Vol. 181, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 27, 1991 Pages 131-137

DNase I HYPERSENSITIVE SITES OF THE 5′ REGION OF THE FIBRONECTIN GENE OF THE LIVER OF THE RAT

Sanjaya Singh and M.S. Kanungo*

Molecular Biology Laboratory, Department of Zoology,
Banaras Hindu University, Varanasi 221005, India

Received September 26, 1991

SUMMARY- We have mapped the DNase I hypersensitive sites (DH-sites) of the 5′ region of the fibronectin (FNT) gene of the liver of the rat. These sites are not detectable in the cerebral hemisphere of the rat. Northern blot hybridization of the gene shows the presence of a 8 kb transcript in the liver which is induced by dexamethasone. Nuclear run on transcription shows that transcription of the FNT gene is much lower than that of the albumin gene in the liver. Gel mobility shift assay using nuclear extract of the liver shows the presence of a trans-acting factor which binds to the cAMP responsive element which overlaps with a DH-site in the region. There is thus good correlation between DH-sites of the FNT gene and its transcription in the liver.

INTRODUCTION - Fibronectins (FNTs) are high molecular weight glycoproteins having two subunits, each of 220 to 250 Kd. They are joined by S-S bonds to form a dimer (1). FNTs play a role in cellular differentiation, migration,cellular adhesion, wound healing, hemostasis and tumor metastasis (2). Hepatocytes synthesize and secrete FNTs into the plasma (3,4). Brain cells are reported not to synthesize FNT(1). A single FNT gene is present per haploid genome in rodents and man (5,6). Subunit variants of FNTs are generated by alternative splicing of the primary transcripts (7,8). The promoter region of the gene contains several transcriptional regulatory elements (9,10) including TATAA and CCAAT, and SPI binding site, GGGCGGG. Also present in this region, and in the first intron are other potential regulatory elements including consensus sequences for heat shock, glucocorticoid and cAMP. Hence it is not surprising that its biosynthesis is subjected to complex regulations during

*To whom reprint requests may be addressed.

0006-291X/91 $1.50

growth, differentiation, oncogenic transformation and physiological responses.

To understand the complex molecular mechanisms involved in the expression of the FNT gene we examined the DNase I hyper-sensitive regions in the 5′ flanking region of the gene of the liver of the rat. The presence of these sites in a gene often correlates with its expression and binding sites for regulatory proteins (11). Transcription of the gene was studied by dot-blot and northern blot hybridizations and nuclear run-on transcription. We report the presence of at least three DH-sites in the 5′ flanking region of the gene. One of them is in the region where a consensus sequence for CRE is present. Using a 25-mer oligo-DNA containing the CRE, we show the presence of a nuclear factor that binds to this sequence.

MATERIALS AND METHODS

Materials - The FNT probes used were (i) 1.2 Kb EcoRI/Pst I fragment derived from a 1.5 Kb genomic clone P-RP1 (9) and (ii) a cDNA clone containing the exons encoding the 10th and 11th type I repeat near the -COOH terminal of the molecule (7). 5′α (^{32}P)dCTP and 5′α (^{32}P)UTP were purchased from BARC, India, DNase I from Pharmacia, Sweden, and restriction endonucleases from New England Biolabs, USA. Other biochemicals were of analytical or molecular biology grade. Male Wistar albino rats of about 25-week were used.

Methods- Isolation of nuclei and DNase I digestion - Nuclei from liver and cerebral hemisphere were purified essentially as described previously (12). Purified nuclei were washed and suspended in 0.34 M sucrose, 15 mM Tris-Cl; pH 7.4, 15 mM NaCl,60 mM KCl, 15 mM β-ME, 0.5 mM spermidine, 0.15 mM spermine and 0.2 mM PMSF at a DNA concentration of 1 mg/ml . The nuclear suspension was preincubated for 5 min at 25°C with 5 mM MgCl2. DNase I was then added and digestion was carried out for 10 min at 25°C, DNA was then purified (13) and digested with restriction enzymes to completion as mentioned in legends for figures.

Gel electrophoresis and Southern blotting - Aliquots containing 20 μg of DNA digested with both DNase I and restriction enzymes (REs) were electrophoresed in 1% agarose gels, transferred onto nytran membrane, and hybridized to 1.2 Kb probe labelled by 32P by random primer method and autoradiographed (5).

Run-on nuclear transcription - It was carried out (14) for 45 min at 25°C and transcripts were purified (15). About 10^7 CPM RNA was then hybridized to plasmids containing the probe, applied onto nytran strips and either autoradiographed or counted for incorporated radioactivity.

Dot and northern blot hybridization -Total cellular RNA was purified (16) from the liver and brain of rats.Dexamethasone, 5 mg/100 g body wt. was administered intraperitonially 24 hours before sacrificing the rats as indicated in the figures. Slot-blot and northern blot hybridizations were carried out using cDNA probe (17).

Gel mobility shift assay- Nuclear extracts were prepared from the liver essentially according to Dignam et al. (18). Gel mobility

shift assay was carried out according to MacDonald et al. (19). The 25-mer DNA (Fig. 5) containing the CRE sequence was cloned in pUC 19 vector in EcoRI/Hind III site. This was then gel purified, labelled at 5′ and used for gel mobility shift assay.

RESULTS

Three hypersensitivesites are seen in the 5′ region of the gene after hybridization of DNA fragments with the 1.2 Kb labelled probe (Fig.1). One is in the CRE region, the second very near to the transcription start site and the third is in the first intron as indicated in the map. The site present in the intron is sensitive to endogenous nucleases as 1.5 Kb band is produced without adding exogenous nuclease. The presence of two sites, one at CRE and another at transcription initiation site was confirmed by carrying out digestion of DNA fragments by EcoRI

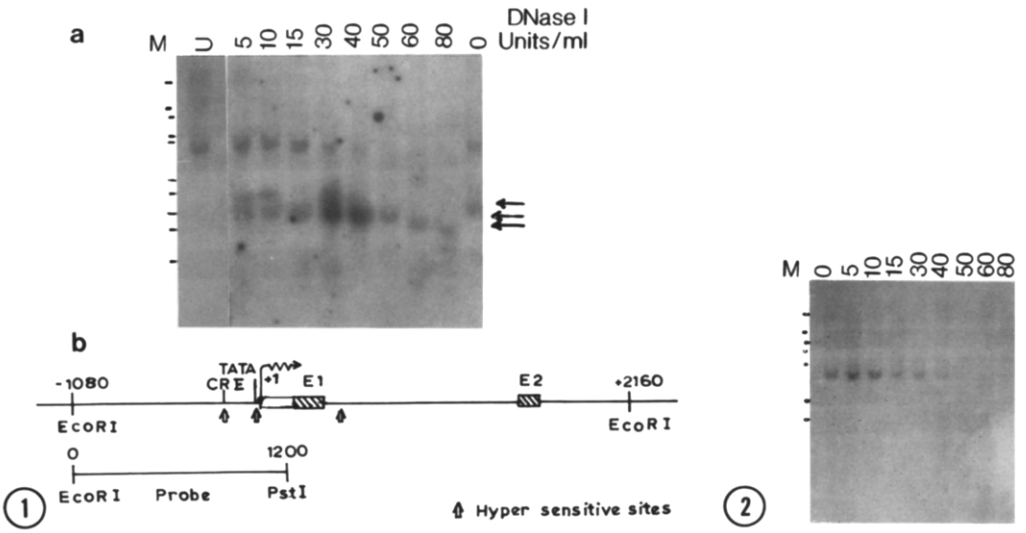

Fig. 1.(a) Nuclease hypersensitive sites in the 5′ region of fibronectin gene of the liver of the rat. Nuclei from liver were digested with DNase I at the concentrations indicated above each lane in the autoradiogram. After digestion, DNA was purified, digested with EcoRI, electrophoresed on 1% agarose gel in TBE buffer, blotted onto nytran membrane and probed with ^{32}P-labelled FNT probe. Arrows on the right side indicate the sites cut by DNase I. M-marker (λ phage DNA digested by EcoRI and Hind III); U-high M.W. DNA digested by EcoRI and treated in similar way as DNase I digested samples.

(b) Map of the region of interest of FNT gene. E1 and E2 represent the exons. The numbers above the line represent the distance in base pairs relative to transcription start site(+1).

Fig.2. DNase I digestion pattern of 5′ region of the FNT gene of the cerebral hemisphere (CH). Nuclei from CH were purified, digested with DNase I, and again digested with EcoRI, and processed as described under Fig 1. M-marker (λ phage DNA digested with Hind III)

/PstI after DNase I digestion, and hybridizing with the same 1.2 Kb probe. The site present in the first intron could not be located as the probe (1.2 kb) does not extend up to that region (data not shown). No DH-site could be located in the gene of the cerebral hemisphere (Fig.2). Before doing this experiment, we had carried out Southern hybridization experiments using restricted fragments of high molecular weight DNA of the liver of the rat obtained after digesting it with different REs to ascertain the homology and restriction map (data not shown).

Dot-blot hybridization shows the absence of transcripts of the gene in the cerebral hemisphere, though it is abundant in the liver (Fig.3). Northern blot analysis shows that the average size of the transcript is about 8 kb (Fig.3) whose level increases significantly after dexamethasone administration. Run-on transcription assay shows that the rate of transcription of this gene in the liver is less than 10% of that of albumin (Table I, Fig.4).

Gel mobility shift assay using the 25-mer oligo-DNA shows the presence of a factor that binds to consensus CRE sequence of the promoter region (Fig.5).

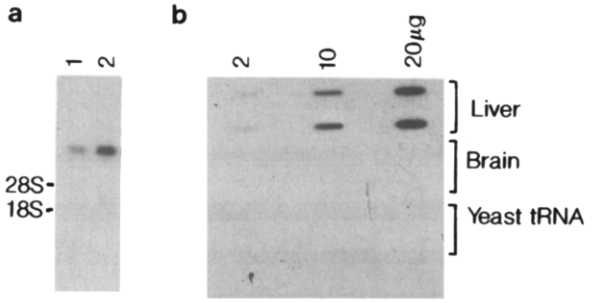

Fig.3.(a) Northern blot hybridization of total RNA to ^{32}P-labelled fibronectin cDNA. Total RNA was isolated from rat liver and fractionated on 1% denaturing agarose gel in formaldehyde, transferred to nytran membrane and hybridised to cDNA of FNT. 1-normal; 2- dexamethasone treated.

(b) Dot-blot hybridization of total RNA to ^{32}P-labelled fibronectin cDNA. 2, 10, 20μg of total RNA from liver and cerebral hemisphere was slot blotted on nytran membrane along with yeast tRNA and hybridised to cDNA of fibronectin.

Table I

CPM (^{32}P) of nuclear transcripts hybridised to nytran bound plasmid DNA (Fig 4)

No. of Expts.	FNT gene 20 µg	Albumin gene 2 µg	pBR322 20 µg
1	47	88	12
2	40	79	10
3	43	92	11
Mean	43.3	86.3	11

Transcriptional activity of the fibronectin gene of the liver of the rat in nuclear run-on assay. Purified nuclei from the liver of adult rats were incubated with ^{32}P-UTP for 45min. Labelled nuclear RNA was isolated and hybridized to nytran strips loaded with plasmids containing the 5′ region of fibronectin gene, albumin gene and pBR322 without any insert. Data are counts from three separate sets of experiments.

DISCUSSION

Nuclease hypersensitive sites are believed to be short nucleosomal free regions which mark sites where access is required for regulatory proteins to specific DNA sequences (20).

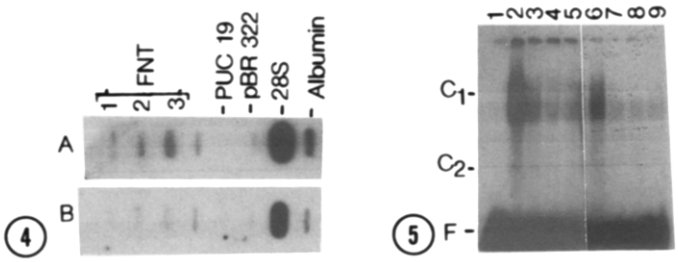

Fig. 4. Measurement of the rate of transcription of fibronectin gene in isolated nuclei of the liver of the rat. Transcripts were hybridised to plasmids containing various genes as indicated. Three concentrations 1-0.2, 2-2, 3-10 µg of plasmid containing the fibronectin gene were spotted. 20 µg of PUC19, pBR322 and 0.1µg of yeast tRNA were used. 2 µg of plasmid containing 28S and albumin cDNA were used. B- with α-amanitin.

Fig. 5. Gel retardation assay for the binding of the CRE of FNT gene to the nuclear extract of the liver of the rat. Nuclear extract was incubated in the presence of ^{32}P-labelled 25-mer double stranded DNA which contained the CRE and was subjected to electrophoresis on non-denaturing 4% polyacrylamide gel. Lane 1-No extract; Lane 2-with 20µg of extract; Lanes 3,4,5 - 1, 2, 4µg of poly dIdC, respectively with 20µg of extract; Lane 6-20µg of extract; Lanes 7,8,9 - 20µg of extract with 10, 50, 100 fold excess of unlabeled 25-mer DNA. C1, C2 nucleoprotein complex; F-free DNA.

It is well established that sites within the promoter region of active or potentially active genes are hyper-sensitive to various nucleases (11, 20,21). The 5' flanking region of the FNT gene of the liver of the rat is nuclease hypersensitive, but in the cerebral hemisphere it is not. Transcripts of the gene are also not detectable in the cerebral hemisphere which indicates that either it is not transcribed or it is too low. A DH- site occurs in the CRE region. It has been reported that the FNT gene is induced by cAMP or by agents which enhance the level of intracellular cAMP (22).It has also been reported that a factor binds to this sequence in different human cell lines (23). We have shown here that a factor is also present in the liver of the rat that binds to this sequence and may regulate transcription of the gene. Our data on run-on transcription of FNT and albumin genes are in agreement with the relative amounts of the two proteins present in the plasma and their turn-over rates. Tamkun and Hynes (24) have suggested that hepatocytes in in vitro culture secrete FNTs at about 4% of the rate of albumin. Dexamethasone is reported to stabilize FNT mRNA (22). This may be the reason for the enhanced signals in the northern blots.

These data clearly show that the expression of the FNT gene in the liver of the rat, in contrast to that of the brain, is due atleast partly to three DH sites, two located 5' to the transcription start site and one at the first intron. One of these sites overlaps with the CRE consensus sequence in the promoter region and binds to a trans-acting factor which may be involved in transcription. It would be of interest to find out if the other two sites also bind to the trans-acting factors to confer transcription competence to the FNT gene.

Acknowledgments. We thank Prof. R.O.Hynes, MIT, Cambridge, U.S.A. for the gift of 1.5 Kb genomic probe of FNT gene and its cDNA. The 25-mer oligo-DNA was kindly supplied by Dr. S.K. Brahmachari, Indian Inst. of Science, Bangalore. This research was funded by a grant from the Dept. of Science & Technology, Govt. of India to M.S.K. S.S. thanks the Council of Scientific & Industrial Research, New Delhi, for a research fellowship.

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