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A liver-specific nuclear protein that binds to the distal promoter element of the rat tyrosine aminotransferase gene

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Using a gel retardation assay and exonuclease III footprinting, we have analyzed sequence-specific DNA-binding nuclear factors which interact with the distal promoter element of the rat tyrosine aminotransferase gene. A factor called $LspA_1$, binding to a sequence that resembles the consensus binding site for the transcription factor Ap-1, was shown to be present in adult rat-liver nuclear protein extracts but not in the extracts from embryonic liver or spleen nuclei.

DNA binding; Liver-specific nuclear protein; Tyrosine aminotransferase gene; (Rat)

1. INTRODUCTION

In adult animals, the rat tyrosine aminotransferase (TAT) gene is selectively transcribed in parenchymal cells of the liver and this transcription is regulated. Three DNase 1-hypersensitive sites in isolated nuclei of TAT-expressing but not of non-expressing cells mark important regulatory DNA sequences [1,2]. Two sites occur within the promoter region (HS sites I and II, respectively [1]). The distal site III at about 2.5 kbp upstream of the promoter which appears specifically upon induction of the gene expression by hormone has been shown to contain two cooperating glucocorticoid response elements [2]. Hormone-dependent DNA-protein interaction was observed within site III in hepatoma cells [3] but the proteins binding within HS sites I and II were not dependent on glucocorticoids [4].

Genomic footprinting revealed cell type-specific (hepatoma vs non-hepatoma cells) binding of ubiquitous nuclear factors to DNA sequences in HS sites I and II [4]. Six DNA sequences protected by proteins binding in vivo and in vitro within promoter HS site I and two sequences within HS site II were observed, but the corresponding nuclear factors have not yet been characterized. It is also unclear whether similar nuclear factors are present in normal rat-liver nuclei.

In order to elucidate how regulatory signals from the glucocorticoid response elements of the rat TAT gene are transduced to its related promoter, we have investigated the rat-liver nuclear factors which bind to the promoter sequences. The finding that 100–300 bp of upstream promoter sequence of other liver-specific genes are sufficient to govern the low-level tissue-specific expression encouraged us to begin studying sequence-specific DNA-protein interactions within the upstream promoter region around HS site I. In a previous study, using gel-retardation and DNase 1 footprinting assays, we have analysed the rat-liver nuclear factors binding to the proximal promoter fragment (from −2 to −210 bp relative to the transcription start) of the rat TAT gene [5–7] and have found that the same nuclear factor related to the CTF/NF1 nuclear protein family binds to elements which contain the (−155)TGGGCCAC-CTTCCAAT(−170) and (−37)AGCCAAT(−43)

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sequences [6,7]. This ubiquitous factor was considerably enriched in the liver nuclear extracts in comparison to the spleen, kidney or embryonic liver extracts [6].

Here, we present data on a protein termed $LspA_1$ which binds to sequences further upstream of the TAT gene (from −210 to −313 bp). Factor $LspA_1$, assayed by gel retardation and ExoIII-footprinting techniques, appears to be a liver-specific nuclear protein.

2. MATERIALS AND METHODS

DNA fragment A (fig.1) that was used as a ^{32}P-probe in gel-retardation and exonuclease III-footprinting assays represents the sequence from nucleotide −210 to −313 of the 5′-flanking region of the rat TAT gene. It was excised with *HpaI* from the plasmid pUTATS2.7 [8] kindly provided by G. Shütz and subcloned into the *Hin*cII site of the pUC12 polylinker. To generate 5′-end-labelled fragment A, the plasmid (pTAT *Hpa*I/12) was cleaved at the *Hin*dIII site, dephosphorylated with calf intestinal alkaline phosphatase, labelled with [γ-^{32}P]ATP using T_4 polynucleotide kinase, redigested with *Eco*RI, and the labelled fragment A purified by agarose gel electrophoresis. The nuclei from adult rat liver, spleen, and embryonic liver were prepared according to Hewish and Burgoyne [9]. Isolated nuclei were extracted with 0.35 M NaCl and solubilized proteins were fractionated by $(NH_4)_2SO_4$ precipitation and DNA-cellulose chromatography essentially as in [10,11] for isolation of the C4 protein fraction except that NaCl instead of $(NH_4)_2SO_4$ was used to elute proteins from the DNA-cellulose. The protease inhibitors PMSF and sodium metabisulfite were added to all buffers at final concentrations of 1 mM. The gel-retardation assay was carried out essentially as described by Fried and Crothers [12] with an equimolar mixture of poly(dA-dT)·poly(dA-dT) and poly(dA)·poly(dT) used as the non-specific competitor DNA. ExoIII footprinting was carried out as described by Cordingley et al. [13]. For both gel-retardation and footprinting assays, identical buffers, competitor DNA and incubation conditions were used.

3. RESULTS AND DISCUSSION

DNA sequences within nucleosome-free chromatin regions are the most likely elements for specific interactions with *trans*-acting regulatory proteins. Fragment A (fig.1) that covers the 5′-side of the DNase 1-hypersensitive site I of the rat TAT gene was used to assay for sequence-specific DNA-binding factors from rat-liver nuclei by the gel-retardation technique. Fig.1 shows the effect of adding nuclear proteins from adult liver (lanes 1–5, panels A and B), spleen (lanes 6–10, panel A) or embryonic liver (lanes 6–9, panel B) on the electrophoretic behaviour of ^{32}P-labelled DNA frag-

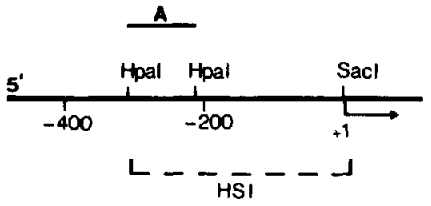

Fig.1. Restriction map of the rat TAT gene 5′-flanking region. Numbers indicate the positions of the nucleotides upstream of the major transcriptional start site (+1). Bracket marks the location of the DNase 1-hypersensitive site I (HSI) that has been mapped by Becker et al. [1]. The short horizontal line shows DNA fragment A used as a ^{32}P-probe in this study.

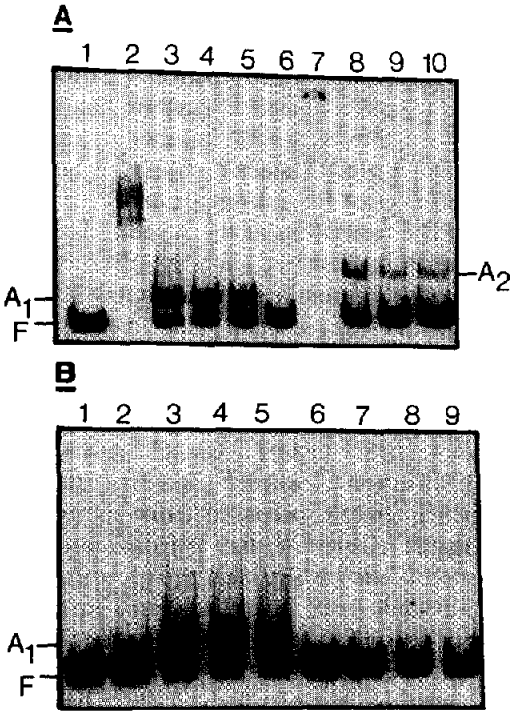

Fig.2. Gel-retardation analysis of nuclear proteins specifically binding to DNA fragment A. Adult rat liver nuclear proteins (A, lanes 2–5, ~90 ng/μl), or spleen nuclear proteins (A, lanes 7–10, ~90 ng/μl) were incubated with ^{32}P-end-labelled fragment A and various concentrations of the non-specific competitor DNA prior to electrophoresis. Lanes: 2,7, no competitor added; 3,8, 25 ng/μl; 4,9, 75 ng/μl; 5,10, 150 ng/μl. In lanes 1,6 of A, no protein was added. On (B) labelled fragment A was incubated with 300 ng/μl of the non-specific competitor DNA and 0, 35, 75, 150 and 250 ng/μl of adult rat liver nuclear proteins (lanes 1–5, respectively) or 40, 80, 150 and 300 ng/μl of embryonic liver nuclear extract (lanes 6–9, respectively).

ment A. Only one distinct band of slower mobility (A_1) was observed. Complex A_1 was obtained using either a constant concentration of the proteins and varying concentrations of non-specific DNA (lanes 2–4, panel A) or a constant concentration of non-specific DNA and increasing amount of proteins (lines 2–5, panel B). When no competitor DNA was added, smeared bands of non-specific complexes were seen (line 2, panel A). To demonstrate the sequence specificity of complex A_1, increasing amounts of unlabelled fragment A or an *Hpa*I-*Sac*I restriction fragment of the rat TAT gene promoter were added to labelled fragment A in the presence of adult rat-liver nuclear extract. Addition of a 10–20-fold molar excess of unlabelled fragment A efficiently competed for the factor whereas a 50–100-fold excess of the *Hpa*I-*Sac*I fragment did not compete (not shown).

With the spleen nuclear extract no bands similar to A_1 were observed, but another unique band, designated A_2, was detected (fig.1A, lanes 8–10). The properties of the spleen nuclear factor responsible for the formation of the A_2-complex are currently being investigated. Whilst spleen represents a terminally differentiated tissue that will never express the TAT gene, embryonic liver tissue contains cells which are competent to switch on TAT gene expression. It was therefore interesting to determine whether factors A_1 or A_2 are present in embryonic liver nuclear extract. To this end, a series of titration experiments were performed with the nuclear extract prepared from embryonic rat liver exactly in the same way as from adult rat liver and spleen. The results clearly demonstrated that in embryonic rat liver nuclear extracts no sequence-specific factor binding to the fragment A is present at all (fig.1B, lanes 6–9; and not shown). Thus, factor A_1 appears to be a liver-specific protein present only in adults; we have termed this protein $LspA_1$ (liver-specific protein A_1). We hypothesize that $LspA_1$ may be a *trans*-acting transcriptional activator whereas factor A_2 may act as a terminal repressor.

In order to determine the nucleotide sequence of the binding site for $LspA_1$, we have used exonuclease III-footprinting technique since it is more sensitive in comparison with commonly used DNase I-footprinting. The DNA-binding nuclear protein fraction from adult rat liver was incubated with fragment A 5′-end-labelled either at the 3′

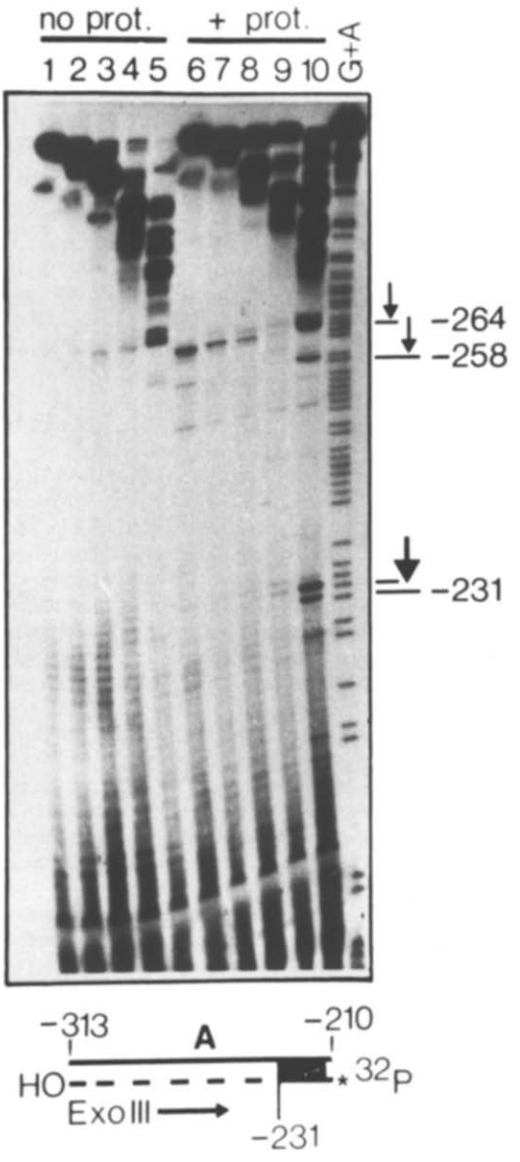

Fig.3. Exonuclease III protection mapping of $LspA_1$ binding to fragment A. The probe labelled as shown in the lower part of the figure was incubated in the absence (lanes 1–5) or presence (lanes 6–10) of adult rat liver nuclear extract (250 ng/ml) plus 300 ng/μl of the non-specific competitor (same conditions as those used in fig.2B, lane 5). Exonuclease III treatments were as follows. Lanes: 1,6, 500 U/μl; 2,7, 1000 U/μl; 4,9, 9000 U/μl; 5,10, 18000 U/μl. Exonuclease III-resistant fragments were electrophoresed in parallel with a Maxam-Gilbert sequencing ladder (lane G + A). Two minor boundaries and the major exonuclease III stop at −231 are indicated by arrows beside the sequence ladder.

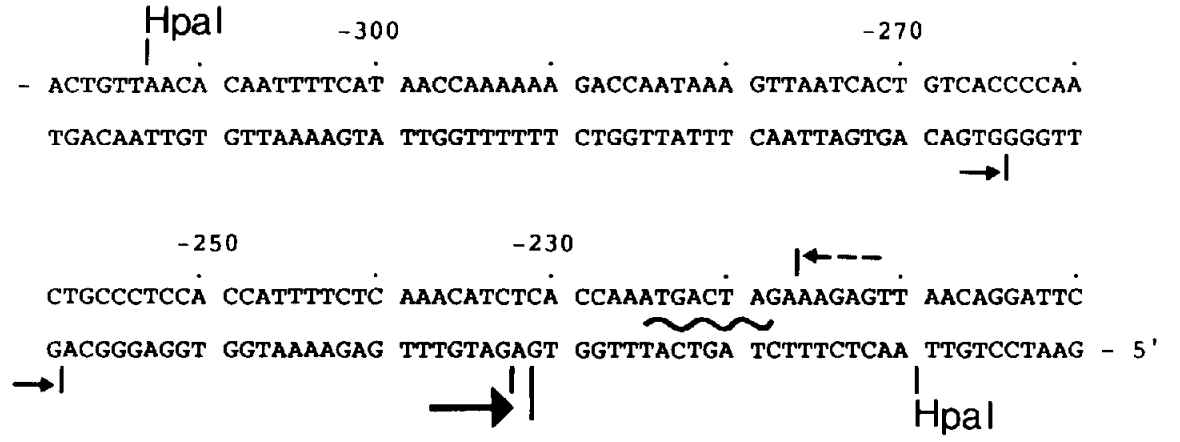

Fig.4. DNA sequence of fragment A showing exonuclease III-protected region. A sequence homologous to binding sites for the Ap-1 [16–18], PEA1 [15] or GCN4 [12] nuclear factors is indicated by wavy underlining.

*Hpa*I site (−210) to map the 5′-border of protein binding on the non-coding strands, or at the 5′ *Hpa*I site (−313) to map the 3′ border on the coding strand. Fig.3 shows that addition of protein to the fragment labelled at the 3′ *Hpa*I site results in predominant termination of exonuclease III digestion at positions −231 and −232 (lane 10). These termination sites are clearly specific for added nuclear protein fraction since corresponding termination is not observed in control digestions of fragment A in the absence of proteins (fig.3, lanes 1–5). As the defined boundary is situated just 20 bp from the 3′-end of fragment A, factor $LspA_1$ binds within the sequence from −231 to −210. When fragment A was labelled at the 5′ *Hpa*I site and used for exonuclease III-footprinting, one termination site was observed at around −216 (dashed arrow, fig.4), but the band could not be resolved well on the sequencing gel because of its proximity to the 3′-end of the fragment (not shown).

Thus, defined boundaries bracket sequences from approximately −215 to −231/−232 as shown in fig.4. The central part of this protected region includes the pentameric sequence TGACT that is found in recognition elements' binding to a closely related family of transcription factors which includes yeast GCN4 [14], mouse transcription enhancer factor PEA1 [15], avian sarcoma virus v-*jun* oncogene [16] and human activator protein 1 (Ap-1) [17]. In addition to functioning as a basic transcription factor, Ap-1 is an enhancer-binding protein that mediates induction by phorbol ester tumour promoters [18]. We have no direct evidence that the factor $LspA_1$ is actually related to the Ap-1. It should be noted, however, that the rat TAT gene belongs to the family of phorbol ester-inducible genes because its expression in the Morris hepatoma 7777 cells could be 4–7-fold increased by the treatment with 12-*O*-tetradecanoylphorbol 13-acetate (Adler, V.V. et al., unpublished). Current research is directed towards testing whether the DNA sequence bound by the factor $LspA_1$ is involved in the induction of the rat TAT gene by phorbol esters.

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REFERENCES

[1] Becker, P.B., Renkawitz, R. and Schütz, G. (1984) EMBO J. 3, 2015–2020.
[2] Jantzen, H.-M., Strähle, U., Gloss, B., Stewart, F., Schmid, W., Boshart, M., Miksisek, R. and Schütz, G. (1987) Cell 49, 29–38.
[3] Becker, P.B., Gloss, B., Schmid, W., Strähle, U. and Schütz, G. (1986) Nature 324, 686–688.
[4] Becker, P.B., Ruppert, S. and Schütz, G. (1987) Cell 51, 435–443.
[5] Lobanenkov, V.V., Adler, V.V. and Klenowa, E.M. (1987) in: Physicochemistry of DNA and Molecular Mechanisms of Genome Functioning, pp.114–115, Mettsniereba, Tbilisi.

[6] Lobanenkov, V.V., Klenowa, E.M. and Adler, V.V. (1988) Proc. Natl. Acad. Sci. USA 298, 746–750.
[7] Klenowa, E.M., Adler, V.V., Gribanovsky, V.A., Gorodetsky, S.I., Bogdanova, N.N. and Lobanenkov, V.V. (1988) Mol. Biol. (Moscow), in press.
[8] Shinomiya, T., Scherer, G., Schmid, W., Zentgraf, H. and Schütz, G. (1984) Proc. Natl. Acad. Sci. USA 81, 1346–1350.
[9] Hewish, D.R. and Burgoyne, L.A. (1975) Biochem. Biophys. Res. Commun. 52, 504–510.
[10] Emerson, B.M. and Felsenfeld, G. (1984) Proc. Natl. Acad. Sci. USA 81, 95–99.
[11] Lobanenkov, V.V., Nicolas, R.H., Plumb, M.A., Wright, C.A. and Goodwin, G.H. (1986) Eur. J. Biochem. 159, 181–188.
[12] Fried, M. and Crothers, D.M. (1981) Nucleic Acids Res. 9, 6505–6525.
[13] Cordingley, M.G., Riegel, A.T. and Hager, G.L. (1987) Cell 48, 261–270.
[14] Struhl, K. (1987) Cell 50, 841–846.
[15] Piette, J. and Yaniv, M. (1987) EMBO J. 6, 1331–1337.
[16] Angel, P., Allgretto, E.A., Okino, S.T., Hattori, K., Boyle, W.J., Hunter, T. and Karin, M. (1988) Nature 332, 166–171.
[17] Lee, W., Mitchell, P. and Tjian, R. (1987) Cell 49, 741–752.
[18] Chiu, R., Imagawa, M., Imbra, R.J., Bockoven, J.R. and Karin, M. (1987) Nature 329, 648–651.