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Sp1-Mediated Transactivation of LamC1 Promoter and Coordinated Expression of Laminin-γ1 and Sp1 in Human Hepatocellular Carcinomas

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The laminin-γ1 chain is present in most basement membranes and is involved in various physiological and pathological processes, including carcinogenesis in the liver. We have investigated the role of the transcription factor Sp1 in the activation of the LamC1 gene, which encodes laminin-γ1, both in hepatocytes and in human hepatocellular carcinomas. DNAse I hypersensitive sites were mapped in the murine LamC1 promoter using early hepatocyte primary cultures in which LamC1 becomes activated. Three hypersensitive sites were found in enhancer-like elements that contain GC-rich regions. Gel-shift analyses showed that specific complexes were resolved using GC-containing oligonucleotides and Faza 567 hepatoma cells, which constitutively express laminin-γ1 at a high level. Increased GC-binding activity was observed using nuclear extracts from early hepatocyte cultures *versus* normal liver. Sp1 overexpression in normal hepatocytes transfected with an Sp1 expression vector induced a marked increased of laminin-γ1 mRNA content and co-transfection of promoter fragments in *Drosophila melanogaster* SL2 cells demonstrated that Sp1 transactivates LamC1. In human hepatocellular carcinomas, Sp1 and laminin-γ1 mRNA were simultaneously expressed at high levels, and gel-shift experiments demonstrated a higher GC-binding activity to Sp1 compared with control livers. *In situ* hybridization indicated that cells exhibiting a high content of laminin-γ1 mRNA were also strongly positive for Sp1 mRNA, including both cancer cells at the invasion front and stromal cells. These results show that Sp1 is involved in the activation of LamC1 that occurs in human hepatocellular carcinomas. *(Am J Pathol 1997, 151:1663–1672)*

Laminins are a family of the major noncollagenous glycoproteins of all basement membranes. They are composed of three genetically distinct chains, α-, β-, and γ-chains, which are held together by disulfide bonds and by a triple-stranded coiled-coil structure. Among these different polypeptides, laminin-γ1 is present in all laminin isoforms, except in laminin-5,[1] and it has a widespread distribution in most basement membranes.[2] Laminins are potent modulators of numerous biological processes in development, including cell proliferation, migration, and differentiation.[3] In adult tissues, laminins influence the maintenance of specific gene expression[3,4] and are involved in various pathological situations, including fibrosis, carcinogenesis, and metastasis.[3] Although laminins are not abundant in the normal adult liver, they are expressed at high levels during liver development,[5] in the early stages of hepatic regeneration,[6] and in fibrotic livers.[7] It has been shown that laminin-γ1 mRNA is abundant in hepatic stellate cells, the major site of matrix formation, but not in normal hepatocytes.[7–9] However, laminin-γ1 begins to be synthesized by hepatocytes after a few hours in culture,[5] and it is expressed at high levels in transformed hepatoma cell lines and in chemically induced rat hepatocellular carcinomas.[5,10] It has been hypothesized that laminin is important during the development and progression of cancer cells in various tissues, including the liver.[3] However, little is known about the mechanisms that regulate the expression of laminin chains during formation of primary and secondary tumors in human livers.

We have recently identified several enhancer and silencer regions in the promoter of the LamC1 gene, which encodes laminin-γ1.[11] We have also shown that a stretch of both CTC- and GC-rich sequences is involved in the expression of laminin-γ1 in a rat hepatoma cell line and in early hepatocyte primary cultures. Sp1 is a nuclear factor that interacts with GC-rich elements and is a member of the C_2-H_2 zinc finger family.[12] It is involved in the regulation of a wide variety of different genes, including the

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early promoter of SV40,[13] genes involved in the proliferative response,[14,15] housekeeping genes,[16,17] and a number of growth factor genes.[18] In addition, GC boxes are present in promoters of many basement membrane genes, including both human and murine LamB1 and LamC1, human nidogen[19] and perlecan,[20] and the bidirectional promoter of the human and murine α1(IV) and α2(IV) collagen genes.[21,22] In the latter, mutations introduced into GC boxes decreased transcriptional activity of both genes at different levels.[21,23] Sp1 has been also shown to be involved in the regulation of procollagen α1(I) and α2(I)[24,25] and fibronectin genes.[26]

In this report, we have investigated whether Sp1 regulates transcription of LamC1, both in hepatocytes and in human hepatocellular carcinomas. We show that GC-rich regions in the LamC1 promoter are important regulatory elements in both hepatoma cells and early hepatocyte primary cultures. We also show that Sp1 induces high levels of endogenous laminin-γ1 mRNA in normal hepatocytes and directly transactivates the LamC1 promoter in *Drosophila* cells. Furthermore, we demonstrate that laminin-γ1 mRNA is expressed at high levels in human hepatocellular carcinomas, which coincides with a high expression and activity of Sp1.

Materials and Methods

Cell and Culture Obtainment

The Faza cell line has been subcloned from the rat hepatoma cell line H4IIEC3 isolated from the Reuber H35 hepatoma.[27] Normal rat hepatocytes were isolated from male Sprague-Dawley rats using the two-step collagenase perfusion method.[28] Primary hepatocyte cultures and hepatoma cells were maintained at 37°C in a mixture of 75% minimal essential medium and 25% medium-199 containing 10 μg/ml bovine insulin, 0.2% bovine albumin, and 10% fetal calf serum. The *Drosophila melanogaster* cell line (SL2) was maintained at 25°C in Schneider's medium complemented with 10% fetal calf serum.

Tissue Samples

Twenty-three liver tissue specimens were from patients with primary hepatocellular carcinoma (HCC). Controls were eight explanted liver samples from cadaveric liver donors not suitable for transplantation due to suspected extra-abdominal infection unrelated to the cause of death. Access to this biopsy material was in agreement with French laws and regulations and satisfied the requirements of the local Ethics Committee. After macroscopic examination by two pathologists, representative samples were fixed in buffered formalin for histopathological routine diagnosis; a part of the fresh material was snap-frozen in isopentane cooled in liquid nitrogen and stored at −80°C until use. Before RNA or nuclear protein extraction, 5-μm frozen serial sections were obtained from the tissue blocks, stained with methylene blue, and observed under light microscopy. Similarly, formalin-fixed, paraffin-embedded material from the same patient was analyzed independently by a different pathologist. Nontumor areas from hepatocellular carcinomas presented extensive portal fibrosis or cirrhosis. Control livers were histologically normal.

Plasmids

A recombinant plasmid pKH 135 containing the promoter region of LamC1 gene (−2000 to +106 bp) fused to the structural part of the gene encoding CAT was constructed.[29] Segment deletion constructs, pKH 151 (−474 to +106 bp), pKH 150 (−294 to +106 bp), pKH 155 (−144 to +106 bp), and pKH 156 (−94 to +106 bp) were also prepared. These promoter segments were subcloned into *Nde*I-*Hin*dIII sites of pSV0CAT.

The expression vector for Sp1 under control of the *Drosophila* actin promoter pPacSp1[30] was a gift from Dr. R. Tjian (University of California at Berkeley, Berkeley, CA). pPac0 was obtained from the pPacSp1 plasmid lacking the 2.1-kb Sp1 cDNA fragment. The rat expression vector for Sp1 under control of the CMV promoter, pRC/CMV/Sp1, was a gift from Dr. G. Suske (Institut für Molekularbiologie und Tumorforschung, Marburg, Germany).

pSB1-CAT (promoterless plasmid), pRSV-CAT (Rous sarcoma virus promoter driving the chloramphenicol acetyl transferase expression), and pRSV-βgal were generous gifts from Dr. M. Yaniv (Institut Pasteur, Paris, France). All plasmids used for transfection were purified by two cesium chloride centrifugation steps.

Cloning of cDNA

For the preparation of Sp1 and laminin-γ1 cDNA probes, reverse transcriptase polymerase chain reaction products from rat and human liver mRNA were cloned into a pTag plasmid vector (R&D Systems, Abingdon, Oxon, UK) and then subcloned in the plasmid pGem 7Z or 4Z (Promega, Charbonnières, France), respectively. Sequencing with the Sequenase version 2.0 DNA sequencing kit (USB, Cleveland, OH) confirmed the identity of Sp1 and laminin-γ1 constructs with the previously published sequence.

Mapping of DNAse I Hypersensitivity Sites in Chromatin

Nuclei from total rat liver and hepatocytes at 4 hours of culture were treated with DNAse I (Boehringer grade II, Boehringer Mannheim, Mannheim, Germany) as previously described.[31] DNA was purified, and 40 μg of DNA was digested with a restriction enzyme that has a unique site in the LamC1 gene (*Pst*I at +260 bp). Subsequently, DNA was electrophoresed for Southern blotting and hybridized with radiolabeled [^{32}P]pKH 156 plasmid (nucleotide −94 to +106 bp in the LamC1 gene).

Nuclear Protein Extracts and Gel Retardation Assays

Nuclear extracts from Faza rat hepatoma cells, hepatocyte cultures, and rat and human liver tissues were prepared, and gel-shift assays were performed as described by Cereghini et al,[32] except that the nuclear extracts were not dialyzed. Binding reactions were carried out in a 15-μl volume containing 1 mmol/L sodium phosphate, pH 7.5, 0.1 mmol/L EDTA, 0.5 mmol/L EGTA, 0.5 mmol/L dithiothreitol, 10% (v/v) glycerol, 2 μg of poly (dI-dC), 1 mmol/L $MgCl_2$, 10 mmol/L spermidine, and 15,000 cpm of ^{32}P-labeled, 5′-end, double-stranded oligonucleotide. Oligonucleotides from the LamC1 promoter were as follows: CL-10, 5′-CGC*CCCCTCCC*ATCTCGCTTCTCTG-GCCC*ACCGCCC*T-3′ (−213 to −178 bp; CTC and GC sequences are in italics), and CL-20, 5′-CCTCT*CCGC-CC*ACCTTGGGC*CCCCTCCC*TCTTACCC-3′ (−414 to −380 bp; GC and CTC sequences are in italics). The Sp1 consensus oligonucleotide was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and was termed CL-Sp1. Double-stranded oligonucleotide CL-8 (5′-GC-CCCTCCCATCTCGC-3′) derived from CL-10 (−212 to −197 bp) was used as a CTC-rich oligonucleotide. Five micrograms of proteins were added to the reaction mixture and were incubated for 15 minutes on ice. For gel supershift assays, binding reaction mixtures were incubated at room temperature for 20 minutes in 10 mmol/L Tris (pH 7.5) buffer with 50 mmol/L NaCl, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, 5% glycerol, and 1 μg of poly (dI-dC); subsequently, 1 μl of TransCruz gel supershift antibody (Santa Cruz Biotechnology) was added and incubated for 45 minutes at room temperature. The DNA-protein complexes were resolved on 6% acrylamide gels in 0.5X TBE (45 mmol/L Tris/borate, 1.25 mmol/L EDTA) at 22 mA for 1 hour and 30 minutes. The gel was then fixed, dried, and subjected to autoradiography.

Transient Transfections and CAT Assays

Hepatocytes were transfected by electroporation using a Gene Pulser apparatus (Eurogentec, Seraing, Belgium) with 10 μg of pRC/CMV/Sp1. *Drosophila* cells were transfected using the calcium phosphate precipitation method.[33] Cells were seeded at 4.5×10^6/60-mm dish and were transfected 24 hours later with 5 μg of CAT reporter and 100 ng of either pPacSp1 or pPac0 plasmids. Cells were harvested 48 hours after transfection. CAT assays were carried out by the method of Gorman et al.[33] Radioactive spots on thin layer chromatography plates were cut out and quantitated by scintillation counting. The ratio of acetylated to total chloramphenicol was displayed as percentage of conversion.

RNA Extraction, Northern Blot, and Dot-Blot Analyses

Total RNA from both cells and liver samples were prepared by using the guanidium thiocyanate/cesium chloride method.[34] Histological analysis of frozen blocks before RNA extraction of large tissue samples ensured that sampling was both macroscopically and microscopically consistent. Only those tissue samples with anatomic pathology features that allowed a matching diagnosis with the pathology report of each patient were homogenized for total RNA extraction. For Northern blot, RNA was resolved by electrophoresis on 1% agarose gels and transferred onto Hybond N^+ nylon membranes (Amersham, Little Chalfont, UK). For dot-blot, each RNA sample was blotted in triplicate at 1, 2.5, and 5 μg/μl in 100 μl of 4X SSC, 4 mol/L formaldehyde, 0.3 mol/L NaAc, and 0.002% methylene blue onto Hybond N^+ nylon membranes using a filtration manifold. cDNA probes were labeled (1×10^8 to 1×10^9 cpm/μg cDNA) using the Rediprime kit (Amersham) and [α-^{32}P]dCTP, 3000 Ci/mmol (Amersham). Hybridizations with cDNA probes were performed in 0.25mol/L Na_2 H PO_4, 12 H_2O; 20 mmol/L H_3 PO_4, pH 7.2, containing 7% sodium dodecyl sulfate (SDS), 1 mmol/L EDTA, and 1% bovine serum albumin for 16 hours at 65°C. Filters were washed in 3X SSC/0.1% SDS followed by 0.1X SSC/0.1% SDS at 65°C. Amersham Hyperfilm-MP films were exposed with enhancing screens at −80°C for 3 and 5 days for laminin-γ1 and Sp1, respectively. For each probe, all samples were run in the same experiment and exposed simultaneously to the same film. Densitometry scanning of the autoradiograms was performed with the Densylab program (Bioprobe Systems, Les Ulis, France). Hybridizations were performed under conditions, probe concentrations, and film exposure times that gave a linear relationship between densitometry signal and amount of RNA loaded (range tested, 1 to 5 μg of RNA; $r = 0.99$). To correct for any minor differences in amount of RNA in each dot, hybridization was performed with a specific 25-mer oligoprobe for 18 S ribosomal RNA on the same filter.[35] Using the densitometry readings obtained with the 18 S oligoprobe, each densitometry signal for laminin-γ1 and Sp1 was normalized to reflect equivalent total RNA in each dot. Values were expressed as laminin-γ1/18 S and Sp1/18 S ratios (mean ± SD). The statistical analysis of the different groups was performed using the Kruskal-Wallis analysis of variance (ANOVA), median test. Comparisons between groups were performed using the Mann-Whitney *U* test. Correlation between laminin-γ1 and Sp1 mRNA expression levels was calculated with the Spearman rank order correlation test.

In Situ *Hybridization*

In situ hybridization with sense and antisense cRNA probes was performed according to the procedure of Milani et al[36] and Musso et al.[37] A 1.5-μg amount of linearized plasmids carrying either the Sp1 or laminin-γ1 inserts downstream of the SP_6 or T_7 promoters was used for *in vitro* transcription with 20 U of RNA polymerase in the presence of 60 μCi of [^{35}S]UTP (1.25 Ci/mmol; SJ 603, Amersham) using the riboprobe *in vitro* transcription system (Promega, Madison, WI) for 1 hour at 40°C. Frozen tissue sections 5 μm thick were fixed in 4% paraformaldehyde/PBS, rinsed in PBS, dehydrated through

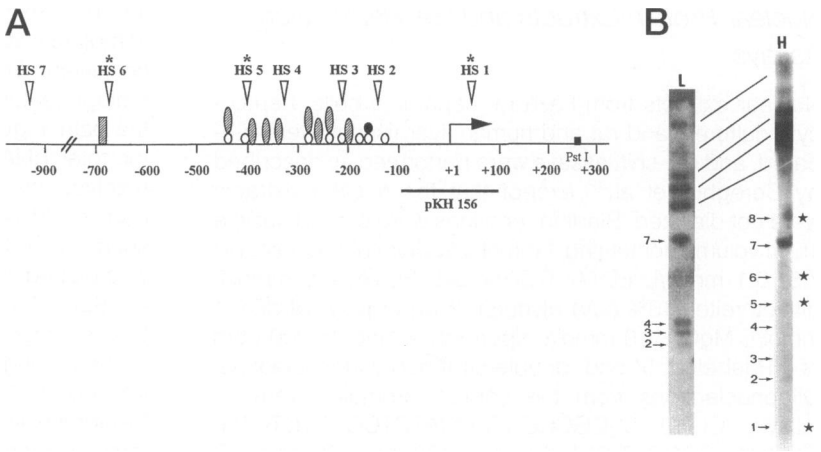

Figure 1. Mapping of DNAse I hypersensitive sites within the LamC1 promoter. **A:** The start site and direction of transcription are indicated by an arrow at position +1. The plasmid pKH 156 containing the fragment of promoter from −94 to +106 bp used as a probe and the *PstI* restriction site are indicated. DNAse I hypersensitive sites (HS) are indicated by vertical **arrowheads**. **Asterisks** denote HS sites present only in 4-hour rat hepatocyte primary cultures. **B:** Nuclei from total rat liver (L) and 4-hours rat hepatocyte primary cultures (H) were treated with DNAse I and purified DNA was digested with *PstI* and subjected to Southern blot analysis. Hypersensitive sites are numbered as indicated in the top of the figure. ○, GC box; ◖, CTC motif; ▮, AP1 site; ●, EGR box.

graded ethanols, air dried, and hybridized as previously described.[37] Afterwards, slides were exposed to ILFORD G5 emulsion (Ilford Anitec, Lyon, France) at 4°C for 2 weeks for Sp1 and laminin-γ1, developed in Kodak D19, fixed with Kodak Unifix (Kodak-Pathé, Chalons-sur-Saone, France), and stained with hematoxylin and eosin (H&E).

Results

Hypersensitive Sites in the LamC1 Promoter Contain Stretches of GC Motifs

DNAse I hypersensitive sites (HS sites) were mapped in the LamC1 promoter using total rat liver and early rat hepatocyte primary cultures, in which LamC1 becomes activated, to identify regulatory sites. In cultured hepatocytes, seven hypersensitive sites were detected in the promoter region (Figure 1). One site, designated HS-1, was located around position +50 bp. The second site, HS-2, was located at position −140 bp near a GC box. The third site, HS-3, was located at position −210 bp within a GC box and close to a CTC box. HS-4 was at position −330 bp, which contained a CTC box. HS-5 was located at position −400 bp, between a CTC box and a GC box. HS-6 was located at position −680 bp near an AP-1-binding site at −687 bp. HS-7 was located at −940 bp. An additional site, HS-8, was located at −1240 bp. Using nuclei from total rat liver, ie, when LamC1 is expressed at low levels, only four hypersensitive sites were found: HS-2, HS-3, HS-4, and HS-7, suggesting that HS-1, HS-5, HS-6, and HS-8 are involved in the regulation of the LamC1 promoter.

Sp1-Related Nuclear Factors Interact with GC Boxes in the LamC1 Promoter

Gel-shift assays were performed with nuclear extracts from Faza hepatoma cells, which constitutively express laminin-γ1 mRNA, to investigate whether specific proteins interact with GC-containing motifs in the LamC1 promoter (Figure 2). Different synthetic double-stranded oligonucleotides were used: CL-10 (−213 to −178 bp), which contains HS-3, and CL-20 (−414 to −380 bp), which contains HS-5. These oligonucleotides have a GC box either downstream or upstream of a CTC sequence, respectively. Two major complexes (A and B in Figure 2) were formed with the CL-20 probe (lane 5) and were competed for by an excess of unlabeled Sp1 consensus oligonucleotide (CL-Sp1; lane 6). Only the A complex was detected after incubation of Faza hepatoma cell

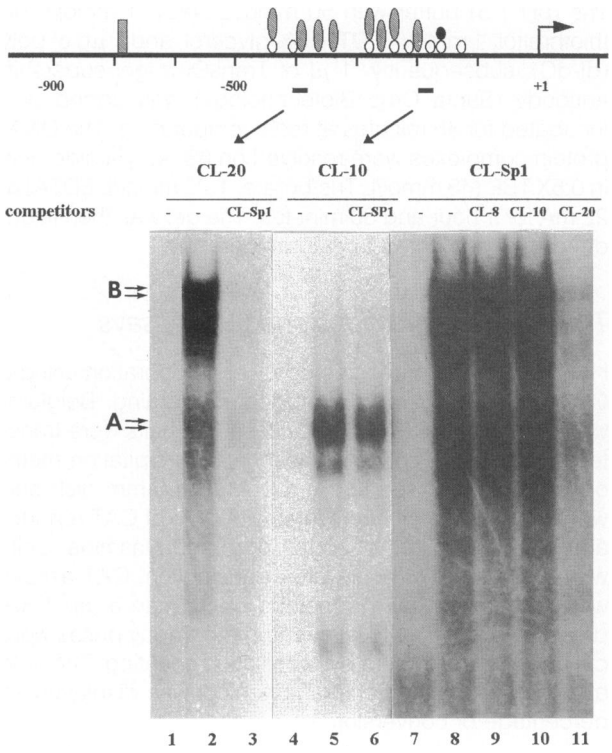

Figure 2. Gel mobility shift assay. Nuclear extracts were prepared from rat hepatoma cells and incubated with ^{32}P-labeled oligonucleotide. **Lanes 1** to **3**, CL-20; **lanes 4** to **6**, CL-10; **lanes 7** to **11**, CL-Sp1. Incubation steps were performed without (−) or with 100-fold excess of cold competitors. **lanes 3** and **6**, CL-Sp1; **lane 10**, CL-10; **lane 11**, CL-20; **lane 9**, CL-8; **lanes 1**, **4**, and **7**, control without nuclear extracts. Complexes A and B are indicated on the left. **Top panel:** The location of both CL-20 and CL-10 sequences within the LamC1 promoter are indicated. ○, GC box; ◖, CTC motif; ▮, AP1 site; ●, EGR box.

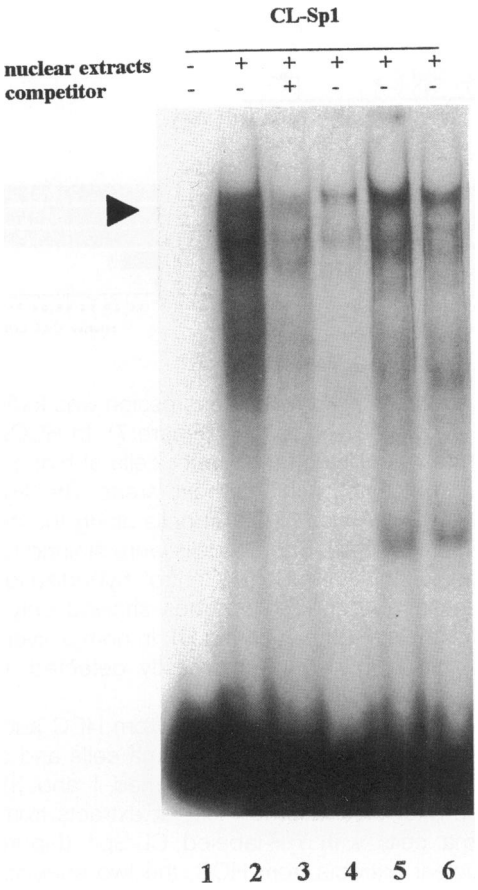

Figure 3. Gel mobility shift assay with ^{32}P-labeled CL-Sp1. Nuclear extracts were prepared from Faza hepatoma cells (**lanes 2** and **3**), normal rat liver (**lane 4**), and hepatocytes immediately after collagenase perfusion (**lane 5**) and after 2 days in primary culture (**lane 6**). **Lane 1**, control without nuclear extracts; **lane 3**, incubation with 100-fold excess of cold competitor; specific complexes are indicated by an **arrowhead**.

nuclear extracts with ^{32}P-labeled CL-10 probe (Figure 2, lane 2), which was not competed with CL-Sp1 (lane 3). The complex formed with ^{32}P-labeled CL-Sp1 oligonucleotide (lane 8) was competed for by an excess of cold CL-20 oligonucleotide (lane 11) but not by an excess of unlabeled CL-10 oligonucleotide (lane 10) or an excess of unlabeled nonspecific CTC-rich oligonucleotide (CL-8; lane 9). This indicates that an Sp1-related protein(s) interacts with GC motifs located in the HS-5 region within the LamC1 promoter.

Sp1 Activates LamC1

To examine whether the pattern of Sp1 binding differs depending on expression levels of the LamC1 gene, nuclear extracts were prepared from rat liver, from freshly isolated or 2-day cultured hepatocytes, and from Faza hepatoma cells. Gel-shift assays were performed with these nuclear extracts and with the Sp1 consensus oligonucleotide CL-Sp1 (Figure 3). The level of the binding activity to CL-Sp1 was high in Faza cells (lane 2) whereas it was low in rat liver (lane 4). Specificity of the binding was achieved by competition with cold oligonucleotide CL-Sp1 (lane 3). In addition, an increased CL-Sp1 binding was observed with nuclear extracts from freshly isolated (lane 5) and 2-day cultured hepatocytes (lane 6) compared with that from rat liver.

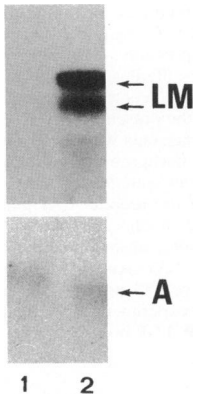

Figure 4. Northern blot analysis of laminin-γ1 and albumin mRNA in hepatocytes. Total RNA was prepared from hepatocytes transfected (**lane 2**) or not (**lane 1**) with 10 μg of pRC/CMV/Sp1 expression vector and analyzed by Northern blot with ^{32}P-labeled laminin-γ1 (LM) and albumin (A) cDNA probes. No change in the steady-state albumin mRNA content is observed between transfected and nontransfected cells (**lanes 2** and **1**, respectively, for albumin). As the staining of laminin-γ1 mRNA species (**arrows**) in transfected hepatocytes was intense, only a short time exposure of the blots is shown. Thus, laminin-γ1 mRNA in control cells is only barely detectable.

To determine whether Sp1 overexpression in normal cells increases LamC1 expression, freshly isolated hepatocytes were transfected by electroporation with 10 μg of the Sp1 expression vector pRC/CMV/Sp1 and cultured for 48 hours. In these conditions, approximately 10 to 20% of hepatocytes were transfected as measured after transfection of a β-galactosidase plasmid pRSV-βgal (not shown). Northern blot analysis was performed (Figure 4), and the relative amount of laminin-γ1 mRNA was determined by densitometric scanning and normalized after hybridization with an 18 S ribosomal RNA probe. In control cells, laminin-γ1 mRNA was detectable, whereas in hepatocytes transfected with the Sp1 expression vector, bands were intensely stained (Figure 4). The densitometric scanning showed a 50-fold increase in laminin-γ1 mRNA content compared with control cells (lane 2 *versus* lane 1). No change was observed in albumin mRNA content in transfected hepatocytes *versus* control cells (Figure 4).

To analyze the region in the LamC1 promoter involved in Sp1 activation, various 5′ deletions from −2000 to +106 bp were constructed from the mouse LamC1 promoter and linked to the CAT reporter gene. These constructs were co-transfected with the plasmid pPacSp1 or control plasmid (pPac0) without Sp1 coding sequences in *Drosophila* Schneider cells that lack endogenous Sp1 (Figure 5). Although basal CAT activity from transfected Schneider cells was only slightly above background for the different constructs co-transfected with pPac0, co-transfection with the Sp1 expression plasmid strongly induced CAT activity. The activity of the longest construct, pKH 135, was increased 40-fold by pPacSp1. Deletion of the fragment from −2000 to −294 bp (pKH 151, pKH 150) also showed high activity. pKH 155 (−144 to +106 bp) showed reduced inducibility. No significant CAT activity was detected using pKH 156, the GC-less

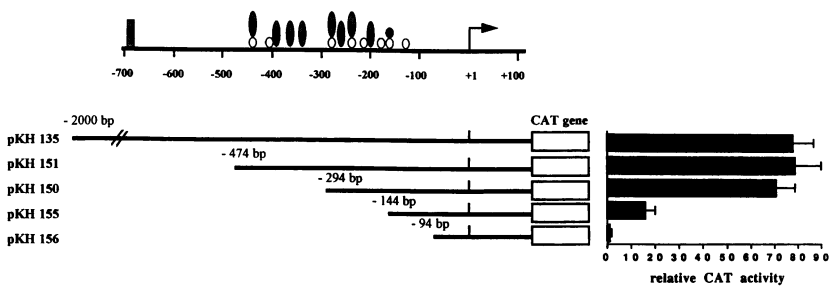

Figure 5. Activity of pKH fragments of LamC1 promoter fragments in *Drosophila melanogaster* cells co-transfected with the expression vector pPacSp1. The transactivation of the different fragments was evaluated by measuring the percentage of chloramphenicol transacylation in the presence of pPacSp1. Co-transfections of pKH fragments with the control vector pPac0 showed only background levels. Controls included co-transfections with either pSB1-CAT (no promoter upstream of the reporter CAT gene; relative CAT activity = 12 ± 4%) and pRSV-CAT (Rous sarcoma virus promoter upstream of the reporter CAT gene; relative CAT activity = 207 ± 11%). The pattern of relative CAT activities did not differ in three independent experiments. ○, GC box; ◖, CTC motif; ▮, AP1 site; ●, EGR box.

fragment from −94 to +106 bp, as found with the control pSB1-CAT. These results suggest that a region between −474 to −144 bp is one of the sites important for Sp1-mediated activation of the LamC1 promoter.

Sp1 and Laminin-γ1 Are Prominently Expressed in Human Hepatocellular Carcinomas

The relevance of Sp1-mediated activation of the LamC1 gene was further studied in human liver biopsies. The steady-state laminin-γ1 and Sp1 mRNA levels were measured by dot-blot in hepatocellular carcinomas (HCCs) and control livers (Figure 6, A and B). Both laminin-γ1 and Sp1 mRNA levels were, respectively, 2.8-fold and 1.8-fold higher in HCCs *versus* liver controls ($P < 0.01$). In HCCs, there was a high correlation between Sp1 and laminin-γ1 mRNA expression ($r = 0.68$, $P = 0.002$; Figure 6C).

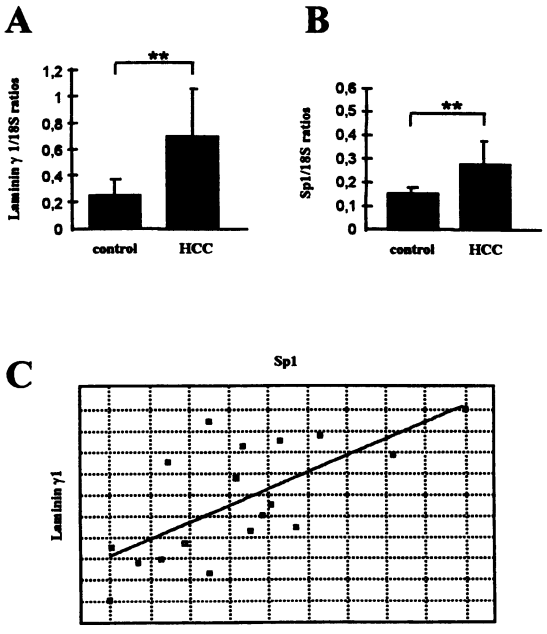

Figure 6. Laminin-γ1 (**A**) and Sp1 (**B**) mRNA content in liver biopsies from control and hepatocellular carcinomas (HCC). Values are expressed as laminin-γ1/18 S and Sp1/18 S ratios. The means ± SD for the two groups were compared by Mann-Whitney U test (**$P < 0.01$). Correlation between laminin-γ1 and Sp1 mRNA expression levels was calculated with the Spearman rank order correlation test in hepatocellular carcinomas (**C**).

Sp1 and laminin-γ1 mRNA expression was further analyzed by *in situ* hybridization (Figure 7). In HCCs, both transcripts were detected in tumor cells at higher levels than in hepatocytes within cirrhotic areas. The signal for laminin-γ1 mRNA was higher in cells along the invasion front (Figure 7, E–G). Stromal cells were stained for both Sp1 and laminin-γ1 mRNA. Control hybridization with sense laminin-γ1 and Sp1 probes showed only background signals (Figure 7, D and H). In normal livers, Sp1 and laminin-γ1 mRNAs were mostly detected in non-parenchymal cells (not shown).

Nuclear extracts were prepared from HCC and compared with those from Faza hepatoma cells and control liver. Two specific complexes (termed I and II) were detected after incubation of nuclear extracts from Faza hepatoma cells with ^{32}P-labeled CL-Sp1 (Figure 8A). With nuclear extracts from HCC, the two specific complexes I and II were detected. The intensity of complexes I and II was higher with nuclear extracts from tumor areas than with those from cirrhotic areas that surrounded the tumors. The intensity of both complexes obtained with nuclear extracts from tumor areas of HCC was similar to that obtained with nuclear extracts from Faza hepatoma cells. In all of these experiments, an additional band appeared to be not specific as it was not competed by an excess of unlabeled CL-Sp1 using extracts from HCC (Figure 8B). Using nuclear extracts from a control liver, bands corresponding to complexes I and II were faint and a faster migrating band was detected (III). To determine whether complexes I and II were related to Sp1, nuclear extracts from HCC, Faza hepatoma cells, and control livers were incubated with radiolabeled CL-Sp1 in the presence of specific anti-Sp1 antibodies (Figure 9). In nuclear extracts from Faza hepatoma cells and tumor areas from HCC, a super-shifted band was obtained, which paralleled a decrease in the staining intensity of complex I. No change in the intensity of complexes II and III was found in all of the samples.

Discussion

In this study we demonstrate that the transcription factor Sp1 is involved in regulating the LamC1 gene. As they are a prerequisite for transcriptional activation,[38] we first identified DNAse hypersensitive sites in the LamC1 pro-

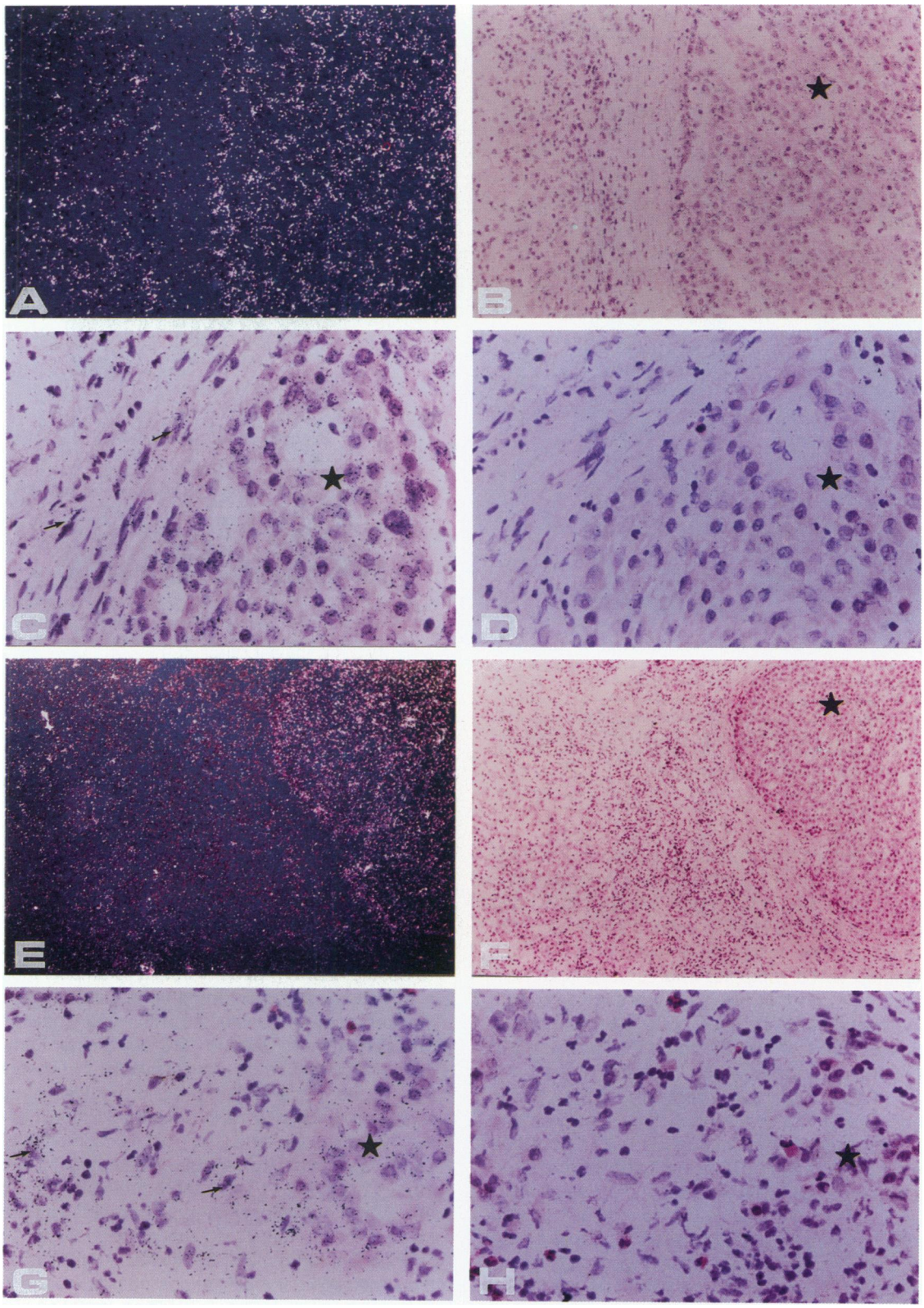

Figure 7. *In situ* hybridization of Sp1 and laminin-γ1 mRNA with ^{35}S-labeled cRNA probes in hepatocellular carcinoma. Sp1 (**A**, dark-field; **B** and **C**, bright-field) and laminin-γ1 mRNA (**E**, dark-field; **F** and **G**, bright-field) are detected in tumor cells (★) at higher levels than in the nontumor liver parenchyma. The signal for laminin-γ1 mRNA delineates the invasion front (**E** and **F**). Sections hybridized with sense Sp1 (**D**, section contiguous to **C**) and laminin-γ1 probes (**H**, section contiguous to **G**) show only background signal. Microphotographs are from H&E-stained microautoradiographs. Original magnification, ×50 (**A** and **B**), ×100 (**C**, **D**, **G**, and **H**), and ×25 (**E** and **F**).

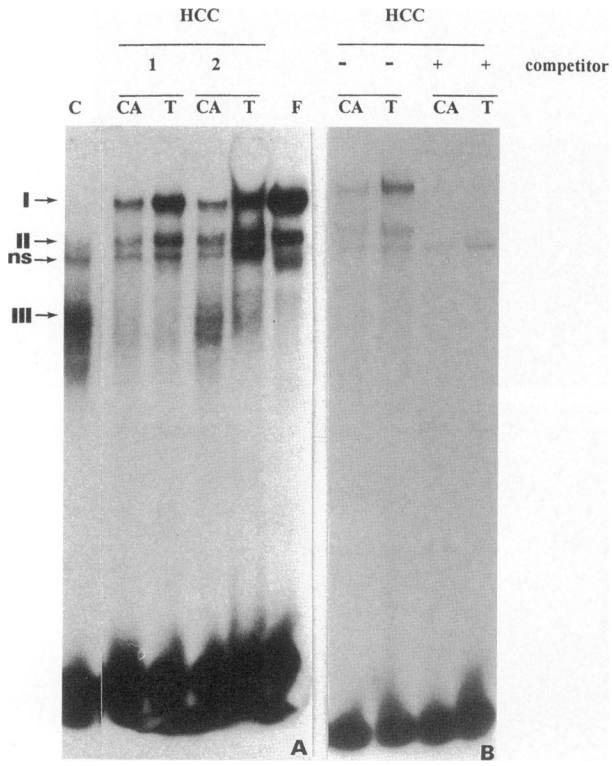

Figure 8. Gel mobility shift assay with ^{32}P-labeled CL-Sp1. **A:** Nuclear extracts were prepared from normal human liver (C) and from cirrhotic (CA) and tumor (T) areas from two hepatocellular carcinomas (HCC 1 and 2). F, nuclear extracts from Faza hepatoma cells; *control without nuclear extracts. **B:** Competition experiment. Nuclear extracts from cirrhotic and tumor areas from hepatocellular carcinoma were incubated (+) or not (−) with 100-fold excess of cold competitor. Formation of I, II, and III specific complexes and a nonspecific (ns) complex are indicated on the left.

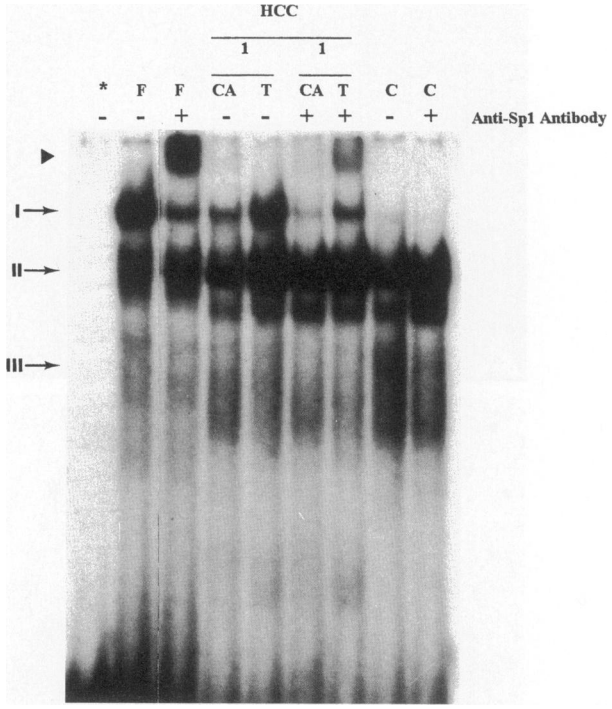

Figure 9. Super-shift experiment. Gel mobility shift assay was performed with ^{32}P-labeled CL-Sp1 and nuclear extracts from Faza hepatoma cells (F), a control liver (C), and a hepatocellular carcinoma (HCC) in the absence (−) or presence of anti-Sp1 antibodies (+). A slower migrating band (**arrowhead**) was detected in the presence of anti-Sp1 antibodies (F and HCC, T). Complexes I, II, and III are indicated on the left. *Control without nuclear extracts; CA, cirrhotic area, T, tumor area.

moter in early hepatocyte primary cultures, in which the gene becomes activated.[5] The hypersensitive sites found in the LamC1 promoter in the region containing GC and CTC sequences are essentially located between −450 and −140 bp. This is consistent with our recent report on transfection of LamC1 promoter fragments in Faza hepatoma cells, showing that *cis*-regulatory elements were located between −474 and −94 bp; a silencer-like element was identified in the −294-bp/−224-bp region and two enhancer-like elements in the −144-bp/−94-bp and −474-bp/−294-bp regions, respectively.[11] Thus, HS-2, HS-4, and HS-5 localize to the enhancer elements and HS-3 to the silencer element. In addition, an Sp1 consensus binding site prevented the formation of the DNA-protein complex formed with the oligonucleotide containing the GC box located at −400 bp within the HS-5 hypersensitive site of the LamC1 promoter, thus suggesting a potential role for Sp1 in laminin gene regulation. Binding activity of Sp1 was correlated with the laminin-γ1 expression level in hepatocytes. Indeed, LamC1 is activated during the disruption of liver by collagenase perfusion and in early hepatocyte primary cultures,[11] that is, while cells spontaneously progress from the G0 to the G1 phase of the cell cycle.[39,40] Accordingly, Sp1 binding activity may be significantly elevated in nuclear extracts from regenerating and newborn rat livers compared with those from normal adult livers.[41] Other studies indicated that Sp1 and Sp3, a member of the Sp1 family, are involved in the regulation of many growth-related cellular genes through the pRb control element, including c-*fos*, c-*myc*, TGF-β1, and TGF-β3 genes.[42–45] Taken together, these results suggest that there is a relationship between cell proliferation and Sp1 expression.

Our results demonstrate that Sp1 directly transactivates the LamC1 gene. First, a dramatic increase of endogenous laminin-γ1 mRNA content was found in normal hepatocytes transfected with a mammalian Sp1 expression vector. Second, GC-containing LamC1 promoter fragments were transactivated in *Drosophila* SL2 transfected with an Sp1 expression vector. Although *Drosophila melanogaster* cells have been shown to express the AP-1 transcription factor complex homologues for *Jun* and *Fos*, no activity has been detected for the homologues of Sp1.[46,47] Thus, the increased CAT activity of the LamC1 promoter constructs indicates that Sp1 is able to transactivate LamC1, whereas the role of AP-1 transcription factor(s) might be minimal in this system. Additional deletions of the 5′ region decreased the transactivation of LamC1 by Sp1. In addition, mutation of one CTC box in the pKH 150 fragment lowered the activity of the promoter by approximately 70% (not shown). A number of factors that recognize CTC sequences in various genes have been identified, including ZF87/MAZ,[48] CTCBF,[49] NSEP1,[50] and Sp1.[15] Interestingly, we have recently shown that the interactions of nuclear factor(s)

on CTC-containing sequences depend on the location of the flanking GC sequences.[11] Consequently, both GC-and CTC-containing sequences may act as positive regulatory elements for Sp1-mediated activation of LamC1.

Data from human liver biopsies support the concept that Sp1 has an important role in the regulation of laminin-γ1 expression, particularly in hepatocarcinogenesis. Laminin γ1 mRNA was 2.8-fold increased in human HCCs compared with control livers. This is in agreement with previous studies showing that laminin-γ1 is produced at high levels in human hepatoma cells[10] and that the steady-state level of laminin-γ1 mRNA is higher in nodules than in nontumor areas of diethylnitrosamine-treated rats.[5] In normal adult human livers, basement membrane components are not abundant,[10] whereas in both fetal and newborn livers, laminin expression and deposition are increased.[5,6] Interestingly, in human liver cancers, Wewer and Albrechtsen[51] have shown an increased deposition of laminins in the sinusoids, and an active process of basement membrane remodeling was suggested in human livers during the development of both primary and secondary cancers.[37] Importantly, the high expression of laminin-γ1 in HCCs correlated with high steady-state Sp1 mRNA levels. In addition, *in situ* hybridization experiments clearly indicated that cells that exhibit a high content of laminin-γ1 mRNA are also strongly positive for Sp1 mRNA. Both stromal cells and cancer cells in HCCs were stained for both mRNA species. Our data agree with and extend previous immunoelectron microscopy studies showing that sinusoidal endothelial cells, neoplastic hepatocytes, and hepatic stellate cells can produce laminins in chemically induced cancers in rats.[5] Accordingly, Rippe et al[52] have recently reported an increase in Sp1 binding activity to the GC-rich boxes of the α1(I) collagen promoter in activated hepatic stellate cells. Gel-shift experiments with nuclear extracts from HCCs showed that Sp1 mRNA levels paralleled a high Sp1 binding activity, which was similar to that found in the hepatoma cell line. Also, other members of the Sp1 family might be involved in the formation of additional complexes that appeared not supershifted by specific anti-Sp1 antibodies. However, the role of these factor(s) in laminin-γ1 expression might be minimal as no change in staining intensity of the complex was found between normal livers and HCCs.

In conclusion, our findings indicate that laminin-γ1 is expressed at high levels in HCCs, through a possible Sp1-mediated transactivation of the gene. In addition, the overexpression of Sp1 in liver cancers probably modulates a large variety of other genes, particularly growth factor genes and those involved in the cell cycle and cell proliferation. However, it can be hypothesized that an increased laminin deposition around tumor hepatocytes influence the balance between differentiation and growth[53] and thus contribute to the negative regulation of cell proliferation. This emphasizes the key role of laminin-hepatocytes interplay during the development of liver cancer.

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