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CHANGES IN EXPRESSION AND CRE BINDING PROTEINS OF THE FIBRONECTIN GENE DURING AGING OF THE RAT

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SUMMARY - The level of plasma fibronectin (pFNT) which is synthesized in the liver and secreted to the plasma has been found to decrease with age. Nuclear run-on transcription, slot-blot, and northern blot analysis also show that the expression of the FNT gene is lower in the liver of old rats. cAMP is known to influence the expression of the gene. Gel mobility shift assay using an oligonucleotide containing the cAMP responsive element (CRE) and nuclear extract of liver shows the presence of trans-acting factors that bind to CRE. These factors change with age. This may be the reason for the lower expression of the gene in the old rat.

INTRODUCTION - Fibronectin (FNT) is a cell adhesion, dimeric glycoprotein (mol. wt. ~4,40,000) that mediates a variety of cellular functions including cell migration, phagocytosis, cytoskeletal organisation, cell adhesion and cell differentiation (1,2). It is present in plasma, interstitial fluids and extra-cellular matrices. FNT mRNA is transcribed from a single gene, but a number of distinct isoforms are generated by alternative splicing of the transcript (3-5). Functions of various forms of FNTs derived from alternatively spliced mRNAs have been studied (6). However, information on the regulation and expression of the gene during aging is not available.

The 5' and of the FNT gene contains clusters of transcriptional regulatory elements including cAMP responsive element (CRE) (7). In our earlier report we have shown the presence of a DH-site in the CRE region of the gene (8). It has

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been shown that the FNT gene is induced by cAMP and by agents which increase intracellular cAMP levels (9).

We have measured the pFNT level by immunoprec-ipitation, the steady state level of FNT mRNA, the rate of its transcription, and have also carried out gel mobility shift assay of the CRE with the nuclear extract of liver to examine the changes which take place in its expression, and the trans-acting factors that bind to the CRE. Our results show that the expression of the gene decreases with age. Also the nuclear factors that bind to the CRE change with age.

MATERIALS AND METHODS

Materials- The probe used was a cDNA clone (3). 5'α^{32}P dCTP and 5' (α^{32}P) UTP were purchased from BRIT, India, and antibodies from Sigma Chemical Co. USA. Male albino rats, 2-(immature),25-(young) and 120-week (old), were used.

Methods- Immunoprecipitation and gel electrophoresis- Immunoprecipitation of pFNT was carried out essentially as described by Hynes (2). Gel electrophoresis was carried out according to Tamkun and Hynes (10).

Run-on nuclear transcription - It was carried out (11) for 45 min at 25° C and the transcripts were purified. It was then hybridized to plasmids containing the probe (8).

Slot and northern blot hybridization - Total cellular RNA was purified from the liver (12). Slot-blot and northern blot hybridization were carried out using the cDNA probe (8).

Nuclear extract and gel retardation assay - A synthetic 25-mer dsDNA

CRE

5' AATTCCCCG**TGACGTCA**CCCGGACA 3'
3' GGGGC**ACTGCAGT**GGGCCTGTTCGA 5'

representing -144 to -166 from the transcription start site (+1) of the FNT gene and containing the consensus sequence for CRE was used for gel retardation assay. It has flanking sequences to enable the cloning at EcoRI/Hind III site in the pUC 19. Nuclear extract was prepared essentially according to Gorski (13). Gel mobility shift assay was carried out according to Verri et al (14). About 10,000 cpm ^{32}P-lebelled dsDNA was incubated for 10 min with 2 µg poly-dI:dC and increasing concentrations of cold 25-mer dsDNA in reaction mixture. The reaction mixture was electrophoresed on 5% polyacrylamide gel in 0.5 X TBE buffer. After electrophoresis, the gels were dried and autoradiographed.

RESULTS AND DISCUSSION - Electrophoresis of purified and immunoprecipitated pFNT in high resolution SDS polyacrylamide gel under reducing condition shows a doublet (Fig.1 lane 1) as reported earlier (2). Immunoprecipitated pFNTs from 2, 20 and 125 week old rats also show a similar pattern (Fig.1, lanes 2,3 & 4). The level of pFNT of 120 week old rats is, however, lower in

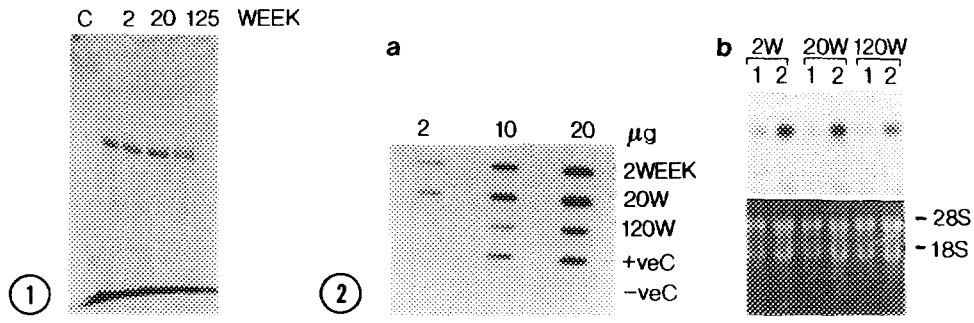

Fig. 1. SDS polyacrylamide gel electrophoretic pattern of immunoprecipitated plasma FNT of rats of different ages. C-control- purified and precipitated fibronectin from adult plasma.

Fig. 2(a). Slot-blot hybridization of total RNA to ^{32}P-labelled FNT cDNA- 2, 10 and 20 µg of total RNA from liver was slot-blotted on nytran membrane and hybridized to cDNA of fibronectin. +ve control: 0.1, 0.5 and 1pg cold probe. -ve control: yeast tRNA.

Fig. 2(b). Northern blot hybridization of total RNA to ^{32}P-labelled FNT cDNA. Total RNA was isolated from rat liver, 5 & 10 µg total RNA from the three ages was fractionated on 1% denaturing agarose gel in formaldehyde, transfered to nytran membrane and hybridized to FNT cDNA. Bottom- Ethidium bromide stained gel.

comparison to those of 2 and 20 week old rats. Antibody was used far in excess for complete precipitation of pFNTs from the plasma. The supernatant obtained was checked for complete precipitation.

Slot-blot hybridization using total cellular RNA purified from the liver shows that the steady state level of FNT mRNA is lower in old rats (Fig.2a). It is contrary to those of culture conditions. It has been shown that in late passage fibroblasts the overall expression of FNT mRNA increases (15), though the protein produced has reduced binding affinity (16).

Northern blot analysis (Fig.2b) also shows that FNT transcripts are lower in 120 week old rats. The average size of the FNT mRNA is ~8 Kb which is the same in the three ages. The size of the mRNA is in agreement with the earlier reports (7,8).

Run-on transcription assay (Fig.3, Table 1) shows that transcription of FNT gene is lower in old rats. In our earlier report (8) we have shown that transcription of FNT gene is less than 10% of that of albumin. One µg of α-amanitin blocks the transcription of the gene. The levels of several mRNAs of the liver decreases between 6 to 29 months of age, e.g. 2µ globulin (85%), aldolase (30%), cytochrome P-450b (50%), superoxide dismutase (30%) (17,18). Several workers have reported changes in

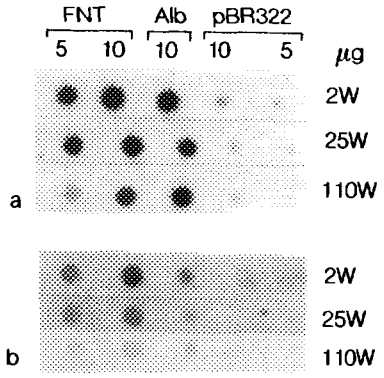

Fig. 3(a). Measurement of the rate of transcription of FNT gene in isolated nuclei of the liver of the rat. ^{32}P-labelled transcripts were hybridized to plasmids containing genes spotted on nytran membrane. (b) Similar experiment as in (a) with 1 μg of α-amanitin run-on reaction mixture.

transcription of specific genes in many organisms as they age (19). The decrease in the rate of run-on transcription in old liver may be due to a decrease in the RNA polymerase II activity, or due to non-availability of the DNA template for transcription because of condensation of the chromatin with increasing age (20). Alternatively, a decrease in the formation of initiation complex for RNA synthesis including the interaction of transcription factors with the DNA may reduce transcription. One such possibility was examined by gel mobility shift assay of CRE.

Since a DH-site was mapped in the CRE region (8), experiments were carried out to find out if regulatory factors are involved. While doing the initial titration, it was found that the same amount of nuclear extract prepared from the liver of old

Table 1

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

No. of expt.	FNT gene 20 μg		Albumin gene 2 μg		pBR322 20 μg	
	20 W	130 W	20 W	130 W	20 W	130 W
1.	47	30	88	64	12	11
2.	40	28	79	63	10	10
Mean	43.5	29	83.5	62.5	11	10.5

Transcriptional activity of the fibronectin gene of the liver of 20 and 130 week old male rats in the nuclear run-on assay. About 10^7 counts of ^{32}P labelled transcripts were hybridized to plasmids containing various genes. Data are counts from two different sets of experiments.

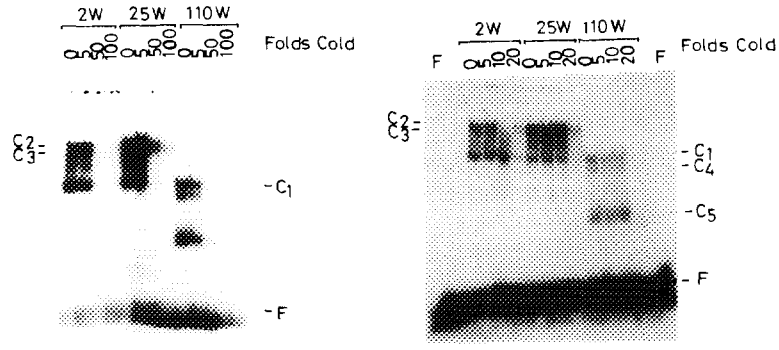

Fig. 4. Mobility shift assay for binding of CRE of FNT gene to proteins in the nuclear extracts of the liver of 2-, 25-, 110 W old rats. ^{32}P-labelled 25-mer dsDNA containing the CRE was incubated with 10 μg of nuclear extract, 2 μg of poly dI-dC and different concentrations of cold CRE as mentioned above each lane in reaction mixture. It was resolved on 5% polyacrylamide gel in TBE buffer. C1-C5 different nucleo-protein complex. F- free DNA.

rats retarded lesser amount of the labelled DNA (data not shown). It indicated that the levels of the factors which bind to CRE are lower in old rats. Titration with poly-dI:dC and cold CRE showed the presence of three CRE binding factors (Fig. 4) in each age. However, two of these are different in old rats. They appear to be smaller in size than those found in the liver of immature and young rats.

The group of Kornblihtt has shown the liver specific DNase I foot-printing pattern of the promoter of human FNT gene. They have shown that the CRE is protected. They have also shown the presence of two factors in the nuclear extract that bind to CRE of the FNT gene (21). Several CRE binding proteins (CREB) have been identified. One CREB is 43 KDa (22) and another is CREB-1. C-jun also binds to the CRE but with a lower affinity than CREB or CREB-1 (23, 24). It has been demonstrated that interaction of CREB with CRE can influence binding of TFIID to the TATA box (25). From available reports, CRE appears to be a multifunctional element. So it appears that different nuclear proteins may mediate transcriptional stimulation through different pathways.

Our data show that the two nuclear factors of the young that do not bind to the CRE in old rats have either lost their affinity for binding to the CRE or are absent. Two factors which are present only in the liver of old rats are either age-specific or have higher affinity for the CRE in old rats. The single

nuclear factor which is present in all the ages seems to be the main CREB. Such alterations in trans-acting factors may be responsible for lower expression of the FNT gene in old rats.

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