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Functional role of TTF-1 binding sites in bovine thyroglobulin promoter

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We have studied the binding of purified TTF-1 on the bovine thyroglobulin gene promoter. DNase I footprinting experiments revealed three binding sites which corresponded in location to the A, B and C sites found in the rat thyroglobulin promoter. Mutants in the A and C regions showing reduced binding of TTF-1, also exhibited largely decreased promoter activity in transient expression experiments in primary-cultured dog thyrocytes. Two mutants in the B site that exhibited a reduced capacity to bind TTF-1 also displayed a drastically affected transcriptional activity in transient assays. As in the rat, sites A and C only are critical for promoter activity, these results suggest that full occupancy of the B site is required for thyroglobulin promoter activity in the cow only.

Transcription factor; TTF-1; *Tg* gene

1. INTRODUCTION

The expression of thyroglobulin (*Tg*) gene is restricted to the follicular cells of the thyroid gland. The expression of the gene is under the positive control of thyrotropin which acts mainly via the intracellular messenger cAMP [1]. This control occurs, at least partly, at the transcriptional level [2,3]. It has been shown that the specific expression of a reporter-gene, the chloramphenicol acetyltransferase (CAT) gene, required only the first 250 bp of the bovine *Tg* promoter when transfected in thyroid cells in primary culture [4]. Likewise, 170 bp of the rat *Tg* promoter were sufficient to direct the cell-type-specific expression of the same reporter gene when transfected in FRTL-5 cells [5].

The control of gene expression depends largely on the binding of transcription factors on promoter sequences. A DNA sequence repeated three times in the rat *Tg* promoter has been shown to interact in footprinting experiments with a thyroid-specific nuclear factor called TTF-1 [6]. The TTF-1 protein has been purified and characterized by cDNA cloning [7].

In this study, we compare the binding of TTF-1 on both rat and bovine promoters and its functional role in the control of the expression of the corresponding genes. Although TTF-1 recognizes three sequences in both promoters in DNase I footprinting assays, only two sequences appear functionally relevant in the rat promoter while occupancy of the three sites seems essential to sustain normal activity of the bovine promoter.

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2. MATERIALS AND METHODS

2.1. *Footprinting experiments*

DNase I protection experiments were performed on *Sau*I end-labelled probes with purified TTF-1 in the presence of 500 μg/ml bovine serum albumin, 0.1% NP-40 and 2% polyvinylalcohol, according to Musti et al. [5]. The DNase I digestion was stopped with 1% SDS, 5 mM EDTA and 50 μg/ml proteinase K. The DNA was then ethanol precipitated and analyzed on a 6% sequencing gel.

2.2. *Mutagenesis of the promoter*

Mutations were introduced in the bovine *Tg* promoter in plasmid pbTgCAT 14 ([4] and Donda et al., in preparation) using the PCR technique [8]. The description of the different substitutions is given Fig. 2. The resulting constructs were partially sequenced to verify the presence of the mutations and to exclude the occurrence of undesired base changes in the PCR-amplified fragments.

2.3. *Cell culture, transfection experiments and CAT assay*

Dog thyrocyte primary cultures were established and maintained as described in [9]. Transfection of primary cultured dog thyrocytes was done as described in [4]. After transfection, cells were maintained three days either in control medium (control conditions) or in control medium supplemented with 10 μM forskolin (Fo conditions). Analysis of CAT activity was affected according to Seed and Sheen [10].

3. RESULTS AND DISCUSSION

3.1. *Localization of TTF-1 binding sites on the bovine* Tg *gene promoter*

Footprints were realized with a 250 bp fragment from the bovine *Tg* promoter and with purified TTF-1. Figure 1A shows the results obtained with the wild-type promoter sequence. Purified TTF-1 generated three

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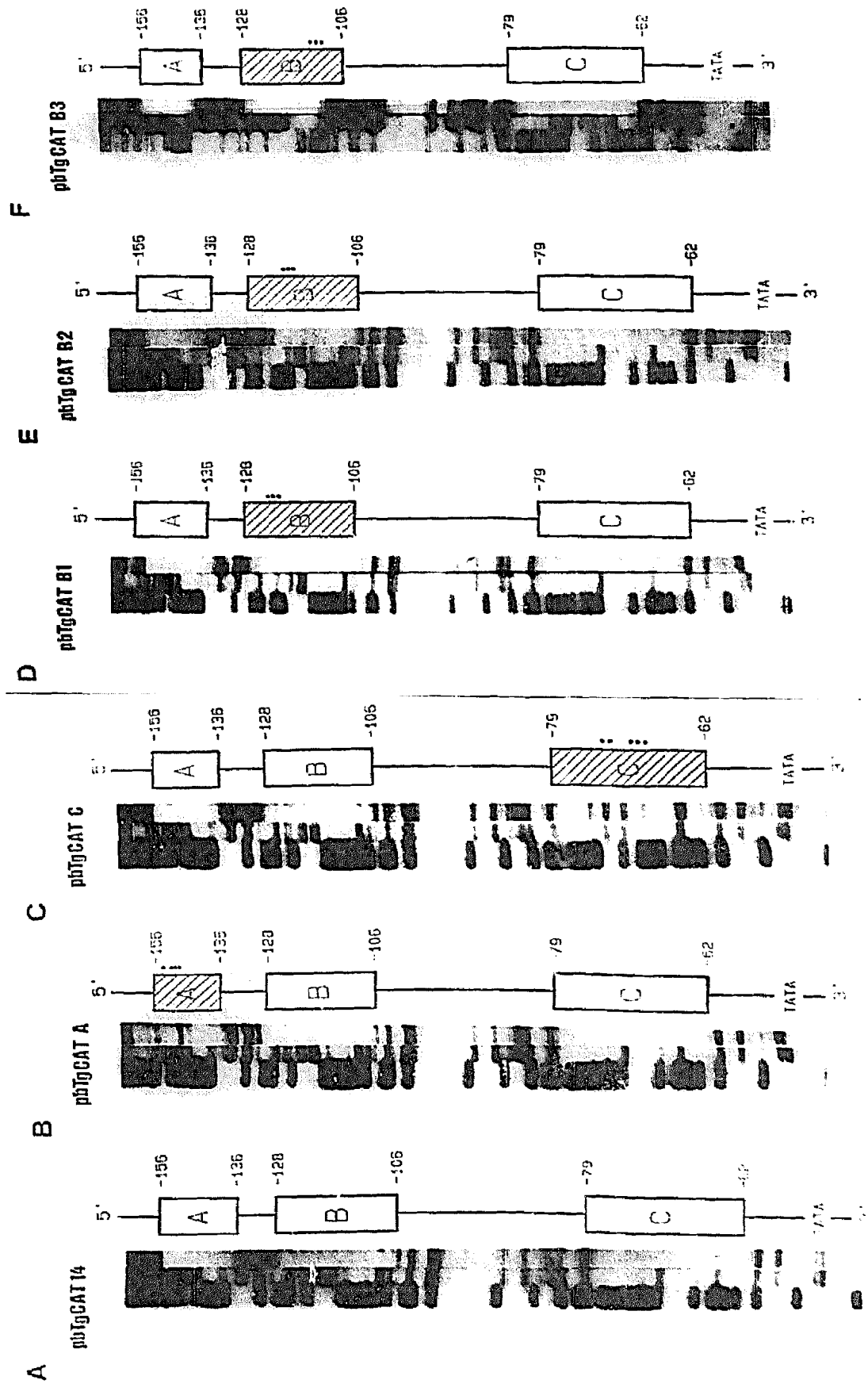


Fig. 1. Interaction of TTF-1 with bovine *Tg* promoter sequences. DNase I footprints using (A) the wild-type pbTgCAT 14 or the mutated (B) pbTgCAT A, (C) pbTgCAT C, (D) pbTgCAT B1, (E) pbTgCAT B2, (F) pbTgCAT B3 bovine *Tg* promoter sequences are shown. The positions of TTF-1 binding sites A, B and C are boxed. The mutated TTF-1 recognition sequences are hatched and positions of mutations are indicated by asterisks (*). First lane: G + A sequencing reaction; second lane: naked DNA; third lane: probe preincubated with 0.5 μl of purified TTF-1.

Table I

Transient expression of pbTgCAT fusion genes in primary cultures of dog thyrocytes

A	pbTgCAT 14		pbTgCAT A		pbTgCAT C	
	Ctl	Fo	Ctl	Fo	Ctl	Fo
Exp. I	1507	34644	547	1692 (5%)	440	5669 (16%)
Exp. II	1317	19611	731	1678 (8.5%)	925	3559 (18%)
Exp. III	1848	18225	414	1239 (7%)	488	2924 (16%)

B	pbTgCAT 14		pbTgCAT B3	
	Ctl	Fo	Ctl	Fo
Exp. I	3171	81307	1340	9287 (11.5%)
Exp. II	6729	82457	1845	13854 (16.8%)
Exp. III	742	25037	483	3466 (13.8%)

Results of the transfection experiments with wild-type and mutated promoters obtained in three separate experiments are shown. Cells were maintained either in control medium (Ctl) or in medium supplemented with 10 μM forskolin (Fo) after transfection (relative activities of mutated promoters A, C and B3 versus 14 wild-type appear in brackets in Fo conditions only. The background value of the assays (≈400 cpm and 900 cpm for panels (A) and (B), respectively) have been deduced.

footprints that correlated in positions, although less in DNA sequences, to the regions A, B and C of the rat *Tg* promoter. So, in vitro, TTF-1 has three similar binding sites on the rat and the bovine *Tg* promoters.

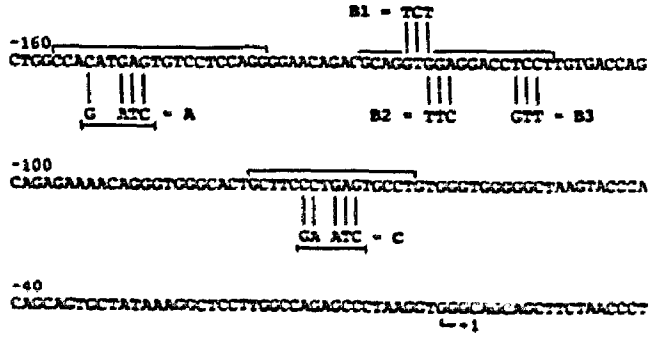


Fig. 2. Bovine *Tg* gene promoter sequence. The first 160 bp of the bovine *Tg* gene promoter are shown. Positions of TTF-1 footprints are indicated by half boxes drawn above the sequence. Mutations introduced in the three TTF-1 DNA-binding sites are indicated and named.

3.2. *Binding of TTF-1 on mutated A and C regions*

Mutations introduced in the A and C consensus sequences in the rat *Tg* promoter drastically decrease the transcription of a reporter-gene in transfection experiments [11]. This effect parallels the lack of binding to TTF-1 in vitro. In order to correlate the binding of TTF-1 with promoter activity in the beef, two mutations were introduced in the A and C regions of the bovine promoter at the −153 to −148 and at the −73 to −68 positions, respectively (Fig. 2). Weak or no binding of TTF-1 was observed respectively on the mutated A region (Fig. 1B) or on the mutated C region (Fig. 1C) in footprinting assays.

Moreover, transfections of primary-cultured dog thyrocytes with the pbTgCAT A or pbTgCAT C constructs, that contained the mutated bovine *Tg* promoters, in the regions A and C respectively, linked to the CAT gene showed that the two substitutions greatly affected promoter activity (Table IA and II). As initially observed in the rat promoter, reduced or lack of binding of TTF-1 to the A and C mutants thus correlates with a dramatic decrease in transcriptional activity.

3.3. *Binding of TTF-1 on mutated B region*

By contrast to what is observed for the TTF-1 A and C sites, mutations introduced in the rat TTF-1B binding site do not impair promoter activity in transfection experiments. Moreover, the rat B region has a much weaker affinity for TTF-1 than the A and C sites [12]. Three bovine promoters containing mutations in the B region were assayed for their capacity to bind TTF-1 in DNase I protection experiments (Fig. 2). As shown in Fig. 1D, the GTG→TCT substitution of the B1 mutation [13] did not prevent the binding of TTF-1 on the B site. On the other hand, the B2 (GGA→TTC substitution) (Donda et al., in preparation) and the B3 (TCC→GTT substitution) (this study) mutated sequences revealed a weaker affinity for TTF-1 and displayed a narrower footprint of the protein on this region of the promoter that correlated with the positions of the mutated residues (Fig. 1E and F). However, none of

Table II

CAT activity of mutated promoters normalized to the value obtained with the wild-type *Tg* promoter (pbTgCAT 14 construct) in the presence of forskolin

Construct	relative CAT activity
pbTgCAT 14	100%
pbTgCAT A	6.8 ± 1.8%
pbTgCAT B1	69 ± 6% (1)
pbTgCAT B2	8 ± 6% (2)
pbTgCAT B3	14 ± 2.8%
pbTgCAT C	16.6 ± 1.4%

These values represent averages obtained in four to six separate experiments. (1), taken from [13]; (2), taken from Donda et al., in preparation.

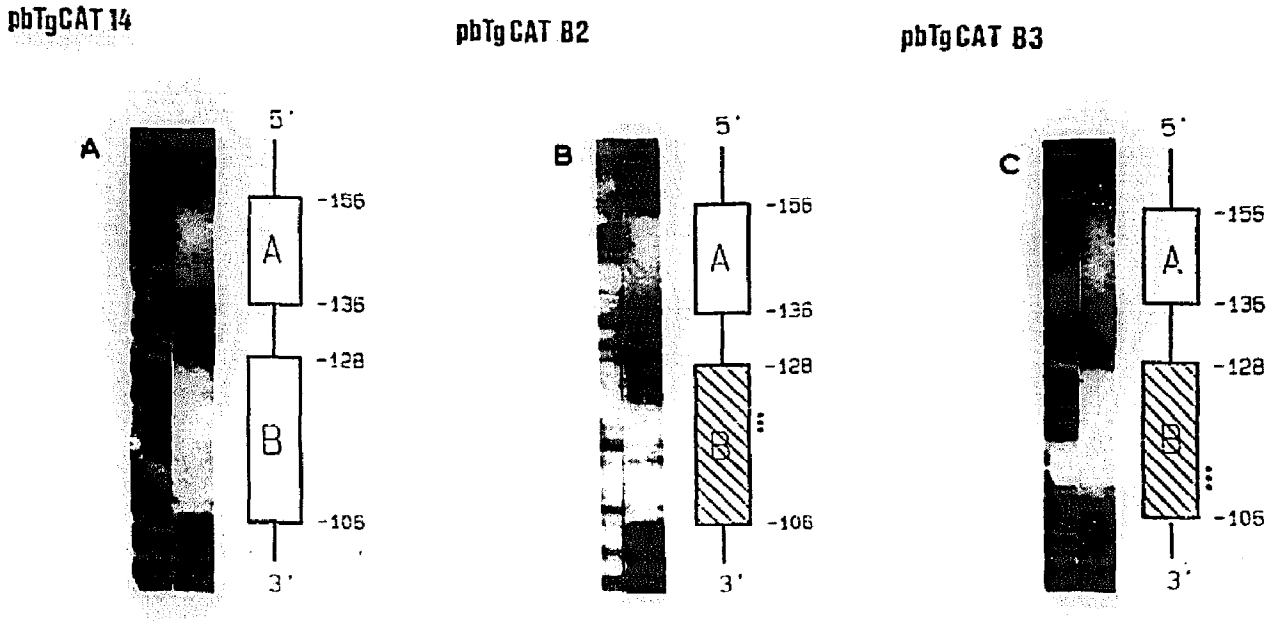


Fig. 3. Interaction of the isolated homeodomain of TTF-1 produced in *E. coli* with bovine *Tg* gene promoter sequences. DNase I footprints using (A) the wild-type pbTgCAT 14 or the mutated (B) pbTgCAT B2 or (C) pbTgCAT B3 bovine *Tg* promoter sequences are shown. The positions of TTF-1 binding sites A and B are boxed. The mutated TTF-1 recognition sequences are hatched and positions of mutations are indicated by asterisks (*). First lane: naked DNA; second lane: probe preincubated with 1 μl of TTF-1 homeodomain produced in *E. coli*.

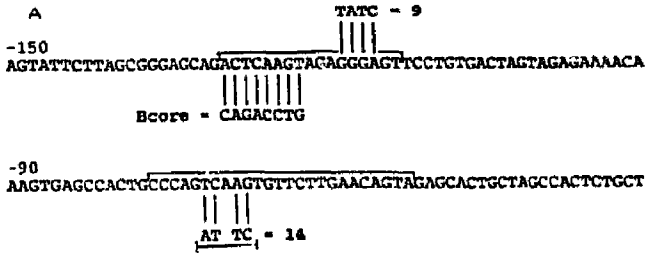
these two mutations completely abolished TTF-1 binding in vitro. Transcriptional activities of the B1 and B2 mutated promoters are displayed elsewhere ([13] and Donda et al. in preparation) and summarized in Table II. Results obtained with the B3 mutated promoter in gene-transfer experiments are shown in Table IB.

To exclude the intervention of other proteins that could have been co-purified with TTF-1, the isolated homeodomain of the TTF-1 protein produced in *E. coli* and which is sufficient to allow binding to DNA in vitro was also used in footprinting experiments. The observed footprints were, as expected, smaller than the ones displayed by the complete protein but the affinity of the homeodomain for the different B mutants paralleled that of the purified protein and, especially, was similarly distorted in the B2 and B3 mutants (Fig. 3). Altered binding of the TTF-1 protein alone could thus account for the observed changes in the B footprints.

Thus, from the three mutants in the bovine B site that were used here, one (B1) displayed an apparently unaffected ability to bind to TTF-1 and performed barely less than the wild-type promoter in a transient assay, and the two others (B2 and B3) showed reduced and distorted TTF-1 footprints and were severely less active than the natural sequence in the functional assay.

3.4. *Transient expression of rat* Tg *promoter-CAT constructs in primary-cultured dog thyrocytes*

In order to verify that the discrepancy observed between the results of gene-transfer experiments with rat and bovine *Tg* promoters mutated in the B region did not result from differences in the systems of cell culture used so far (FRTL-5 cell line for the rat promoter [11] and dog thyrocytes in primary culture for the bovine



B

	pTACAT 3		Bcore mutant		pTACAT 9		pTACAT 14	
	Ctl	Fo	Ctl	Fo	Ctl	Fo	Ctl	Fo
Exp.I	UD	7395	402	8306	546	7103	ND	ND
Exp.II	1537	21795	3890	34078	5403	39691	34	1462
Exp.III	1404	15615	2291	20304	3988	20856	624	1835
Exp.IV	168	5488	ND	ND	653	11588	UD	535

Fig. 4. (A) Rat Tg gene promoter. A 120 bp fragment of the rat *Tg* gene promoter is shown. Positions of TTF-1 B and C footprints are indicated by half boxes drawn above the sequence. Mutations introduced in the TTF-1 DNA binding sites are indicated and named. The plasmid constructs containing these mutations were described elsewhere [6,11]. (B) Transient expression of rat *Tg* promoter in primary-cultured dog thyrocytes. The results obtained in four separate experiments are shown. (ND: not done. UD: undetectable, background value (deduced): ≈450 cpm routinely).

promoter [9]), we decided to transfect the wild-type rat promoter and some of the pTACAT mutants (described in [6,11] and in Fig. 4A) in primary-cultured dog thyrocytes. Results are presented in Fig. 4B. The presence of forskolin in the medium increased the transcriptional activity of wild-type pTACAT 3 construct 10-to-30-fold.

The results obtained with the mutated sequences were similar to those obtained in FRTL-5 transfected cells [11], in that the C mutant (pTACAT14) exhibited reduced promoter activity, while both B mutants (Bcore and pTACAT9) did not. Thus, the functional difference observed between rat and bovine *Tg* promoters mutated in their B regions in transfection experiments reflects a real functional disparity between these promoters. On the other hand, transfection of bovine promoter-containing constructs in FRTL-5 cells allowed the same conclusions to be drawn (data not shown). Paradoxically, although in the rat the three TTF-1 sites are much more conserved in terms of DNA sequences, they display better conservation of function in the cow.

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