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In vitro transcription of the mouse whey acidic protein promoter is affected by upstream sequences

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The promoter sequences from -175 to +10 of the mouse whey acidic protein (WAP) gene are a target for sequence-specific binding of nuclear proteins [(1987) Nucleic Acids Res. 15, 2103-2121]. Using in vitro transcription assays based on nuclear extracts, transcription factors were shown to bind to these sequences and promoter upstream sequences were found to stimulate transcription.

Transcription; Sequence specificity; Promoter binding; Gene promoter

1. INTRODUCTION

Whole cell [1] or nuclear [2] extracts can mediate transcriptional initiation by RNA polymerase II from gene promoters which are not expressed in the respective cell type in vivo [3-6]. This most likely reflects a greater permissiveness of in vitro systems which have lost components present in vivo and contain general factors which are neither cell- nor tissue-specific, but sufficient to initiate and sustain transcription from cloned DNA templates. Thus, soluble transcription systems from non-homologous cell types can be used to test the functionality of DNA templates with different sequences, and to search for gene-specific regions whose effects are manifested by interactions with protein factors involved in transcription.

We used these properties of in vitro transcription systems for an analysis of transcriptional elements in the mouse whey acidic protein (WAP) promoter [7]. The mouse WAP gene has been shown to be expressed preferentially in the mammary gland of lactating animals under the influence of steroid and peptide hormones. Low-level expression can also be observed in non-mammary tissue of wild-type and transgenic mice [8] suggesting that the WAP promoter is also subject to the action of general transcription factors. Since transfection assays into tissue culture cells did not yield information about transcriptional elements within the WAP promoter, soluble in vitro transcription systems appeared to be an alternative. Here, we demonstrate that in vitro transcription from the WAP promoter is initiated accurately in several cell types and that sequences upstream of the promoter, which bind specific nuclear proteins [9], augment transcription.

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2. MATERIALS AND METHODS

2.1. *Plasmids*

The mouse WAP gene template [7] was isolated as a 640 bp *Hin*fI fragment (-410 to 230), treated with mung bean nuclease and cloned into the *Sma*I site of plasmid pTZ18R (Pharmacia). In the resulting plasmid, named WAP-HH640, the *Hin*dIII site of the polylinker was located downstream of the promoter. The plasmid HCMV-Pro contains the promoter (-55 to 7) of the human cytomegalovirus (HCMV) immediate early 1 (IE1) gene [10] cloned into the *Bam*HI site of pUC13. HCMV-WAP1 contains the *Dra*I fragment (-175 to -25) of the mouse WAP gene

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linked to the HCMV IE1 promoter of HCMV-Pro. HCMV-WAPII contains the *Dra*I fragment in reverse orientation.

2.2. *DNA fragments*

The following DNA fragments were prepared from the WAP gene 5′ flanking region: *Dra*I-*Dra*I (-175 to -25), *Dra*I-*Xba*I (-175 to -88), *Xba*I-*Kpn*I (-88 to 24). The DNA fragments were prepared from a low-melting agarose gel.

2.3. *Preparation of nuclear extracts*

Nuclear extracts were prepared from mammary epithelial (T47D), Burkitt Lymphoma (Raji), HeLa and B-cells according to Dignam et al. [2] and the protein concentrations were assayed according to Bradford [11].

2.4. *In vitro transcription assays*

The incubation mixture for the transcription reaction (25μl) contained 12 mM Hepes at pH 7.9, 10% glycerol, 1.6 mM dithiothreitol, 0.12 mM EDTA, 60 mM KCl, 2 mM $MgCl_2$, 5 U placental ribonuclease inhibitor (Amersham), 300 μM ATP, CTP and GTP, 30 μM UTP, [^{32}P]UTP (100 000 cpm/pmol), 70 μg nuclear protein, and 0.5 μg template DNA. After 45 min incubation at 30°C, the reaction was stopped and processed as described [2]. The RNA was subjected to electrophoresis in a 4% sequencing gel. In the competition assay, 35 μg nuclear protein and 0.125 μg DNA template were used. Nuclear proteins were incubated for 15 min with competitor DNA. Upon adding template and nucleotides, incubation was continued for 45 min. In each assay using specific fragments, the amount of competitor DNA was adjusted to 200 ng using poly(dI-dC).

2.5. *Transfection and transient expression*

Transfection of plasmids HCMV-Pro, HCMV-WAPI and HCMV-WAPII into T47D and HeLa cells was performed using the standard calcium phosphate precipitation technique. CAT activities were measured with [^{14}C]chloramphenicol by standard procedures.

3. RESULTS AND DISCUSSION

Our initial attempts to derive transcription extracts from homologous tissues involved mouse mammary glands. We found that nuclear extracts prepared from this tissue were not capable of correctly initiating transcription from the WAP gene and other cellular and viral gene templates. This prompted us to examine nuclear extracts from a mammary cell line and other human cell lines for their transcriptional activity. Run-off transcription assays were performed with nuclear proteins from different cell types which contain transcription factors capable of promoting accurate transcriptional initiation from gene templates [12]. The plasmid WAP-HH640 containing the mouse WAP promoter was truncated at the *Hin*dIII site in the polylinker, which is located about 260 nucleotides 3′ of the start site of transcription, and at the *Eco*RI site in the polylinker, which is located about 440 nucleotides 5′ of the start site. We focused on this region of the WAP gene because sequences further upstream of the promoter did not affect in vitro transcription (not shown). In the in vitro transcription assay run-off transcripts with a size of 263 nucleotides were generated by nuclear proteins from T47D, Raji, HeLa and B-cells (fig. 1A). This indicates that the WAP promoter can be activated in vitro by proteins from cell types derived from different lineages. This is in contrast to the intact animal where the WAP promoter is preferentially active in the lactating mammary gland [8].

Using nuclear extract from the human mammary cell line T47D we investigated several transcriptional properties of the WAP promoter. Transcription from the WAP promoter is sensitive to 0.5 μg/ml α-amanitin, indicating that it is dependent on polymerase II (fig. 1B). Efficiency of in vitro transcription can be dependent on the concentration of Mg^{2+} [1,2]. The WAP promoter is transcribed best in the presence of 6 mM $MgCl_2$, but is also active over a wide concentration range of Mg^{2+} (fig. 1C). The ability of the in vitro system to initiate transcription at the start site used in vivo was confirmed by using templates truncated at the *Bam*HI or *Xba*I sites which result in run-off transcripts of 233 and 239 bp, respectively (fig. 1D, lanes 3,4).

Having established a polymerase II-dependent in vitro transcription system for the WAP gene, we analyzed its promoter sequences for their ability to modulate transcription. Using restriction enzymes we deleted specific sequences from the promoter and analyzed the resulting templates using nuclear extracts from T47D cells. Deleting sequences upstream of the *Sst*I site at −354 did not alter the level of run-off transcripts (fig. 1D, lane 2). Additional deletion of sequences between −88 and −354 resulted in a significant reduction of in vitro transcripts (fig. 1D, lane 4). Restricting the template with *Xba*I at −88 leads to the loss of DNA sequences which are recognized by nuclear protein from several cell types including mammary epithelial cells [9], and concomitantly leading to the destruction of additional sites of protein-DNA interaction located over the *Xba*I site. This suggested that the sites of protein-DNA interaction in the WAP promoter are involved in transcriptional stimulation in vitro.

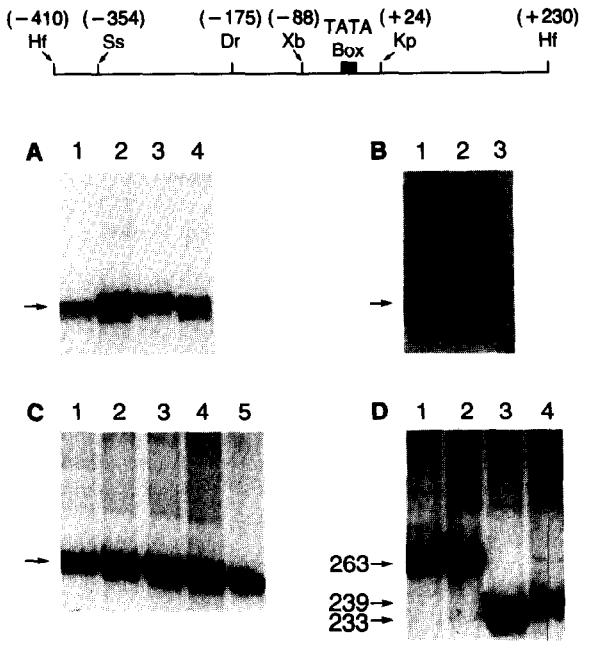

Fig.1. In vitro transcription from WAP gene promoter templates. In (A-C) we used as template the plasmid WAP-HH600 cut with *Hin*dIII and *Eco*RI. (A) Run-off transcription assay with nuclear extract from Raji (lane 1), B-cells (lane 2), T47D cells (lane 3) and HeLa cells (lane 4). The arrow indicates the transcript of 263 nucleotides. (B) Transcription in nuclear extract from T47D cells in the absence (lane 1) or presence of 0.5 μg/ml (lane 2) or 200 μg/ml α-amanitin (lane 3). (C) Effect of Mg^{2+} on transcription from the WAP promoter in T47D extract. Lanes: (1) 2 mM, (2) 4 mM, (3) 6 mM, (4) 8 mM, (5) 10 mM Mg^{2+}. (D) Transcription from WAP deletion mutants in T47D extract. The following templates were used (lanes); (1) WAP-HH600 cut with *Eco*RI and *Hin*dIII, (2) cut with *Sst*I and *Hin*dIII, (3) cut with *Eco*RI and *Bam*HI, (4) cut with *Xba*I. The numbers indicate the size of the respective transcripts in nucleotides. The map at the top shows the WAP promoters and important restriction sites are indicated. HF, *Hin*fI; Ss, *Sst*I; Dr, *Dra*I; Xb, *Xba*I; Kp, *Kpn*I.

An alternative way of studying the role of DNA-binding proteins in the transcriptional activation of promoters involves competition assays with specific DNA fragments [13,14]. As shown in fig. 2, incubation of the extract with fragments from the WAP gene promoter prior to adding the template resulted in decreased transcription. The *Dra*I-*Dra*I (−175 to −25) fragment which contains several protein binding sites [9] competed well at low excess (fig. 2, lane 3-5). Effective reduction of transcription by the *Dra*I-*Xba*I (−175 to −88)

Fig.2. Effect of specific competitor fragments on the in vitro transcriptional activity of the WAP promoter. The plasmid WAP-HH600 was cut with *Eco*RI and *Hin*dIII and used as template in T47D cell nuclear extract in the absence of competitor DNA (lane 1), 200 ng poly(dI-dC) (lane 2), 50, 100 and 150 ng *Dra*I-*Dra*I fragment (lanes 3-5), 50, 100 and 150 ng *Xba*I-*Kpn*I fragment (lanes 6-8) and 50, 100 and 150 ng *Dra*I-*Xba*I fragment (lanes-9-11).

and *Xba*I-*Kpn*I (−88 to 24) fragments was observed at higher concentration of competitor DNA (fig. 2, lanes 6-11). The presence of nonspecific competitor DNA [poly(dI-dC)] led to an increase of transcription. These data show that specific transcription factors interact with the WAP promoter upstream region. A significant feature of the *Dra*I-*Xba*I fragment is a conserved sequence motif [consensus sequence: TGGCAAGSCTGGGCST-(G)YTCTCTCT(NTG)TGGCAARA] which can be found in similar positions in the promoters of four whey protein genes and which is recognized by nuclear proteins in the mouse WAP [9] and rat α-lactalbumin gene [15]. This suggests that this conserved sequence is involved in transcriptional regulation of the WAP and other milk protein

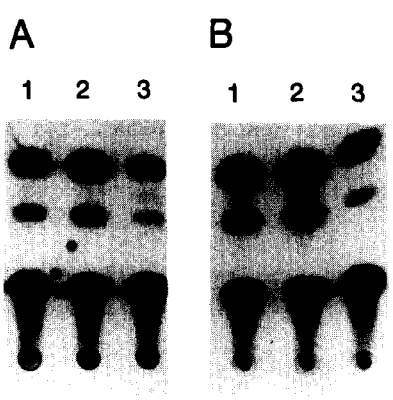

Fig.3. Transient expression of the HCMV IE1 gene promoter in the presence or absence of the WAP upstream region. Lanes 1-3 show CAT expression in T47D (A) and HeLa cells (B). CAT activity directed by plasmids HCMV-WAPI (lanes 1), HCMV-WAPII (lanes 2) and HCMV-PRO (lanes 3).

genes. The protein binding sites in the WAP and α-lactalbumin genes contain similar sequences (TGGCARNNWCKKC) [15] which resemble part of the nuclear factor 1 binding site and which is recognized by purified nuclear factor 1 from HeLa cells (not shown). Therefore, nuclear factor 1-like proteins can be involved in the transcriptional regulation of some milk protein genes. Recently, it has been shown that nuclear factor 1 binding sites can stimulate transcription in vivo [16,17] and in vitro [18].

Since the WAP promoter upstream region activates the homologous promoter, we addressed the question as to whether this region can also enhance transcription from a heterologous promoter. The *Dra*I-*Dra*I fragment in which the WAP TTTAAA box is absent, was linked to the promoter of the human cytomegalovirus (HCMV) immediate early 1 (IE1) gene. We chose the IE1 promoter because it can be activated by the IE1 enhancer, which like the WAP upstream region contains nuclear factor 1-like binding sites [19]. In in vivo transfection assays in T47D and HeLa cells we observed a 3–5-fold stimulatory effect by the WAP upstream region on the HCMV promoter in both orientations (fig. 3).

Our results suggest that upstream sequences of the mouse WAP promoter, which are recognized by nuclear proteins [9], have the capability of increasing transcription in vitro. This region also enhances transcription from the HCMV promoter in vivo, indicating that the WAP gene sequence between −175 and −25 can act as a functional promoter upstream element. Since we could not measure activity of the upstream region in the context of the WAP TTTAAA box and surrounding sequences in transient expression assays, it is possible that this TTTAAA box is a target for transcriptional repression.

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