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Vitamin E dietary supplementation inhibits transforming growth factor β1 gene expression in the rat liver

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Overexpression of transforming growth factor β1 (TGFβ1) and increased transcription of pro-collagen type I, are known to represent major events implicated in the development of liver fibrosis under either experimental or clinical conditions. Here we report that long-term dietary vitamin E supplementation in animals undergoing an experimental model of liver fibrosis (induced by chronic treatment of rats with carbon tetrachloride) results in a net inhibition of both hepatic TGFβ1 and α2 (I) procollagen mRNA levels. Moreover, of striking interest is the observation that vitamin E supplementation per so down-modulates basal levels of TGFβ1 mRNA in the liver of untreated animals, suggesting that a dietary regimen rich in vitamin E may potentially interfere with both the initiation and progression of the fibrosclerotic processes.

Liver fibrosis; TGFβ1; α2 (I) Procollagen; Vitamin E

1. INTRODUCTION

Transforming growth factor-β1 (TGFβ1), a pleiotropic peptide involved in the regulation of cell metabolism and growth, also plays a key role in tissue remodelling following necrosis and inflammation mainly through the synthesis of extracellular matrix components [1–10]. During the fibrotic response its overexpression in the liver is associated with increased transcription of procollagen type I [11–16]. The parallel rise of these factors is now generally accepted as a relevant feature of the development of liver fibrosis [11–16] and finds its explanation in the existence of TGFβ1-activated elements in the type I collagen gene [17,18]. Necrosis, inflammation and fibrosis are readily reproducible events in the rat during chronic intoxication with the prooxidant hepatotoxin carbon tetrachloride (CCl_4) and such a model is extensively used for experimental purposes [8,12,14].

We have previously shown that dietary vitamin E supplementation, by increasing the liver content of the vitamin, can afford protection against liver fibrosis induced in the rat by chronic treatment with CCl_4 [19]. This protective effect of vitamin E is most likely related to the antioxidant effect of the vitamin well characterized in the acute model of CCl_4 intoxication [20–24]. In order to investigate the effect of vitamin E membrane enrichment on liver expression of TGFβ1, the mRNA levels of the cytokine as well as of α2 (I) procollagen were determined during CCl_4-induced liver fibrosis in animals fed either on a standard or on a vitamin E supplemented diet.

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2. MATERIALS AND METHODS

Vitamin E (α-tocopherol), guanidinium thiocyanate, formamide, formaldehyde and salmon sperm DNA were purchased from Sigma Chemical company. Ethidium bromide and agarose gel for electrophoresis were from Bio-Rad (Richmond, California, USA). Yeast RNA was from Boehringer (Mannheim, Germany) whereas sodium dodecyl sulphate (SDS), carbon tetrachloride and all the other reagents were from Merck (Darmstadt, Germany). Autoradiographic film was provided by Kodak Ltd., England. Multiprime DNA labelling system kit was provided by Amersham (Milano, Italy).

Young male Wistar rats (Nossan, Correzana, Italy), weighing 40–50 g at the beginning of the experiment, were used. The rats were divided initially into two experimental groups: the first group was fed throughout the experiment with a standard pelletted diet (diet no. 48, F. lli Piccioni, Brescia, Italy) containing 50 mg of vitamin E (as α-tocopheryl-acetate) per kg of diet (hereafter referred to as control diet); the second group was fed on the same diet supplemented in vitamin E up to 250 mg per kg of diet. After three weeks of feeding the animals of these two groups (130–140 g b.wt. at this stage) differed only in their hepatic vitamin E content that was three times higher in the rats fed on the supplemented diet (90 ± 15 vs. 32 ± 9 nmol/g liver [19]). At this point half of the animals of each group were submitted to a cirrhosis induction protocol for 5 weeks as described by Ehrinpreis et al. (animals injected intraperitonsally three times a week either with 0.15 ml of CCl_4, diluted in mineral oil, or with the vehicle alone) [25]. The rats were then sacrificed under diethyl ether anaesthesia 24 h after the last treatment and liver samples were immediately frozen in liquid nitrogen and stored at −80°C.

RNA extraction and Northern blot analysis were performed as follows. Frozen livers were homogenized in a glass-teflon homogenizer

and total RNA was isolated using a acid guanidinium thiocyanate/phenol/chloroform technique. RNA concentration was determined by measuring the absorbance of the RNA solution at 260 nm. Total RNA was subjected to electrophoresis on a formaldehyde-containing 1.5% agarose gel and transferred to a nylon membrane. Membranes were prehybridized for 16–18 h at 42°C in a solution containing 50% formamide (v/v), 5× SSC solution pH 7.0 (20× SSC stock solution contains NaCl 17.53% w/v, and sodium citrate 8.92%, w/v), 0.1% SDS (w/v), 5× Denhardt's, 100 μg/ml salmon sperm DNA, 500 μg/ml yeast RNA. Hybridization with ^{32}P-labelled full-length TGFβ1 and α2 (I) procollagen cDNA probes [26,27] was carried out in 50% formamide (v/v), 5× SSC, 0.1% SDS (w/v), 1 × Denhardt's at 42°C overnight. After hybridization membranes were washed with 2 × SSC, 0.5% SDS (w/v) for 20 min at room temperature and with 0.1 × SSC, 0.1% SDS (w/v) for 20 min at 52°C. Membranes were exposed to autoradiographic film at −80°C with intensifying screens. The α-actin mRNA levels were not taken as a reference since they proved to be strongly increased by the treatment with the hepatotoxin [28]. Autoradiograms were quantified by densitometrical scanning using a laser densitometer (LKB 2022 ultroscan) and all values were normalised against the relative ethidium bromide stained total RNA.

Western blot analysis was performed employing a rabbit polyclonal antibody that was kindly provided by Dr. Dennis Wang (Dept. Physiology and Pharmacology, University of Queensland, Queensland, Australia). This antibody, cross-reacting with rat, was raised against a peptide corresponding to carboxyterminal 10 amino acids of human TGFβ1. 150 μg of proteins of the crude tissue extract obtained by an acidic/ethanol procedure according to Assoian et al. [29], were subjected to a 15% polyacrylamide SDS gel electrophoresis under reducing conditions [30] and transferred to polyvinylidene difluoride microporus membranes using a Bio-Rad Trans-Blot Semi Dry Cell [31]. Excess binding capacity of membranes was blocked by overnight incubation at 4°C with 5% bovine serum albumin in TBS (10 mM Tris-HCl pH 7.4, 150 mM NaCl). The blots were then incubated successively with polyclonal antibody diluted 1:200 for 2 h at room temperature, with biotinylated antirabbit IgG diluted 1:2000 for 1 h and, finally, with streptavidin-biotinylated horseradish peroxidase complex (Dako spa, Milano, Italy). Between incubations the membranes were washed extensively with TBS containing 0.1% Tween 20. Immunoreactive bands were then visualized by reaction with 3,3'-diaminobenzidine. The molecular weight marker proteins were localized by brief staining of blots with 0.1% Amido black in 25% isopropanol containing 10% acetic acid.

3. RESULTS AND DISCUSSION

The protective effect exerted by vitamin E supplementation against CCl_4-induced liver necrosis is a well established event [20–24]. Recently we found that the same procedure afforded also protection against fibrosis induced in the male Wistar rat after five weeks of chronic CCl_4 i.p. dosing [19]. This observation was strongly suggestive of a modulating effect of the natural antioxidant in the expression of fibrogenic cytokines.

As shown in Fig. 1, while the steady-state level of TGFβ1 mRNA remained low in control rat liver after five weeks of intoxication with CCl_4 there was a four times increase in the hepatic amount of TGFβ1 mRNA. The stimulated expression of the cytokine was paralleled in the CCl_4-treated animals by high levels of α2 (I) procollagen mRNA (Fig. 2) that is almost undetectable in normal liver [12]. As already mentioned in the introduction, the concomitant rise of these two factors represents a relevant event in the development of liver fibrosis [11–16]. Densitometric scanning of Northern blots

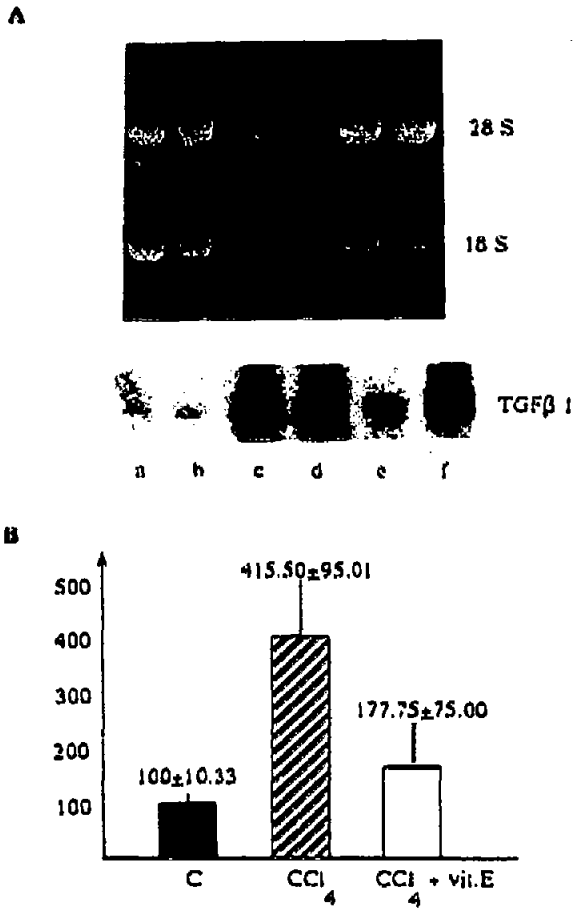

Fig. 1. Northern blot analysis of TGFβ1 expression in fibrotic liver. (A) Levels of TGFβ1 mRNA (bottom panel) in the liver of control rats (lanes a,b) and of rats treated respectively with CCl_4 (c,d) and with CCl_4 and vitamin E (e,f). The ethidium bromide-stained gel is shown in the top panel. (B) Quantitation of TGFβ1 mRNA levels in the liver of control rats (C) and of rats treated respectively with CCl_4 (CCl_4) and with CCl_4 and vitamin E (CCl_4 + Vit E). Results are expressed as percent of the control and are means ± S.D. of four separate experiments.

from four separate experiments showed more than 50% decrease in TGFβ1 mRNA steady-state content in the liver of rats receiving a dietary supplementation of α-tocopherol during chronic intoxication with the haloalkane (Fig. 1). Together with the reduced expression of the cytokine, in the liver of CCl_4 treated but α-tocopherol supplemented animals the mRNA for α1 (I) procollagen was significantly lowered (approx. 75% as to the CCl_4-treated group taken as 100%, Fig. 2).

The described antioxidant treatment has been demonstrated to efficiently counteract the toxic effect of carbon tetrachloride by inhibiting the stimulated oxidative breakdown of membrane polyunsaturated fatty acids [20–24]. Thus, our data suggest a role for oxidative mechanisms in the stimulation of TGFβ1 and α2 (I) procollagen gene expression. Consistent with this interpretation is the stimulation of collagen gene transcription induced in cultured human foetal fibroblasts by

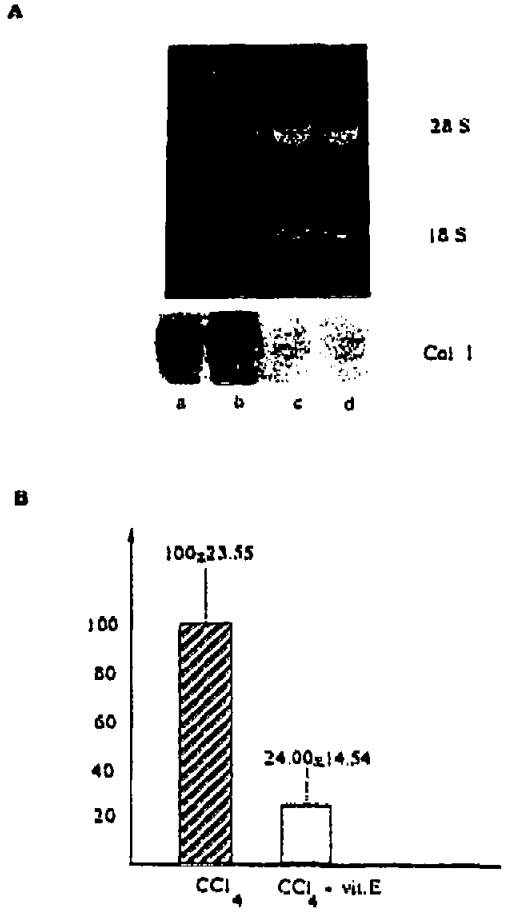

Fig. 2. Northern blot analysis of α2 (I) procollagen expression in fibrotic liver. (A) Levels of α2 (I) procollagen (bottom panel) in the liver of rats treated respectively with CCl_4 (lanes a,b) and with CCl_4 and vitamin E (c,d). The ethidium bromide stained gel is shown in the top panel. (B) Quantitation of α2 (I) procollagen mRNA levels in the liver of rats treated respectively with CCl_4 (CCl_4), and with CCl_4 and vitamin E (CCl_4 + Vit E). Results are expressed as percent of the CCl_4 group and are means ± S.D. of four separate experiments.

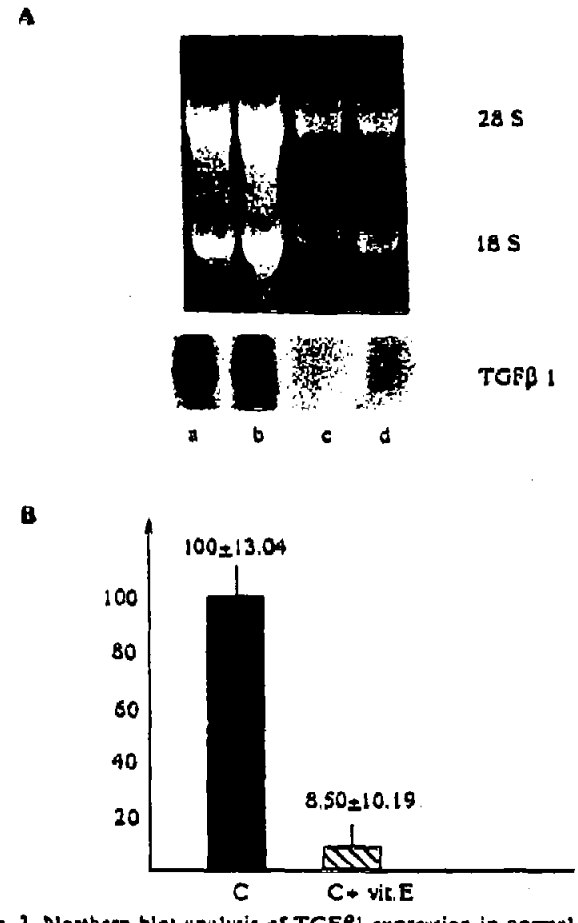

Fig. 3. Northern blot analysis of TGFβ1 expression in normal liver. (A) Levels of TGFβ1 mRNA (bottom panel) in the liver of control rats (lanes a,b) and of rats treated with vitamin E (c,d). The ethidium bromide-stained gel is shown in the top panel. (B) Quantitation of TGFβ1 mRNA levels in the liver of control rats (C) and of rats treated with vitamin E (Vit E). Results are expressed as percent of the control and are means ± S.D. of six separate experiments.

ascorbate dependent lipid peroxidation [32] and its inhibition by α-tocopherol and other antioxidants directly added to the cell incubation medium [33].

On the other hand, one can argue that the lowering effect of α-tocopherol on the TGFβ1 mRNA levels and consequently on α2 (I) procollagen transcripts is merely a consequence of the prevention of CCl_4-induced necrosis exerted by the vitamin. Even if the latter possibility cannot be excluded, the hereafter reported data on the down modulation of basal TGFβ1 mRNA levels by dietary α-tocopherol definitely support a direct action of vitamin E on TGFβ1 expression. Fig. 3 shows the Northern blot analysis of TGFβ1 mRNA extracted from the liver of supplemented rats compared to that from animals fed the control diet. The results concerning TGFβ1 mRNA levels under different experimental conditions (see Figs. 1 and 3) are supported by Western blot analysis (Figs. 4 and 5). As shown in Fig. 4, using purified human TGFβ1 as a standard (Genzyme Corp., Cambridge, MA, USA), under reducing conditions, the polyclonal antibody employed allowed the detection, as the major form, of a polypeptide of 14.45 kDa which corresponds to mature monomeric TGFβ1. The evaluation of immunoblots of this immunoreactive 14.45 kDa peptide in the crude acid liver extracts from different experimental groups suggests a pattern of changes in agreement with TGFβ1 mRNA modifications (Fig. 5).

The high liver concentrations of α-tocopherol reached by dietary supplementation appear to down-modulate the gene expression of a cytokine most likely playing a key role in liver fibrogenesis [11–16]. Even if its chain-breaking antioxidant activity seems likely to be involved, the exact molecular mechanisms by which vitamin E exerts the effects here described are yet to be clarified.

The present research will be extended by assessing the effect of α-tocopherol on the gene expression of other fibrogenic cytokines and later on, various human diseases characterized by fibrotic processes will be consid-

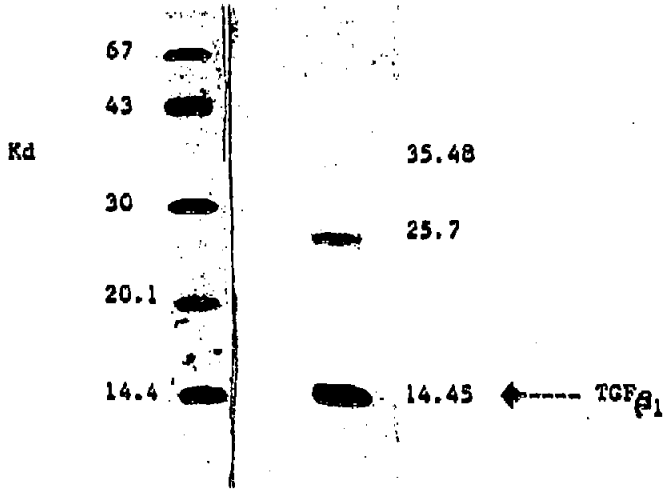

Fig. 4. Western blot detection of purified TGFβ1 compared with molecular weight standards.

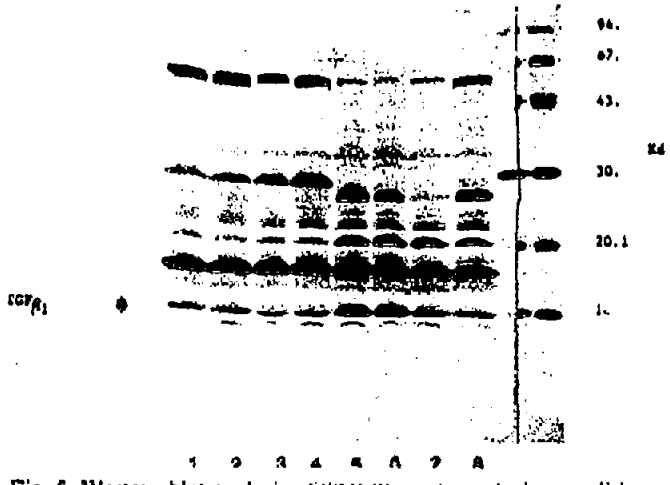

Fig. 5. Western blot analysis of TGFβ1, under reducing conditions, in the crude acid extracts of liver samples from different experimental groups. Levels of immunoreactive TGFβ1 peptide (14.4 kDa) in the liver of control rats (lanes 1 and 2), rats treated with vitamin E (lanes 3 and 4), rats treated chronically with either CCl_4 alone (lanes 5 and 6) or with CCl_4 plus vitamin E (lanes 7 and 8).

ered. In fact, the most important suggestion which can be drawn from the reported data is the possibility to control by dietary vitamin E certain pathways responsible for the initiation and worsening of fibrosis occurring not only in the liver but also in the lung, kidney and arterial walls.

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REFERENCES

[1] Sporn, M.B., Roberts, A.B., Wakefield, L.M. and de Crombrugghe, B. (1987) J. Cell Biol. 105, 1039–1045.
[2] Sporn, M.B. and Roberts, A.B. (1988) Nature 332, 217–219.
[3] Roberts, A.B. and Sporn, M.B., in: Handbook of Experimental Pharmacology. Peptide Growth Factors and Their Receptors (M.B. Sporn and A.B. Roberts, Eds.), Springer, Heidelberg, 1990, pp. 419–472.
[4] Kovacs, E. (1991) J. Immunol. Today 12, 17–23.
[5] Wahl, S. (1991) Immunol. Res. 10, 249–254.
[6] Roberts, A.B., Sporn, M.B., Assoian, R.K., Smith, J.M., Roche, N.S., Wakefield, L.M., Heine, U.I., Liotta, L.A., Falanga, V. and Fauci, A.S. (1986) Proc. Natl. Acad. Sci. USA 83, 4167–4171.
[7] Ignotz, R.A. and Massagué, J.J. (1986) Biol. Chem. 261, 4337–4345.
[8] Armendariz-Borunda, J., Seyer, J.M., Kang, A.H. and Raghow, R. (1989) FASEB J. 4, 215–221.
[9] Bissel, D.M., Friedman, S.L., Maher, J.J. and Roll, F.J. (1990) Hepatology 11, 488–498.
[10] Weiner, F.R., Giambrone, M.A., Czaja, M.J., Shah, A., Annoni, G., Takahashi, S., Eghbali, M. and Zern, M.A. (1990) Hepatology 11, 111–117.
[11] Czaja, M.J., Weiner, F.R., Flanders, K.C., Giambrone, M.A., Wind, R., Biempica, L. and Zern, M.A. (1989) J. Cell. Biol. 108, 2477–2482.
[12] Nakatsukasa, H., Evarts, R.P., Hsia, C. and Thorgeirsson, S.S. (1990) Lab. Invest. 63, 171–180.
[13] Nakatsukasa, H., Nagy, P., Evarts, R.P., Hsia, C., Marsden, K. and Thorgeirsson, S.S. (1990) J. Clin. Invest. 85, 1833–1843.
[14] Miao, S., Bao-En, W., Annoni, G., Degli Esposti, S., Biempica, L. and Zern, M.A. (1990) Lab. Invest. 63, 467–475.
[15] Castilla, A., Prieto, J. and Fausto, N. (1991) New Engl. J. Med. 324, 933–940.
[16] Brenner, D.A. (1991) Hepatology 14, 740–741.
[17] Rossi, P., Karentsy, G., Roberts, A.B., Roche, N.S., Sporn, M.B. and Crombugghe, B. (1988) Cell (Cambridge, MA) 52, 405–414.
[18] Ritzenthaler, J.D., Goldstein, R.H., Fine, A., Lichtler, A., Rowe, D.W. and Smith, B.D. (1991) Biochem. J. 280, 157–162.
[19] Parola, M., Leonarduzzi, G., Biasi, F., Albano, E., Biocca, M.E., Poli, G. and Dianzani, M.U. (1992) Hepatology, in press.
[20] Poli, G., Albano, E., Biasi, F., Cecchini, G., Carini, R., Bellomo, G. and Dianzani, M.U., in: Free Radicals in Liver Injury (G. Poli, K.H. Cheeseman, M.U. Dianzani and T.F. Slater, Eds.), IRL Press, Oxford, 1985, pp. 207–215.
[21] Poli, G., Albano, E. and Dianzani, M.U. (1987) Chem. Phys. Lipids 45, 117–142.
[22] Albano, E., Carini, R., Parola, M., Bellomo, G., Gorin-Gatti, L., Poli, G. and Dianzani, M.U. (1989) Biochem. Pharmacol. 39, 2717–2735.
[23] Poli, G., Cottalasso, D., Pronzato, M.A., Chiarpotto, E., Biasi, F., Corongiu, F.P., Marinari, U.M., Nanni, G. and Dianzani, M.U. (1990) Cell Biochem. Funct. 8, 1–10.
[24] Biasi, F., Albano, E., Chiarpotto, E., Corongiu, F.P., Pronzato, M.A., Marinari, U.M., Parola, M., Dianzani, M.U. and Poli, G. (1991) Cell Biochem. Funct. 9, 111–118.
[25] Ehrinpreis, M.N., Giambrone, M.A. and Rojkind, M. (1980) Biochim. Biophys. Acta 629, 184–193.
[26] Derynck, R., Jarrett, J.A., Chen, E.Y., Eaton, D.H., Bell, J.R., Assoian, R.K., Roberts, A.B., Sporn, M.B. and Goeddel, D.V. (1985) Nature 316, 701–705.
[27] Genovese, C., Rowe, D. and Kream, B. (1984) Biochemistry 23, 6210–6217.
[28] Rojkind, M. and Greenwel, P., in The Liver: Biology and Pathobiology (I.M. Arias, et al., Eds.), Raven Press, New York, 1988, pp. 1269–1285.
[29] Assoian, R.K., Komoriya, A., Meyers, C.A., Miller, D.M. and Sporn, M.B. (1983) J. Biol. Chem. 258, 7155–7160.
[30] Laemli, U.K. (1970) Nature 277, 680–685.
[31] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[32] Geesin, J.C., Gordon, J.S. and Berg, R.A. (1990) Arch. Biochem. Biophys. 278, 350–355.
[33] Houghlum, K., Brenner, D.A. and Chojkier, M. (1991) J. Clin. Invest. 87, 2230–2235.