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Binding of multiple factors to the MMTV promoter in crude and fractionated nuclear extracts

Michael G.Cordingley and Gordon L.Hager*

Hormone Action and Oncogenesis Section, Laboratory of Experimental Carcinogenesis, Building 37, Room 3C19, National Cancer Institute, Bethesda, MD 20892, USA

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ABSTRACT

Hormone activation of MMTV transcription results in the establishment of a tightly bound transcription factor complex at the promoter (Cordingley et al., Cell 48, 261-270, 1987). We have characterized two fractionable binding activities which participate in this complex. One factor, previously identified as the mouse homologue of NF-1 (or CTF), protects sequences -82 to -56 from exonuclease III digestion in vitro. Sequences protected by a second factor (-42 to -4) span the TATA box of the promoter, suggesting that the binding activity in this fraction is equivalent to the HeLa cell transcription factor TFIID (Sawadogo and Roeder, Cell 43, 165-175, 1986). The downstream boundary of exonuclease protection by the putative TATA-binding factor is -4; DNaseI footprinting of this fraction, however, showed additional protection of discrete sites downstream of the cap site. The apparent concentration and promoter-specific binding activity of both factors is unaffected by hormone treatment of the cells.

INTRODUCTION

The promoter of mouse mammary tumor virus is activated by a variety of steroid receptors. We recently demonstrated by in vivo exonuclease III footprinting that transactivation of MMTV by the glucocorticoid receptor leads to the formation a high affinity protein complex over the promoter (1). Formation of this complex results in a broad ExoIII-resistant "footprint" in MMTV chromatin, with boundaries at -82 and +12 with respect to the site of transcription initiation. One component of this initiation site complex was tentatively identified (1) as the mouse cell homologue of nuclear factor 1 (NF-1). This protein, originally characterized as an adenovirus replication factor (2,3), is therefore rigorously shown to be a transcription factor for MMTV. This result supports the putative identity (4) of NF-1 and CTF (CAT-box binding transcription factor), a protein which stimulates transcription

from a number of promoters in vitro (4,5). Recruitment of such factors onto promoter DNA may be a common mechanism in enhancer activation, and their characterization will be central to understanding the processes involved in transcription initiation and its regulation.

In this study we have utilized the sensitive in vitro exonuclease III footprinting assay (1,6) to examine binding of factors to the MMTV promoter region in crude and fractionated nuclear extracts, and to identify a second component of the hormone dependent initiation site complex. Binding activities were fractionated by phosphocellulose column chromatography. A mouse cell equivalent of NF-1, is eluted from phosphocellulose by 0.5M KCl. We conclude that this protein is responsible for the upstream boundary of the in vivo promoter complex.

A second factor protects sequences containing the TATA homology of the MMTV promoter; although present in 0.5M KCl fractions, it is optimally eluted by 1.0M KCl. This factor, previously referred to as F-i (1), protects sequences -42 to -4 from exonuclease digestion. F-i is probably equivalent to TFIID, a factor from HeLa cells which binds to the TATA region of the adenovirus major late promoter (AMLP) and is an essential component in reconstituted in vitro transcription reactions with this promoter (7,8). Phosphocellulose fractions containing this binding activity were shown by DNAseI footprint analysis to protect TATA containing sequences (-45 to -20); extensive weaker protection at discrete positions occurred as far downstream as position +37. This protein fraction clearly contains factor(s) which make multiple DNA contacts in the region of the TATA box and cap site of the promoter.

Glucocorticoid activation of MMTV thus involves receptor-mediated formation of a promoter bound complex composed of at least two factors, NF-1 (CTF) and F-i. Both factors are detectable in apparently equivalent quantity and affinity in nuclear extracts from hormone-free or hormone-treated cells, suggesting that secondary modifications of NF-1 or F-i are not critical to the assembly of the factor complex. Identification of the proteins that are assembled into a preinitiation complex by glucocorticoid receptor provides a basis for further exploration of the mechanism

of steroid transactivation. Steroid receptors may interact directly with components of the transcription initiation complex; alternatively, receptor binding may perturb the nucleoprotein template such that accessibility for the preinitiation complex is altered. Identification of the components of the complex will considerably facilitate experimental tests of these models.

MATERIALS AND METHODS

Preparation and Fractionation of Nuclear Extracts

Crude nuclear extracts were prepared from 34I cl.101, an MMTV-induced murine mammary carcinoma cell line (9) essentially as described by Dignam and Roeder (10). This protocol yielded crude extract at a concentration of approximately 10 mg/ml. The extract was fractionated by P11 phosphocellulose chromatography as described by Dignam et al (10) to yield four subfractions; flow through (5 mg/ml), 0.3M KCl (3 mg/ml), 0.5M KCl (1 mg/ml) and 1M KCl (200 μg/ml). The fractions eluted from the column by KCl were dialyzed for 3 hours against buffer D, which contained 20mM Hepes pH 7.9, 0.1M KCl, 0.2mM EDTA, 0.5mM DTT, 0.5mM phenylmethylsulfonyl fluoride (PMSF), and stored in small aliquotes at -80°C.

Footprinting Assays

Exonuclease III footprinting: Binding reactions and exonuclease III digestion of protein-DNA complexes were essentially as described previously (1) with the exception that extracts (5 μl) were incubated with 10 fmoles 5' terminally labelled DNA probe in 50 μl of 10mM Tris-HCl (pH 7.5), 50mM NaCl, 1mM DTT and 5% glycerol containing 1 μg poly dI.dC (PL-Pharmacia) as nonspecific competitor nucleic acid.

DNaseI Footprinting: Extract (30 μl) was incubated with 10 fmoles probe DNA in 50 μl at a final concentration of 12mM Hepes (pH 7.9), 0.12mM EDTA, 60mM KCl, 12% glycerol, 0.5mM DTT and 20 μg/ml poly dI.dC (PL-Pharmacia). DNase treatment and sample preparation were essentially as described by Jones et al (5). Digestion products from footprinting reactions were analyzed on 5% sequencing gels in parallel with Maxam-Gilbert sequencing ladders (11).

Preparation of probes: Probe DNA's were prepared from plasmid pM50 (1) which contains the -223(HaeIII)/+107(BamHI) MMTV promoter fragment (with a synthetic EcoRI linker at position -223) cloned between the BamHI and EcoRI sites of pSP65 (Bethesda Research Laboratories). DNA was cleaved with either BamHI (noncoding strand probe) or EcoRI (coding strand probe), dephosphorylated with bacterial alkaline phosphatase and 5' terminally labelled using T4 polynucleotide kinase. The 330 base pair probe fragment was released by cleavage at the other flanking restriction site with the corresponding restriction endonuclease (BamHI or EcoRI) and purified by electrophoresis on a 5% polyacrylamide gel.
Competitor DNA: Plasmid pd167 was kindly provided by Dr T. Kelly and consists of the terminal 67 base pairs of the Adenovirus 2 genome cloned between the BamHI and EcoRI sites of pUC8. Plasmid pM50 is described above.

RESULTS

To detect potential transcription regulatory proteins which bind to the promoter of mouse mammary tumor virus, we used the in vitro exonuclease III footprinting assay. In this technique, 5'-terminally labelled DNA fragments containing MMTV promoter sequences are incubated in the presence of suitable non-specific competitor DNA with crude nuclear extracts prepared by the protocol described by Dignam et al (10). After incubation, protein-DNA complexes are detected by digestion with E. coli exonuclease III (ExoIII). Specifically bound proteins physically obstruct the 3' to 5' processive digestion of DNA by the exonuclease, and create novel termination products which may be distinguished from natural termination events by comparing the array of digestion products with those created by digestion of the probe DNA fragment in the absence of added extract.

Promoter binding activities in crude nuclear extracts

In experiments designed to detect nuclear factors binding to the MMTV promoter region we utilized probe fragments spanning 330 bases of the promoter region, which were terminally labelled at position -223 or position +107. The former probe serves to detect the upstream boundary of protein binding sites on the coding strand while the latter can be used to detect the down-

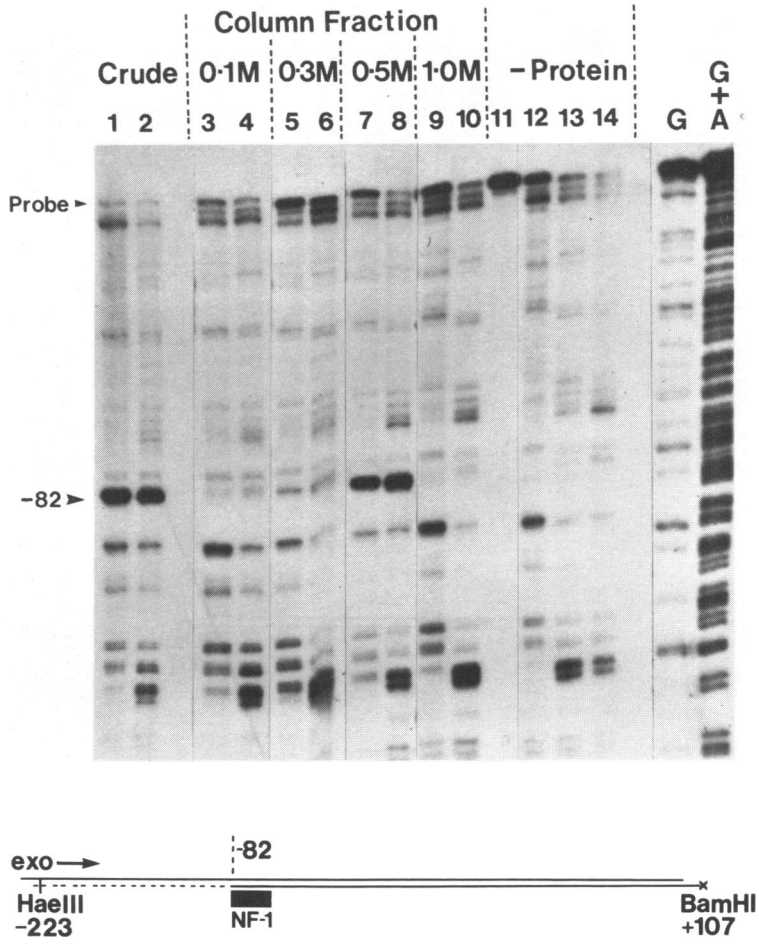
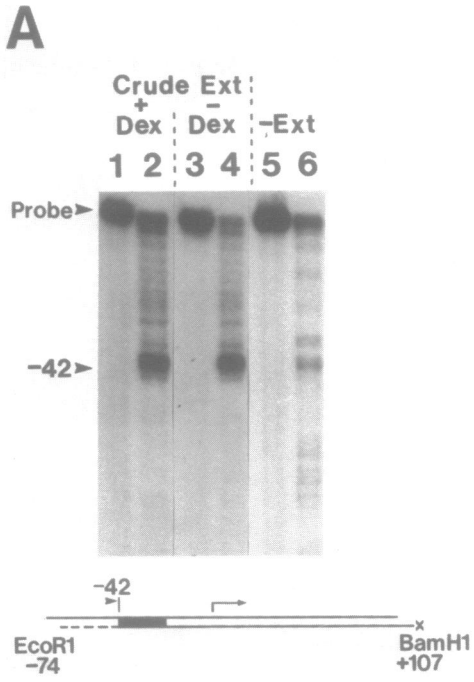


Figure 1. Exo III footprint analysis of crude and fractionated nuclear extract.
The -223/+107 HaeIII-BamHI promoter fragment labelled at the 5' terminus of the non-coding strand was used as probe. Binding conditions and exonuclease III digestion conditions are described in Materials and Methods. ExoIII termination products were analysed on a 5% sequencing gel, and compared with fragments generated by sequence analysis of the same probe (lanes labelled G and G+A). Probe DNA was incubated with the following fractions: 40 μg unfractionated nuclear extract (lanes 1 and 2); 5 μl of phosphocellulose column eluate fractions, 0.1M KCl (lanes 3 and 4), 0.3M KCl (lanes 5 and 6), 0.5M KCl (lanes 7 and 8), 1.0M KCl (lanes 9 and 10); no added protein (lanes 11-14). Protein-DNA complexes were digested with exonuclease III at the following concentrations: 200 units/ml (lanes 1, 3, 5, 7, 9, 13), 1000 units/ml (lanes 2, 4, 6, 8, 10, 14), and 40 units/ml (lane 12). Lane 11 contains undigested probe.


A
Crude Ext
+
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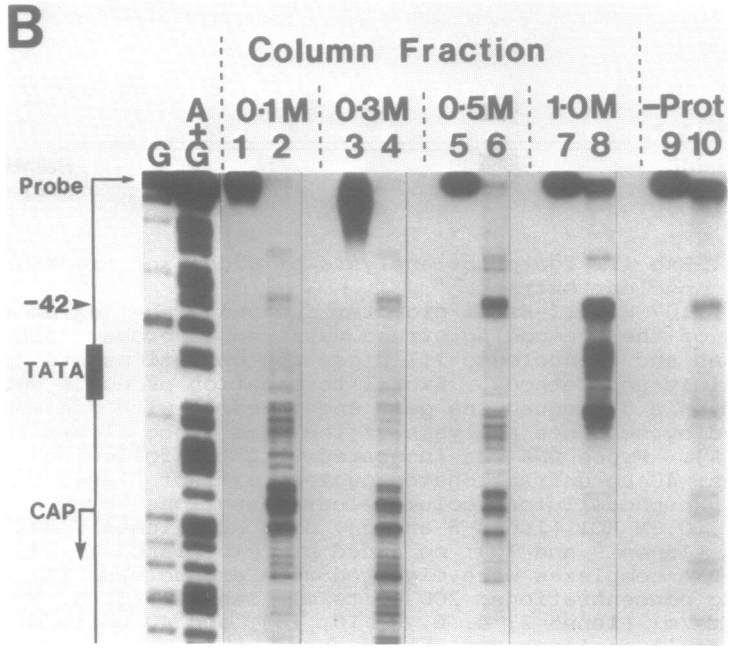
Dex
−Ext
1 2 3 4 5 6
Probe
−42
−42
EcoR1
−74
BamH1
+107


B
Column Fraction
A
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0·1M
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−Prot
1 2 3 4 5 6 7 8 9 10
Probe
−42
TATA
CAP

stream boundary of protected regions on the non-coding strand. Figure 1 shows the results of an exonuclease III assay in which crude nuclear extract (lanes 1 and 2) was probed with the -223/+107 promoter fragment terminally labelled at +107 (BamHI). A single major exonuclease III boundary at position -82 corresponds to ExoIII termination at the upstream boundary of sequences which bind NF-1. This boundary is highly resistant to increased exonuclease concentrations and is not observed in the absence of added extract (lanes 11-14). No other extractdependent termination products are observed with this probe; proteins potentially bound to sites distal to the NF-1 binding site cannot be detected.

To detect putative binding events downstream from NF-1, probe DNA was further cleaved with EcoRI* at position -74. This cleavage site is in the central portion of the NF-1 recognition sequence and enables detection of proteins bound downstream from this site (Fig. 2A). Nuclear extracts from hormone-treated and hormone-free cells were incubated with this probe and subjected to ExoIII digestion. In the presence of nuclear extract (lanes 2 and 4) exonuclease digestion resolves a prominent boundary at position -42 which is not detected in control digestions of protein-free probe DNA (lane 6). From this experiment we deduce that sequences downstream from -42 are protected by binding of a second nuclear factor. Furthermore, since equivalent protection

Figure 2. ExoIII footprint analysis of downstream promoter binding activity.
The -74/+107 EcoRI*-BamHI promoter fragment labelled at the 5' terminus of the non-coding strand was used as probe. Binding and exonuclease III digestion conditions are described in Materials and Methods. ExoIII termination products were analysed on a 5% sequencing gel and compared with sequence markers (lanes labelled G and A+G) of the same probe fragment.
Panel 2A. Probe DNA was incubated with 40μg crude nuclear extract from cells treated with 2μM dexamethasone (lanes 1 and 2), from untreated cells (lanes 3 and 4), or no added protein (lanes 5 and 6). Protein-DNA complexes were digested with 40 units/ml exonuclease III (lanes 2, 4, 6) or not digested (lanes 1, 3, 5).
Panel 2B. Probe was incubated with 5 μl of phosphocellulose column fractions as follows: 0.1M KCl (lanes 1 and 2), 0.3M KCl (lanes 3 and 4), 0.5M KCl (lanes 5 and 6), 1.0M KCl (lanes 7 and 8), no added protein (lanes 9 and 10). Protein-DNA complexes were digested with exonuclease III at 40 units/ml (lanes 2, 4, 6, 8, 10), or not digested (lanes 1, 3, 5, 7, 9).

is observed with both extracts it appears that the binding activity of this factor is unchanged in hormone treated cells.

Further experiments were carried out using a probe labelled at the 5'end of the coding strand at position -223. ExoIII footprint analysis of crude extract using this probe is shown in Figure 4, lanes 1-4. Two extract-dependent ExoIII boundaries are generated. The first (lane 2) is proximal to the site at which ExoIII initiates digestion and is relatively labile to exonuclease III, being disrupted by high levels of enzyme (lanes 3 and 4). The -42 boundary is also labile to high levels of ExoIII (results not shown). Comparison of this boundary with the reaction products of partial G-specific chemical cleavage of the probe fragment (lane "G") located this termination site at position -4. Detection of the boundary at -4 is also associated with the observation of high levels of pretermination in the region +50 to +20. These sites of termination appear to correspond largely to enhancements of natural termination sites (lane 8) and are not observed at increased levels in fractions enriched for the -4 boundary (lane 5; see below). This effect is probably due to the low levels of enzyme required to detect specific termination at -4.

At increased levels of exonuclease, the -4 boundary is not detected, and a major, highly resistant boundary appears at position -55. The -55 boundary corresponds to the downstream limit of ExoIII protection by NF-1 at -55 (1). ExoIII footprint analysis of crude nuclear extracts therefore demonstrates two promoter binding activities. NF-1 protects upstream promoter sequences -82 to -55 and a second activity protects sequences -42 to -4, a region that encompasses the TATA sequence. Interaction of the second activity with TATA sequences appears to be of considerably lower affinity than that of NF-1 with its binding site as evidenced by its lability during digestion with high levels of exonuclease.

Competition of promoter binding activities

To confirm the identity and sequence specificity of the binding activities observed in crude nuclear extracts, we carried out competition experiments. The results of an experiment where binding of the factor which protects upstream promoter sequences

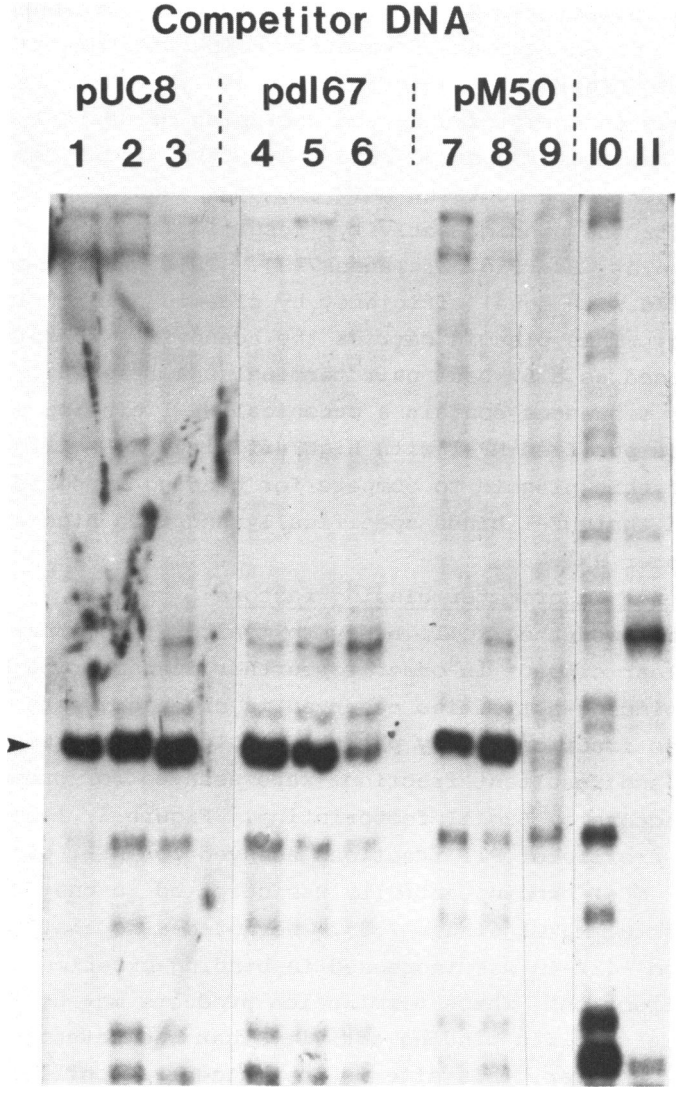


Figure 3. Competition of NF-1 binding to MMTV promoter DNA. Nuclear factor 1 binding in crude nuclear extract was assayed as described in Figure 1. Competitor plasmid DNAs were included in the binding reactions at 0.2 μg/ml (lanes 1, 4, 7), 2.0 μg/ml (lanes 2, 5, 8) and 20 μg/ml (lanes 3, 6, 9). Specific competitor DNAs (see Materials and Methods) were puc8 (lanes 1-3), pdl67 (lanes 4-6) and pM50 (lanes 7-9). Protein-DNA complexes were resolved by digestion with 1000 units/ml ExoIII (lanes 1-10) or 200 units/ml (lane 11). Lanes 10 and 11 contain probe DNA incubated in the absence of added extract.

-82 to -55 (putatively NF-1) was assayed in the presence of increasing concentrations of specific competitor DNA's are presented in Figure 3. Lanes 1-3 indicate that detection of the -82 boundary is unaffected by the inclusion of up to 1 microgram of unlabelled vector plasmid pUC8 DNA in the binding assay. Plasmid pM50 (which contains MMTV promoter sequences) successfully competed for the binding activity, resulting in reduced ExoIII termination at -82 (Fig. 3, lanes 7-9). This binding activity was competed with equal efficiency by plasmid pdl67 (Fig. 3, lanes 7-9). This plasmid carries the adenovirus 2 replication origin cloned as a 67 base pair terminal DNA fragment. The adenovirus sequences contain a canonical NF-1 binding site (3) which binds purified NF-1 with high affinity (K_d = $2x10^{-11}$). The ability of this plasmid to compete for binding supports our conclusion that NF-1 binds specifically and with high affinity to MMTV promoter DNA.

Fractionation of promoter binding factors

We utilized the ExoIII assay to monitor fractionation of the crude nuclear extract in order to further characterize MMTV promoter binding activities observed in crude extracts. Nuclear extract was fractionated by phosphocellulose P11 column chromatography and resultant fractions were assayed for promoter binding factors by ExoIII footprinting. Figure 1, lanes 3-10 shows the profile of P11 fractions assayed for NF-1 binding activity. NF-1 binding activity was detected in the fraction eluted with 0.5M KCl (lanes 7,8). We noted also that termination at position -42/-40 was increased in binding experiments with the 1.0M KCl fraction. These termination products may be the result of the same specific binding activity that was detected in crude extracts. However, this site is in the central portion of the probe fragment, and termination products could be generated in this region due to simultaneous digestion of the probe from each 3' terminus. Digestion by ExoIII proceeds from each end until the fragment is rendered single stranded. Variability in the position at which the converging exonucleases meet can result in generation of artifactual termination products in this region.

The same fractions were therefore assayed for the presence of the -42 binding activity with the -74/+107 probe (Fig. 2B).

This binding activity was separable from NF-1 binding activity, eluting principally in the 1.0M KCl fraction (lane 8). We note, however, that significant binding activity was detected in the P11 0.5M fraction (lane 6). Although material which eluted in the 1.0M KCl fraction causes high levels of ExoIII termination at position -42, two other areas of termination centered around positions -33 and -22 appear to be associated with this fraction. Unlike the termination product -42, activities associated with these boundaries are were not observed in crude extracts. Whether distinct DNA-binding proteins can be associated with these termination events remains to be elucidated.

A number of extract-dependent boundaries at positions downstream (-5 to +1) were detected in the 0.1M KCl (flow through) fraction (lane 2), however we have been unable to substantiate the existence of a protein factor in FT fractions which binds sequences downstream of this position. No Exo III terminations corresponding to the downstream boundary of a putative protein binding domain are evident in ExoIII assays with probe DNA labelled on the opposite strand.

Concomitant NF-1 and TATA-factor binding

Using -223/+107 probe end-labelled at -223 on the coding strand to detect the downstream boundary of protein binding domains, P11 fractions were ExoIII footprinted. As with the assay using the +107 terminally labelled probe (Fig. 1) NF-1 binding activity was found to elute from the column with 0.5M KCl (results not shown). However contrary to expectation, detection of the downstream boundary of the TATA-binding activity (-4) was high in the same fraction (Fig. 4, lanes 5-7). Most of the TATA-binding activity is present in the 1.0M KCl fraction when the short probe lacking NF-1 binding sequences is used in the assay (Fig. 2B). However, detection of this factor by the complete promoter probe, in which NF-1 sequences are directly upstream from TATA sequences, is highest in the 0.5M KCl fraction. One explanation for this result is that the presence of large amounts of NF-1 binding activity in the 0.5M fraction, and the concomitant occupation of the closely adjacent NF-1 binding site on the probe DNA, stabilize the interaction of the TATA-binding factor with DNA. It is quite feasible that the TATA-binding

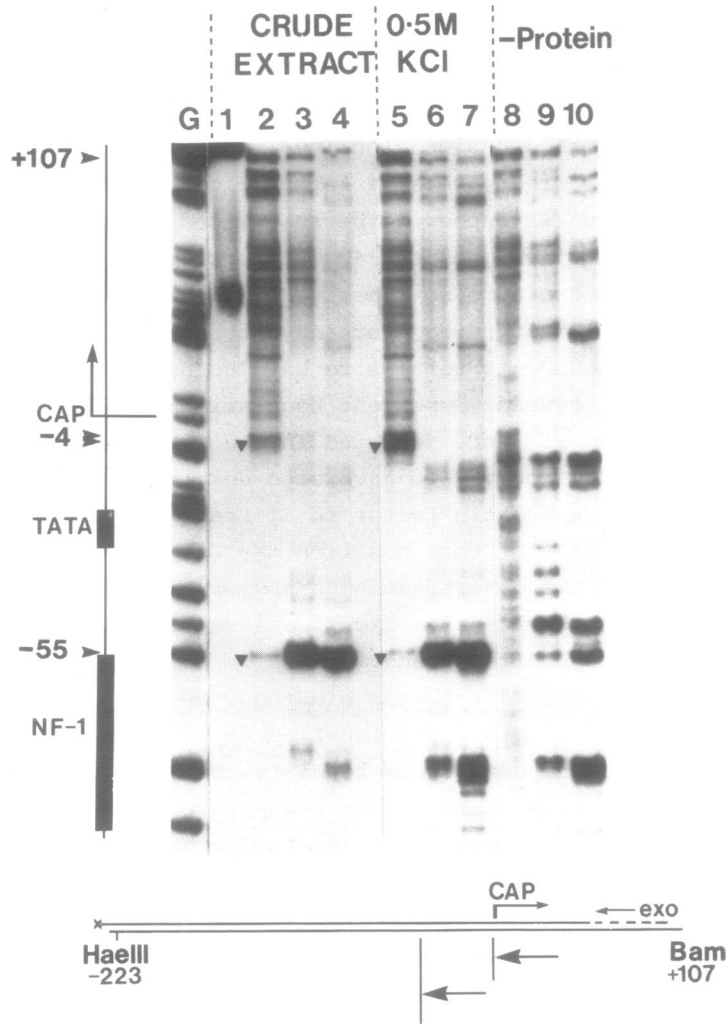
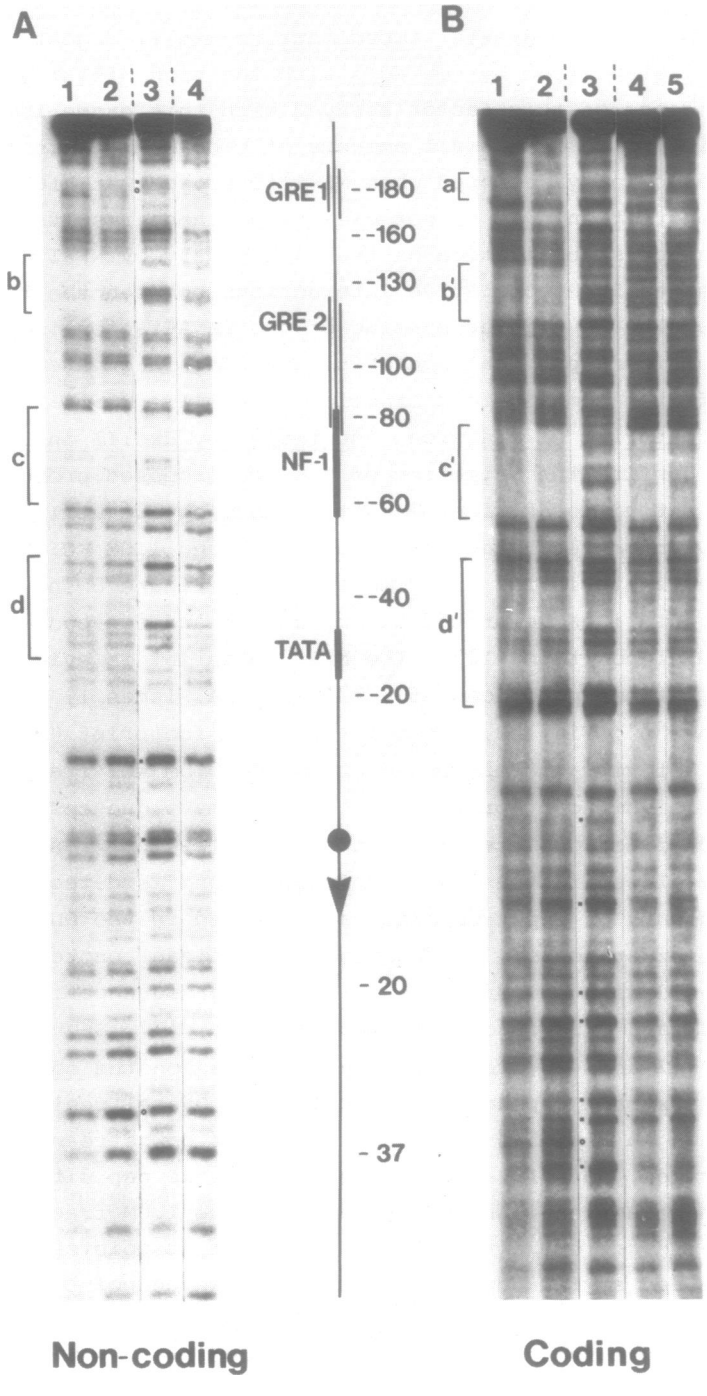


Figure 4. ExoIII detection of the downstream boundaries of promoter binding activitites.
The -223/+107 HaeIII-BamHI promoter fragment labelled at the 5' terminus of the coding strand was used as probe. Binding conditions and exonuclease III digestion are described in Materials and Methods. ExoIII termination products were analysed on a 5% sequencing gel and compared with sequence markers (lane labelled G). Probe DNA was incubated with 40 μg unfractionated nuclear extract (lanes 1-4), 5 μl of phosphocellulose column 0.5M KCl fraction (lanes 5-7), or no added protein (lanes 8-10). Protein-DNA complexes were digested with exonuclease III at 40 units/ml (lanes 2, 5, 8), 200 units/ml (lanes 3, 6, 9), or 1000 units/ml (lanes 4, 7, 10). Lane 1 contains untreated probe.

factor, whose interaction with DNA is labile to high levels of ExoIII, presents a greater impediment to ExoIII digestion when laterally stabilized by contacts with the high affinity NF-1. The abundance of this factor assayed with this probe is therefore misleading since even small amounts of binding of this factor may be preferentially detected when NF-1 is present at high levels in the same fraction. It is not clear from these results whether the apparent stabilization of the -4 boundary by NF-1 binding reflects genuine cooperative interactions between the two factors which results in tighter binding of the TATA-factor, or merely non-significant physical contacts resulting in greater resistance of the factor-complex to disruption by ExoIII.

DNaseI footprint of fractions identified by ExoIII analysis

To confirm the detection of the two proposed promoter binding activities by independent methodology we utilized the -223/+107 promoter fragment and the 0.5M and 1.0M KCl P11 fractions in DNaseI footprinting experiments (12). Figure 5 shows the DNaseI footprints generated on the coding and non-coding strands labelled at position +107. The NF-1 footprint is evident on both strands between positions -57/-79 (Fig. 5A, lanes 1, 2, 4, footprint "c"; Fig. 5B, lanes 1, 2, 4, 5, footprint "c'"). The binding activity is more abundant in the 0.5M KCl fraction although significant protection of the same sequences is evident with 1.0M fraction material. This indicates that residual NF-1 activity elutes in the 1.0M KCl fraction and is detected when large amounts of input material are assayed in the DNaseI footprinting experiments. In addition, significant protection of sequences downstream from the NF-1 binding sequence is evident in both 1.0M and 0.5M fractions (footprint d, d'). Sequences between -47 and -20 are uniformly protected on the coding strand. However, extract dependent differences in DNaseI resistance (indicated in Fig. 5) are evident at sites downstream from -20 on both strands, notably at positions around the cap site. Bound DNA with both fractions, but especially the 1.0M fraction exhibit further protection, although less distinct at points beyond the cap site and as far downstream as +37 at which point several protected bases are observed and increased sensitivity is observed on both strands with the 0.5M fraction.


A
B
1 2 3 4
1 2 3 4 5
GRE1
GRE 2
NF-1
TATA
-180
-160
-130
-100
-80
-60
-40
-20
-20
-37
a
b
c
d
a'
b'
c'
d'
Non-coding
Coding

DNase1 footprinting therefore confirms the existence of a binding activity in both 0.5M and 1.0M KCl fractions which is responsible for protection of TATA sequences. The results complement those from ExoIII footprinting and suggest that a protein binds to the TATA homology resulting in local protection of TATA sequences and lower levels of protection for considerable distance downstream.

In addition, two further regions of DNase1 protection (a and b, b'; A and B, lanes 1,2) were attributable to a binding activity in 0.5M P11 material but not in the 1M P11 fraction (A, lane 4 and B, lane 4 and 5). The significance of these protected regions has not yet been determined. Protection of the region -115/-140 (b and b') and of -175/-185 (coding strand; a) is observed. Discreet changes in DNase1 sensitivity at non-coding strand sequences corresponding to coding strand footprint "a" are also evident.

DISCUSSION

Exo III footprint analysis in combination with DNase1 footprinting has allowed us to detect MMTV promoter binding activities in crude and fractionated nuclear extracts. Our aim was to identify putative participants in the stable transcription factor promoter complex that we have observed in vivo. The characterization of factors which are recruited to the active promoter during hormone activation is an important step in

Figure 5. DNase1 footprint analysis of promoter binding fractions.
Protein fractions were incubated with the -223/+107 promoter fragment probe 5' end-labelled on the non-coding strand (panel 4A) or 3' end-labelled on the coding strand (panel 4B). Binding and DNase1 cleavage conditions were as described in Materials and Methods.
Panel 5A. Probe DNA was incubated with 0.5M KCl phosphocellulose column fraction (lanes 1 and 2), 1.0M KCl column fraction (lane 4), or no added protein (lane 3). Protein-DNA complexes were digested with 0.2 μg/ml DNase1 (lanes 2, 3, 4), or 0.05 μg/ml (lane 1).
Panel 5B. Probe DNA was incubated with 0.5M KCl phosphocellulose column fraction (lanes 1 and 2), no added protein (lane 3), or 1.0M KCl column fraction (lane 4 and 5). DNase1 cleavage was performed with 0.05 units/ml (lanes 1 and 4), or 0.2 units/ml (lanes 2, 3, 5).

understanding the process by which steroid receptors regulate transcription initiation. Identification of the molecular participants in an active promoter complex may facilitate analysis of molecular interactions in promoter complex formation and subsequent reconstruction of MMTV transcriptional regulation in a cell free system.

Two binding activities were identified which protect proximal promoter sequences from ExoIII in in vitro binding assays. The first factor protected sequences -82 to -55, which encompass the NF-1 consensus binding sequence (-76)TGGANNNNNAT/GCCA(-64) from exonuclease digestion. This component of the initiation site complex was previously identified from in vivo studies with MMTV chromatin as the mouse homologue of NF-1 (1). A second factor was identified which protected sequences -42 to -4 from ExoIII digestion in vitro. This factor was present in both hormone treated and control cell nuclei in apparently unmodified concentration. Thus, as was observed for NF-1 (1), this factor is present in the uninduced cell nucleus in a form competent to bind DNA, although it is bound to the MMTV promoter in vivo only after hormone stimulation. These binding activities were fractionated by phosphocellulose column chromatography and found to be enriched in the 0.5M KCl (NF-1) and 1.0M KCl (-42/-4 protection) step fractions.

Footprint analysis of these fractions with DNaseI showed that both fractions exhibited protection of coding strand sequences -45 to -20 which contain the TATA homology. Furthermore a lesser degree of protection occurred at discrete sites extending downstream over the cap site. The upstream boundary of the DNaseI footprint of this binding activity corresponds well with the limit of ExoIII protection at -42. However, the relatively uniform protection to DNaseI does not extend beyond -20. Instead, weak protection of discrete non-contiguous cutting sites occurs over a large downstream region. ExoIII protection extends to -4 at which a discrete boundary to low levels of exonuclease can be observed.

While this factor binds to and protects sequences in the vicinity of the TATA homology most strongly, it seems likely that the protein comes into close contact with DNA sequences outside

this immediate region and extending downstream over the cap site. One can envisage that while ExoIII digestion is blocked at -4 by a protein-DNA contact, regions within the protected sequences might still be susceptible to DNaseI. Likewise, if multiple loose DNA contacts contribute to the extended region of protection to DNaseI by this factor it is probable that the ExoIII may partially penetrate this region before termination at -4. The observation that sites internal to the region -42 to -4 are termination sites in DNA bound with the 1.0M KCl salt step material (Figure 2B, lane 8) also suggests that this protein makes multiple DNA contacts over a large region such that it may be partially displaced by ExoIII digestion before the enzyme detaches from the template. Further analysis of the binding of more highly fractionated material are necessary to further interpret binding of this factor to the TATA-cap site region of the promoter.

The TATA-binding activity we have identified in murine cells is most likely equivalent to the factor TFIID which was originally defined as a HeLa cell transcription factor component of a protein fraction required for reconstituted in vitro transcription from the AMLP (7). This activity, as with the TATA-binding activity we have described, was in the 1.0M KCl eluate of a P11 column and has subsequently been further characterized (8). Highly purified preparations of the binding activity exhibited a footprinting activity which protected the TATA region strongly while protecting sequences downstream to +30 in a fashion which suggested that DNA protein contacts were occurring along one face of the DNA helix. Other workers have detected factors which interact with TATA sequence of promoters; Weinman and colleagues were able to footprint binding activity in the 0.5M KCl P11 fraction which protected the -65 to -10 in the AMLP (13). Mutations in the TATA homology of the promoter abolished protection of the -30 to -10 region. In addition TATA binding factors for the Drosophila heat shock gene promoter have been detected in transcription extracts (14) and in crude nuclear extracts (6). Furthermore, an activity important for efficient transcription initiation was implicated in the formation of preinitiation complexes on TATA containing promoter fragments (15). This

activity was fractionated from HeLa S100 transcription extracts and eluted from Heparin Agarose with 1.0M KCl. A further activity was also implicated with flow through fractions. In this regard it is interesting to note that we detected a flow-through dependent boundary near the cap site of the MMTV promoter. Although we have not substantiated the existence of a binding activity by other experimentation, it is tempting to speculate that a binding activity may be present in this protein fraction and may be responsible for the discrepancy between the downstream ExoIII boundary of the promoter complex in vitro (-4) and in vivo (+12).

Of particular interest is our observation that similar levels of protection of sequences containing the TATA homology is observed with both 0.5M and 1.0M KCl fraction material when a probe containing an intact NF-1 binding site is used in the assay. In contrast, the 1.0M material yielded far more protection in an ExoIII assay in which the probe lacks the upstream NF-1 binding site (Fig. 2B). This suggests that although the major proportion of TATA-binding activity resides in the 1.0M fraction, binding in the presence of NF-1 which is present at high levels in the 0.5M binding fraction is increased. Thus TATA protection in the 0.5M fraction appears to reflect efficient binding of small amounts of a factor whereas high levels of the same factor do not result in higher levels of DNaseI or ExoIII protection by the 1.0M KCl fraction. This effect could be caused by interaction of NF-1 with the factor binding downstream resulting in a more stable interaction of the factor with DNA. A similar phenomenon has been convincingly demonstrated for TFIID and the upstream stimulatory factor (USF) of the AMLP (8). Further experiments will establish if cooperative binding occurs between NF-1 and factors binding at the MMTV TATA box and cap site. Cooperative interactions between promoter binding factors may be central to the assembly of a tightly bound initiation site complex like that observed in vivo.

The results we have presented confirm that equivalent TATA binding activity (-42/-4 protection) is found in nuclear extracts from both hormone-treated and hormone-free cells. Thus, as was found to be the case with NF-1 (1), hormone treatment results in no detectable change in abundance or binding affinity of this

factor. It is clear that cooperative interactions between these two factors in the nucleus of a hormone-free cell is not sufficient to establish the promoter complex we have observed in vivo since neither factor is stablily bound to the MMTV promoter in hormone-free cells (1). Binding of the glucocorticoid receptor at the GRE is therefore either directly or indirectly responsible for the participation of these factors in a stable promoter complex in vivo. We have speculated (1,16) that the establishment of this complex may be dependent upon protein-protein contacts between receptor and transcription factors which it recruits to target promoters. However, direct interactions between the glucocorticoid receptor and transcription factors remain to be demonstrated. Receptor-mediated changes in local nucleoprotein structure (16) which permit factor binding may also be involved in promoter activation.

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*To whom correspondence should be addressed

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