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QUARTERLY

The bovine tyrosine hydroxylase gene associates *in vitro* with the nuclear matrix by its first intron sequence★✪✱

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Recently we have shown that *in vitro* binding of the proximal part of the human tyrosine hydroxylase gene to the nuclear matrix is correlated with its transcriptional activity. The strongest binding potential was predicted by computing for the first intron sequence (Lenartowski & Goc, 2002, *Neurosci Lett.;* 330: 151–154). In this study a 16 kb fragment of the bovine genomic DNA containing the tyrosine hydroxylase gene was investigated for its affinity to the nuclear matrix. Only a 950 bp fragment encoding the distal part of the first intron, second exon and a few nucleotides of the second intron bound to the nuclear matrix. The binding was independent of the tissue-specific tyrosine hydroxylase gene activation. The fragment was subcloned and sequenced. Computer search pointed to one potential intronic matrix attachment region with two AP1-like sites embedded in the sequence. We conclude that even if the position of the matrix binding region is conserved among the tyrosine hydroxylase genes in mammals, its tissue specificity and/or function is not preserved or is achieved by different mechanisms.

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✱*Bos taurus* tyrosine hydroxylase gene, exons 1 and 2 and partial cds. – GenBank accession number AF510493

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Abbreviations: S/MAR, scaffold/matrix attachment region; TH, tyrosine hydroxylase; NM, nuclear matrix.

Structure and function are interdependent also at the level of the cell and its substructures. Differential functioning of a given DNA sequence in specialized tissues is believed to be determined by its nuclear structure. Tissue-specific DNA methylation, nuclease sensitivity, nucleosome phasing and transcription factors are accompanied by an equally specific internal proteinaceous nuclear scaffold called the nuclear matrix (NM). As shown earlier the protein composition of the NM differs among tissues (Fey & Penman, 1988; Bidwell *et al.*, 1993; Korosec *et al.*, 1997), among differentiation stages of the same tissue (Dworetzky *et al.*, 1990) and between normal *vs.* tumor tissue (Fey & Penman, 1988; Getzenberg *et al.*, 1991; Partin *et al.*, 1993; Bidwell *et al.*, 1994). As a result some of the chromatin domains determined by scaffold/matrix attachment regions (S/MARs) were demonstrated to be tissue-specific (Ciejek *et al.*, 1983; Robinson *et al.*, 1983; Gasser & Laemmli, 1986).

Tyrosine hydroxylase (TH) is the first and rate-limiting enzyme of the catecholamine biosynthetic pathway. Kumer and Vrana in their review (1996) have documented that TH and its gene are subjected to multilevel regulation specific for both the developmental stage and the tissue and homeostatic stimuli. *Cis*-acting regulatory elements involved in transcriptional regulation have been carefully studied especially in the mouse and rat (Kumer & Vrana, 1996; Schimmel *et al.*, 1999). The regulatory elements which are located in the promoter region are similar in sequence and localization in human (Coker *et al.*,1988), rat (Coker *et al.*, 1988; Cambi *et al.*, 1989), bovine (D'Mello *et al.*, 1989) and mouse (Iwata *et al.*, 1992) TH genes. However, elements located in the first intron (Meloni *et al.*,1998; Albanese *et al.*, 2001) and in the 3′ flanking region (Gandelman *et al.*, 1990; Wong *et al.*, 1995) were shown to be specific for the human gene. Our results of an *in vitro* binding assay and computer search suggested that the first intron of the human TH gene is also involved in tissue specific binding to the nuclear matrix (Lenartowski & Goc, 2002).

In the present study we have investigated if the bovine TH gene is associated with the NM. The results suggest that its first intron has S/MAR properties which are not correlated with the transcriptional activity of the gene.

MATERIALS AND METHODS

Materials. The recombinant phage Lambda$_{DASHII}$TH carrying 16 kb of bovine DNA containing the TH gene was obtained from Dr. B. B. Kaplan and Dr. A. E. Gioio (Medical Center, University Pittsburgh, PA, U.S.A.). Bovine liver and adrenal glands were taken from local slaughter houses and kept at 4°C. After saline perfusion the tissues were stored at –75°C.

Probes. *Eco*RI fragments (9 and 7 kb, Fig. 1) of the insert cloned in the Lambda$_{DASHII}$TH were subcloned into the pUC19 vector. After amplification their DNA was isolated by alkaline lysis and purified through a CsCl+EtBr gradient. The plasmid DNA was digested with *Eco*RI and electrophoresed. The fragments of the bovine DNA were isolated and subsequently restricted with *Xho*I or *Bam*HI. About 100 ng of the fragments were labelled at their 3′ ends with DIG-11ddUTP using terminal transferase according to the Boehringer Mannheim protocol.

Isolation of nuclei. The method of Berezney & Coffey (1977) was used.

Nuclear matrix preparation and* in vitro *binding. The modified method of Mirkovitch *et al.* (1984) was used for nuclear matrix extraction. Briefly, 10^7 cell nuclei were suspended in 150 μl of 3.75 mM Tris/HCl, pH 7.4, 0.05 mM spermine, 0.125 mM spermidine, 20 mM KCl, 1% thiodiglycol, 1 mM sodium tetrathionate, 0.1% digitonin, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.05 mM aprotinin and leupeptin each, 1 μg/μl pepstatin A and heated at 37°C for 45 min.

Then 7 ml of 5 mM Hepes/NaOH, pH 7.4, 2 mM EDTA, pH 8.0, 10 mM lithium 3′,5′-diiodosalicylate, 100 mM lithium acetate, 2 mM KCl, 0.25 mM spermidine with the same protease inhibitor cocktail as above were added dropwise. After 10 min incubation at room temperature histone-depleted nuclei were recovered by centrifugation and washed 3 times with digestion buffer (20 mM Tris/HCl pH 7.4, 20 mM KCl, 70 mM NaCl, 10 mM $MgCl_2$, 0.05 mM spermine, 0.125 mM spermidine). The pellet of the nuclear matrices was suspended in 150 μl of digestion buffer. Two types of *in vitro* binding reactions were performed. In the A reactions 1–10 μg of sheared *Escherichia coli* DNA and 75 units of each restriction enzyme were added to the NM preparations; after 15 min the binding was started by adding labeled probes. After 2 h the same portion of fresh enzymes was added and the reaction continued for a further 2 h. In the B reactions 75 units of each enzyme were added twice at 0 and 4^{th} h of 24 h restriction, then the matrices were pelleted and the supernatant fraction was removed. The 4 h type B binding reaction was carried out in fresh 150 μl portion of restriction buffer, first 15 min only in the presence of 0.5–10 μg of sheared *E. coli* DNA and then with labeled probe. Each type of the restriction and binding reaction was performed at 37°C, 100 ng of the probes were added. The final pellet and supernatant fractions were collected and saved for DNA isolation.

Isolation of DNA bound to the matrix and released to the supernatant. Pellets of nuclear matrices were incubated in 15 mM Tris/HCl, pH 7.4, 50 mM EDTA, 1% SDS, 1 mg/ml proteinase K and supernatants were adjusted with EDTA, SDS and proteinase K to the same final concentrations. After over night incubation at 30°C phenol:chloroform:isoamyl alcohol extraction was performed and DNA was precipitated with ethanol and dissolved in TE.

Visualization of bound and unbound DNA. The matrix bound DNA and the DNA released to the supernatant were electrophoresed in 1% agarose gel with EtBr in 0.5×TBE. Southern blotting of the DNA to Hybond N^+ nylon membrane (Amersham) was followed by application of DIG antibodies conjugated with alkaline phosphatase (Boehringer Mannheim) according to the manufacturer's protocol. The membranes were exposed to Rad-Free sheet (Schleicher and Schuell) containing chemiluminescent substrate for alkaline phosphatase and to X ray film.

DNA sequencing. The *Nae*I/*Eco*RI, *Eco*RI/*Cfr*42I and *Cfr*42I fragments of the bovine DNA covering the +24/+2094 region of the TH gene were cloned into pBluescriptII KS+/– vector and sequenced from both strands with (–21) M13 forward and M13 reverse primers using the BigDye Primer Cycle Sequencing Kit (Perkin Elmer) according to the manufacturer's protocol. Sequencing products were separated in a 4% PAGE gel on an ABI Prism^{TM} 377 DNA Sequencer. Data were analysed using the DNA Sequencing Analysis and Sequence Navigator programs (Perkin Elmer).

RESULTS

Cell nuclei isolated from bovine liver and adrenal medullae were thermally stabilized and their proteins were crosslinked using sodium tetrathionate. Then histones were extracted with a mild detergent (10 mM lithium 3′,5′-diiodosalicylate). The DNA that after such extraction remains associated with the nuclear matrix was digested with restriction enzymes. The nuclear matrices obtained in this way were used for *in vitro* binding reactions with fragments of bovine DNA containing the TH gene (Fig. 1). Two types of binding reactions (A, B) were performed. They differed in the length of restriction period, simultaneous or separate reactions of restriction and binding and the presence of endogenous competitor DNA during the binding reaction.

The DNA bound to the nuclear matrix and that released to the supernatant fraction was isolated and analyzed. The amount and origin of the DNA in the supernatant fractions differed between the two types of the binding reaction. The DNA isolated from the pellet fractions consisted of these parts of endogenous DNA and labeled probe which bound to the nuclear matrix. The amount of total DNA from the adrenal medulla (n = 10) and liver (n = 6) remained in the NM preparations was 79.8±2.9% and 81.2±5.7%, respectively (data from control experiments in which restriction reactions lasted 4 h and no exogenous competitor DNA was added).

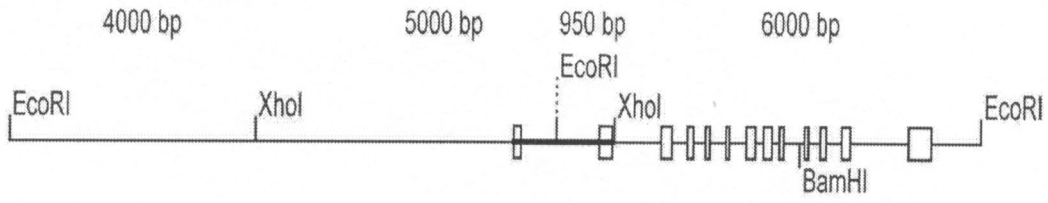

Figure 1. Restriction map of a 16 kb genomic fragment of bovine TH gene.

Numbers above give *Eco*RI/*Xho*I fragment sizes in bp. Rectangles denote exons. The sequenced part of the fragment is marked as a thick bar.

The region of bovine genomic DNA used in the *in vitro* binding experiments covered the entire TH gene and about 8.3 kb of its proximal and 0.8 kb of its distal sequences (Fig. 1). We assumed that a fragment binds the NM, i.e. it has S/MAR properties, if at least half of its probe remains in the pellet fraction. Most of the *in vitro* binding experiments were performed with *Eco*RI/*Xho*I digested matrices and probes. Their results are summarized in Table 1.

We have never observed association of the 4000 and 5000 bp probes with the adrenal medulla NM (Fig. 2b, Table 1). The same tendency but less distinct was seen for the liver nuclear matrices (Fig. 3, Table 1). According to our assumption neither the adrenal medulla (Fig. 2a) nor the liver (Fig. 3) matrices bound the 6000 bp fragment containing most of the TH coding sequence. Only the 950 bp fragment enclosing the distal part of the first intron, second exon and few base pairs of the second intron showed S/MAR properties in

Table 1. Summary of the *in vitro* binding experiments with *Eco*RI/*Xho*I digested nuclear matrices and probes.

S, supernatant; P, pellet. Number of experiments in which at least one half of the added probe was found in the pellet or supernatant fraction, respectively, are given.

Tissue	Adrenal medulla								Liver							
Probe [bp]	4000		5000		953		6000		4000		5000		953		6000	
DNA fraction	S	P	S	P	S	P	S	P	S	P	S	P	S	P	S	P
Reaction type A[1]	9	0	6	0	0	8	-	-	3	0	3	0	3	0	3	0
Reaction type B[2]	12	0	8	0	4	8	8	0	3	3	4	2	0	8	4	1
Σ	**21**	**0**	**14**	**0**	**4**	**16**	**8**	**0**	**6**	**3**	**7**	**2**	**3**	**8**	**7**	**1**

[1]4 h of simultaneous restriction and binding in the presence of both endogenous [bovine DNA released to the supernatant fraction (17.5±4.8 μg for the adrenal medulla NM and 20.2±10.1 μg for the liver)] and exogenous (0.5–10 μg of sheared *E. coli* DNA) competitor DNA; [2]24 h of restriction + 4 h of binding in the presence of exogenous competitor DNA.

both studied tissues (Figs. 2b and 3, Table 1). It constitutes a weak S/MAR as its *in vitro* affinity for NM disappeared when the same sequence was part of a 4300 bp *Eco*RI/*Bam*HI fragment (not shown).

We have subcloned and sequenced fragments of the bovine DNA from +24 bp in the first exon to +2094 bp in the second intron of the TH gene (GenBank accession number AF510493). The MAR-Wiz program (http://www.futuresoft.org/MAR-Wiz/), designed for finding potential S/MARs, was applied to the sequence. Also in this search, like in the *in vitro* binding assay, the sequences outside the 950 bp *Eco*RI/*Xho*I fragment (preceding the +725 bp and following the +1678 bp) did not show any NM binding potential. Inside the 950 bp fragment bound *in vitro* by the matrices MAR-Wiz found on both strands three *ORI* sequences, three topoisomerase II sites and two stretches longer than 20 nucleotides lacking G nucleotides on one strand, and predicted one attachment region (Fig. 4a).

Additional structural NM binding motifs were defined by Boulikas (1995). Figure 4b shows their presence in the sequence of the 950 bp fragment. Inside the 200 bp sequence of the predicted S/MAR two AP1-like elements were also found.

DISCUSSION

Our preparation method produced matrices very rich in DNA. The DNA retained in the pellet fraction corresponds to a nearly 1000-fold excess over the used amount of the longest probe (6000 bp) and an over 5600-fold excess over the shortest one (950 bp). The high content of DNA in the nuclear matrices resulted partially from using infrequently cutting restriction enzymes (*Eco*RI, *Xho*I, *Bam*HI). Additionally, some of the restriction sites can be protected by NM proteins against endonuclease attack as was observed by Käs & Chasin (1987) and Greenstein (1988). It was also shown (Mirkovitch *et al.*, 1984; Berezney & Bucholtz, 1981) that after thermal and chemical crosslinking treatments the matrices should be enriched in the protein component. We have confirmed (Lenartowski *et al.*, 1997) by the results of SDS/PAGE experi-

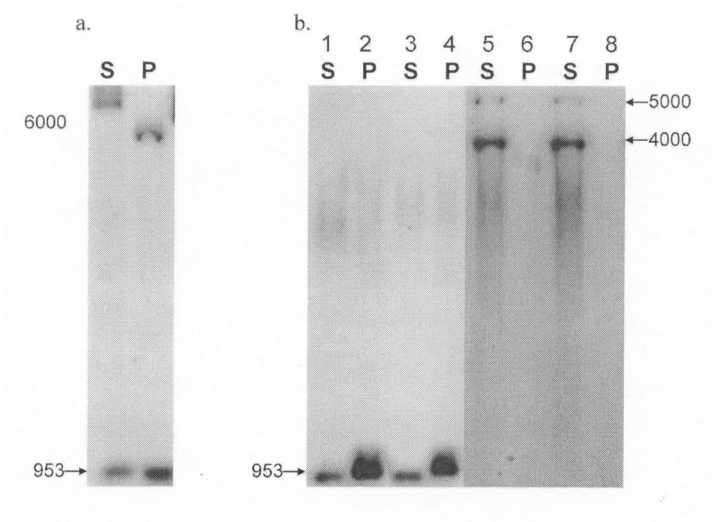

Figure 2. *In vitro* binding assay type B with bovine NM from adrenal medullae.

S and P, DNA isolated from supernatant and pellet fraction, respectively. **a**. Reaction with 950 + 6000 bp probes and 0.5 μg of competitor DNA. Whole amount of supernatant DNA and one third of pellet DNA were loaded onto the gel. For unknown reasons the 6000 bp probe had different mobility in S and P lanes. **b**. Reaction with 950 bp (lanes 1–4) and 4000 + 5000 bp probes (lanes 5–8) with 5 μg (lanes 1, 2, 5, 6) or 1 μg (lanes 3, 4, 7, 8) of *E. coli* DNA as an exogenous competitor. Equal parts of DNA from both fractions were analyzed.

ments that the matrices obtained in our experiments contained a wide range of proteins (13–205 kDa). Thus conditions were created

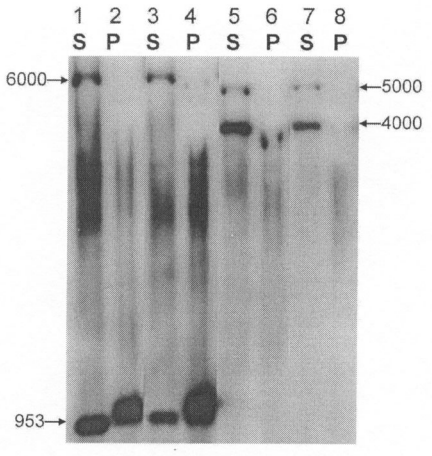

Figure 3. *In vitro* binding assay type B with the NM from bovine liver and 950 + 6000 bp (lanes 1–4), and 4000 + 5000 bp probes (lanes 5–8).

Exogenous competitor (5 μg, lanes 1, 3, 5, 7 or 1 μg, lanes 2, 4, 6, 8) was added. Equal parts of DNA from both supernatant (S) and pellet (P) fractions were analyzed.

in which on the one hand a huge amount of endogenous DNA is retained in the NM forming complexes with the proteins normally loosely associated with the nuclear scaffold, but on the other hand the diversity of the NM proteins better represented the variety of intranuclear DNA–protein interactions.

The TH gene is active in the adrenal medullae and silent in the liver (Kilbourne *et al.*, 1991). We checked by *in vitro* binding assay if its DNA is bound to the nuclear matrix of both tissues and if there are any tissue specific differences in its NM affinity. Sixteen thousand base pairs of bovine genomic DNA enclosing the TH gene were assayed. We found that the 950 bp *Eco*RI/*Xho*I fragment enclosing the distal part of the first intron, second exon and a few base pairs of the second intron binds to the matrices from both tissues. Binding of the same fragment of the TH gene by the nuclear matrices from the liver and adrenal medullae does not necessarily mean that association of the chromatin with the NM is not involved in the tissue specific regulation of the bovine TH gene. The results of our Southwestern experiments suggested that at least one of the nuclear matrix proteins specific for the adrenal medulla binds the 7 kb *Eco*RI region of the bovine TH gene enclosing the 950 bp fragment (Lenartowski *et al.*, 1997). Thus, different tis-

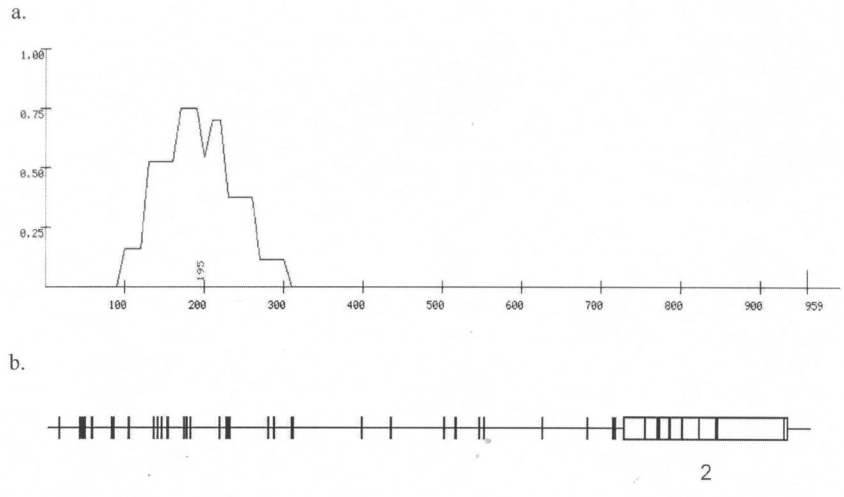

Figure 4. Computer analysis of the 950 bp fragment of the bovine TH gene.

a. MAR-Wiz search. Window width of 100 bp and 10 bp slide distance were used. x- and y-axis shows the distance from the beginning of the 950 bp fragment and the matrix association potential counted by the program respectively. **b**. Search for S/MAR structural motifs as defined by Boulikas (1995). Numerous trihomonucleotides, 7–12-nucleotide long streches of purines or pyrimidines, $(RY)_{2\text{-}4}$, $(CT)_3$, $(CA)_{1\text{-}2}$, $(CG)_{1\text{-}2}$, $(AG)_{1\text{-}3}$ were found and shown as vertical lines. The position of the second exon (D'Mello *et al.*, 1989) is denoted by box and number 2.

sue specific NM proteins could be involved in the binding of the same DNA region.

The sequencing and subsequent MAR-Wiz search of the +24/+2094 bp fragment of the bovine TH gene also predicted the existence of a potential matrix attachment region inside the first exon. The length of the putative S/MAR is about 200 bp which is in agreement with the experimental data for the matrix binding regions of other genes (Cockerill & Garrard, 1986; Gasser & Laemmli, 1986). We checked that the NM proteins from bovine liver and adrenal medullae retarded, in a tissue-specific manner, the gel mobility of this 200 bp intronic fragment of the bovine TH gene (Lenartowski *et al.*, unpublished). Further experiments will be performed to identify the NM proteins involved in the binding of the 200 bp S/MAR fragment of the bovine TH gene.

A further sequence search showed that the putative 200 bp TH S/MAR contained more structural elements for NM binding and two AP1-like sites. As van Wijnen *et al.* (1993) have shown that the AP1 factor can be associated with the nuclear matrix, thus raises the possibility that AP1 is involved in the bovine TH gene interaction with NM or that an enhancer colocalizes with S/MAR in the first intron of the TH gene. Coexistence of enhancers and S/MARs is well documented (Boulikas, 1995). Several examples of functional enhancers containing AP1 sites which are localized in the first intron have been published (Katai *et al.*, 1992; Gauss *et al.*, 2002; Seshasayee *et al.*, 2000; Cohn *et al.*, 2001; Lu *et al.*, 2001; Joaquin *et al.*, 2002). All of them mediate tissue-specific expression of their genes. If the same is true in the case of the TH gene, it would give good explanation why proper expression of the gene has never been observed in transgenic animals which always had a reporter gene under control of only the 5′ upstream region of the TH gene.

The S/MAR localized in the first intron of the TH gene was also found by the MAR-Wiz program in the human sequence. However, *in vitro* binding of the human TH sequence to the bovine nuclear matrices was tissue specific to the adrenal medulla matrix only. Both a computer analysis and the *in vitro* binding assay suggested that more than one S/MAR existed in the –2300/+2300 region of the human TH gene (Lenartowski & Goc, 2002). The putative strongest S/MAR was found in the first intron. This intron contained a microsatellite repeat, which is characteristic for primate TH genes and is involved in the transcriptional regulation of the human gene (Meloni *et al.*, 1998; Albanese *et al.*, 2001). Apart from AP1-like sites no other sequence similarities were found between bovine and human TH putative intronic S/MARs and neither sequence is A+T-rich. A computer search revealed putative S/MARs and AP1 sites also in the first intron of rat and mouse TH genes (accession numbers M23598 and AF415235). Thus it seems that there is an evolutionary conservation of the position of this particular S/MAR in the TH genes. The role of the colocalized AP1-like sites and their competition or cooperation with the NM proteins involved in intronic S/MAR binding remain to be elucidated.

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