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DNA recognition element required for PUF-I mediated cell-type-specific transcription of the rat prolactin gene

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ABSTRACT

The cell-type-specific transcription of the prolactin gene *in vitro* is mediated through the interaction of prolactin upstream factor I (PUF-I) with a 28 basepair region of the gene promoter (-63 to -36) which contains an 18 bp A+T-rich imperfect palindrome (-63 to -46). Base substitutions were introduced into 16 of the 18 palindromic residues by targeted saturation mutagenesis. The GH_3 binding and *in vitro* transcription assays of the mutated promoters showed that base substitutions within the 5'-ATATTCA-3' sequence located at -52 to -46 were detrimental to PUF-I binding and its cell-type-specific transcriptional enhancement activity. Transcription assays of the mutated promoters performed with several nonpituitary-derived extracts demonstrated that a distal TATA box located from -59 to -53 promotes initiation at -27. Thus, the cell-type-specific *cis*-acting element required by PUF-I for DNA recognition is immediately adjacent to a general TATA sequence. Base substitutions that decreased +1 transcription and PUF-I binding concomitantly increased -27 initiation of RNA *in vitro*. We suggest that PUF-I binding in pituitary cells potentiates +1 transcription and represses alternative TATA box activity for initiation events occurring at -27. This is the first known report of a eukaryotic DNA binding protein that has both an activator and repressor activity for a single transcription unit.

INTRODUCTION

Eukaryotic cell-specific transcriptional regulation of RNA polymerase class II genes is mediated by the precise interaction of tissue-specific *trans*-acting proteins with distinct *cis*-acting elements of the gene (1). These high precision events at the *cis*-acting elements influence the general transcriptional machinery in a positive or negative manner presumably through protein-protein interactions. Little is known concerning the molecular rules that regulate the exact interaction between the DNA regulatory sequences and tissue-specific protein factors.

To obtain a deeper understanding of the promoter- or cell-type-specific transcription factors, several of these proteins have been purified (for example, 2-9). A pituitary-specific factor GHF-1 (2) or Pit-1 (3) was recently purified and cDNA clones were obtained. Convincing evidence was presented by both laboratories that this factor confers the pituitary phenotype to cells. However, there was disagreement concerning the specificity of the Pit-1/GHF-1 for prolactin gene transcription (2). It is clear that the factor present in GH_3, which we refer to as the prolactin upstream factor I (PUF-I), is required for the enhanced GH_3 cell-type-specific transcription of the rat prolactin gene *in vitro* (10,11). In addition, it was shown that purified PUF-I augmented prolactin transcription through a specific interaction with a region of the promoter located from -63 to -36 which contains an imperfect A+T-rich palindrome (11).

In RNA from GH_3 cells and pituitaries, two prolactin transcripts are detectable by primer extension assays; a major RNA that is initiated at the +1 (proximal) cap site and a minor RNA that is initiated at a -27 (distal) start site (10, 12). The -27 RNA is initiated from within the proximal 5'-TTATAA-3' box located at -29 to -24. Deletional mutagenesis of the prolactin promoter demonstrated that the 18 bp palindromic sequence of the PUF-I binding region is a complex *cis*-acting element required for both enhanced cell-type-specific transcription of the major +1 RNA and a TATA activity for initiation of the minor -27 RNA (12). Interestingly, the imperfect palindrome stimulated GH_3-specific *in vitro* transcription of the major (+1) transcript and decreased the initiation of transcription at -27 (12). These data suggested that PUF-I binding to the prolactin promoter enhanced +1 transcription and, in the process, occluded the distal TATA activity of the A+T-rich region of the PUF-I binding region.

To genetically dissect the *cis*-acting cell-type-specific PUF-I element and the distal TATA box in the prolactin promoter, the imperfect palindrome was targeted for saturation mutagenesis. In this paper we report the analysis of 30 mutations within the palindrome region using pituitary and nonpituitary transcription and DNAase I protection assays. We found that the cell-type-specific transcription of the prolactin gene *in vitro* was

dependent on an A+T-rich sequence from -52 to -46. Base substitutions within this region also decreased PUF-I binding to the promoter. The distal TATA element was mapped to a position immediately adjacent to the PUF-I recognition element.

EXPERIMENTAL PROCEDURES

Preparation of nuclear extracts.

GH_3 cells were grown in Ham's F-10 medium supplemented with 10% fetal bovine serum and 2.5% horse serum. HeLa cells were cultured in Eagle's minimal essential medium (Irvine Scientific) with 10% fetal bovine serum and PC12 cells were grown in RPMI medium 1640 (Gibco) with 10% serum. The cells were harvested with 0.03% EDTA in Puck's saline, and the nuclear extracts were prepared according to the procedure of Dignam et al. (14).

DNAase I footprint analysis.

The DNA template used for footprint analyses (10) was the HaeIII/SalI fragments of the rat prolactin DNA which contains the region from -174 to +39. The DNA fragments were labeled at the 5' end of the coding strand with T4 polynucleotide kinase (Pharmacia LKB Biotechnology, Inc.). Protein-DNA binding reactions were carried out in a total volume of 40 μl containing variable amounts of crude GH_3 nuclear extract in 20 mM HEPES, pH 7.9, 1.0 mM EDTA, 1.0 mM DTT, 0.1 M KCl, 10% glycerol, 20,000 cpm of labeled prolactin DNA (0.5 to 1.0 ng) and 1.0 μg of poly (dI-dC)·(dI-dC). After incubation on ice for 30 minutes, 2.0 μl of freshly prepared DNAase I mix containing 10 μg DNAase I per ml, 50 mM $CaCl_2$ and 200 mM $MgCl_2$ was added to start the reaction. The digestions were terminated in 60 seconds by the addition of 60 μl of the stop mix composed of 67 mM EDTA, 0.5 M Na Acetate and 83 μg tRNA per ml. After phenol-chloroform extraction, the DNA fragments were precipitated and analyzed by sequencing gel electrophoresis followed by autoradiography.

In vitro transcription.

The conditions used for in vitro transcription and primer extension assays have been described previously (10). The various prolactin DNA temples were prepared using CsCl density gradient centrifugation. The test templates yielded a 83 nt primer extension product and the reference prolactin template

produced a 72 nt product. In the nonpituitary assays, plasmid HTXB (kindly provided by J. Corden) was also included as a positive transcription control. This plasmid contains three copies of the adenovirus major late promoter-cap site and produced a 54 nt primer extension product in the assays.

Construction of the PUF-I binding region mutations.

The general method utilized was the phosphorothioate-modified DNA technique (15). The HindIII/XbaI prolactin DNA fragment from -420 to +600 was transferred from pUC-based plasmids to a Bluescript plasmid (Stratagene) which was renamed pHSXB. Single stranded DNA was prepared from pHSXB according to supplier recommendations (Stratagene).

The following oligonucleotide was synthesized using an Applied Biosystems DNA Synthesizer:

5'- GATGCCTGATTATATATATATTCATGAAGG -3'

Degeneracy was introduced into the central 18 nt (underlined) by programing the machine to insert the correct base at these positions approximately 97% of the time and one of the other three bases about 3.0% of the time. The degenerate oligonucleotide was gel purified and annealed to the single-stranded template. From this point, the base alterations were introduced using the Amersham In Vitro mutagenesis system. Transformation generated 520 colonies which were pick individually, grown overnight and stored at -20°C as glycerol stocks.

The plasmids containing base substitutions were selected by hybridization of a prolactin PUF-I binding region oligonucleotide (wild-type sequence) with minipreparations of DNA dot blotted to nitrocellulose filters (Schleicher and Schuell). The filters were prehybridized in 6X SSC (1X: 15 mM sodium citrate and 150 mM NaCl); 10X Denharts (1X: .02% Ficoll, .02% polyvinylpyrrolidone and .02% BSA); 0.2% SDS and 100 μg salmon sperm DNA per ml for 15 minutes at 67°C. The oligonucleotide probe (30mer), labeled using T4 polynucleotide kinase, was added to the prehybridization solution (10^6 CPM/ml) and the filters were hybridized for 30 minutes at 67°C and cooled to room temperature. The filters were then washed for five minutes in

three changes of 6X SSC, dried and autoradiographed by exposure to X-ray film at room temperature.

The plasmids which showed a lower level of hybridization signal relative to a wild-type control plasmid were noted and the filters were washed again at 42°C with 6X SSC. After autoradiography, the filters were washed at 65°C and reexposed to film for the final autoradiograph. Plasmids exhibiting a diminished hybridization signal after the first and successively more stringent washes were chosen as candidates for sequencing. This selection procedure was approximately 60% efficient in identifying recombinants with altered nucleotides in the central 18 bp region. The point mutations were verified by the dideoxy chain termination method of DNA sequencing (16) using minipreparations of DNA (17).

RESULTS

PUF-I binding region mutations.

To determine the nucleotide requirements for PUF-I binding and cell-type-specific enhancement of prolactin transcription, the 18 bp imperfect palindrome (Table 1) within the 28 bp PUF-I binding region was targeted for saturation mutagenesis. The 30 mutations listed in Table 1 were obtained by degenerate oligonucleotide directed mutagenesis and hybridization screening using a wild-type palindromic probe (See Experimental Procedures for details). The screening of 520 clones yielded 119 candidates for sequencing from which 71 mutated plasmids were acquired. From these 71 clones, the mutated templates in Table 1 were selected for transcription and binding assays. The number assigned to each clone in Table 1 is used in this paper to identify each mutation.

GH_3 In vitro transcription assays of mutated templates.

It was previously demonstrated that the PUF-I present in GH_3 nuclear extract mediated enhanced cell-type-specific transcription of the prolactin gene through a specific interaction with the -63 to -36 region of the promoter (11). In addition, deletional mutagenesis suggested that the palindromic region of the PUF-I binding region had a vital role in cell-type-

TABLE 1
PUF-I BINDING REGION MUTATIONS

No.	Mutated Template	-67 tgcc **T G A T T A T A T A T A T A T T C A** tgaa -42
1.	Δ62(G→A),53(T→C)	**A** C
2.	Δ61(A→C),58(A→C)	**C** **C**
3.	Δ61(A→C),54(A→T)	**C** **T**
4.	Δ60(T→C)	**C**
5.	Δ58(A→C),51(T→G)	**C** **G**
6.	Δ58(A→G)	**G**
7.	Δ58(A→C)	**C**
8.	Δ57(T→C)	**C**
9.	Δ56(A→G),55(T→A)	**G A**
10.	Δ56(A→G)	**G**
11.	Δ56(A→C)	**C**
12.	Δ55(T→G)	**G**
13.	Δ54(A→T)	**T**
14.	Δ54(A→C)	**C**
15.	Δ54(A→G),50(A→T)	**G** **T**
16.	Δ54(A→G),47(C→A)	**G** **A**
17.	Δ53(T→A)	**A**
18.	Δ53(T→C)	**C**
19.	Δ52(A→G)	**G**
20.	Δ50(A→T)	**T**
21.	Δ50(A→C)	**C**
22.	Δ49(T→G)	**G**
23.	Δ49(T→A)	**A**
24.	Δ49(T→C)	**C**
25.	Δ48(T→C)	**C**
26.	Δ48(T→A)	**A**
27.	Δ48(T→A),47(C→T)	**A T**
28.	Δ48(T→A),47(C→A)	**A A**
29.	Δ47(C→T)	**T**
30.	Δ46(A→T)	**T**

The altered base in the DNA sequence targeted for saturation mutagenesis (bold and in capital letters) is shown for each of the plasmids containing mutated prolactin promoter.

specific transcription of the prolactin gene (12). To assess the functional effects of the base changes within the palindromic region, each of the plasmids listed in Table 1 were assayed for transcriptional competence using GH_3 nuclear extracts. The transcription of each test template (T) was referenced to a wild-type prolactin template (R) which generated a smaller primer extension product (Figure 1, see Experimental Procedures for details). The prolactin promoter initiates two transcripts *in vivo*, the major product starts at +1 and a minor transcript begins at -27 (12). The same pattern of transcripts was obtained

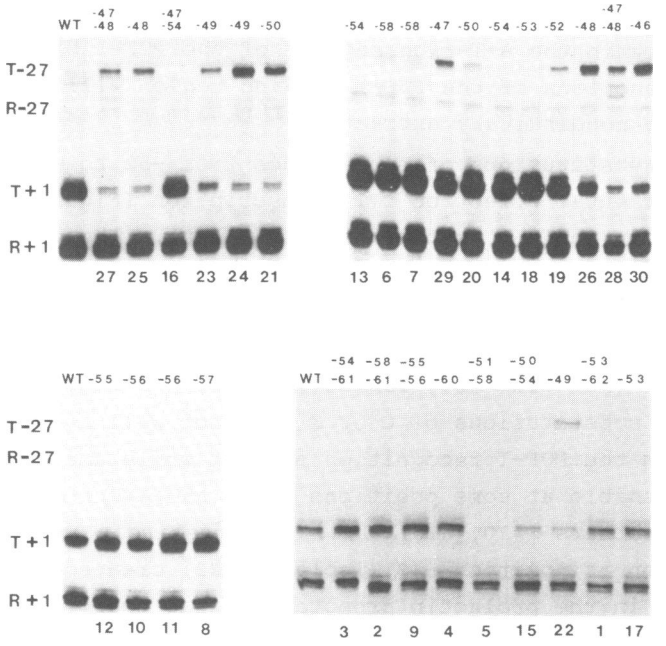


Figure 1. GH_3 transcription assays of mutated PUF-I binding regions. The in vitro transcription reactions were performed as described in the Methods. The number of each mutated template assigned in Table 1 is indicated below each lane. The position of the base substitution is indicated above each lane. Each assay contained 200 ng of a reference template (R) and 200 ng of a test template (T) with the altered nucleotide(s). Transcription initiated at the major +1 cap site for the reference DNA (R+1) and the test DNA (T+1) are indicated. The minor RNA initiated at -27 from the reference (R-27) and test template (T-27) are also indicated. The only difference between the reference and test templates (other than the single or double point mutations) is the presence of an eleven basepair SalI linker inserted into the AvaII site of the first exon (Cao, et al., 1987). T+1 and T-27 RNA are eleven nucleotides larger than R+1 and R-27 in the primer extension assays. WT = wild type prolactin promoter present in the test and reference templates.

in vitro for the reference and test templates (R+1, R-27 and T+1, T-27, respectively, Fig. 1).

Base substitutions of the nucleotides at positions -62 to -53 (Table 1) had no effect on the efficiency of RNA initiated at the +1 site (T+1, Figure 1). Some of these mutations clearly diminished -27 RNA from the test template (T-27, Figure 1, Mutations # 16, 13, 14 and 18). These results indicated that

position -53 and -54 were important for the TATA function residing within the A+T-rich segment of the PUF-I binding region. The TATA functions of the PUF-I binding region will be addressed later using nonpituitary extracts.

Base substitutions of nucleotides from -52 to -46 (Table 1) had a detrimental effect on T+1 transcription (Figure 1, T+1, Summarized in Table 2, mutations # 19 - #30). The effects ranged from a slightly inhibitory (#19) to a strongly negative (#21, #24, #23, #25, #27, and #28) influence on +1 transcription. The exception to this general pattern was the A to T change at position 50 (mutation #20, Tables 1 and 2, and Figure 1). Generally, substitutions of C or G were not well tolerated at any base within the PUF-I recognition element while changes to A or T were permissible at some positions [#20, Δ50(A→T)] but were not allowed at others [#30, Δ46(A→T)]. Interestingly, the T to A substitution at position -49 (Table 1, #23) created a perfect palindrome in the prolactin promoter but was poorly tolerated transcriptionally (Figure 1, T+1 #23). Other mutations which severely disrupted the symmetry (Mutations 1-3, Table 1), but did not substitute bases within -52 to -46, had no effect on function Figure 1). These results indicated that sequences upstream of -52 do not appear to be important for PUF-I recognition of the prolactin promoter and there is no evidence that the imperfect symmetry defines the sequences with which PUF-I interacts.

Another striking result was the concomitant increase in the distal T-27 transcription in the mutations that decreased T+1 transcription (Table 2 and Figure 1, T-27, mutations #19, #21-30. Mutations # 5 [Δ58(A→C),51(T→G)] and #15 [Δ54(A→G),50(A→T)] were both double point mutations that decreased +1 transcription but demonstrated no increase in -27 RNA. The probable reason for this result is that the distal TATA box was also mutated by the base substitutions at -58 and -54. As will be shown later in DNAase I footprint assays, the mutations that severely reduced T+1 transcription and increased T-27 RNA also diminished PUF-I binding. These results suggested that the increase in initiation of -27 RNA was inversely proportional to PUF-I's ability to bind to the A+T-rich region of the prolactin promoter.

TABLE 2
SUMMARY OF MUTATIONAL EFFECTS ON GH_3 PROLACTIN TRANSCRIPTION

No.*	Position	Base Substitution	Transcription +1	Transcription -27
14.	-54	A → C	NE	-
17.	-53	T → A	NE	NE
18.	-53	T → C	NE	-
19.	-52	A → G	-	+
5.	-51	T → G	--	ND
	-58	A → C		
15.	-50	A → T	-	ND
	-54	A → G		
20	-50	A → T	NE	NE
21.	-50	A → C	---	++
22.	-49	T → G	--	++
23.	-49	T → A	--	+
24.	-49	T → C	---	+++
25.	-48	T → C	---	++
26.	-48	T → A	-	+++
27.	-48	T → A	-	++
	-47	C → T		
28.	-48	T → A	--	++
	-47	C → A		
29.	-47	C → T	-	+
30.	-46	A → T	---	+++

The values of the transcriptional effects were determined by scintillation counting of the relevant bans excised from the gels illustrated in Figure 1. (+) = an increase in RNA levels; (-) = a decrease in RNA levels; NE = no effect; ND = not determined. For +1 transcription, a ratio of the counts in the test to reference bands of 1.0 = no effect (NE), 0.8 = (-), 0.6 = (--) and 0.4 or less = (---) The ratios of the -27 RNA were not determined because of uncertainties in the excision of the referece bands. The above values for the levels of increase are, therefore, estimates based on the relative density of the test template -27 RNA product.
* See Table 1.

In summary, the GH_3 transcription assays of the 30 mutations listed in Table 1 showed that positions -52 to -46 are important for the enhanced level of +1 prolactin gene transcription mediated by the PUF-I interaction. Additionally, the concomitant increase in -27 prolactin transcription appears to be a very sensitive assay for decreases in PUF-I function due to base changes within its recognition element.

Nonpituitary transcription assays of the mutated templates.

Since the PUF-I binding region contains a pituitary-specific function (mediated by PUF-I) and a TATA-like activity, transcriptional assays using the mutated templates in nonpituitary extracts should result in much different

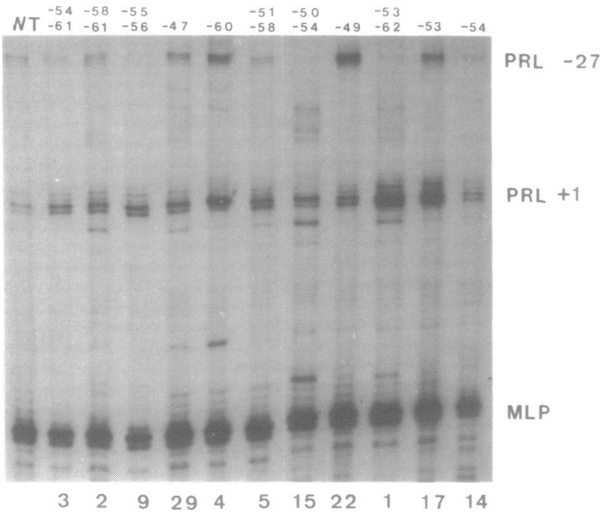


Figure 2. HeLa transcription assays of PUF-I binding region mutations. The in vitro transcription reactions were performed as described in the Methods. Each transcription contained 400 ng of the prolactin template (PRL) and 100 ng of the HTX-B plasmid which contains three copies of the adenovirus major late promoter (MLP). The number of the mutated template (from Table 1) is indicated below the lanes and the position of the mutations is shown above the lanes as in Figure 1. The prolactin templates generate an 83 nt (+1) and 110 (-27) nucleotide primer extension product. The MLP template generates a 54 nt primer extension product in these assays.

transcription patterns compared to those generated by CH_3 extracts. Figure 2 illustrates an example of the functional assays of the altered templates using HeLa nuclear extracts. Although there was variability in the levels of +1 RNA, it appears that +1 initiation was not severely affected by any of the palindrome mutations (Fig. 2, PRL +1) which indicated that the base substitutions in the PUF-I binding region did not affect nonpituitary transcription promoted by the proximal TATA box. This is consistent with deletion mutagenesis which demonstrated that the PUF-I binding region had no influence on +1 transcription in nonpituitary extracts (12). The differences in the +1 transcriptional level illustrated in Fig. 2 were due to variable transcription in each assay as indicated by the variation of the levels of RNA transcribed by the major late promoter reference template (Fig. 2, MLP).

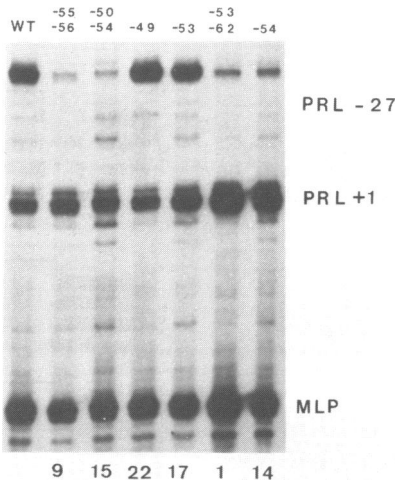
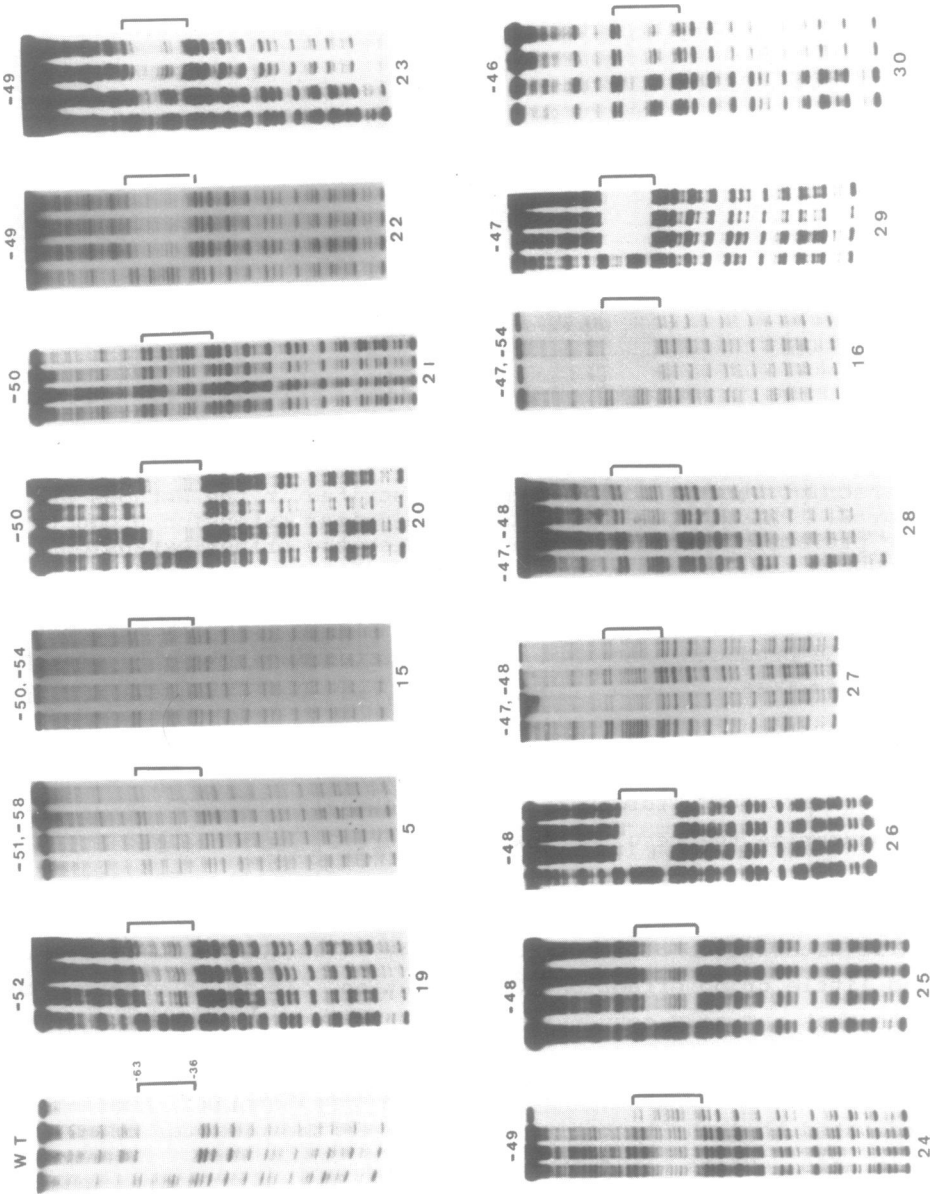


Figure 3. PC12 transcription assays of PUF-I binding region mutations. The lanes are labeled as in Fig. 1. The transcription assays were done as described in the Methods and Fig. 2. PRL +1 and -27 indicate the primer extension products of the prolactin templates. MLP indicates the primer extension products derived from the adenovirus reference template.

In nonpituitary transcription assays in vitro, the wild-type template produced RNAs that were initiated about equally from the -27 and +1 start sites (Figs. 2 and 3, WT, PRL +1 and -27). If a mutation resulted in a decrease of -27 RNA relative to +1 RNA, we assumed that the base change was detrimental to the function of the -27 promoter (for example see #9 and #15, PRL -27, Fig. 2). To summarize the results shown in Figure 2, base changes at positions -58 (#5), -56 (#9), -55 (#9), -54 (#14), -53 (#1), and -50 (#15) showed detrimental effects on transcription of -27 RNA while alterations of bases at -60 (#4), -49 (#22) and -47 (#29) had little or no effect on -27 initiation. It is interesting to note that a T to C conversion at position -53 was detrimental to -27 initiation while an a T to A substitution at the same position had no effect (compare #1 and #17, Fig. 2). These data indicated that the tentative boundary for the promoter of -27 initiation events was from -59 to -53.

Figure 3 illustrates an example of the results of transcription assays using some of the altered templates in nonpituitary nuclear extract prepared from rat PC12 cells. The


-49
23
-49
22
-50
21
-50
20
-50,-54
15
-51,-58
5
-52
19
-63
-36
WT
-46
30
-47
29
-47,-54
16
-47,-48
28
-47,-48
27
-48
26
-48
25
-49
24

results were consistent with the Hela assays. Four of the mutated templates demonstrated impairment of -27 initiation in the PC12 assays (Fig. 3, mutations #9, #15, #1 and #14). Similar results were also obtained using another rat nuclear extract prepared from hepatoma cells (H7777), data not shown. Thus, three different nonpituitary transcription assays indicated that the -59 to -53 region contained the sequences necessary for TATA activity located within the PUF-I binding region. From these data, we concluded that, within the imperfect palindrome of the prolactin promoter, the PUF-I recognition element and the alternative TATA box are immediately adjacent to each other.

DNAase I footprint analyses.

Does the decreased cell-type-specific transcriptional activity of the mutated templates using GH_3 transcription extracts correlate with a diminution of PUF-I binding to the prolactin promoter? To address this question, DNA fragments containing the altered binding regions were assayed for the specific DNA-PUF-I interaction using GH_3 nuclear extract. Figure 4 illustrates footprint experiments using 15 of the templates which contained base substitutions within the -52 to -46 region compared to a wild type promoter. The weakness of the footprint correlated well with the decrease of +1 transcription and increase of -27 RNA. For example, mutation #19 had a slightly weakened footprint consistent with the small decrease of +1 and slight increase of -27 transcription. On the other hand, mutation #21 showed a severely weakened footprint consistent with the large decrease in +1 transcription and increase in -27 RNA.

There were two interesting inconsistencies that are under

Figure 4. DNAase I protection assays of the PUF-I binding region mutations. The footprints were performed as described in the Methods and Cao, et al., 1987. The number of the mutation assigned in Table 1 are indicated below each group and the position of the base substitutions are shown above the 4 lanes of each autoradiograph. The proteins used in the binding reaction were (from right to left): Lane 1, 30 μg of BSA; Lane 2, 1 μl of GH_3 nuclear extract and 20 μg of BSA; Lane 3, 2 μl of extract and 10 μg BSA; Lane 4, 3 μl of nuclear extract. The -63 to -36 region protected by PUF-I binding is indicated by the brackets. WT refers to the wild-type prolactin promoter.

current investigation. The weakness of the footprint for mutation #25 was not consistent with the strong decrease in +1 transcription and increase in -27 initiations (Figure 1). The two base substitutions at positions -54 and -50 in mutation #15 demonstrated a severely weakened footprint and significant diminution of +1 transcription (Figures 4 and 1). However, single point mutations at -54 (#14) or -50 (#20) had little or no effect on PUF-I binding or +1 transcription (Figure 1). These results seem to suggest the cooperativity of residues at -54 and -50 in the binding and function of PUF-I. Overall, however, these binding data supported the proposition that nucleotides from -52 to -46 are important for promoter recognition by PUF-I.

DISCUSSION

Base substitutions were introduced into 16 of the 18 targeted positions within the PUF-I binding region of the prolactin gene promoter. The in vitro mutagenesis technique using a degenerate oligonucleotide combined with the phosphorothioate-modified DNA technique was highly efficient for the introduction of mutations at each position. The screening method was fast and approximately 60% efficient for selecting mutated plasmids.

The binding and functional assays of the mutations selected for this study strongly indicated that PUF-I binding and its cell-type-specific transcriptional enhancement activity required at least the 5'-ATATTCA-3' sequence located from -52 to -46. This sequence is centrally located within the PUF-I protected region (-63 to -36) and differs slightly from the prolactin core element, 5'-ATATATTCAT-3' (-54 to -45), proposed by Nelson, et al. (13), to be responsible for the binding and function of the GC cell-type-specific Pit 1 factor. The functional assays presented in this paper showed that mutations of nucleotides -62 to -53 were not detrimental to PUF-I function. Position -54 was assayed using all three base substitutions and position -53 was tested with two of the possible base alterations (Table 1 and Figure 1). While it is possible that a T→G change at -53 will be detrimental to PUF-I function, it appears likely that -54 and -53 residues are not vital to PUF-I mediated transcriptional

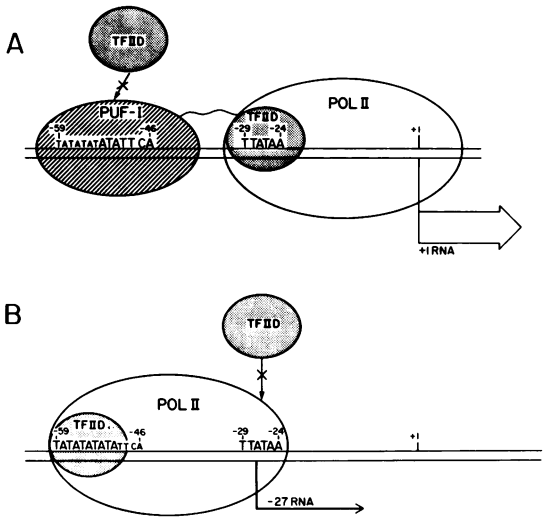


Figure 5. A model proposed for pituitary-specific prolactin gene transcription. A and B represent the two transcription events in pituitary cells. The thickness of the +1 and -27 arrows indicate the relative levels of transcription from the +1 and -27 cap sites. PUF-I = prolactin upstream factor I; Pol II = RNA polymerase II transcription complex; TFIID = Transcription factor IID, a TATA binding factor (18).

enhancement. The single A→G substitution at -52 had a small negative effect on the function and binding of PUF-I and thus appears to be the 5′ border of its recognition element. The -46 position was the 3′ border of the area targeted for mutagenesis in this study. Since the A to T change at this position had severe detrimental effects on transcription and promoter binding, it seems likely that the PUF-I recognition element will extend further in the 3′ direction toward the proximal TATA box. The ATATTCAT consensus sequence (13) suggests that, at a minimum, the T residue at -45 will be important. This suggestion is currently under investigation.

The nonpituitary transcription assays of the altered templates indicated that the 5′-TATATAT-3′ sequence located from -59 to -53 promotes initiation at -27. The position of this cis-acting element relative to the -27 cap site is consistent with its TATA function. The comparative functional assays using GH_3 and nonpituitary transcription systems indicated that the dual

elements of the 5' flanking region of the prolactin gene are immediately adjacent in the prolactin promoter. It is, indeed, remarkable that the PUF-I and the TATA-binding proteins can precisely distinguish between these two A+T-rich elements.

The observation that the PUF-I recognition element and the TATA-like element are very close to one another supports the suggestion that PUF-I binding interferes with the distal TATA element's interaction with the general transcription initiation apparatus (12). Consistent with this hypothesis, several of the single base substitutions in the PUF-I recognition element decreased the PUF-I binding, reduced the level of GH_3 +1 transcription and increased the level of -27 RNA. It seems reasonable to speculate that assembly of the transcription complex at the distal TATA was more efficient with these mutated templates because PUF-I could not efficiently bind to the A+T-rich region. In support of this idea, Cao et al., (11) demonstrated that purified PUF-I increased +1 and decreased -27 RNA in reconstituted cell-type-specific transcription assays. The present paper demonstrated that the shift toward increased levels of -27 RNA in the GH_3-specific transcription assays of PUF-I recognition element mutations (illustrated in Figure 1) is very sensitive for monitoring productive PUF-I interactions with the promoter. This approach will be very useful in the dissection of the remainder of the PUF-I recognition element and determining the functional regions of the PUF-I protein as well.

A model to account for the major and minor transcription events in GH_3-specific <u>in vitro</u> transcription assays is shown in Figs. 5A and 5B. The less frequent occurrence is initiation of -27 RNA which is accomplished by the assembly of an RNA polymerase II transcription complex at the distal TATA box and cap site (Fig. 5B). The more frequent +1 initiation event is shown Fig. 5A which illustrates PUF-I interacting with the A+T-rich region of the promoter. This binding leads to an enhanced level of +1 transcription through, perhaps, protein-protein interactions with the RNA polymerase II transcription complex assembled at the proximal TATA (wavy line, Fig. 5B). An additional consequence of PUF-I binding is the occlusion of the distal TATA box from the TATA factors. Because of either a

greater abundance of PUF-I or a higher affinity of PUF-I for the prolactin promoter, there is a lower usage of the distal TATA in GH_3 in vitro transcription. This is the first known proposal for a eukaryotic transcription factor that is able to enhance transcription from one start site and repress initiation from an alternative cap site.

This unusual functional arrangement of the cis-acting promoter elements also permits a rare comparison of cell-free in vitro and in vivo promoter function. Previous studies demonstrated that the RNA initiated at -27 is a minor contribution to the total prolactin RNA in GH_3 cells and the rat pituitary (12). The similar pattern of initiation events in vivo and in vitro is consistent with the notion that PUF-I is active in pituitary cells and, more importantly, that its in vitro role as a cell-type-specific transcription factor is probably an accurate reflection of a similar function in the rat lactotroph.

In summary, we have shown that single and double point mutations within the PUF-I binding region alter the ability of the PUF-I to interact with DNA and to enhance cell-specific transcription of the prolactin gene. The recognition element for PUF-I interaction is a 7 nt or larger sequence which is adjacent to a 7 nt A+T-rich element that is apparently responsible for the alternative TATA activity of the promoter region.

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