Expression of laminin γ 1 in cultured hepatocytes involves repeated CTC and GC elements in the LAMC1 promoter

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Laminin γ 1 chain is present in all basement membranes and is expressed at high levels in various diseases, such as hepatic fibrosis. We have identified *cis*- and *trans*-acting elements involved in the regulation of this gene in normal rat liver, as well as in hepatocyte primary cultures and hepatoma cell lines. Northern-blot analyses showed that laminin γ 1 mRNA was barely detectable in freshly isolated hepatocytes and expressed at high levels in hepatocyte primary cultures, as early as 4 h after liver dissociation. Actinomycin D and cycloheximide treatment *in vivo* and *in vitro* indicated that laminin γ 1 overexpression in cultured hepatocytes was under the control of transcriptional mechanisms. Transfection of deletion mutants of the 5' flanking region of murine LAMC1 gene in hepatoma cells that constitutively express laminin γ 1 indicated that regulatory elements

were located between –594 bp and –94 bp. This segment included GC- and CTC-containing motifs. Gel-shift analyses showed that two complexes were resolved with different affinity for the CTC sequence depending on the location of the GC box. The pattern of complex formation with nuclear factors from freshly isolated and cultured hepatocytes was different from that obtained with total liver and similar to that with hepatoma cells. Southwestern analysis indicated that several polypeptides bound the CTC-rich sequence. Affinity chromatography demonstrated that a M_r 60 000 polypeptide was a major protein binding to the CTC motif. This polypeptide is probably involved in the transcriptional activation of various proto-oncogenes and extracellular matrix genes that are expressed at high levels in both hepatoma cells and early hepatocyte cultures.

INTRODUCTION

Laminins, the major non-collagenous glycoproteins of all basement membranes, are potent modulators of numerous biological processes in development, including cell proliferation, migration and differentiation [1,2]. In adult tissues, laminins play a role in the maintenance of specific gene expression [3,4] and are involved in a variety of pathological situations, including fibrosis and metastasis [2]. Laminin 1, originally identified from the Engelbreth–Holm–Swarm mouse tumour, contains three disulphide-linked genetically distinct chains: α 1, formerly M_r 400 000 A chain; β 1, formerly M_r 210 000 B1 chain; and γ 1, formerly M_r 200 000 B2 chain [5]. Other laminins include genetically distinct chains, e.g. α 2 (merosin), β 2 (S-chain), α 3, β 3 and γ 2 (K-chains) [6]. Interestingly, laminin γ 1 chain has a general widespread distribution and seems to be ubiquitous in most basement membranes [7,8].

Unco-ordinated expression of laminin chains has been reported in various tissues and cell cultures [9,10]. Thus normal adult liver contains both β 1 and γ 1 chains of laminin, but lacks the α 1 chain [10]. It has been shown that laminin γ 1 mRNA is abundant in hepatic stellate cells, the major site of matrix formation, but not in normal hepatocytes [11–14]. Interestingly, synthesis of laminin occurs in hepatocytes after a few hours in culture [14,15]. This expression parallels the activation of a set of specific genes, including cytoskeleton genes and growth-related genes, e.g. proto-oncogenes [16,17]. Laminin γ 1 is also expressed at high levels in transformed hepatoma cell lines and during chemically

induced hepatocarcinogenesis in the rat [14,18]. This unique pattern of laminin gene expression in the liver led us to investigate the transcriptional regulation of LAMC1 gene, which codes for laminin γ 1 in both cultured normal hepatocytes and hepatocarcinoma cell lines.

Transcriptional regulation of gene expression is a complex process that involves chromatin structure, DNA methylation and both *cis*- and *trans*-acting elements [19,20]. *Cis*-acting elements, i.e. promoter and regulatory elements, interact with *trans*-acting factors, which are either ubiquitous or gene- and/or tissue-specific [20]. The 830 bp murine LAMC1 promoter exhibits particular features, including the lack of TATA and CAAT boxes, one AP-1 motif, 9 'GC' boxes and a stretch of nine copies of a consensus sequence CCC(G,T)CCC(A,T)CCT [21]. In the present study, we show that CTC- and GC-rich regions located between –200 and –450 bp are involved in the regulation of LAMC1 gene transcription in both hepatoma cells and early hepatocyte primary cultures.

MATERIALS AND METHODS

Plasmids

A recombinant plasmid pKH 135 was constructed containing the promoter region of LAMC1 gene (–2000 to +106) fused to the structural part of the gene encoding CAT [21]. Segment deletion constructs, pKH 152 (–594 to +106), pKH 151 (–474 to

Abbreviation used: CAT, chloramphenicol acetyltransferase.

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+106), pKH 150 (−294 to +106), pKH 154 (−224 to +106), pKH 155 (−144 to +106) and pKH 156 (−94 to +106) were prepared. These promoter segments were subcloned into *Nde*I–*Hind*III sites of pSVOCAT.

A mutant construct, pKH 150M, was generated by replacing a 10 bp sequence of pKH 150 (5′-GCCCCTCCCA-3′ at −212 to −203 of the LAMC1 gene) with a 10 bp *Bgl*II linker sequence (5′-GAAGATCTTC-3′). Two sets of primers were prepared: two primers containing the 10 bp *Bgl*II linker sequence at the end of overlapping positions; and two primers from the 5′ portion of the CAT gene and from the PBR322 sequence proximal to the LAMC1 promoter of pKH 150, respectively. Two PCR products with these sets of primers using pKH 150 as template were digested with *Bgl*II and ligated to each other to replace the wild-type sequence. The ligated fragment was then cut by *Nde*I and *Hind*III and replaced the *Nde*I and *Hind*III wild-type LAMC1 promoter sequence (−294 to +106) of pKH 150.

Cell and culture preparation

The Faza cell line has been subcloned from the rat hepatoma cell line H4IIEC3 isolated from the Reuber H35 hepatoma [22]. Normal rat hepatocytes were isolated from male Sprague–Dawley rats by the two-step collagenase perfusion method [23]. Primary hepatocyte cultures and hepatoma cells were maintained in a mixture of 75% minimum essential medium and 25% medium-199 containing 10 µg/ml bovine insulin, 0.2% bovine albumin and 10% fetal calf serum. For the study of actinomycin D effects, the antibiotic was injected at 1 µg/g intraperitoneally to the animal 1 h before starting the perfusion and 2 µg/ml was added to both perfusion solutions and cell culture media [16]. Cycloheximide and emetine were also used in the same conditions both at the concentration of 50 µg/g for injection of animals and 60 µg/ml for perfusion solutions and media.

Northern blot analysis

Total RNA was extracted from hepatocytes by using the guanidinium–thiocyanate/caesium chloride method [24]. RNA (10 µg) was subjected to electrophoresis in a denaturing 1.1 M formaldehyde/agarose gel and transferred onto Hybond-N sheets (Amersham, Arlington Heights, IL, U.S.A.). Filters were soaked in hybridization buffer containing 3×SSC, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 0.1% BSA and 0.1% SDS, then hybridized with the same solution, containing 10% (w/v) dextran sulphate and ³²P-labelled cDNA probe. The LAMC1 cDNA probe was a 1.5 kb *Eco*RI–*Hind*III fragment of P7 cloned from a F9 cell cDNA library [25].

Transient transfections and chloramphenicol acetyltransferase (CAT) assays

All plasmids used for transfection were purified by two cycles of caesium chloride centrifugation. Subsequently, DNA was introduced into Faza cells using the calcium phosphate precipitation technique [26]. CAT plasmids (10 µg) were cotransfected with 10 µg plasmid containing the β-galactosidase gene (pRSV-β gal) to normalize transfection efficiency. Cells were glycerol shocked with 15% (v/v) glycerol for 2 min, 5 h after the addition of plasmids. Cells were allowed to grow for 24 h before harvest. After scraping in phosphate-buffered saline, cells were pelleted and then lysed by sonication in 150 µl of 0.1 M Tris buffer, pH 7.5. An aliquot (40 µl) was removed for the β-galactosidase assay [27], and the remaining sample was treated for 10 min at

65 °C. CAT activity was assayed with either the procedure of Gorman et al. [26] or the extraction method [28]. Briefly, after centrifugation to remove cell debris, 90 µl aliquots from each supernatant fractions were mixed with 145 µl of 0.1 M Tris buffer, pH 7.5, 5 µl of chloramphenicol (50 mM, Sigma), 5 µl of [¹⁴C]acetyl-CoA (0.42 mM), and 5 µl acetyl-CoA (4.4 mM, Pharmacia LKB Biotechnology). Vials were incubated at 37 °C and counted at different times up to 24 h. The final ratios used data obtained during the linear phase of CAT conversion. The CAT activity of pKH 135 was arbitrarily defined as 100%. Each CAT construct was transfected and assayed in duplicate in three independent experiments to confirm reproducibility.

Gel mobility shift

Nuclear extracts were prepared from Faza rat hepatoma cells and rat hepatocyte cultures. Cells were first homogenized in 0.5 M sucrose, 15 mM Hepes, pH 8, 60 mM KCl, 15 mM NaCl, 2 mM 2-mercaptoethanol and 1 mM PMSF, and washed in the same buffer. Nuclear proteins were prepared as described by Dignam et al. [29]. Complementary double-stranded oligonucleotides were synthesized on an Applied Biosystems oligonucleotide synthesizer (Table 1). They were subsequently annealed and end-labelled with [γ-³²P]ATP and polynucleotide kinase (Boehringer, Mannheim). Nuclear extracts were mixed with 20000 c.p.m. of double-stranded oligonucleotides in buffer I containing 12 mM Hepes, pH 8, 4 mM Tris/HCl, pH 8, 50 mM NaCl, 1 mM MgCl₂, 1 mM dithiothreitol, 20% (v/v) glycerol and 2 µg poly(dI-dC) [30]. After incubation for 15 min at 4 °C, protein and DNA complexes were resolved on polyacrylamide gels (10% gel) in 0.045 M Tris/0.045 M boric acid/0.2 M EDTA, pH 8, containing 3% (v/v) glycerol and electrophoresed at 150 V for 6–7 h at 4 °C. For competition experiments, binding reactions were performed in the presence of excess of competitor homologous or heterologous double-stranded oligonucleotides.

Southwestern analysis

Southwestern analysis was performed according to the procedure of Landschulz et al. [31]. Briefly, nuclear extracts were first electrophoresed on an SDS/polyacrylamide gel (8% gel). After a denaturation–renaturation step in 247 mM Tris, pH 8, with 192 mM glycine and 4 M urea for 1 h, proteins were transferred onto Hybond-N sheets. The filters were blocked with 5% (w/v) non-fat dry milk in buffer containing 20 mM Hepes, pH 8.5, and 1 mM dithiothreitol for 1 h at room temperature. The filters were then incubated with ³²P end-labelled double-stranded oligonucleotides (2×10⁶ c.p.m.) for 1 h at room temperature, in binding buffer containing 20 mM Hepes, pH 8.5, 5 mM MgCl₂, 1 mM dithiothreitol, 50 mM NaCl, 0.2 mM EDTA and 1% (w/v) non-fat dry milk. In parallel, one set of filters was incubated in the presence of 1000-fold excess of unlabelled oligonucleotides. The sheets were washed, dried and exposed to a Kodak X-Omat film.

Purification of DNA-binding proteins

DNA-binding proteins were purified on an oligonucleotide Sepharose chromatography column according to the method of Kageyama et al. [32] with slight modifications [33]. Briefly, the purification procedure was performed at 4 °C with the addition of 1 mM PMSF, 0.5 mM dithiothreitol and 1 mM EDTA to all buffers. Nuclear extracts in buffer P (20 mM Hepes, 17% (v/v) glycerol, 1.5 mM MgCl₂) containing 50 mM NaCl were passed through a DEAE–Sephacel column. After washing, bound pro-

Table 1 Nucleotide sequences of oligonucleotides used in this study

GC and CTC sequences are underlined; CI-8M corresponds to CI-8 with a substitution mutation (AAGATCTTC replacing CCCCCCCCA).

Oligonucleotide	Sequence	Localization in LAMC1
CI-8	5'-GCCCTCCATCTCGC-3'	—197 to —212
CI-8M	5'-GAAGATCTTCTCGC-3'	
CI-10	5'-CGCCCTCCATCTCGCTTCTGGCCACGCGCCT-3'	—178 to —213
CI-20	5'-CCTCTCGGCCACCTTGGGCCCTCCCTCTTACCC-3'	—380 to —414
CI-30	5'-ATTGGGGGGGGGGGAGC-3'	
CI-31	5'-CCAGGCCGCCCGCCGCGC-3'	—158 to —177

teins were eluted with 1 M NaCl, diluted with buffer P to 50 mM NaCl, applied to a heparin-Sepharose column (BioRad) and eluted with 1 M NaCl. The oligonucleotide affinity column was prepared as described by Kadonaga and Tjian [34]. After precipitation with 0.33 g/ml ammonium sulphate, heparin-Sepharose-purified proteins were first dialysed against buffer PB containing 25 mM Hepes, 15 mM MgCl₂, 100 mM KCl and 0.01% NP-40, and then applied to a CI-8 oligonucleotide column equilibrated in buffer PB containing 20 µg/ml herring sperm DNA as non-competitive DNA. After extensive washing, bound proteins were eluted with buffer PB containing 1 M KCl. This last step of purification was performed twice. The purified material was then resolved on an SDS/polyacrylamide electrophoresis gel (10% gel) under reducing conditions.

RESULTS

Expression of laminin γ1 mRNA in cultured hepatocytes

The steady-state levels of laminin γ1 chain mRNA were studied by Northern-blot in the Faza hepatoma cell line, which is known to express laminin at high levels, and in normal adult hepatocytes

immediately after their isolation and in primary culture (Figure 1A). Laminin γ1 mRNA was abundant in Faza hepatoma cells (lane 4) and barely detectable in freshly isolated normal hepatocytes (lane 1). In primary culture, hepatocytes expressed high levels of laminin γ1 chain mRNA 4 h after cell seeding (lane 2). The steady-state level of laminin γ1 mRNA was higher in 24 h than in 4 h hepatocyte cultures (lane 3).

The steady-state levels of laminin γ1 mRNA were studied in freshly isolated hepatocytes and in primary cultures in the presence of drugs that prevent either gene transcription or mRNA translation, i.e. actinomycin D and cycloheximide respectively. Injection of actinomycin D to the rats 1 h before liver dissociation and addition of the drug to the perfusion solutions and culture media completely blocked expression of laminin γ1 mRNA in both freshly isolated cells and in 4 h primary cultures (Figure 1B). Blockage of laminin γ1 mRNA expression in hepatocytes was also observed after injection of cycloheximide into the rat (Figure 1B). In a second set of experiments, drugs were added only in culture media after cell preparation (Figure 1C). In the presence of 2 µg/ml actinomycin D, laminin γ1 mRNA was barely detectable in 4 h hepatocyte culture (lane 9), whereas after 24 h laminin γ1 transcripts became undetectable (lane 11). In contrast, addition of 60 µg/ml cycloheximide had no effect on the steady-state levels of laminin γ1 mRNA in both 4 h and 24 h hepatocyte cultures (lanes 10 and 12).

These experiments show that hepatocyte isolation induces expression of the LAMC1 gene. They suggest, in addition, that this expression could be related to transcriptional mechanisms, which requires rapid synthesis of regulating factor(s) during liver enzymatic perfusion and cell isolation.

Figure 1 Expression of laminin γ1 chain mRNA in hepatocytes

(A) Northern-blot analysis of laminin γ1 mRNA in freshly isolated hepatocytes (lane 1), in 4 h (lane 2) and 24 h (lane 3) hepatocyte primary cultures, and in Faza hepatoma cells (lane 4). Total RNA (10 µg) was resolved on 1% (w/v) agarose gel and transferred onto nitrocellulose sheets that were subsequently hybridized with a specific ³²P-radiolabelled LAMC1 complementary DNA probe. A major 8 kb mRNA species was detected, with a minor 6 kb mRNA species in 24 h hepatocyte cultures only (arrows). (B) Effects of treatment *in vivo* with actinomycin D (lanes 5 and 6) and cycloheximide (lanes 7 and 8) on the steady-state laminin γ1 mRNA levels. Actinomycin D (1 mg/kg) and cycloheximide (60 mg/kg) were injected intraperitoneally into rats 1 h before hepatocyte isolation and added to all the solutions and cell culture media at concentrations of 2 and 60 mg/ml, respectively. Hepatocytes were maintained in culture for 4 h (lanes 5 and 7) and 24 h (lanes 6 and 8) in the presence of drugs. (C) Effects of actinomycin D (lanes 9 and 11) and cycloheximide (lanes 10 and 12) on the steady-state laminin γ1 mRNA levels in hepatocyte primary cultures. Hepatocytes were isolated from normal adult liver and subsequently cultured for 4 h (lanes 9 and 10) and 24 h (lanes 11 and 12) in the presence of actinomycin D and cycloheximide at the concentrations of 2 and 60 µg/ml respectively.

Deletion analysis of the LAMC1 promoter

Potential regulatory elements responsible for governing transcription of the LAMC1 gene were identified in Faza rat hepatoma cells, which constitutively express high levels of the laminin γ1 mRNA. A series of 5' deletions from —2000 bp of the transcription start site to +106 bp were constructed from the mouse LAMC1 promoter and linked to the CAT reporter gene (Figure 2). These constructs were transfected into Faza cells and subjected to CAT assay after 24 h. The longest two constructs, i.e. pKH 135 and pKH 152, exhibited strong transcriptional activities. Truncation of the upstream region from —594 bp to —474 bp and from —474 bp to —294 bp (the latter contains a stretch of five CTC boxes) resulted in decreased CAT activities after transfection of pKH 151 and pKH 150 respectively. Transfection of either pKH 154 or pKH 155 constructs, i.e. from —224 bp to +106 bp and —144 bp to +106 bp respectively, resulted in higher CAT activities than with the pKH 150

Figure 2 Deletion analysis of the LAMC1 promoter

Schematic representations of a series of deletion plasmids and their corresponding CAT activities. The pKH plasmid (10 µg each) was transfected into Faza hepatoma cells by using calcium phosphate precipitation. A β-galactosidase-containing plasmid was used to monitor and to normalize transfection efficiency. Relative CAT activities are given as percentages of pKH 135 activity. Data are the averages of duplicate assays in a typical experiment. The pattern of CAT activities did not differ in three independent experiments; the S.D. was < 24.7% for pKH 152; < 11.7% for pKH 151, pKH 154 and pKH 155; < 8.2% for pKH 150 and pKH 156; and no CAT activity was detectable with pKH 158. Shaded rectangle, AP1; hatched ellipse, CTC box; ○, GC box; ●, EGR box.

Figure 3 CAT assay with a mutated CTC binding site

pKH 150M, which corresponds to pKH 150 containing a 10 bp substitution mutation replacing the 5'-GCCCTCCCA-3' at -212 to -203 of the LAMC1 gene with a 10 bp *Bgl* linker sequence (5'-GAAGATCTTC-3'), was transfected into Faza hepatoma cells. The chloramphenicol acetyltransferase activity was determined 48 h later. Each assay included controls with 0.01 m-units or without chloramphenicol transferase (lanes 1 and 2 respectively), wild-type plasmid pKH 150 (lane 3), or mutated pKH 150M (lane 4). The percentage acetylation is shown below the autoradiograph, which represents a typical experiment. The pattern of CAT activities did not differ in four independent experiments.

construct. The -224 bp to -94 bp segment was found to contain four GC boxes and a 5'-CGCCCCGC-3' motif, the complement of 5'-GCGGGGCGC-3', which recognizes the *egr* gene family products [35]. Further deletion to -94 bp gave a marked decrease in CAT activities.

The functional role of CTC boxes was analysed further by transfection of pKH 150M plasmid, which corresponds to pKH 150 with a 10 bp substitution mutation at -212 bp to -203 bp of the LAMC1 promoter. Comparison of the relative CAT activity, as measured by the percentage conversion of the non-acetylated to the acetylated form of the wild-type compared with the pKH 150M mutant, showed that this construct was approx.

Figure 4 Gel mobility shift assay

(A) Nuclear extracts (5 µg) were prepared from Faza hepatoma cells incubated with the following ³²P-labelled double-stranded oligonucleotides: Cl-10, Cl-8, Cl-20, Cl-30 and Cl-31. DNA-protein complex formation was resolved by electrophoresis in a non-denaturing 10% gel. A and B complexes are indicated. (B) The specificity of factors binding to the CTC site was analysed with ³²P-labelled double-stranded Cl-8M, which corresponds to the mutated/substituted sequence in pKH 150M (see Figure 3). Asterisks indicate assays without nuclear extract.

40 % less active (Figure 3). Taken together these data suggest that CTC- and GC-rich sequences positively regulate the activity of the promoter region of LAMC1.

Figure 5 Gel mobility shift assay

Nuclear extracts (5 μ g) prepared from Faza hepatoma cells were incubated with 32 P-labelled CI-10, CI-20 or CI-30 double-stranded oligonucleotides without (—) or with 100-fold excess of the following unlabelled competitors: CI-10, CI-8, CI-20 and CI-30. DNA-protein complex formation was resolved by electrophoresis in a non-denaturing 10% gel. A and B complexes are indicated. Asterisks indicate controls without nuclear extracts.

Figure 6 Gel mobility shift assay

Nuclear extracts (5 μ g) were prepared from Faza hepatoma cells, normal liver, freshly isolated hepatocytes (0 h) and 4 h and 24 h hepatocyte primary cultures and incubated with 32 P-labelled CI-10, CI-20, CI-8 and CI-30 double-stranded oligonucleotides. DNA-protein complex formation was resolved by electrophoresis in a non-denaturing 10% gel. A and B complexes are indicated. Minus sign indicates controls without nuclear extracts.

Different factors bind to the CTC- and GC-containing motifs

To investigate the nuclear factor(s) that may interact with GC- and CTC-containing motifs, synthetic double-stranded oligonucleotides were prepared (Table 1). CI-8 and CI-30 corresponded to CTC and GC consensus motifs. Oligonucleotides termed CI-10 and CI-20 corresponded to sequences in the promoter region containing a GC box either downstream or upstream of the CTC motif. Oligonucleotide CI-31 contained the complement of 5'-

GCGGGGGCG-3', which recognizes the *egr* gene family products.

A major complex, termed A, was detected by gel-shift assay after incubation of Faza cell nuclear extracts with 32 P-labelled CI-10, CI-8, CI-20, CI-30 and CI-31 probes (Figure 4A). The staining intensity of complex A was higher with the CI-10 probe than with other probes. Slower-migrating complexes, termed B, were also resolved. The migration profiles of B complexes were similar when CI-10 and CI-20 were used on the one hand, and

CI-30 and CI-31 on the other. With CI-8 two bands were resolved. Additional complexes between A and B were also detected, particularly with CI-20. The specificity of nuclear factor binding to the CTC motif was tested by using mutations within the CTC site. Gel-shift assay showed that CI-8M was ineffective in binding hepatoma cell nuclear factors, whereas complex A was predominantly resolved by using CI-8 (Figure 4B).

Both A and B complexes obtained with radiolabelled CI-10 were competed for by an excess of unlabelled CI-20, whereas those obtained with radiolabelled CI-20 were not competed for by unlabelled CI-10 (Figure 5A). Because both CI-10 and CI-20 contain CTC and GC motifs, competition experiments were also performed with excess of unlabelled CI-8 and CI-30 (Figures 5B and 5C). Formation of complex A with radiolabelled CI-10 was competed for by an excess of unlabelled CI-8, but not CI-30. Complex B obtained with both radiolabelled CI-10 and CI-20 was competed for by unlabelled CI-30. To investigate whether B complex is related to GC boxes present in both CI-10 and CI-20, competition experiments were performed with CI-30 probe (Figure 5D). Complex B and faster-migrating bands were competed for by an excess of CI-30, CI-20 and CI-8, but not by CI-10.

Taken together, these data indicate that nuclear factors involved in the formation of A and B complexes may recognize both GC and CTC motifs. In addition they suggest that nuclear factors involved in the formation of the A complex interact predominantly with the CTC motif and have more affinity for CI-10 than CI-20, whereas those involved in the B complex recognize both CTC and GC motifs and have more affinity for CI-20 than CI-10.

Complex formation in cultured hepatocytes

To investigate whether the pattern of formation of both A and B complexes was different depending on the activation of LAMC1 gene, nuclear extracts were prepared from total liver, i.e. when laminin $\gamma 1$ mRNA is barely detectable, and hepatocytes immediately after their isolation and after 4 h and 24 h in primary cultures, i.e. when laminin $\gamma 1$ mRNA is expressed at high levels (Figure 6). Gel-shift experiments performed with nuclear extracts from total livers and CI-10, CI-20, CI-8 and CI-30 probes showed different migration profiles than those performed with Faza cells: with CI-10 probe an intense slower-migrating complex was resolved between A and B complexes, whereas additional bands comigrating with the A complex were also detected; with CI-20 probe a slower-migrating band than the B complex was resolved; CI-8 probe gave rise to a doublet comigrating with A complex that was also detectable with CI-10 probe; A and B complexes were only slightly detectable with CI-30 probe. The pattern of complex formation with nuclear factors from freshly isolated hepatocytes was different from that obtained with total liver but similar to that observed with Faza cells by using CI-10, CI-20, CI-8 and CI-30 probes. The intensities of the A and B complexes were decreased with nuclear extracts from 4 h and 24 h cultured hepatocytes.

Characterization of nuclear factors binding to the CTC motif

Polypeptides interacting with the CTC-rich sequence were further characterized by Southwestern analyses. Nuclear extracts from both Faza hepatoma cells and normal livers were resolved by gel electrophoresis, then transferred onto filters that were incubated with radiolabelled CI-10 probe (Figure 7). Three polypeptides of M_r 45000, 55000 and 100000 were found in both samples, the intensity of the bands being weaker with liver nuclear extracts.

Figure 7 Characterization of polypeptides interacting with CTC-containing sequence

Nuclear extracts (50 μ g) were prepared from normal rat liver (lane 1) and Faza hepatoma cells (lane 2), resolved by SDS/PAGE (8% gel) and transferred onto nitrocellulose sheets. The sheets were subsequently incubated with 32 P-labelled double-stranded CI-10 probe (100000 c.p.m. per ml). The positions of molecular mass standards (kDa) are marked at the left.

Figure 8 Purification of CTC-binding proteins

(A) Affinity chromatography. Lane 1: standard molecular masses (kDa). Lane 2: CI-8-binding proteins (arrows) purified from normal liver by affinity column chromatography were resolved by SDS/PAGE (8% gel) and Amido Black staining. (B) Gel mobility shift assay. Lane 1: bound proteins (arrows) were eluted from a CI-8 affinity chromatography column and subsequently incubated with 32 P-labelled CI-8 double-stranded oligonucleotides. Lane 2: control without nuclear extracts.

Two additional polypeptides of M_r 50000 and 60000 were detected in Faza cell nuclear extracts.

Because CI-8 recognizes predominantly proteins involved in the A complex in liver nuclear extracts (Figure 6), proteins were purified on a double-stranded CI-8 affinity chromatography column. Polyacrylamide gel electrophoresis showed that a polypeptide of M_r 60000 was eluted from the column (Figure 8A). A gel-shift experiment with a radiolabelled CI-8 probe indicated that this protein formed the A complex (Figure 8B).

DISCUSSION

Only few results are available on the molecular mechanisms involved in the transcriptional regulation of basement membrane genes in various diseases, such as glomerular nephritis, diabetes and liver fibrosis. In the liver, laminin is abundant during maturation of the organ and accumulates in the adult during the capillarization process that occurs in alcoholic cirrhosis and hepatocarcinogenesis [11,36]. The capability of hepatocytes for expressing laminin has been documented *in vivo*, in fetal and newborn livers and in fibrotic adult livers, as well as *in vitro*, in normal hepatocyte primary cultures and in several hepatoma cell lines [11–15,18,37]. Our data indicate that laminin $\gamma 1$ mRNA is not expressed when gene transcription or protein synthesis is blocked before and during liver disruption. This suggests that regulating factor(s) that are required for the transcriptional machinery must be synthesized rapidly for efficient activation of LAMC1 gene. These factor(s) could be related to an immediate early gene family that encodes transcription factors such as c-Myc, c-Myb and the Egr and Jun/Fos families that govern the expression of a variety of delayed early genes and regulate the growth response by driving cells through the G1-phase of the cell cycle [38]. Interestingly, sequential activation of proto-oncogenes, e.g. the *c-fos*, *c-myc*, *jun* family and *c-Ki-ras*, occurs in hepatocytes after two-thirds hepatectomy, during liver disruption by collagenase perfusion and in primary cultures [16,17]. In addition, replication factor C, which is associated with proliferating cell nuclear factor, is rapidly recruited in the nucleus within the G0/G1 transition and early G1-phase in early hepatocyte cultures [39].

Laminin $\gamma 1$ mRNA expression in hepatoma cells involves several regions within the 2 kb promoter. Transfection of LAMC1 promoter fragments in these cells indicated that regulatory elements are located between –594 bp and –94 bp. The Ap-1 site located at –650 bp is probably not involved in LAMC1 transcription in Faza hepatoma cells, whereas several sequences in the –480 bp to –175 bp region were identified that might bind specific regulatory factors, including Sp1 and immediate early gene products coded for by the *egr*-family [35]. In addition, nine CTC motifs were identified in this region. CTC sequences are present in the 5'-flanking region of various genes coding for extracellular matrix proteins and proto-oncogenes, which are all overexpressed in hepatoma cells and hepatocyte primary cultures but not in normal adult liver (Table 2). These motifs have been reported to act as transcription activators in several genes. Thus mutations in the CTC motif within the bidirectional promoter of collagen $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ resulted in reduced transcription

from both genes [40,41], whereas mutations in the first intron of the $\alpha 2(\text{IV})$ gene decreased transcription of the $\alpha 1(\text{IV})$ gene [40]. A long CTC tract is also necessary for ETS-2 transcription and acts as transcription activator using a heterologous promoter [42]. Interestingly, in human prothrombin [43] and human apolipoprotein AII gene enhancer [44], a CTC motif located adjacent to other liver-specific regulatory elements, e.g. HNF-1, might be important to confer tissue-specific enhancer activity [43]. DesJardins and Hay [45] have shown that the differential expression of the two promoters of *c-myc* was governed by a cluster of five CTC boxes. In addition, a CTC motif in an inverted orientation located 53 bp upstream of the second promoter is required for the transcription of the second promoter and inhibits the first promoter of *c-myc*. The present study with deleted and mutated segments of the promoter suggests that the integrity of the CTC-rich region is required to promote LAMC1 activity. Enhanced transcription activity in smaller promoter segments probably involves three GC boxes, which may recognize Sp1, and one EGR box. In addition, gel mobility shift analyses showed that nuclear factors bound to CTC-containing sites, depending on the location of the flanking GC motif. Nuclear factors involved in the formation of the A complex interacted predominantly with the CTC motif, whereas those involved in the B complex recognized both CTC and GC motifs. Furthermore competition experiments indicated that these nuclear factors have more affinity for the CTC motif than for the GC motif. Consequently it can be hypothesized that there is an interplay between CTC and GC elements, GC boxes being predominant in the activation of truncated CTC-less LAMC1 promoter. However, the role of both the Sp1 and the EGR family in the regulation of LAMC1 gene expression requires further analysis. Indeed, Sp1 was detected by immunohistochemistry in fetal and newborn rat livers, particularly at the level of both immature hepatocytes and haematopoietic cells [46]. Recently we found Sp1 gene expression and binding activities in early adult hepatocyte cultures and in both Hep G2 and Faza hepatoma cell lines (J. Liétard and B. Clément, unpublished work). In the present study, the pattern of complex formation in the gel-retardation assay with GC- and CTC-containing oligonucleotides and nuclear extracts from either hepatoma cells or hepatocyte primary cultures was different from that with nuclear extracts from total liver. These observations, which could be related to post-translational modification of nuclear factors [47], emphasize that changes in binding activity of CTC and GC sites are correlated with increased expression of the LAMC1 gene.

Southwestern analysis showed that several nuclear factors bound to CTC-containing sequences, whereas by affinity chro-

Table 2 CTC sequences in 5' regions of genes induced in early hepatocyte primary cultures

Sequence	Location*	Gene	Reference
CCCTCCCATCT	731	Bovine <i>Ha-ras</i> 1	52
CCCTCCCACCT	25	Rat <i>Ha-ras</i>	53
CCCTCCCACTC	104	Mouse <i>N-myc</i>	54
CCCTCCCCTTT	220	Mouse <i>c-myc</i>	55
CCCTCCCCCTT	327	Mouse <i>c-fos</i>	56
(CCCTCCCC) ₆	277–443	Human LAMC1	57
(CCCTCCC) ₂	348 and 874	Mouse LAMB1	Y. Yamada, unpublished work
(CCCCCTCCC) ₂	483	Human collagen IV	58
CCCCTCCCC	275	Mouse collagen IV	59, 59a
(CCCT(A/C)CC(T/C)) ₄	269–370	Rat fibronectin	60
CCCTTCCCC	293	Human fibronectin	61

* According to the original publication of the sequence.

matography a major M_r 60000 polypeptide was found to interact with the CTC motif. A number of factors that recognize CTC sequences in various genes have been identified, including Sp1 [45,47], CTCBF [48] and NSEP1 [49]. Recently, a human zinc-finger protein designated ZF87 [50] or MAZ [51] has been cloned and found to bind CTC sequences in the mouse *c-myc* promoter. We have tested whether ZF87 was involved in complex A formation. Use of a bacterially synthesized ZF87 protein and Cl-10 oligonucleotide in both Southwestern and gel-shift experiments indicated that this nuclear factor probably does not interact with the CTC sequence in the LAMC1 promoter, although ZF87 mRNA was detectable in both hepatocyte primary cultures and hepatoma cell lines (results not shown). Thus further characterization of the CTC-binding factor(s), particularly the M_r 60000 polypeptide, is required for an analysis of their role in LAMC1 expression.

We thank Michel Raymondjean (INSERM U-129, Hôpital Cochin, Paris) and Pierre Savagner (ENS, Paris) for their constant help and fruitful discussions. We also thank David Hall (Thomas Jefferson University, Philadelphia, PA, U.S.A.) for providing MBP:ZF87 fusion protein, and Yannick Laperche (INSERM U-99, Hôpital H. Mondor, Créteil) for critical review of the manuscript. This work was supported by the Institut National de la Santé et de la Recherche Médicale and Association pour la Recherche contre le Cancer. F.L. was a recipient of a fellowship from La Ligue Nationale contre le Cancer (Comité des Côtes d'Armor).

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