Volume 312, number 1, 47–52 FEBS 11690 November 1992


# Differential expression of keratan sulphate proteoglycans fibromodulin, lumican and aggrecan in normal and fibrotic rat liver

N.B. Krull and A.M. Gressner

*Department of Clinical Chemistry and Central Laboratory, Philipps University, W-3550 Marburg, Germany*

Received 13 August 1992

In this study we investigated in rat liver the expression of genes coding for the core proteins of fibromodulin, lumican and aggrecan. By means of Northern analysis and in situ hybridization we present evidence for their differential transcription during liver fibrogenesis. Whereas no fibromodulin expression could been detected, both lumican and aggrecan transcripts were found displaying different time-courses of expression during the fibrogenic process. Based on studies performed in non-hepatic tissues, these proteoglycans are considered to have keratan sulphate glycosaminoglycan side chains. The expression of the respective core protein genes in liver is unexpected since published data have shown neither keratan sulphate nor its synthesis de novo in this tissue. The results also point to a putative role of aggrecan in the modulation of the inflammatory process in the liver.

Keratan sulphate; Proteoglycans; Lumican; Fibromodulin; Aggrecan; Expression

## 1. INTRODUCTION

Available studies support the notion that liver tissue is devoid of the expression of keratan sulphate glycosaminoglycans. The presence of keratan sulphate could not be demonstrated by means of the classical analytical–chemical procedures [1–3] and only trace amounts of de novo synthesis of a keratan sulphate-like glycosaminoglycan have been reported several years ago [4]. Furthermore, recent immunocytochemical studies failed to demonstrate the synthesis of this special type of glycosaminoglycan in cultured rat liver fat-storing cells [5] which have been identified previously as the major contributor to glycosaminoglycan production in the liver [6–8]. By use of Northern blot hybridization, in situ hybridization and RNase protection assay we were able to detect expression of genes encoding lumican and aggrecan core proteins in the healthy rat liver and during various stages of the development of thioacetamide-induced liver fibrosis. The results are surprising since these proteoglycans are considered to have keratan sulphate chains as deduced from their structure in non-liver tissues [9–11]. It is suggested that aggrecan, the most abundant (large aggregating) proteoglycan in cartilage where its core protein is densely substituted with many keratan sulphate and chondroitin sulfate chains is present also in normal and fibrotic liver albeit with an altered carbohydrate structure.

*Correspondence address:* A.M. Gressner, Abteilung Klinische Chemie und Zentrallaboratorium, Klinikum der Philipps-Universität, Baldingerstr., W-3550 Marburg, Germany. Fax: (49) (6421) 28 5594.

## 2. MATERIALS AND METHODS

### 2.1. *Animals*

Female virgin Uje:WIST rats (University of Jena, Germany), initial age 3 months, initial body weight 150 g, 3 animals per group, were maintained on a standard chow diet (type VTD-1, Berlin-Biesenthal, Germany). Lighting was kept constant at a 12-h light/dark cycle. For induction of fibrosis 0.03% (w/v) of thioacetamide (TAA; Merck AG, Darmstadt, Germany) was administered in drinking water for a maximum of 3 months without withdrawal prior to sacrifice [12]. At 2 weeks, 1, 2 and 3 months since the onset of TAA treatment a group of animals was sacrificed, liver tissue was shock-frozen in liquid $N_2$ and stored at −80°C until use.

### 2.2. *Genomic DNA extraction and Southern transfer*

Genomic rat DNA was extracted according to the method of Woodhead et al. [13]. 10 μg per lane were digested with different restriction enzymes (*Eco*RI, *Hin*dIII and *Bam*HI, all from Gibco BRL, Berlin, Germany) and separated by agarose gel electrophoresis. After depurination and denaturation by standard procedures, gels were transferred to Hybond-N membrane (Amersham-Buchler, Braunschweig, Germany) by vacuum transfer (TransVac, Hoefer, San Francisco, USA). Hybridization and washing of the filters were performed according to Church and Gilbert [14].

### 2.3. *Total RNA extraction and Northern blot analysis*

Total RNA was extracted from frozen liver tissue employing the guanidinium isothiocyanate method and centrifugation through CsCl gradient as described by Chirgwin et al. [15]. Extracted total RNA (25 μg per lane) was separated electrophoretically on formamide/formaldehyde-agarose gels, transferred to Hybond-N membrane and hybridized with [$^{32}$P]dCTP-labelled cDNA probes ([α-$^{32}$P]dCTP, 3000 Ci/mmol; NEN-DuPont, Dreieich, Germany) of specific activity > 1 × $10^9$cpm/μg, concentration > 1 × $10^6$ cpm/ml hybridization solution, at 65°C for 48 h. Blots were washed at stringencies varying as indicated for a given experiment. For Northern blot analysis, entire cDNAs of chicken lumican (donation of J. Hassell, Univ. of Pennsylvania, USA) and of bovine fibromodulin (gift of A. Oldberg, Univ. of Lund, Sweden) were used as probes.

2.4. *In situ hybridization*

2.4.1. Probes

cDNA for rat aggrecan was provided by K.L. Dreher (Weis Center for Research, Danville, USA). The cDNA fragment was subcloned into the pBSK-KS II vector (Stratagene Cloning Systems, La Jolla, USA); construction of the probe is shown in Fig. 1. After linearization of the plasmid with appropriate restriction endonucleases single-stranded RNA probes complementary (anti-sense probe) and anti-complementary (sense probe; negative control) to the corresponding gene transcripts were obtained by in vitro transcription using T3 and T7 polymerases (Stratagene Cloning Systems). The probe was labelled with [$\alpha$-$^{35}$S]CTP (1000–1500 Ci/mmol, NEN-DuPont) to a specific activity of $> 5 \times 10^8$ cpm/$\mu$g.

2.4.2. Hybridization and autoradiography

Serial cryostat sections, uniform thickness 8 $\mu$m, were lyophilized at −70°C for 2 h, then fixed in 4% paraformaldehyde/PBS, pH 7.4, for 10 min, at 4°C. Prior to hybridization slides were acetylated in 0.5% of acetic anhydride in 0.1 M triethanolamine, pH 8.0, for 10 min at room temperature, rinsed in 2 × SSC and air-dried.

In situ hybridization was performed as described by Tecott et al. [16]. In brief, sections were prehybridized at 52°C for 2 h in 50% formamide/0.02 M Tris, pH 7.5/0.3 M NaCl/0.001 M EDTA/0.1 M dithiothreitol/1 × Denhardt's solution/0.5 mg per ml yeast tRNA/0.1 mg per ml poly(A)/0.5 mg per ml sonicated salmon sperm DNA. Hybridization was carried out at 25°C below the calculated $T_m$ in 0.05 ml of mixture per section (composition as described above, with dextran sulphate added to a final concentration of 10% and $1 \times 10^6$ cpm of the radioactively labelled probe). After 16 h of incubation slides were rinsed in 4× SSC/0.02 M $\beta$-mercaptoethanol at room temperature followed by a stringency wash in hybridization buffer lacking dextran sulphate, labelled probe, t-RNA, poly(A) and salmon sperm DNA, at 5°C under the $T_m$ calculated, for 10 min. Decrease of the background due to non-specific binding was achieved by a 30 min digestion of mismatched sequences with RNase A (0.02 mg/ml) and RNase T1 (2500 Kunitz U/ml), at 37°C, in 0.5 M NaCl/0.01 M Tris, pH 7.5/0.001 M EDTA. After a wash (30 min at 37°C) in this buffer in the absence of the enzymes and a brief rinse in 2 × SSC, the slides were subjected to a second stringency wash in 0.1 × SSC, at 50°C for 15 min, with subsequent equilibration in 0.1 × SSC at room temperature.

Air-dried slides were dipped into NTB 2 nuclear emulsion (Kodak-Pathé, Paris, France) and exposed for 11 days at 4°C. Development was performed with Kodak D19 developer, for 2 min; afterwards, slides were rinsed in 1% acetic acid and fixed in ADEFO fixer (ADEFO, Nuremberg, Germany) for 15 min. After extensive washing in distilled water, slides were counter-stained with Cresyl violet, air-dried, cleared in xylol for 10 min and mounted in Entellan (Merck AG, Darmstadt, Germany). Sections were evaluated both by bright-field transmission and dark-field reflectance microscopy (Nikon Epiphot).

2.5. *Immunocytochemistry*

Monoclonal antibody BMA 0370 (Boehringer-Mannheim, Germany) detecting human macrophages was used. The APAAP staining method as described by Cordell et al. [17] was applied.

## 3. RESULTS

Based on the results obtained, fibromodulin is shown to be a single-copy gene in the rat genome (Fig. 2). The homology to its bovine counterpart is greater than 80% – washing of the rat genomic blot at stringencies up to 0.2 × SSC/0.1% SSC at 65°C for 30 min did not remove the signals. No hybridization with the liver tissue RNA could be demonstrated (data not shown).

The mRNA for lumican, also represented by a single-copy gene in the rat genome, has a homology of only about 60% to the chicken homologue (Fig. 3A). Washing at a stringency of 2 × SSC/0.1% SDS at 65°C for 30 min completely removed the signals observed at the

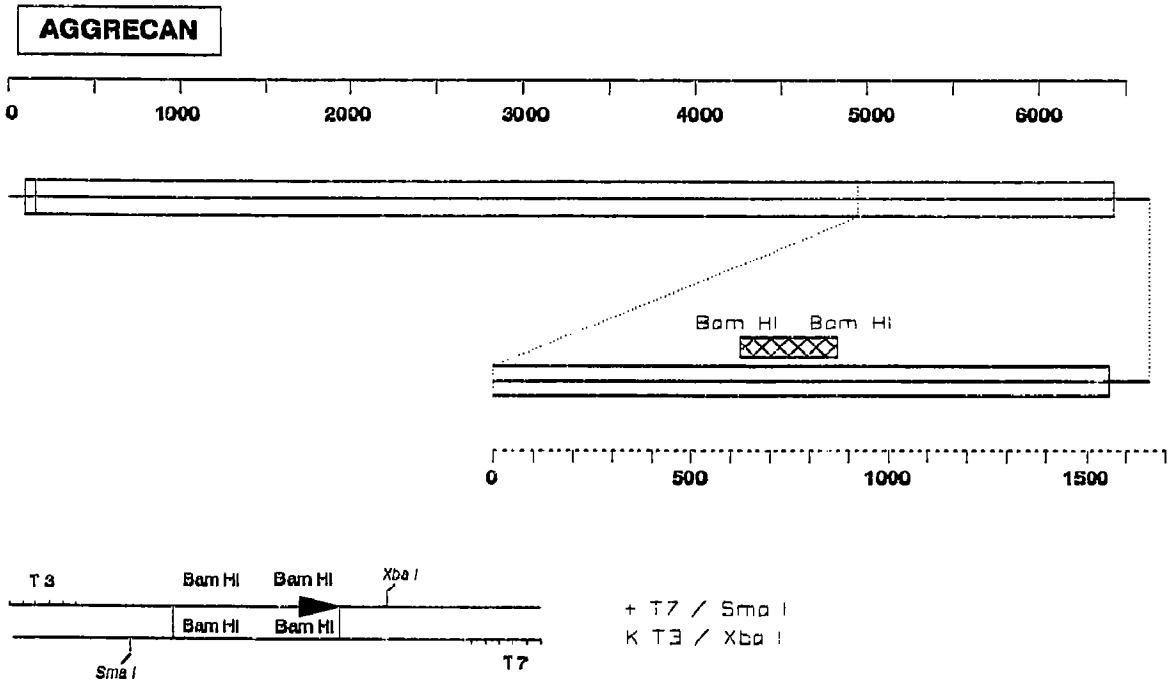


Fig. 1. Graphic description of the aggrecan cDNA construct used for in situ hybridization showing the gene structure. Empty bars representing the ORF of the gene, shaded bars the fragment subcloned. Direction of the subcloning with respect to $T_3$ / $T_7$ is depicted by use of an arrowhead.

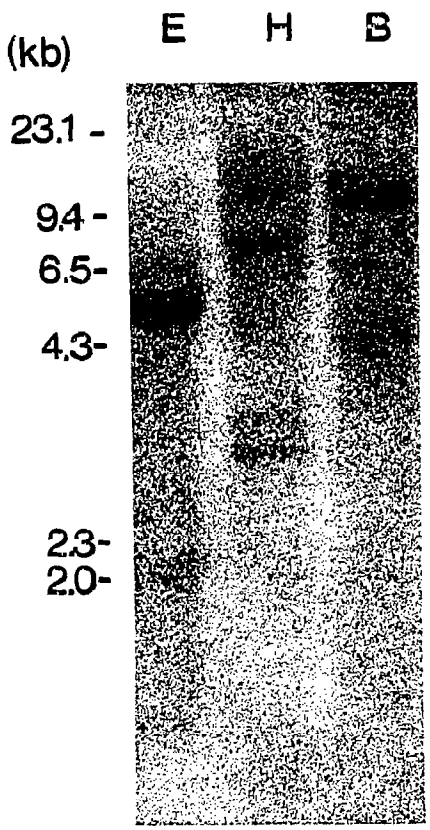


Fig. 2. Genomic blot of fibromodulin. 10 μg of rat genomic DNA each were digested with *Eco*RI (E), *Hin*dIII (H) and *Bam*HI (B), respectively. Asterisks mark the cross-hybridization of the fibromodulin probe with satellite DNA.

genomic as well as Northern blots. Weak hybridization signals of an almost constant intensity throughout the time-course of the fibrotic process could be detected in the rat liver tissue (Fig. 3B). A control RNA extracted from whole rat eyes revealed a closely spaced doublet of approximately 1.3 kb in length. The lumican probe from liver hybridized to the upper part of this doublet.

In normal liver tissue, aggrecan expressing cells are found scattered predominantly in the vicinity of the Glisson's capsule (Fig. 4). Their proportion as well as the intensity of the signal increase during the acute inflammatory phase of the liver damage, i.e. within the first two weeks of TAA treatment. Expressing cells are localized bordering the periportal fields, more rarely close to the orifices of large branches of the portal vein. During the following repair phase the expression intensity decreases continuously but remains present even in fully developed bridging fibrosis at the interface between the septa and the proliferating nodes and at the limiting plate. The immunocytochemical staining performed in parallel with the monoclonal antibody BMA 0370 (marker for human sessile tissue macrophages, reacts with Kupffer cells of the rat liver) showed good correlation up to 2 months of TAA treatment. In the later phase, characterized histomorphologically by transition to cirrhosis, divergence occurred. Cells of the limiting plate of small regeneration nodes showed strong hybridization signals whereas no immunostaining was present. This may be caused by the loss of antigenic epitopes of the expressing cells. Alternatively, other cell types, e.g. certain subtype(s) of the hepatocytes, start expressing aggrecan at this time point.

## 4. DISCUSSION

Although keratan sulphate is considered to be absent from liver tissue [1–3], we present evidence that genes coding for proteoglycans described as keratan sulphate-bearing are expressed in rat liver. Moreover, this expression seems to be regulated differentially during the course of liver fibrosis. This apparent discrepancy might have several possible explanations. First, it is possible that analytical-chemical methods applied to detect keratan sulphate failed to do so due to their relatively low sensitivity. Second, biglycan and decorin have been reported to possess varying glycosaminoglycan chains depending on the kind of the tissue investigated [18]. Thus, it might be possible that lumican and/or fibromodulin, belonging to the same gene family, do possess carbohydrate chains in the liver other than keratan sulphate. For aggrecan, in contrast, varying lengths of the hyaluronic acid (hyaluronan) binding region (HABR), the site of attachment of keratan sulphate side chains, have been described in different species. In rat, this region is distinctly shorter or even missing compared to other species studied [9]. Thus, rat liver tissue may truly be depleted of keratan sulphate, although core proteins of proteoglycans described as keratan sulphate-substituted in other tissues are indeed present. Although there is transcription present, a putative translation block also cannot be ruled out. Further investigation will elucidate which hypothesis gains more support.

As already mentioned, fibromodulin, lumican, biglycan and decorin are supposed to have arisen from a common ancestral gene [10,11,19]. Both biglycan and decorin are expressed in the rat liver, albeit at different basal levels, with an increase paralleling the development of fibrosis [20]. Lumican is also expressed in the rat liver, but at quite constant levels throughout the fibrotic process. This finding is concordant with that of Blochberger et al. [11], who found low expression levels of lumican in healthy chicken liver. The observation that lumican hybridizes in rat liver to the upper part of a closely spaced doublet compared to the RNA extracted from whole rat eyes (similarly to the observations described in chicken) is most probably due to a differential splicing of lumican mRNA with predominant occurrence of one of both splicing types specific for the tissue given. Investigations are under way to detect the rat homologue of the lumican gene, to determine the

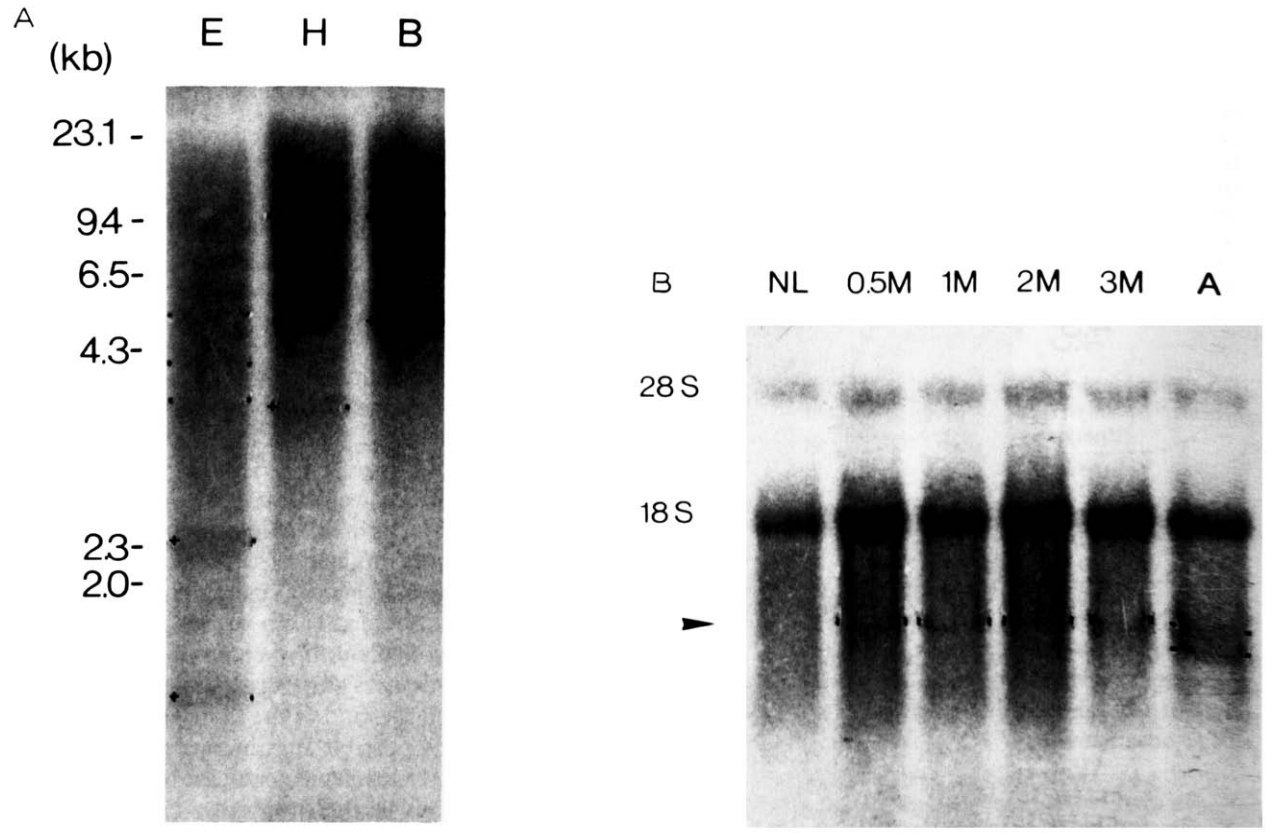


Fig. 3. Genomic blot of lumican (A) and the appropriate Northern blot (B) depicting the time-course of lumican expression at various stages of the development of liver fibrosis (NL = normal liver; following time-points in months, i.e. 0.5, 1, 2 and 3; A = whole rat eyes). Abbreviations used for the genomic blot are as in Fig. 2.

expressing cell type(s) and to resolve the spatial expression pattern by means of in situ hybridization.

On the contrary, no detectable fibromodulin expression was found in rat liver. One possible explanation is that the expression levels of this proteoglycan lie below the sensitivity limit of Northern blotting. Alternatively, rat liver can be truly devoid of fibromodulin expression, which would support the hypothesis that irrespective of the structural similarities proteoglycans are differentially regulated and exert quite specialized functions depending on the kind of tissue and, eventually, even on the developmental stage.

Aggrecan was detected by means of in situ hybridization in the Kupffer cells of the liver. Its levels of expression are extremely low in the healthy tissue but increase during a narrow period of acute inflammatory response to toxic liver damage. Low amounts, restricted to the cells localized at the limiting plate of proliferating nodes, remain detectable until the development of cirrhosis. Based on preliminary results obtained by an RNase protection assay, the expressing cell type is the Kupffer cell. This finding is of particular interest: though aggrecan has been described as the major constitutive proteoglycan of the bone matrix and cartilage and put in connection with their aberrant development [21,22], no aggrecan expression has been reported so far in liver tissue or in connection with the inflammatory process. In this context, though not wishing to overinterpret the observation, certain facts concerning the molecular organization of aggrecan could allow insight into its probably multifunctional nature. Aggrecan has been described as a molecule possessing distinct structural domains; whether these domains are capable of independent function is not clear. The molecular organization of its G3 domain resembles that of a group of cell adhesion molecules, known as 'LECAMs' [23], which are involved in mediating and modulation of inflammatory processes. From the parallel with the induction of aggrecan expression during the inflammatory phase as well as these structural properties it is tempting to postulate a possible involvement of aggrecan in the modulation of the inflammatory reaction following liver injury. Further experimental work will clarify the question of the mode of induction of aggrecan expression as well as the levels of aggrecan translation. The ligand involved will also have to be identified. Should

our hypothesis prove correct, aggrecan could be a good target for medical intervention in terms of controlling the inflammatory conditions in acutely injured liver.

*Acknowledgements:* We are thankful to Mrs. A. Lehnhardt, Dept. of Pathology of the Philipps University of Marburg, for technical assistance in immunocytochemical methods. Further, we are indebted to the Institute of Hormone and Fertility Research, Hamburg, Section of Molecular Biology (Head Dr. R. Ivell), for use of research facilities.

## REFERENCES

[1] Delbrück, A. (1968) Z. klin. Chem. u. klin. Biochem. 6, 460–466.
[2] Galambos, J.T. and Shapira, R. (1973) J. Clin. Invest. 52, 2952–2962.
[3] Stuhlsatz, H.W., Vierhaus, S., Gressner, A.M. and Greiling, H. (1983) in: Structural Carbohydrates in the Liver (Popper, H., Reutter, W., Gudat, F. and Köttgen, E. eds.) pp. 650–651, MTP Press, Lancaster.
[4] Gressner, A.M., Pazen, H. and Greiling, H. (1977) Hoppe Seyler's. Z. Physiol. Chem. 358, 825–833.
[5] Ross, R.S., Eyken Van, P., Desmet, V.J. and Gressner, A.M. (1992) in: Molecular and Cell Biology of Liver Fibrogenesis (in press) [Proceedings of International Falk Symposium, Marburg, Germany, January 1992] (Gressner, A.M. and Ramadori, G., Eds.), Kluwer Academic Publishers, Dordrecht/Boston/London.
[6] Schäfer, S., Zerbe, O. and Gressner, A.M. (1987) Hepatology 7, 680–687.
[7] Arenson, D.M., Friedman, S.L. and Bissell, D.M. (1988) Gastroenterology 95, 441–447.
[8] Gressner, A.M. and Schäfer, S. (1989) J. Clin. Chem. Clin. Biochem. 27, 141–149.
[9] Neame, P.J., Christner, E. and Baker, J.R. (1987) J. Biol. Chem. 262, 17768–17778.
[10] Oldberg, A., Antonsson, P., Lindblom, K. and Heinegard, D. (1989) EMBO J. 8, 2601–2604.
[11] Blochberger, T.C., Vergnes, J.P., Hempel, J. and Hassell, J.R. (1992) J. Biol. Chem. 267, 347–352.
[12] Müller, A., Machnik, F., Zimmermann, T. and Schubert, H. (1988) Exp. Pathol. 34, 229–236.
[13] Woodhead, J.L., Fallon, R., Figueiredo, H., Langdale, J. and Malcolm, A.D.B. (1986) in: Human Genetic Diseases. A Practical Approach (Davies, K.E. ed.) pp. 51–64, IRL Press.
[14] Church, G.M. and Gilbert, W. (1984) Proc. Natl. Acad. Sci. USA 81, 1991–1995.
[15] Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294–5299.
[16] Tecott, L.H., Eberwine, J.H., Barchas, J.D. and Valentino, K.L. (1987) in: In Situ Hybridization (Valentino, K.L., Eberwine, J.H. and Barchas, J.D. eds.) pp. 3–24, Oxford University Press.
[17] Cordell, J.L., Falini, B., Erber, W.N., Ghosh, A.K., Abdulaziz, Z., MacDonald, S., Pulford, K.A., Stein, H.A. and Manson, D.Y. (1984) J. Histochem. Cytochem. 32, 219–229.
[18] Fisher, L.W., Hawkins, G.R., Tuross, N. and Termine, J.D. (1987) J. Biol. Chem. 262, 9702–9708.
[19] Fisher, L.W., Heegaard, A.M., Vetter, U., Vogel, W., Just, W., Termine, J.D. and Young, M.F. (1991) J. Biol. Chem. 266, 14371–14377.
[20] Meyer, D.H., Krull, N., Dreher, K.L. and Gressner, A.M. (1992) Hepatology 16, 204–216.
[21] Doege, K., Sasaki, M., Horigan, E., Hassell, J.R. and Yamada, Y. (1987) J. Biol. Chem. 262, 17757–17767.
[22] Wong, M., Lawton, T., Goetinck, P.F., Kuhn, J.L., Goldstein, S.A. and Bonadio, J. (1992) J. Biol. Chem. 267, 5592–5598.
[23] Drickamer, K. (1988) J. Biol. Chem. 263, 9557–9560.