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RAT PIT-1 STIMULATES TRANSCRIPTION *IN VITRO* BY INFLUENCING PRE-INITIATION COMPLEX ASSEMBLY

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Summary: The anterior pituitary-specific transcription factor, Pit-1, activates prolactin, growth hormone, TSHβ, growth hormone receptor genes and autoregulates the *pit*-1 gene. Its mechanism of transcription activation is unknown. Using immobilized DNA templates and order-of-addition transcription assays, it is shown that Pit-1 is required during pre-initiation complex assembly to activate the prolactin gene *in vitro*. Using prolactin promoters containing point mutations in the distal TATA box, it is also demonstrated that Pit-1 activation *in vitro* is not mediated simply by repressing the upstream, alternative promoter. Experiments show that a preformed class II pre-initiation complex is refractory to Pit-1 influence. The data indicate that Pit-1, and perhaps other members of the POU-protein family, activate transcription by influencing the type pre-initiation complex assembled on target promoters.

The transcription factor, Pit-1, is required for differentiation of three of the five parenchymal cell types in the anterior pituitary glands of mammals (1-4). A member of the POU-homeodomain class of developmental regulatory proteins (5), Pit-1 was discovered as an activator of prolactin (6,7) and growth hormone (7,8) genes *in vivo* and *in vitro*. It is also capable of autoregulating its own gene (9-11) and can transactivate the TSH β (4) and growth hormone releasing hormone receptor genes (12,13) in transient transfection assays. Pit-1's molecular mechanism of action in pituitary gene activation and cell differentiation is unknown. As a first step in the elucidation of the molecular events in Pit-1-mediated transcription stimulation, evidence is reported that shows Pit-1 acts during RNA polymerase II transcription complex assembly and that a preformed class II complex is refractory to Pit-1 influence.

MATERIALS AND METHODS

Nuclear Extracts, Pit-1 Purification and Transcription Assays. GH_3 cells were grown, harvested, nuclear extracts prepared and Pit-1 protein was DNA-affinity purified as previously described (14). Cell free transcription assays were performed as previously described (6). Immobilized template assays were done according to the protocol described by Arias and Dynan (15). All prolactin gene fragments [754 bp, both wild-type and distal TATA box mutant, Δ56[A->G], 55[T->A], contained Pit-1 binding sites, distal and proximal TATA boxes, transcription start sites [major +1 and minor -27], the first exon and a portion of the first intron was the transcription template. Figure 1 illustrates the pertinent parts of prolactin promoter used in these experiments. The DNA fragments [-175 to +579] were biotinylated [dATP, BRL] using a

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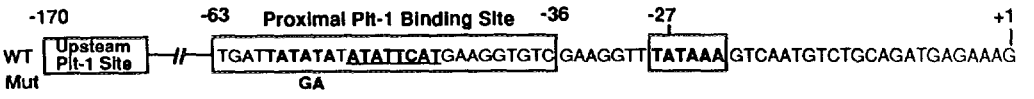

Figure 1. Prolactin Promoter. Alternative cap sites are indicated by +1 and -27. The proximal TATA box is boxed with bold sequence. The proximal Pit-1 binding site, as shown by DNAaseI footprinting (6) is boxed with the Pit-1 recognition octamer (19) in bold and underlined, and the distal TATA box in bold. A distal Pit-1 binding site at about -170 is shown. Mut indicates the altered residues in the Δ56[A–>G], 55[T–>A] mutation that inhibits function of the distal TATA box (19).

Klenow fill-in (16) of the 5' end after cleavage of plasmid pHS1 [an SK^+ plasmid containing the *Hae*III/*Xba*I prolactin fragment from pHXS420 (17)] at the *Hind*III site in the Bluescript polylinker next to the prolactin *Hae*III site. After biotinylation, the 754 bp fragments were isolated by cleavage with *Xba*I and electroelution from low-melting point agarose [BRL]. Sixty μg of biotinylated fragment was linked to 3 ml of packed streptavidin agarose beads [BRL].

The immobilized prolactin template [20 μl of beads with 300 ng of linked prolactin DNA] was incubated in 20 μl GH_3 or HeLa nuclear extract and 10 μl of buffer C (18) for 60 minutes at 30°C. The beads were then pelleted by gentle microcentrifugation, and washed two times with TM buffer [10 mM Hepes, pH7.5, 10% glycerol, 50 mM K-glutamate, 0.1 mM EDTA, and 12 mM $MgCl_2$]. After the final wash, the beads were resuspended in TM buffer [48.5 μl], and 1.0 μl of 25 mM rNTP mix [Pharmacia], 0.1 μl of RNAsin [BRL] plus 0.4 μl of 0.1M DTT were added for another 60 minute incubation at 30°C. In control experiments, omission of rNTPs in the second incubation resulted in undetectable levels of nascent prolactin RNA [data not shown]. After the final incubation, 150 μl of stop solution [0.2M NaCl, 20 mM EDTA, 1% SDS, and 37.5 μg tRNA] was added and the nucleic acids were extracted with 200 μl of chloroform:phenol [3:4]. The aqueous phase was removed and the beads [at the interface] and the organic phase was back-extracted by addition of 50 μl of TM, 45 μl of 5M NaCl and 4.5 μl of 0.2 M EDTA. The nucleic acids in the combined aqueous phases were precipitated overnight at -20°C after addition of three volumes of ethanol. After microcentrifugation, the nucleic acid pellet was washed in 70% ethanol, dried and redissolved in 0.3 M Na_2OAc, and reprecipitated by addition of ethanol and storage overnight at -20°C.

RNA Assay. Nascent prolactin RNA was assayed by primer extension, gel electrophoresis, autoradiography (6) and the [^{32}P] in individual bands was quantitated with a Betagen Betascope. All of the DNA test templates in these experiments [Figures 2 and 3] generated RNA that is 11 nt longer than the endogenous prolactin RNA that is sometimes present in the GH_3 nuclear extract. The test templates were derived from constructions that contain an additional *Sal*I linker in the first exon of the prolactin gene (17).

RESULTS

To begin obtaining an understanding of the role of Pit-1 in transcription activation, an immobilized template protocol was used in order-of-addition assays of Pit-1-reconstituted transcription in Pit^- HeLa nuclear extracts. In this assay, affinity purified rat Pit-1 or buffer was added either during the initial preincubation [complex assembly step] or with addition of rNTPs [transcription step]. Figure 2 illustrates a typical assay and Table 1 lists the quantitation of individual bands using a wild-type prolactin promoter. For comparison, lanes 4 and 5 show an immobilized and non-immobilized assay, respectively, using GH_3 nuclear extract. Lanes 1-3 show that Pit-1 is capable of stimulating transcription only if it is present during assembly of the class II transcription complex [compare lanes 1-3, and Table 1]. Pit-1 induces a 3.5-fold increase

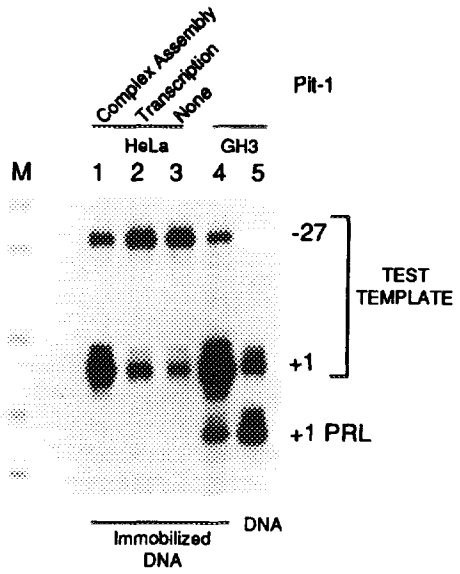

Figure 2. Pit-1 potentiates transcription by favorable interactions during preinitiation complex assembly. Lanes 1 and 2 are immobilized transcription assays using HeLa nuclear extracts that were either supplemented with rat affinity-purified Pit-1 during complex assembly preincubation, or during the transcription stage [indicated above each lane]. Lane 3 is an assay using HeLa extracts without any addition of Pit-1. +1 PRL indicates the primer extension product [72 nt] of prolactin RNA present in the nuclear proteins. The product of the test template [immobilized and non-immobilized] are indicated by +1 [83 nt] from the major initiation site and by -27 from the minor start site [see Materials and Methods and text for further discussion].

in +1 prolactin transcripts if is available during the complex assembly step [Figure 2, lanes 1, 2 and Table 1].

Consistent with previous non-immobilized assays (14), the immobilized template assays also show a two fold decrease in -27 initiation in the HeLa assays when Pit-1 is present during complex assembly [compare lanes 1 and 2, Figure 2]. This is most likely explained by the overlap of the proximal Pit-1 binding site [-36 to -66] with the distal TATA box at -56 [see Figure 1 and (19)]. It was previously shown that Pit-1 binding concomitantly increased +1 and decreased -27 prolactin RNA transcription (19). Thus, the increase in +1 transcription in the experiment illustrated in Figure 2 could be explained, at least partially, by occlusion of the distal TATA box in prolactin

TABLE 1

Lanes	Proteins	Counts[a] -27RNA	Counts[a] +1RNA
1	HeLa + rPit-1 [complex assembly step]	1747	5708
2	HeLa + rPit-1 [transcription step]	3877	1572
3	HeLa	3642	1320
4	GH_3 [Immobilized assay]	1306	20326
5	GH_3 [Nonimmobilized assay]	nc[b]	2413

a = quantitated with a Betagen Betascope.
b = not counted.

promoter occupied by Pit-1. Prolactin promoter occupancy by Pit-1 could have the effect of increasing the availability of basal factors to the proximal core promoter. To address this possibility, a prolactin promoter with a mutation in the distal TATA homology, Δ56[A->G], 55[T->A] that has previously been shown to significantly decrease initiation events at the -27 start site without affecting +1 RNA transcription mediated by Pit-1 activation [see Figure 1 and (17), was used in the assay. A 754 bp restriction fragment from the Δ56[A->G], 55[T->A] plasmid (19) was immobilized on streptavidin agarose beads and assayed for transcription. Similar to a wild-type prolactin promoter, experiments using the mutated template show a 3.7-fold enhancement of +1 transcription when Pit-1 is available for pre-initiation complex assembly but not at later steps in transcription [lanes 1-3, Figure 3; Table 2].

DISCUSSION

Using the prolactin promoter, it was demonstrated that Pit-1 can mediate a positive influence on transcription in the context of class II transcription complex if Pit-1 participates in its assembly. A pre-assembled complex in the absence of Pit-1 is refractory to Pit-1 effects in the assays. There are a number of steps in RNA polymerase II transcription that Pit-1 could exert its influence. It was a concern that the increase in +1 transcription could be explained by occlusion of the distal TATA box, thereby providing additional basal transcription components [perhaps TFIID complex] to the proximal core promoter. Immobilized assays using templates with a disabled upstream TATA box ruled out the possibility that the Pit-1 influence is purely the result of blocking access to the distal TATA box. These combined results indicate that Pit-1 mediates its positive influence on

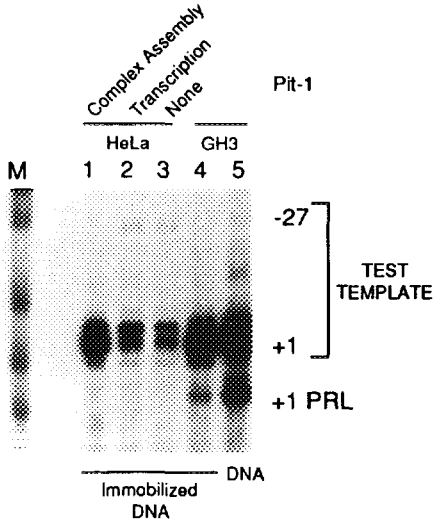

Figure 3. Pit-1 activation is not mediated by occlusion of the distal core promoter. This experiment was done exactly as described in Figure 2 except that a prolactin DNA template that contained a two base pair substitution in the distal TATA box, Δ56[A->G], 55[T->A] Figure 1, was used. The figure is labeled the same as Figure 2. Note that the mutation in the prolactin promoter significantly reduces -27 initiation events on the test template while the pattern of potentiation by Pit-1 at the proximal promoter is the same as with wild-type promoter in Figure 2.

TABLE 2

Lanes	Proteins	Counts[a] -27RNA	Counts[a] +1RNA
1	HeLa + rPit-1 [complex assembly step]	439	28898
2	HeLa + rPit-1 [transcription step]	775	7778
3	HeLa	827	6105
4	GH_3 [Immobilized assay]	521	64964
5	GH_3 [Nonimmobilized assay]	881	164018

a = quantitated with a Betagen Betascope.

mammalian RNA polymerase II transcription by somehow promoting pre-initiation complex assembly or stability, rather than a later step such as promoter clearance or elongation. Although nuclear extracts are depleted of chromatin (18), these experiments have not ruled out the possibility that preincubation in nuclear extracts prevents Pit-1 binding to the prolactin promoter in subsequent incubations.

These observations suggest that Pit-1 promotes the assembly of a quantitatively and/or qualitatively different pre-initition complex in HeLa extracts. The working hypothesis is that Pit-1 mediates enhanced transcription by attracting a different subset of basal factors through contacts with proteins comprising the TFIID complex, possibly TAFs (20). Pit-1 bound to DNA could also modify basal factors to increase complex stability or enhance closed to open complex conversion.

Interestingly, Pit-1 has very specific distant requirements for activation of the prolactin gene [Smith and Liu, et al, submitted]. This is similar to the CRP protein in *E. coli* that, as an activator, stimulates initiation of transcription in similar, but distinctly different ways depending on which of its two preferred sites it is bound (21). Thus, it is possible that Pit-1 bound to its proximal site in the growth hormone promoter [-66 to -96] could, in theory, function by a different mechanism than it does when it occupies the prolactin promoter [-36 to -63]. Understanding these issues is fundamental to Pit-1's function as a transcription activator and, equally important, its role in the differentiation of pituitary cells.

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