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Developmental extinction of liver lipoprotein lipase mRNA expression might be regulated by an NF-1-like site

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The molecular mechanism underlying the extinction of lipoprotein lipase (LPL) expression in rat liver during development was investigated. A mouse (BWTG3) and a rat (7777) hepatoma, both of which exhibit characteristics of fetal hepatocytes, were found to contain LPL mRNA, whereas the more differentiated human (Hep G2 and Hep 3B) or rat (Fa32) hepatoma cell lines did not. Somatic cell hybrids between LPL-producing hepatoma cells and non-LPL-producing cells, such as adult rat hepatocytes or fibroblasts, exhibited extinction of LPL gene expression. Assay of expression of nested deletions in the 5′ regulatory sequences of the LPL gene in the Hep G2 cell line and in BWTG3 cells localized sequences involved in the suppression of LPL production to a region between −591 and −288 relative to the transcription initiation site. A site with sequence homology to a glucocorticoid responsive element (GRE) was shown not to play an important role in the extinction process. A novel transcription factor, termed RF-1-LPL, was shown to bind to an NF-1-like site in this region. In contrast to neonatal animals, in adult animals an additional protein complex (RF-2-LPL), was formed on the NF-1-like site, suggesting that this sequence might recruit a *trans*-acting factor involved in the extinction of LPL gene expression in adult rat liver.

Atherosclerosis; NF-1/CTF; Lipoprotein lipase; Transcription; Gene expression; Extinction

1. INTRODUCTION

Lipoprotein lipase (LPL), which catalyzes the hydrolysis of triacylglycerols of lipoprotein particles, is a secreted enzyme with a relative molecular mass of 57,000 and functions as a homodimer in vivo (reviewed in [1]). After secretion LPL becomes bound to glycosaminoglycans on the luminal surface of extrahepatic capillary endothelium, where it hydrolyzes core triglycerides in triglyceride-rich lipoproteins, such as chylomicrons and very low density lipoproteins (reviewed in [1]). The released free fatty acids are either oxidized to generate ATP in muscle, stored in adipose tissue, or secreted in milk by the mammary gland. LPL therefore occupies a pivotal position in both lipoprotein and energy metabolism. LPL synthesis is induced during differentiation in a number of cell types and tissues including adipocytes, monocytes, skeletal and cardiac muscle (reviewed in [1]). Adult rat liver normally synthesizes hepatic lipase but not LPL, whereas in neonatal rat liver LPL mRNA and protein are produced [2,3]. During development the expression of LPL becomes, however, extinguished in rat liver. The present study aims at investigating the molecular mechanisms involved in the extinction of neonatal rat liver LPL production. Hepatoma cell lines, which are still capable of producing LPL were identified and analysis of somatic cell hybrids obtained from fusion of such LPL-producing hepatoma cells with other non-LPL-producing cells suggests that suppression of LPL synthesis in adult liver cells involves a developmentally regulated specific *trans*-acting extinguisher. A region in the 5′ upstream regulatory sequences (URS) of the LPL gene was shown to possibly mediate neonatal extinction of LPL gene transcription. In this region a NF-1 like sequence was identified, which forms different protein complexes with liver nuclear extracts from adult versus neonatal animals. It is speculated therefore that the extinction process of the LPL gene involves interaction of a nuclear protein with the NF-1-like site.

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2. EXPERIMENTAL

2.1. *Cell culture and transfections*

The human hepatoblastoma cell lines, Hep 3B and Hep-G2, the human epitheloid carcinoma cell line, HeLa, and the mouse teratocarcinoma cell line, F9, were obtained from the American Type Culture Collection. The mouse hepatoma cell line, BWTG3 [4], and the rat hepatoma cell lines, 7777 [5] and Fa32 [6,7], a derivative of the H4IIEC$_3$ line [8], were described previously. The BS 140 and BS 181 p10 cells are cell hybrids formed between malignant mouse hepatoma

(BWTG3) and normal rat skin fibroblasts [9]. The LB 2100 and LB 2041 are cell hybrids between the mouse hepatoma line BWTG3 and adult rat hepatocytes were described previously [10]. Each of the hybrids retained at least one copy of each rat chromosome ([10,11] and unpublished data). Cells were maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere of 5% CO_2, 95% air [12,13]. For the somatic cell hybrids the medium was supplemented with 100 μM hypoxanthine, 4 μM aminopterin, and 16 μM thymidine.

Hela, F9, BWTG3, and Hep-G2 cells were transfected at 60–70% confluency. A total of 5 μg of plasmid DNA was transfected by the calcium phosphate coprecipitation procedure (10 h contact time). After washing with PBS, fresh medium was added and cells were harvested after a 16 h incubation. Chloramphenicol acetyl transferase (CAT) activity was determined on cell extracts, after normalizing to β-galactosidase activity (derived from cotransfected β-galactosidase plasmid), as described by Gorman et al. [14].

2.2. *Recombinant plasmids*

pHLPL-26 contains a human LPL cDNA clone [12,15]; genomic restriction fragments containing various lenghts of the LPL 5′ upstream sequences were subclones of clone B-1 [15]. The LPL 5′ upstream sequences were cloned into the polylinker of the promotor-less expression vector KS$^+$-0-CAT [16] and a scheme of these constructs can be seen in Fig. 2A. KS$^+$-PN-CAT contained a fragment spanning −1719 to +136, KS$^+$-XN-CAT a fragment from −852 to +136, KS$^+$-AN-CAT a fragment from −230 to +136 of the LPL genomic sequence (− refers to 5′ upstream sequences as defined in [15]. Finer deletion mutants KS$^+$-XN-1-CAT (−827 to +136), KS$^+$-XN-2-CAT (−768 to +136), KS$^+$-XN-4-CAT (−591 to +136), and KS$^+$-XN-5-CAT (−288 to +136) were used for detailed mapping. All constructs were examined by restriction mapping and partial sequencing. KS$^+$-0-CAT was used as a negative control, while KS$^+$-SV40-CAT, containing the SV-40 promotor and a CMV-driven β-galactosidase plasmid served as a positive control and a control for transfection efficiency, respectively. The GR-DBD prokaryotic expression vector was described by De Vos et al. [17,18].

2.3. *Analysis of RNA*

RNA was isolated by the guanidine isothiocyanate method and analyzed either by electrophoresis through 1.2% agarose–formaldehyde gels followed by capillary transfer to nylon membranes, or by dot blot hybridization as described [12]. Transcripts were quantitated by densitometric tracing of autoradiographs following hybridization to a ^{32}P-labeled 1.36 kb *Eco*RI fragment of the human LPL cDNA clone (hLPL-26; specific activity of 1×10^9 cpm/μg) [12]. A rat [19] or a human apo E [12], and a chicken β-actin [20] clone were used as controls.

2.4. *Nuclear extracts and gel retardation assays*

Nuclear extracts from cell cultures and tissues were prepared exactly as described by Dignam et al. [21] and by Gorski et al. [22], respectively. Final protein concentrations were typically 2–4 mg/ml. Construction of the GR-DBD prokaryotic expression vector and production of GR-DBD fusion proteins with protein A (GRF1) was performed exactly as described by De Vos et al. [17,18]. DNA–protein binding assays were performed as follows in a 20 μl reaction mixture. Nuclear extracts (1–3 μl) were incubated for 20 min at room temperature with 2 μg poly(dI:dC) and radiolabelled probe in TM buffer (25 mM NaCl, 20 mM Tris-HCl pH 7.4, 1 mM MgCl2, 0.5 mM EDTA, 5 mM DTT, 10% glycerol). DNA–protein binding, and subsequent separation of DNA–protein complexes on a 5% polyacrylamide gel were performed essentially as previously described [23–25]. As probes to study the LPL-NF-1-like site a synthetic double-stranded 30 bp oligonucleotide (wild-type LPL; 5′-GATCCATCTTGCCAATGTTAAAACACCAGA-3′) spanning from nucleotide −514 to −487 of the human LPL gene 5′URS was used. Other oligonucleotides include LPL-m1 (5′-GATCCATCTTGCCAATGTCGAAACACCAGA-3′), and LPL-m2 (5′-GATCCATCTTGCGCATGTTAAAACACCAGA-3′). For the delineation of the glucocorticoid response element (GRE) overlapping DNA fragments spanning the GRE were used initially (−665 to −419 and −522 to −227 and −144 to +17). Later a 23 bp synthetic oligonucleotide containing the GRE was used as probe (5′-AGCTGGGACGCAATGTGTGTCCC-3′). In some control experiments a 21 bp oligonucleotide representing the third GC box of the SV-40 promotor was used.

2.5. *DNase I footprinting assays*

DNase I footprinting reactions were performed essentially as described by Jones et al. [26] using nuclear extracts isolated from rat livers (see above). In short, probes end-labelled on one strand were prepared by PCR amplification of plasmid DNA using one cold primer and one labelled primer. These labelled DNA fragments were incubated in a total volume of 50 μl containing 10 mM Tris-HCl pH 7.9, 50 mM KCl, 6.25 mM $MgCl_2$, 0.05 mM EDTA, 1 mM DTT, 8.5% v/v glycerol, 2% polyvinylalcohol, and 1 μg poly(dI:dC]) with 10–20 μg nuclear extract. After incubation for 20 min at room temperature, 50 μl of 5 mM $CaCl_2$, 20 mM $MgCl_2$ was added, then 4 μl of freshly prepared DNase I diluted in 50 mM $MgCl_2$ was added. Digestion was allowed for 60 s at room temperature, after which 100 μl stop buffer (20 mM EDTA, 200 mM NaCl, 1% w/v SDS) was added. Reactions were analyzed on 8% polyacrylamide/8 M urea sequencing gels after extraction with phenol chloroform and isopropanol precipitation.

3. RESULTS AND DISCUSSION

3.1. *LPL expression in hepatoma cell lines*

LPL mRNA is, like α-fetoprotein (AFP), expressed in fetal but not in adult liver [3,27]. Indeed, shortly after birth, liver LPL gene expression is extinguished. It was shown that hepatoma cell lines that exhibit adult characteristics, though they also produce AFP, such as the two human cell lines Hep G2 and Hep 3B do not produce any detectable LPL mRNA, whereas the mouse fetal hepatoma line BWTG3 (producing AFP) expressed substantial amounts of LPL mRNA (Fig. 1; see also [28]). In the present study these analysis were extended to two rat cell lines. In fact, rat Fa32 hepatoma cells, which display adult characteristics and does not produce AFP, lack LPL mRNA, whereas the less differentiated 7777 cell line (which produces AFP) still produces LPL mRNA (data not shown). The production of LPL by hepatoma cell lines expressing fetal traits is consistant with hepatocytes being the source of LPL in the neonatal liver, and thus such cell lines are a useful in vitro model systems to study the control of liver LPL gene expression. These data support the notion that LPL production is a fetal hepatocyte trait. LPL would thus belong to a class of hepatic proteins which like AFP are expressed during fetal hepatic tissue differentiation and are repressed once full tissue maturation is reached [29–31]; however, a strict correlation between LPL and AFP expression is not observed since some hepatoma cells (Hep G2, Hep 3B) only express AFP.

3.2. *LPL mRNA is extinguished in hybrids between LPL-producing and non-producing cells*

Although we had previously shown that the production of LPL in rat liver could be modified in vivo by treatment with either glucocorticoid or thyroid

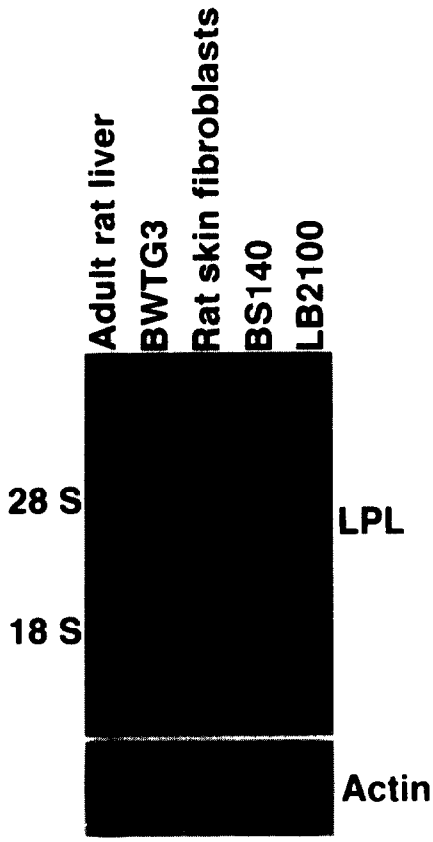

Fig. 1. LPL mRNA in hepatoma cell lines (lanes 1 and 2) and in cell hybrids (lanes 3–5). Twenty μg of total RNA was analysed by Northern blot hybridization. Mouse LPL mRNA, which represents a single species, is indicated by an arrowhead; Lane 1, adult rat liver RNA; lane 2, BWTG3 RNA; lane 3, rat skin fibroblast RNA; lane 4, BS140 RNA; lane 5, LB 2100 RNA. Description of the hybrids can be found in section 2.

hormone [3], several observations suggest that factors other than these hormones might be involved in the neonatal extinction process. First, prolonged hypothyroidism in the adult rat did not restore liver LPL production (Staels and Auwerx, unpublished data). Second, glucocorticoid hormone did not influence LPL production in BWTG3 cells [28]. Although the absence of a response of the LPL gene to glucocorticoids in BWTG3 cells could be due to the absence of a receptor, the activation of an LTR-CAT construct by addition of dexamethasone to BWTG3 culture medium ([28] and not shown) argues against this last possibility. The availability of hepatoma cell lines, expressing LPL, however, provided a good tool to study the molecular mechanism of this extinction. Data obtained from studies of cell hybrids derived from the fusion of LPL-producing mouse hepatoma cell line, BWTG3, with non-LPL-producing rat cells argued strongly against hormonal factors being the sole cause of extinction. In fact two different types of hybrids were tested for the presence of LPL mRNA (Fig. 1): hybrids derived from the fusion of the BWTG3 with cells of a non-hepatic lineage, such as rat fibroblasts, or hybrids derived from the fusion of BWTG3 with hepatic cells at a different stage of development, such as adult rat hepatocytes. BWTG3 × rat skin fibroblasts hybrids, (BS 140 and BS 181p10), both of which have retained at least one copy of each rat chromosome [11], contained no detectable LPL mRNA, arguing for an extinction of LPL gene expression by a factor provided by the rat fibroblast genome (Fig. 1). Two hybrid lines derived from the fusion of BWTG3 with adult rat hepatocytes (LB 2100 and LB 2181; 10), were also analyzed for LPL mRNA production (Fig. 1). LPL gene expression was likewise extinguished in these two hybrid lines, which also retained at least one copy of each mouse and rat chromosome. LPL expression thus appears to be extinguished not only in hybrids formed between cells of different histiotypes (BWTG3 hepatoma cells × fibroblasts) but also in hybrids formed between cells of the same lineage but at different developmental stages (BWTG3 hepatoma cells × adult hepatocytes). This situation is in contrast with that of the AFP gene, which is not extinguished in BWTG3 × hepatocyte hybrids [10]. Since glucocorticoid or thyroid hormones negatively modulate expression of LPL in vivo [3], it is conceivable that LPL extinction in these hybrids could be caused by the presence of hormones in the tissue culture medium. This hypothesis seems, however, unlikely, since similar results (absence of LPL mRNA) were obtained when these hybrid cells were grown in medium from which these hormones were removed by adsorption with charcoal.

By using similar somatic cell and microcell hybrid studies it has been shown previously that the tissue-specific extinction of several genes is mediated in *cis* by specific loci, which also implicates trans-acting factors in this process [32,33]. Examples of such tissue specific extinction include the genes for α-fetoprotein [7,34,35], tyrosine aminotransferase [33,36,37], the T cell antigen receptor [38] or albumin [7,32,39]. The LPL gene might therefore be another example of a gene regulated by extinguisher factors produced by non-LPL-expressing cells.

3.3. *Localization of DNA sequences responsible for extinction*

The existence of *cis*-acting regulatory sequences within the 5′ upstream region of the LPL gene that could respond to the *trans*-acting extinguisher was next explored. Expression vectors, in which nested deletions of the 5′ LPL regulatory sequences (Fig. 2A; [15]) were used to drive the expression of the reporter gene CAT, were transiently transfected into Hep G2 and BWTG3 cells (Fig. 2B). The deletion of a region between −852 and −230 (relative to the major transcription initiation site described by Deeb and Peng [15] resulted in the consistent expression of the 5′-LPL-CAT reporter gene in the non-LPL-expressing Hep G2 cells (KS^+-AN-

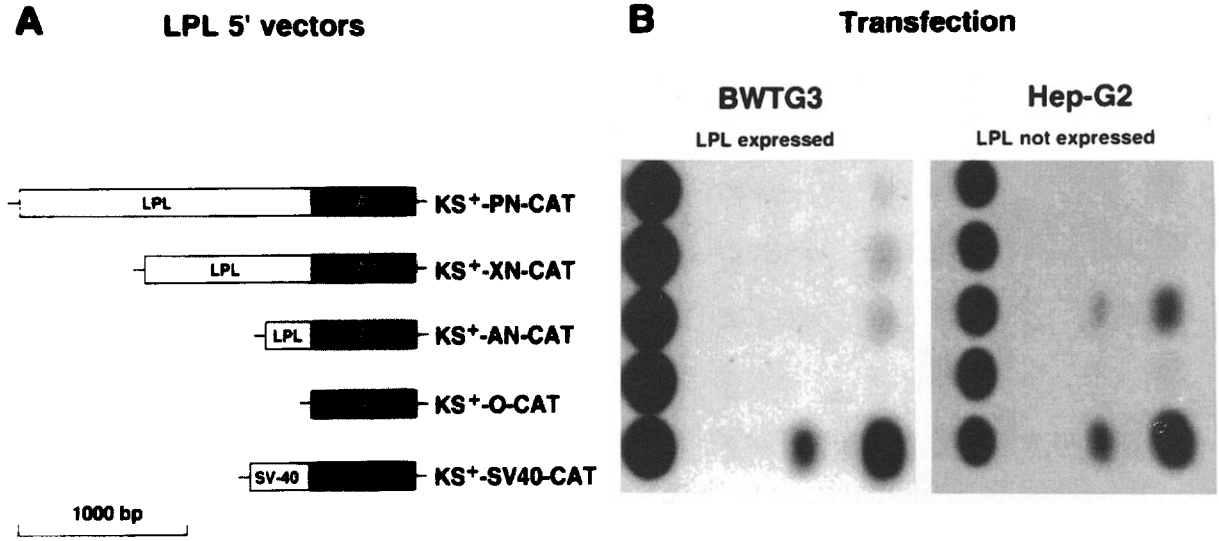

Fig. 2. Localization of extinguisher-responsive sequences in the LPL gene. (A) Structure of some of the reporter constructs used in the transient transfection experiments. Genomic restriction fragments containing various lengths of the LPL 5′ upstream sequences were cloned into the polylinker of the vector KS+-SV0-CAT as described. (B) Representative transfection experiment in either BWTG3 or Hep G2 cells localizing the extinguisher-responsive element to a region between −852 and −230 of the LPL 5′ end. Ten μg of each plasmid (depicted in panel A) was cotransfected with 5 μg of CMV-β-galactosidase and CAT activity determined as described.

CAT; Fig. 2B and Table I). In addition, deletion of the same region (between −852 and −230; KS+-AN-CAT) allowed the expression of LPL-CAT hybrid genes in different non-LPL-producing cell lines, such as HeLa cells or F9, mouse teratocarcinoma cells (data not shown). Deletion of this sequence had no major influence on the expression of 5′-LPL-CAT constructs in the LPL-expressing neonatal BWTG3 cells (Fig. 2B & Table I). Transfection with constructs containing small deletions defined the region important for extinction to a region between −591 and −288 (Table I). These results indicate that, as in other systems previously described, extinction could occur by interaction of extinguisher proteins with specific DNA motifs (−591 to −288) in the promotor–enhancer region of the gene.

3.4. *Identification of transcription factor binding site to the 5′URS of LPL implicated in extinction*

By homology searches we could identify 4 potentially important regulatory elements in the region between −591 and −288 (Fig. 3) i.e. a potential GRE (GGGACGCAATGTGTG at −468/−455) [40], an NF-1 like site (CCATCTTGCCAATGTTAAAACACCAGA at −514/−487) [41–43], and one HNF-3 binding site (CATTGACTTTG at −440/−430)[44]. It is important to note that none of these sites except for the NF-1 like site was protected by DNase I footprinting techniques using up to 40 μg of liver nuclear extracts (data not shown). The absence of a footprint on the HNF-3 site made it highly unlikely that the liver-enriched factor, HNF-3 was important in the regulation of this extinction process. Due to the low abundance of steroid receptors in nuclear extracts, steroid response elements are only rarely protected in DNase I footprinting assays using nuclear extracts. Therefore, we investigated whether this GRE could be delineated by electrophoretic mobility shift assay (EMSA) utilizing a bacterially synthesized recombinant protein composed of the DNA-binding domain of the glucocorticoid receptor. By using overlapping DNA sequences of the LPL 5′URS, we could map the GRE to the region between −468/−455 (Fig. 3). The presence of a glucocorticoid receptor binding site was confirmed by using a short double stranded oligonucleotide, representing strictly

Table I

Expression of deletion mutants of the LPL 5′URS in different hepatoma cell lines (Hep G2 and BWTG3; section A) and after treatment with glucocorticoid hormones (in BWTG3 cells either in the presence or absence of 10 μM dexamethasone; section B).

A.	Hep G2	BWTG3
KS+-AN-CAT	830 ± 61 (4)	143 ± 15 (4)
KS+-XN5-CAT	739 ± 72 (3)	105 ± 20 (3)
KS+-XN4-CAT	79 ± 10 (3)	126 ± 8 (3)
KS+-XN2-CAT	71 ± 16 (3)	91 ± 9 (3)
KS+-XN-CAT	88 ± 10 (4)	120 ± 11 (4)
KS+-PN-CAT	100 (4)	100 (4)
B.	**− Dexamethasone**	**+ Dexamethasone**
KS+-XN-CAT	92 ± 12 (3)	110 ± 18 (3)
KS+-PN-CAT	100 (3)	85 ± 7 (3)

Values represent mean ± S.D. of several transfection experiments (the number in brackets indicates the number of independent experiments). Numbers were corrected for transfection efficiency and weighed against the expression of KS+-PN-CAT in unstimulated cells (which was set arbitrarily at 100).

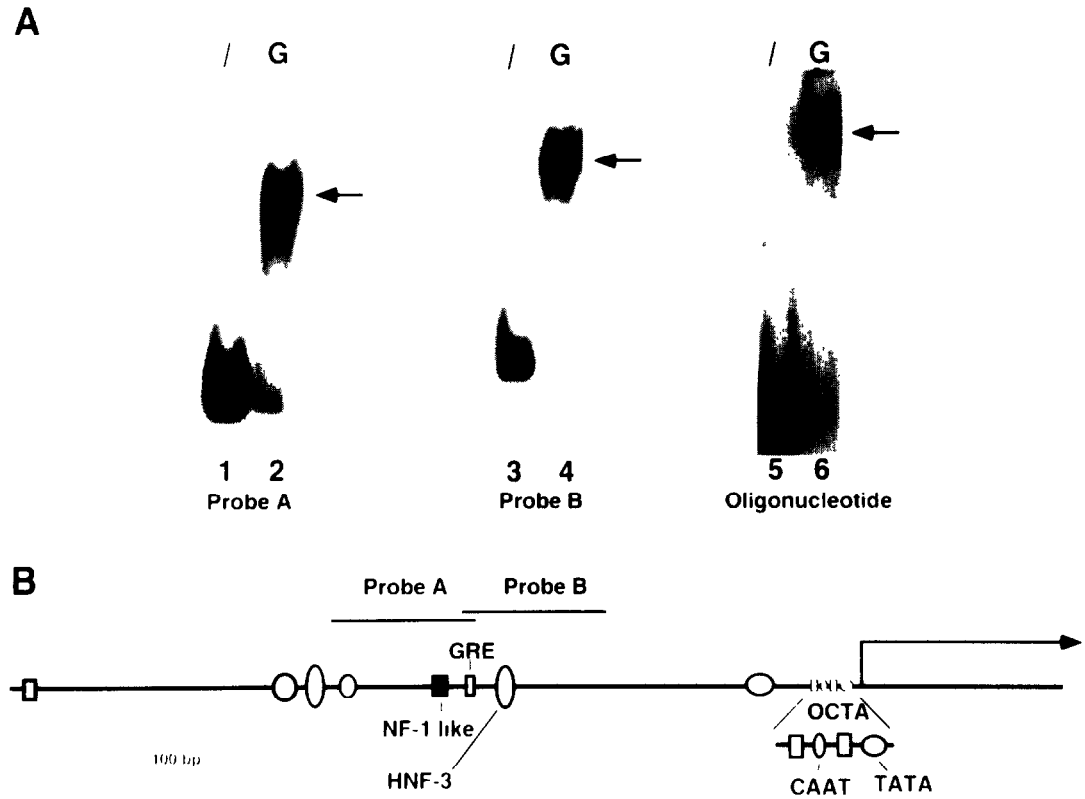

Fig. 3. Identification of a binding site of the GRE to the LPL 5′ region. (A) Gelshift (EMSA) assay showing the binding of the DNA binding domain of the glucocorticoid receptor to the LPL 5′ URS. Lanes 1, 3, 5: no extract added (/); lanes 2, 4, 6: GRF-1, the glucocorticoid receptor DNA binding domain fusion protein with protein A (G) . Labelled DNA fragments used as probes (probes A and B) in lanes 1–4 are depicted in the figure in panel B. In lane 5 and 6 a radiolabelled 23 bp oligonucleotide representing only the GRE was used as probe. Gelshift assays were performed exactly as described in section 2. (B) Graphic representation of a part of the 5′URS of the LPL gene. The potential responsive elements, as well as the probes used in panel A, are indicated. The DNA fragments used as probes are highlighted above the figure.

the LPL-GRE (Fig. 3). Although these data demonstrated the presence of a GRE, its functional significance in liver cells was unclear. LPL gene expression in BWTG3 cells was not affected by glucocorticoid hormones, despite the presence of a functional glucocorticoid receptor [28]. Furthermore, cotransfection of a glucocorticoid receptor expression vector (either in the presence or absence of dexamethasone in the culture medium) with the KS^+-XN-CAT reporter construct had no effect on CAT expression in LPL expressing BWTG3 hepatoma cells (Table I). The NF-1-like site was therefore further tested for its possible role in the extinction process.

3.5. *A novel factor RF1-LPL binds to an LPL-NF-1-like site in the −591/−288region*

Utilizing DNase I footprinting, this NF-1-like site, which showed strong sequence homology to a typical CCAAT box [41–43], was in fact the only site protected by employing liver nuclear extracts from either neonatal or adult animals (Fig. 4A). It was interesting to note that the extent of protection was consistently greater when liver extracts of adult animals were used relative to extracts from neonatal animals. In addition, when higher amounts of nuclear extracts were used an extension of the footprint became evident in extract from adult rats (Fig. 4A; compare lane 7 to lane 8). This extention was more pronounced with extracts from adult animals when compared to neonatal animals. Since this NF-1-like site is a potentially important previously undescribed *cis*-acting element, we set out to analyse the proteins that interacted with it in more detail by EMSA (Fig. 4B). Upon incubation of a double-stranded oligonucleotide, encompassing the sequence protected by DNase I footprinting analysis, with liver nuclear extracts of neonatal animals, a specific protein–DNA complex could be observed. In contrast, when liver nuclear extracts from adult animals were used a second complex of slower mobility could be visualized in addition to the complex present in neonatal animals. Both of these complexes were competed by a 30-fold molar excess of unlabelled wild-type LPL oligonucleotide. It was interesting to note that LPL-m2 oligonucleotide, in which the CCAAT box itself was mutated competed as efficiently as the wild-type LPL oligonucleotide, suggesting that the CCAAT sequence was not very important in the binding of this factor. In contrast competition experiments with a 30-fold excess of LPL-m1

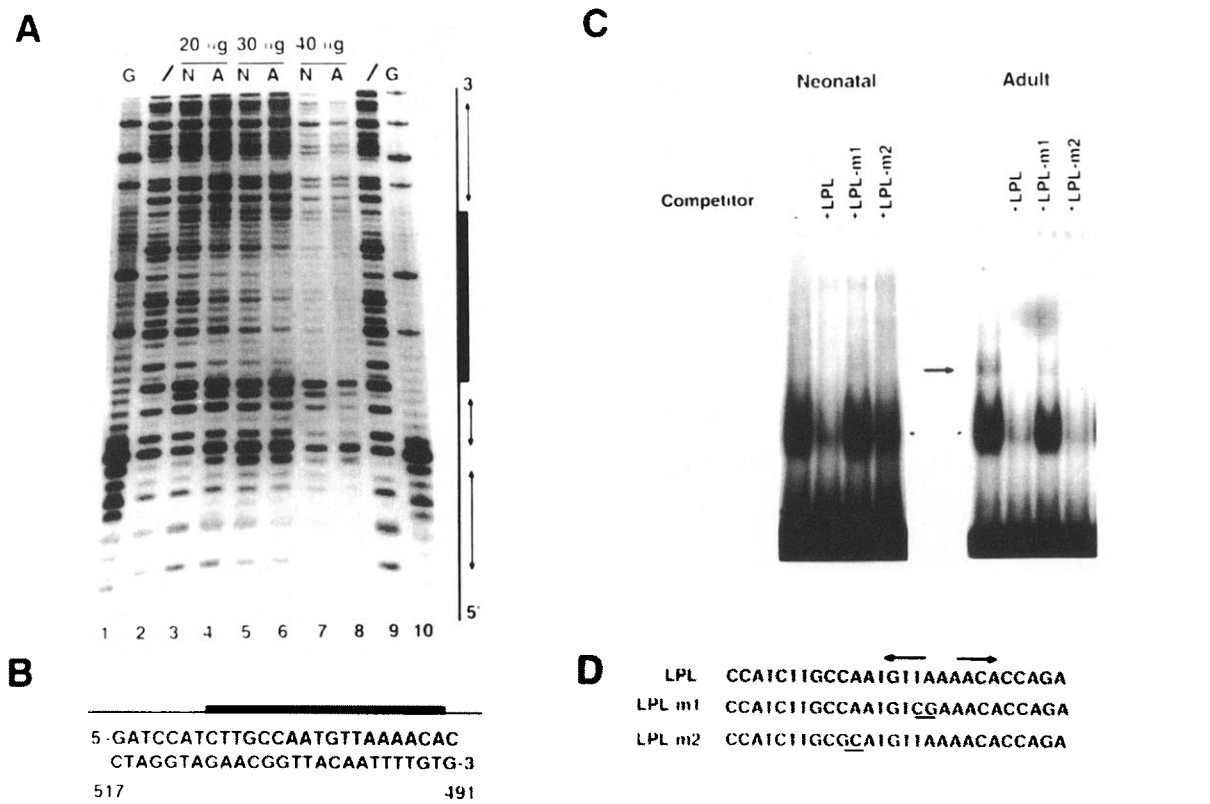

Fig. 4. An additional factor binds to the LPL-NF-1 site in adult vs. neonatal animals. (A) DNase I footprint experiment. The footprint (indicated by the shaded bar) was consistently stronger, when equivalent amounts of neonatal (N) and adult (A) extracts were compared. Several hypersensitive sites are evident and are indicated by an asterisk (*). G depicts a G sequence reaction and / represents the degradation pattern when no nuclear extracts were added. When 40 μg of liver extract was used an extension of the footprint became evident towards both 3' and 5' ends (see arrows). (B) Sequence of the footprinted region. (C) Gel shift experiment showing an additional complex present in adult animals. Unlabelled oligonucleotide representing either the LPL NF-1-like site or 2 mutations (LPL-m1 and LPL-m2) of this site were used in the competition experiments. (D) Oligonucleotides used in the competition experiments. The palindromic sequence is indicated by arrows. Mutated base pairs in LPL-m1 and LPL-m2 are underlined.

oligonucleotide containing a 2-bp substitution in another region of the protected site, resulted in a loss of competition.

These data suggest that this additional factor binding to this LPL-NF-1 site (present in adult animals only), might be a transacting factor or extinguisher implicated in the extinction or negative regulation of LPL gene expression. At present the factors binding to this site in neonatal, as well as in adult animals are being characterized in more detail in the laboratory. It is not excluded that this factor might interact with the GRE lying in close proximity, thereby controlling the extinction process.

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