the general functional properties of the LPL promoter, including its differentiation-linked induction during adipocyte differentiation, we have cloned and fused different deletion mutants of LPL intragenic, 5′-flanking, and 3′-flanking sequences to a CAT reporter gene. Transient transfections of such constructs into preadipocytes and adipocytes defined a region at positions −1718 to −52 (Fig. 2B) as important for adipocyte-specific LPL expression. This result is in accordance with the work of Hua et al. (26), who demonstrated that *cis*-regulatory regions of importance for tissue specificity are located outside the immediate promoter region (−100 to +188) of the murine LPL promoter. The sequence of this region is extremely well conserved between human and murine species. Previato et al. (34a) have shown that the human LPL promoter contains an OCT-1 site at position −46. 5′-deletion construct −461, containing the OCT-1 site, had approximately five times higher activity than did construct −461, which lacked this binding site; this was true for both adipocytes (3T3-L1 cells) and hepatocytes (HepG2 cells), indicating that OCT-1 is important for the transcriptional regulation of LPL and that the tissue specificity of LPL gene expression is dependent on factors other than OCT-1. With this knowledge, we transfected a series of 5′-deletion mutants spanning from kb −4 to base −52 (Fig. 2C), and several positive and negative functional elements could be demonstrated. One striking feature is the strong negative element present at kb −4 to base −827, which reduces the activity to a level similar to that of construct −52 (Fig. 2C). This finding led us to seek compensatory positive elements elsewhere; for this purpose, a DNase I hypersensitivity assay was used essentially as described by Gimble and Max (18). Three DNase I-hypersensitive regions were identified, and genomic fragments containing these sites were cloned into construct −4 kb 3′ of the CAT gene. Two of these regions, DHS 2 and 3 (Fig. 3), contain sequences that can counteract the strong negative element present from kb −4 to base −827. Thus, DHS 2 and 3 are two DNase I-hypersensitive regions identified in LPL-expressing THP-1 cells with the ability to counteract the negative element(s) present at kb −4 to base −827 when constructs are expressed in 3T3-F442A adipocytes. Considering the limitations of the methods used, it seems likely that intragenic sequences as well as sequences located in the 3′-flanking region together with 5′-flanking sequences contribute to transcriptional regulation of the LPL gene. This is a situation that is analogous to that one described for the human adenosine deaminase gene (2).

Having determined this general organization of the regulatory sequences involved in transcriptional regulation of the LPL gene, we stably transfected 3T3-F442A preadipocytes with various 5′-deletion mutants, enabling us to compare induction patterns of the different constructs during adipocyte differentiation. As demonstrated in Fig. 4, two major breakpoints of an LPL-like induction pattern were observed at sites LP-α (−702 to −666) and LP-β (−468 to −430). The experiment in Fig. 4 was designed to demonstrate the ability of various 5′-deletion constructs, stably transfected into preadipocytes, to be induced during adipocyte differentiation; thus, the level of induction of CAT activity relative to day −1 was measured. Compared with the transient transfections shown in Fig. 2, these two experiments measure different aspects of LPL gene regulation; hence, no direct comparison can be made with regard to differences in CAT activity between constructs in these two experiments. It is, however, interesting to note that the major positive elements −430 to −400 and −196 to −168 defined by transient transfections (Fig. 2C) are different from LP-α and LP-β, as defined by the ability of these regions to confer an adipocyte differentiation-linked activation to a reporter gene construct (Fig. 4 and 11) when stably transfected into preadipocytes. Apart from differences in methodology, these findings indicate that LP-α and LP-β could function as gates that modulate transcriptional activity during adipocyte differentiation. Since LP-α and LP-β induce low activity in preadipocytes (non-LPL-expressing cells) and high activity in adipocytes (LPL-expressing cells), this ability clearly includes some components of tissue specificity. Previato et al. (34a) have identified the region from −724 to −92 of the human LPL promoter as having lower activity in hepatocytes (non-LPL-expressing HepG2 cells) than in adipocytes (LPL-expressing 3T3-L1 cells); LP-α and LP-β are located within this region.

LP-α and LP-β contain a motif with striking similarity to the consensus sequence for HNF-3 binding sites (Fig. 7) (7).

Further experiments showed that LP-α and LP-β could completely compete for each other's DNA-protein interactions (Fig. 5), whereas a nonspecific probe did not. When adipocyte, preadipocyte, and liver nuclear extracts were compared in a gel shift assay for the presence of specific interactions with LP-α and LP-β, a clear similarity was found between the adipocyte and liver extracts (Fig. 8). It was also demonstrated that a lower amount of proteins bound to LP-α and LP-β in the preadipocyte extract (Fig. 6). When fractions from a heparin-Sepharose column were compared, several interactions present in adipocyte and liver extracts could not be found in preadipocytes (Fig. 8A). We cannot determine whether this result is due to an inability of preadipocytes to express these proteins or whether it is due to an overall lower level of these proteins, as a consequence of which some of the proteins are expressed at a level below that of the detection limit of the assay. Nevertheless, both quantitative and qualitative differences are present when adipocyte and preadipocyte extracts are compared (Fig. 6 and 8). In a competition experiment, it was shown that several of the interactions present in adipocyte and liver extracts (Fig. 8B and C) could be competed for by a double-stranded oligonucleotide identical to the one used by Lai et al. (30) for affinity purification of HNF-3α. To our knowledge, this is the first time that a nuclear protein with DNA-binding specificity similar to that of HNF-3 has been identified in adipocytes. However, some of the interactions are not influenced by the presence of the HNF-3-binding oligonucleotide, illustrating that proteins with different binding specificities also contribute to these interactions. Results of DNase I and exonuclease III in vitro protection assays performed with an adipocyte extract demonstrate that the HNF-3 consensus sequence is included in the protected fragment over LP-α (Fig. 10).

These experiments show that LP-α and LP-β interact with nuclear proteins that are enriched during adipogenesis and that some of these interactions have a binding specificity similar to that reported for the HNF-3 family of transcription factors. This group of transcription factors has recently been shown to also include the *Drosophila* homeotic fork head gene (30, 42, 43). Fork head plays a fundamental role in embryonic development of hindgut and foregut; during late embryonic development, the gene is expressed in a variety of tissues (42). Hence, a DNA-binding protein with extensive homologies to the HNF-3 family has been shown to be of great importance for correct cellular differentiation in *Drosophila* cells. It is possible that factors similar in DNA-binding specificity to the HNF-3/fork head family of transcription factors, but with no immunological identity with either HNF-3α or β (Fig. 9), interact with LP-α and LP-β in the LPL promoter and that these interactions are part of a differentiation switch that controls LPL transcription during adipocyte differentiation. The observation that there is an enrichment of proteins that interact with LP-α and LP-β during adipocyte development would fit into this scheme. This hypothesis is also supported by the experiment shown in Fig. 11, which demonstrates that LP-α and LP-β can confer an adipocyte differentiation-linked expression pattern to a heterolog promoter. It should be emphasized that the LPL gene is not expressed in the liver under normal circumstances (13, 45); hence, the similarities in gel shift pattern and DNA-binding specificity between adipocyte and liver extracts suggest that other *cis*-regulatory elements and DNA-binding proteins are also involved in directing adipocyte-specific LPL induction. We should, however, bear in mind that after administration of tumor necrosis factor α and during fetal development, significant levels of LPL-specific mRNA can be found in the liver (14, 41), demonstrating the ability of the liver to express transcription factors necessary for LPL expression.

The data presented in this report delimit two regions of the LPL promoter, LP-α and LP-β, which we show to be two *cis* elements important for transcriptional activation of the LPL gene during adipocyte differentiation in vitro. Since LPL is one of the first genes to be activated during adipocyte development, analysis of the nature of the transcription factors interacting with LP-α and LP-β could provide valuable information about the early cellular events that initiate the adipocyte differentiation scheme. Future investigations will focus on this issue.

ACKNOWLEDGMENTS

We thank P. Carlsson, T. Edlund, and M. Yaniv for sharing plasmids with us; H. Green for providing 3T3-F442A cells; James E. Darnell, Jr., for making HNF-3α and -β antisera available; and M. Hermansson for excellent technical assistance. We also thank P. Carlsson for valuable discussions and J. M. Gimble for critical reading of the manuscript.

This work was supported by grants from the Swedish Medical Research Council, HM King Gustav V and HM Queen Victoria Foundation, and Inga-Britt and Arne Lundberg Research Foundation.

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