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Two Independent Pathways for Transcription from the MMTV Promoter

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The influence of progesterone receptor (PR) and glucocorticoid receptor (GR) on transcription from the mouse mammary tumour virus (MMTV) promoter was analyzed using cell-free transcription of DNA templates with a G-free cassette. Preincubation of the templates with either PR or GR stimulates the rate of transcription initiation 10–50 fold, whereas the recombinant DNA binding domain of GR is inactive. Mutations that inactivate the nuclear factor I (NFI) binding site, or NFI depletion of the nuclear extract, decrease basal transcription without influencing receptor-dependent induction. Recombinant NFI, but not its DNA-binding domain, restores efficient basal transcription of the depleted extract. Recombinant OTF1 or OTF2, but not the POU domain of OTF1, enhance MMTV transcription independently of NF1. In agreement with this finding, NFI and OTF1 do not cooperate, but rather compete for binding to the wild type MMTV promoter, though they have the potential to bind simultaneously to properly oriented sites. Our results imply the existence of two independent pathways for MMTV transcription: one initiated by NFI and the other dependent on octamer transcription factors. Only the second pathway is stimulated by steroid hormone receptors *in vitro*.

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INTRODUCTION

Steroid receptors are ligand inducible eukaryotic transregulators that can activate or repress transcription of specific genes. In most cases hormone induction is mediated by binding of the hormone receptors to well-defined DNA sequences called hormone responsive elements (HREs) [1]. Though the molecular details of the interaction between receptors and HRE sequences have been studied extensively, the mechanisms by which binding of the receptors to the HRE brings about changes in the transcriptional efficiency of an adjacent promoter remain unclear. Probably cooperation with other transcription factors or with the basal transcriptional machinery is required. This cooperation could occur through protein–protein interactions, direct or indirect, or could be mediated by alterations in chromatin structure [2].

In cells carrying stably integrated copies of the mouse mammary tumor virus (MMTV) DNA, expression from the viral promoter is strictly dependent on glucocorticoid or progesterone treatment and this behaviour can be reproduced by transient transfection experiments [3, 4]. Induction by both hormones is mediated by the interaction of their receptors with a set of four imperfect palindromic HREs located between −190 and −75 upstream of the transcription start site [4–6]. In minichromosomes the MMTV-LTR region is organized in nucleosomes precisely positioned along the DNA sequence with a nucleosome covering the HREs [7]. Though direct evidence for receptors bound to the HREs of MMTV in the intact cells has been elusive, it is generally accepted that such an interaction takes place after hormonal treatment and that it is essential for induction. Induction is accompanied by changes in chromatin structure which becomes hypersensitive to MPE and DNaseI over the HREs [7]. Concurrently, other transcription factors bind to the MMTV promoter downstream of the HREs [8].

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Immediately adjacent to the HREs is a palindromic site with affinity for the transcription factor NFI [9]. Though it has not been formally proven that NFI is one of the factors bound to the MMTV promoter after hormonal induction of intact cells, the footprints observed in this region with the purified NFI are very similar to those found *in vivo* [8, 10] and NFI acts as a transcription factor on the MMTV promoter [11]. However, we have not found synergistic binding of hormone receptors and NFI to the MMTV promoter [11]. Moreover, in a cell-free transcription assay, purified progesterone receptor enhances transcription from the MMTV promoter in the presence of a competitor oligonucleotide containing the consensus sequence for NFI binding and is also able to activate transcription of a MMTV template lacking the NFI binding site [12]. These findings and the regular structure of MMTV chromatin led to the notion that hormonal induction could be mediated partly by facilitation of NFI access to the nucleosomaly organized MMTV promoter [1, 8, 13].

In addition to NFI, other transcription factors can also mediate hormonal induction of the MMTV promoter. Between the NFI binding site and the TATA box there are two degenerate octamer motifs that are recognized by the transcription factor OTF1/Oct1 [14]. Mutation of these motifs drastically impairs the response to either glucocorticoids or progesterone in transfection assays [11, 14, 15]. Under cell-free conditions binding of the purified hormone receptors to the HREs facilitates the interaction of purified OTF1 with the octamer motifs [14, 16]. This synergistic DNA binding is likely to be responsible for the enhancement of transcription from the MMTV promoter observed in the presence of purified progesterone receptor, since induction can be inhibited by addition of competitor oligonucleotide with the octamer consensus sequence or by mutations in the octamer motifs [14]. How OTF1 recruits the general transcriptional machinery is not completely clear, although it has been shown to mediate the effects of cellular and viral transactivators [17–20].

To further our understanding of the interplay of various transcription factors on the MMTV promoter we have used purified proteins and recombinant variants of NFI and octamer transcription factors in a cell-free system derived from HeLa cell nuclei [21], that efficiently mediates induction by purified progesterone [12] or glucocorticoid receptor (GR). By depleting the extract from NFI activity we show that the complete NFI, but not its DNA binding domain, acts as a basal transcription factor on the naked MMTV promoter. Induction by either hormone receptor *in vitro* is independent of NFI. Under cell-free conditions both purified OTF1 and OTF2 added to the depleted extract activate transcription independently of NFI. There is no indication for synergistic binding of OTF and NFI to the wild type template. The cell-free assay reported here should be useful for dissecting the functional interactions between the various factors on the MMTV promoter.

EXPERIMENTAL

DNA

Template-plasmids for the cell-free transcription were derived from $p(C_2AT)_{19}$ [22] and contained stretches of the GR-MMTV or the Adenovirus Major Late (AdML)-promoter fused upstream to a region encoding a G-free transcript of defined length. The indicated mutations in the factor binding sites were derived from sequences described [14]. A schematic representation of the constructions is given in Fig. 1(A). Oligonucleotides have the following sequences: NFI and "Octc" as described [11]; ENFB, AATTCCTTTT TTTGGATTGA AGCCAATCGG ATAATGAGG; (Octc)2, GATCCTATGA TTTGCATAAG CATTTGCATA AGACTA. The GNO-oligonucleotide contains the MMTV sequences from −83 to −32 with 9 additional nucleotides added to the 5′-end to generate a perfect HRE palindrome.

[Fig. 1(A–C) Opposite]

Fig. 1. PR and GR induce transcription from the MMTV promoter. (A) DNA templates. Schematic representation of the MMTV template plasmids. The numbers indicate the distance in nucleotides upstream of the correct start site (+1). The binding sites for known transcription factors, hormone receptor (HR), NFI, OTF and TFIID, are boxed and the transcript lengths are given for correct initiation events. A 20 nucleotides longer satellite band originates from read-through transcripts starting upstream of the G-free cassette. (B) Analysis of cell-free transcripts. Cell-free transcription reactions were performed with 25 ng of the hormone-responsive plasmid pMMTV 240/380 ("360nt") and 12.5 ng of the control plasmid pMMTV80/280 ("260nt") as templates, according to the indicated schedule (top). PR (6 ng) or GR (72 ng) were added 15 min prior to the start of transcription and incubation with the template DNA was performed in the absence (lanes 1 and 3) or in the presence of 8% PEG (lanes 2, 4 and 5) for 15 min at 250°C. The strong signal corresponds to correctly initiated transcripts and the weaker signal to read-through transcripts. (C) Dependence of specific transcription on receptor concentration. Increasing amounts of GR or PR were added to analogous transcription reactions as described in Fig. 1(B) (data not shown). For quantitation, densitometric data of the specific hormone responsive transcript (360 nt) were normalized against the internal control (260 nt) to exclude variations during the purification of the samples. The obtained values of induction (ratio of normalized level of the 360 nt transcript to a control reaction without receptor) are displayed for PR and for GR against the amount of protein.

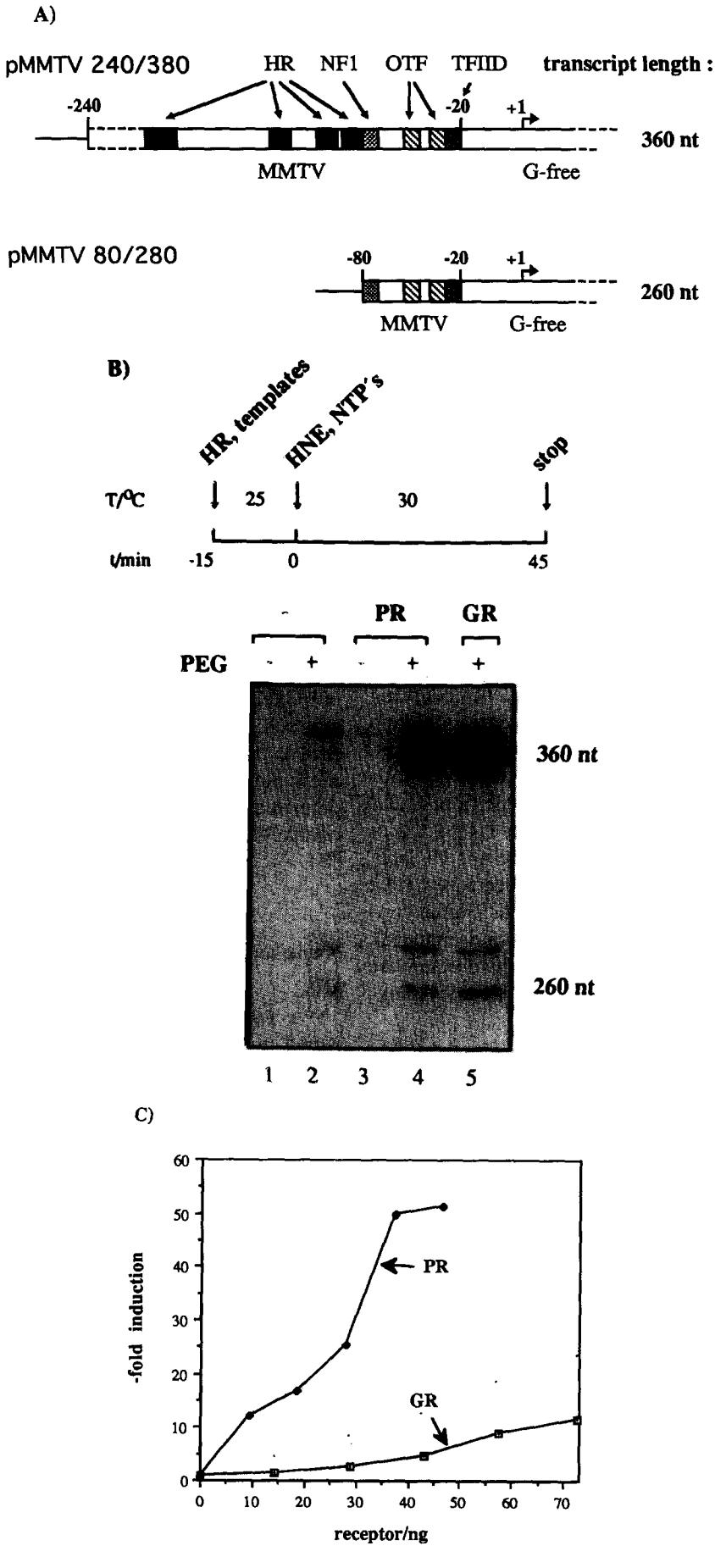

Fig. 1(A–C)–*legend opposite.*

Preparation of HeLa-cell nuclear extract (HNE) and the transcription factors

HeLa cell nuclear extracts were prepared essentially as described [21]. The recombinant transcription factors vNF1; the DNA-binding domain of NF1, vNF1-DBD (aa 4–240); vOTF1; the POU-domain, vOTF1-POU (aa 1–23 and 269–440); and vOTF2, were prepared via a vaccinia virus expression system as described [23, 24]. For some experiments nOTFI, purified from Namalwa cells was used [25]. The purification of vNFI-DBD and OTF1-POU domain was as described [26]. Recombinant NFI was purified essentially as described for endogenous HeLa NFI [27]. The PR was purified from rabbit uteri [4] and the GR from rat liver [28]. The DNA-binding domain of GR (aa 432–523) was obtained from a *Staphylococcus aureus* expression system as described [29].

DNA-affinity matrix preparation and HNE depletion

Specific DNA-affinity resin was obtained by coupling of double-stranded oligonucleotides containing the relevant factor recognition sites to CNBr-activated sepharose 4B (Pharmacia) as described [30], with some modifications. Inactivation of surplus coupling sites was performed with 1M Tris buffer and the coupling efficiency was monitored by spectrophotometric determination of the DNA content in the various buffers. Typically, a resin contained approx. 13 fmol oligonucleotide per μl of settled volume. For depletion, HeLa cell nuclear extracts were inverted with 1/4 to 1/2 of their volume of DNA-affinity resin at 4°C for 10–14 h. The supernatant was then aliquoted and refrozen. The resin was inverted for 1 h with anologous buffer containing 500 mM NaCl to elute the bound material. Depleted and complete HNE as well as the eluate were assayed for factor content by Gel Mobility Shift Analysis as given below.

Gel Mobility Shift Analysis

HNE depletion and DNA-binding activity in the various factor preparations were determined by gel mobility shift experiments essentially as described [29] NF1- and OTF-binding was assayed in a final volume of 22 μl of reaction mixture based on HNE buffer C [21] supplemented with 100 ng/μl poly-dI · dC, 3 mg/ml BSA, 0.05% NP 40 and 100 mM (Na/K)Cl. All steps were performed at 4°C. The protein was preincubated with the competitor for 10 min (20 μl) followed by the addition of [^{32}P]-end labeled specific oligonucleotide (10,000 cpm in 1 μl) and further incubation for 15 min. Complexes were resolved by gel electrophoresis, visualized by autoradiography and quantitated by densitometry.

The binding assays with the GNO-oligonucleotide were performed under the same conditions except that NP40 was omitted.

Cell free transcription

The assay follows the original description [22] with few modifications. A standard reaction mixture (final volume: 25 μl) contained 16.3 mM HEPES, 8 mM Tris (pH 7.9 at 4°C), 60–80 mM (Na/K)Cl, 7.3 mM $MgCl_2$, 0.44 mM EDTA, 0.8 mM (DTT, DTE), 40 ng/μl BSA, 7.4% (w/v) glycerol, 4% (w/v) polyethylene glycol 20000 (PEG), 0.4 mM ATP, 0.4 mM UTP, 20 μM CTP, 11.4 kBq/μl α-[^{32}P]CTP (15 TBq/mmol), 0.16 mM 3′-*O*-methyl-GTP, 9.8 mM creatin phosphate, 0.4 U/μl RNase T_1, 10 ng/μl calf thymus DNA, approx. 1.8 μg/μl HNE protein, plus the transcription factors and template plasmids as indicated in the figure legends. Transcription was then allowed to proceed for 45 min at 30°C. Preincubation of the steroid hormone receptors with the template plasmids was carried out for 15 min at room temperature (volume: 12.5 μl) in 5mM HEPES, 16 mM Tris (pH 7.9 at 4°C), 80–120 mM NaCl, 0.8 mM EDTA, 0.8 mM DTE, 8% (w/v) glycerol, 8% PEG, 80 μg/ml BSA and 4 ng/μl calf thymus DNA, before HNE and nucleotide mix was added. The reactions were finally stopped by addition of 200 μl stop buffer (0.1 M sodium acetate (pH 6), 10 mM EDTA, 0.1% SDS, 125 μg/ml total yeast RNA). Samples were then phenol/$CHCl_3$ extracted twice before precipitation with ethanol. Transcripts were separated on a 6.5% denaturing polyacrylamide gel, visualized by autoradiography and quantitated by densitometry. Induction was determined with respect to the transcription of the control-template.

RESULTS AND DISCUSSION

The transcription assay: effect of progesterone (PR) and GR

We have shown previously that PR purified from rabbit uterus stimulates transcription from the MMTV promoter in HeLa nuclear extracts and that this effect depends on the integrity of the octamer motifs [12, 14]. Using a similar HeLa nuclear extract and templates containing G-free cassettes [22] of different lengths [Fig. 1(A)] we can now obtain a better stimulation of transcription by the progesterone receptor and detect a clear induction by GR purified from rat liver [Fig. 1(B)]. Optimal transcription is observed when "macromolecular crowding" is generated by the addition of polyethylene glycol (PEG; [31]). Under these conditions, the extent of induction is dependent on the concentration of added receptor and maximal induction is 50-fold for the PR and 12-fold for the GR [Fig. 1(C)]. The difference in the level of induction observed with PR and GR is probably due to the quality of the two receptor preparations. Quantitation of receptor content was based on bound hormone ligand, assuming one molecule of steroid bound per receptor monomer [12]. However, when PR and GR were compared in terms of

their ability to shift a GRE/PRE oligonucleotide in the gel retardation assay, the PR was considerably more efficient (data not shown), correlating with its higher transcriptional activity. The reasons for the differences in DNA binding and transcriptional activity of both receptor preparations are unknown and could reflect posttranslational modification, interaction with other proteins, or protein stability. The fact that other groups have reported high transcriptional induction with recombinant GR [32] suggests that the differences are not intrinsic to the receptor molecules.

To make sure that the effect of hormone receptors was due to binding of the receptor proteins to the HRE we included in the reaction mixture a control template containing the sequences of the MMTV promoter up

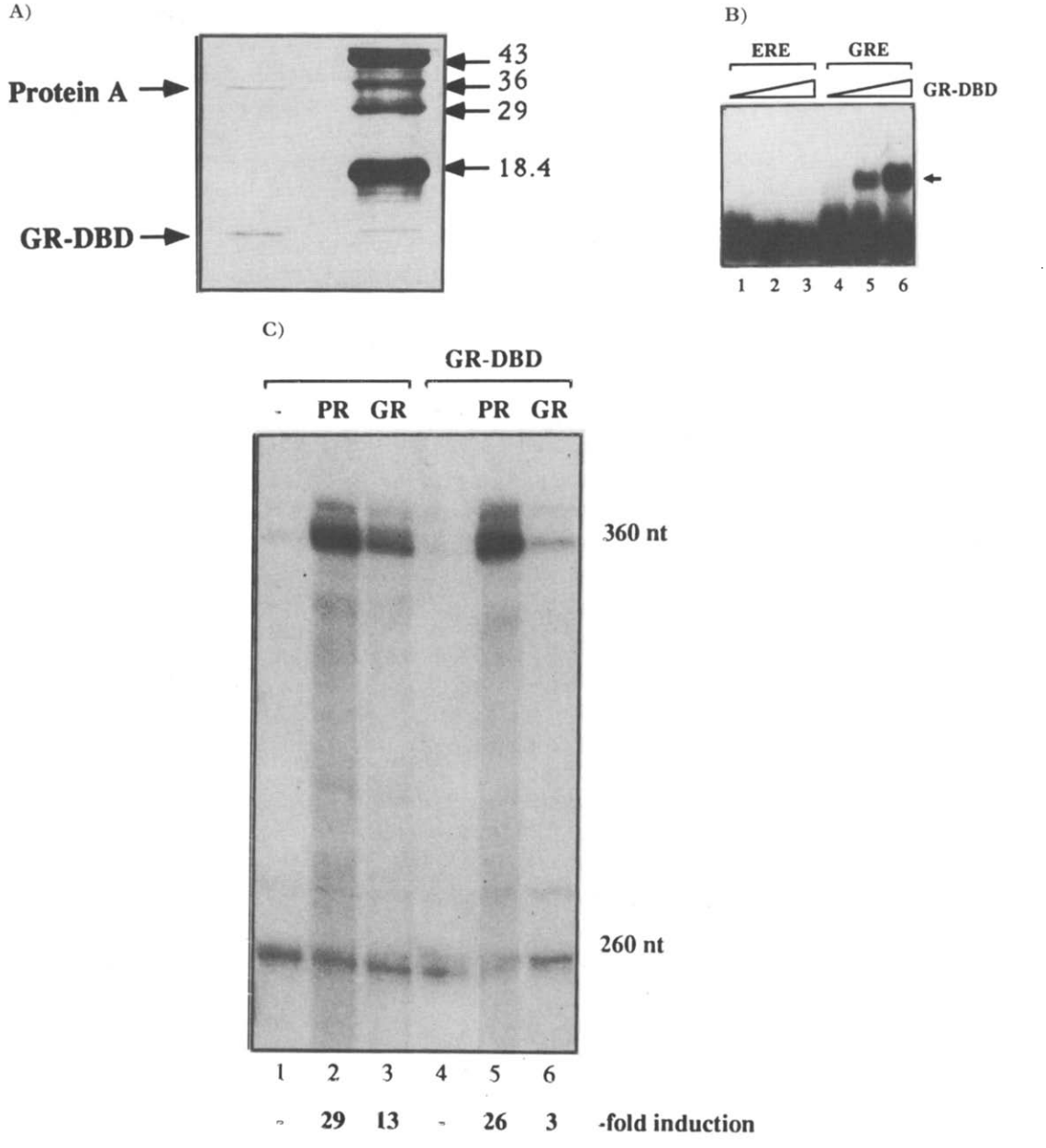

Fig. 2. The GR-DBD is not able to induce MMTV transcription. (A) SDS–PAGE of recombinant GR-DBD. The purified recombinant protein GR-DBD migrates with an approximate molecular weight of 15 kDa on 10% SDS–PAGE. The molecular weight markers are: ovalbumin, lactate dehydrogenase, carbonic anhydrase and lactoglobulin. (B) Band shift assay of DNA binding specificity of GR-DBD. The purified GR-DBD binds specifically to an oligonucleotide comprising the GRE consensus sequence (lanes 4–6—1, 3 and 7 μl of the protein preparation) but not to the ERE consensus (lanes 1–3—corresponding amounts) in gel mobility shift experiments. (C) Influence of GR-DBD on basal and receptor-dependent transcription. In transcription reactions as described in Fig. 1(B) an additional preincubation step with 2.5 μl (50 ng) of the GR-DBD has been included (15 min at 25°C), followed by incubation without added receptor (lane 4), with 42 ng PR (lane 5), or with 55 ng GR (lane 6). Control lanes without addition of the GR-DBD are displayed on the left (lanes 1–3). The corresponding values of induction are shown underneath the lanes.

to −80 and thus lacking the HRE region [Fig. 1(A)]. Transcription of this template was not influenced by addition of PRs or GRs, supporting the specificity of the receptor-mediated induction [Fig. 1(B)].

The DNA binding domain of GR is not sufficient for transactivation in vitro

Using this sensitive assay we asked whether the DNA binding domain of the GR, that has been reported to be sufficient for transactivation in gene transfer experiments [33], was able to activate transcription from the MMTV promoter *in vitro*. To this end we expressed the DNA binding domain of the glucocorticoid receptor (GR-DBD) in *Staphylococcus aureus* as a fusion protein with protein-A [34]. After affinity chromatography on immunoglobulin-sepharose and cleavage of the protein-A moiety, the recombinant protein was purified by DNA cellulose chromatography. The purified preparation yielded a dominant band of molecular weight 15 kDA in SDS–PAGE along with some residual protein A [Fig. 2(A)]. This protein binds as a homodimer selectively and with high affinity to a synthetic GRE/PRE [20] [Fig. 2(B)], but is not able to activate transcription from the MMTV promoter *in vitro* [Fig. 2(C)]. When added together with the intact hormone receptors, the DNA binding domain inhibits induction by GR, but not by PR, suggesting that either it forms inactive heterodimers with native GR [Fig. 2(c)] or competes for the DNA binding site, or both. We conclude that a homodimer of the intact receptor, but not of its DNA binding domain, is able to activate transcription *in vitro*. The apparent contradiction with previous gene transfer experiments performed with canonical GREs [33] could be due to the use of slightly shorter GR fragments in these studies or to the lower DNA binding affinity of GR-DBD for the MMTV-HRE. Alternatively, a synergistic interaction between GR molecules bound to the four sites of the HRE, that is important for optimal induction *in vivo* [4], could require domains of GR not present in GR-DBD or other factors absent from our nuclear extract. Of course, it is also possible that bacterially expressed GR-DBD is not modified posttranslationally in the appropriate way.

Involvement of NFI in MMTV transcription

The requirement of NFI for transcription of the MMTV promoter has been previously reported [11, 12]. Using the new transcription assay we confirm the deleterious effect of mutations on the NFI binding site on transcription from the MMTV promoter [Fig. 3(A)]. Mutation of both halves of the NFI binding site reduces transcription to about 1/3 of the level observed with the wild type MMTV promoter [Fig. 3(A)]. The relatively small effect of these mutations compared to previously published results [12] is not due to residual NFI binding, but probably reflects the macromolecular crowding effect of PEG. However, none of these mutations reduced the extent of induction observed after addition of either GRs or PRs [Fig. 3(A), GR data not shown], confirming previous results [11]. These data show that induction of MMTV transcription *in vitro* by either PR or GR is independent of NFI and are in apparent contradiction to those reported for the ovalbumin promoter [35]. This discrepancy may result from the different array of HREs and NFI binding sites in the two promoters. It is known that when the distance between these two sites is appropriate one observes a functional synergism between them in transient transfection assays [36]. We conclude that although NFI and the hormone receptors have the potential to synergize under certain conditions, they do not synergize on the MMTV promoter.

The HeLa cell nuclear extract can be depleted of more than 90% of its NFI binding activity by incubation with a NFI consensus oligonucleotide linked to sepharose. In the depleted extract transcription from the MMTV promoter decreases drastically, and can be

[Fig. 3(A–C)—Opposite]

Fig. 3. Role of NF1 in MMTV transcription. (A) Influence of mutations in the NFI binding site on MMTV-transcription. Transcription reactions have been carried out as described in Fig. 1, but in addition to the wild type (wt) MMTV promoter, we used templates containing mutations in the NF1 binding site. Either the promoter distal half (NFd), the promoter proximal half (NFp) or both halves (NFt) of the NFI binding site were mutated [11] in the pMMTV 240/380 template as schematically indicated. Reactions were performed after preincubation with PR or control buffer. (B) Complementation of a NFI-depleted extract with recombinant NFI fractions. HNE was depleted of NFI by incubation with the NFI-oligonucleotide resin (HNE-NF1, see experimental section) and used for cell-free transcription experiments. Reactions contained 40 ng of pMMTV 80/280 and, as an internal control, 10 ng of pML$(C_2AT)_{19}$ that yields a 380 nt transcript. The depleted extract was tested without added factors (lane 4) or after complementation with equivalent DNA-binding activities of either recombinant intact NFI (21 ng of vNFI, lane 1), recombinant NFI DNA binding domain (100 ng of vNFI-DBD, lane 3) or the corresponding buffer (c, lane 2). A control reaction with non-depleted extract is included (lane 5). The densitometric evaluation of the data is shown at the bottom. (C) Influence of recombinant NFI on basal and receptor-dependent transcription. Cell-free transcription (as described in Fig. 1) were performed with the NF1-depleted HNE (HNE-NFI) without NFI (lane 2, 4 and 6) or after complementation with 10.5 ng vNFI (lanes 1, 3, 5). The reactions were preincubated with either buffer (lanes 1 and 2), 45 ng of PR (lanes 3 and 4) or 58 ng of GR (lanes 5 and 6). Appropriate control reactions with non-depleted extract have been included (lanes 7–9).

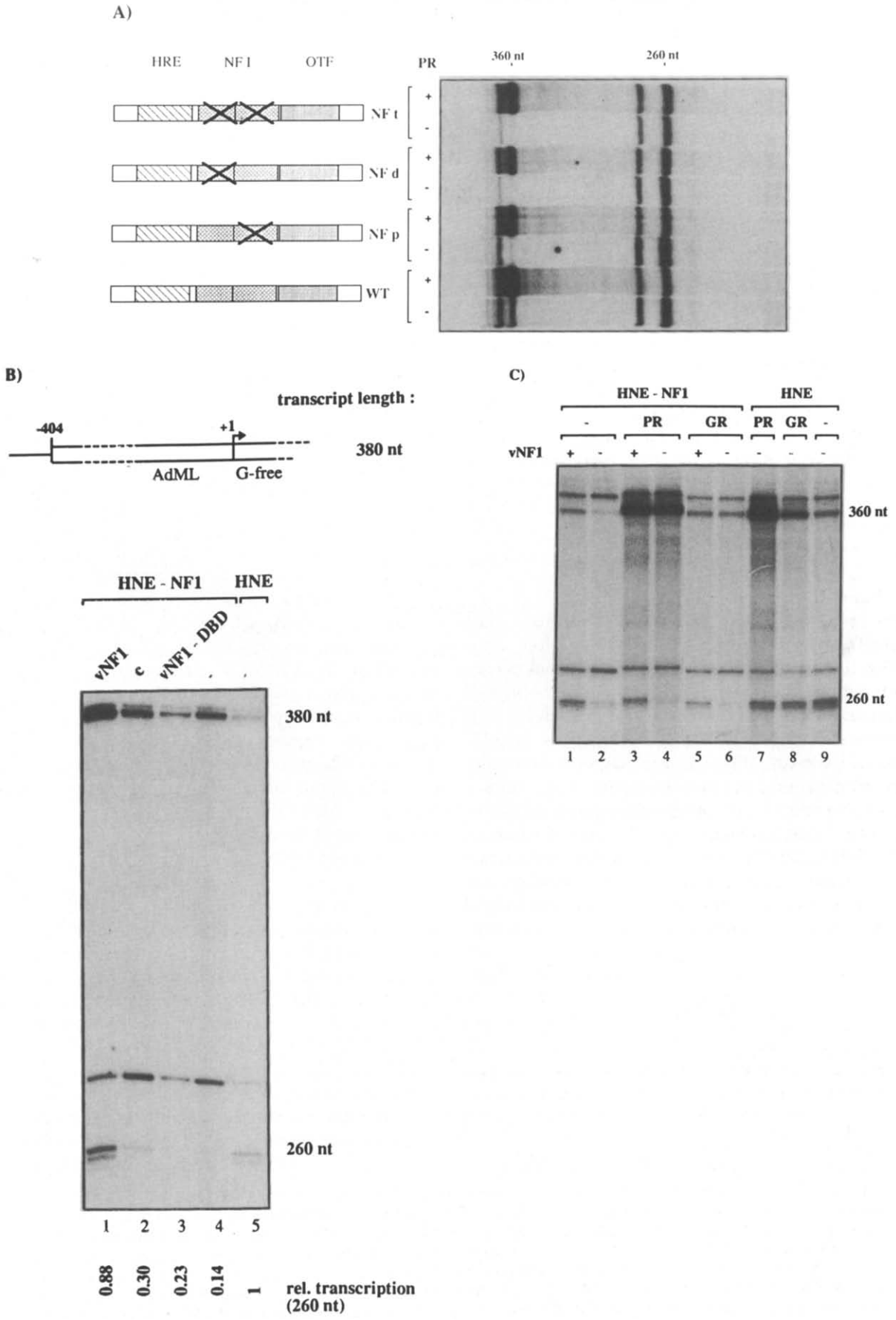

Fig. 3(A–C)—*legend opposite.*

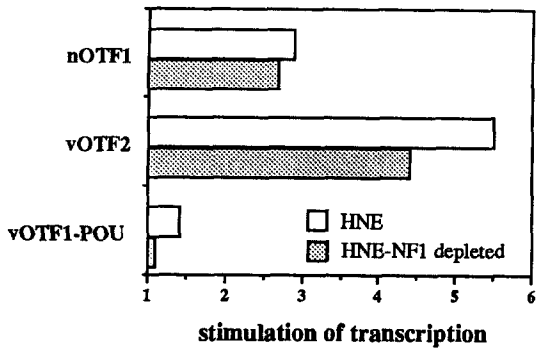

Fig. 4. Role of octamer transcription factors in intact and NF1-depleted HeLa nuclear extract. Influence of octamer transcription factors on receptor-independent transcription. Either NFI depleted extract or intact nuclear extract, were used to test the influence of purified or recombinant octamer transcription factors (OTF1, OTF2 or the POU domain of OTF1, equivalent amounts in terms of DNA binding) on transcription of the pMMTV 80/280 template (260 nt transcript). As internal control, 10 ng of pML$(C_2AT)_{19}$ that yields a 380 nt transcript, were included in each reaction. The autoradiograms of the gel retardation assay were quantitated by densiometry. The figure represents the average of four separate experiments corrected for variations by reference to the internal adenovirus major late promoter control.

partly restored by adding back purified NFI [Fig. 3(B)]. The read-through signals as well as transcription from the Adenovirus Major Late promoter are independent of NFI. Purified wild type NFI obtained from recombinant vaccinia virus infected HeLa cells [23] restores the transcriptional activity of the MMTV promoter, without affecting transcription from the Adenovirus Major Late promoter [Fig. 3(B), lanes 1 and 2]. This effect is not observed when the recombinant DNA binding domain of NFI, containing amino acids 4–240 [23, 27], is added to the depleted extract [Fig. 3(B), lane 3]. The amount of truncated protein added was equivalent to the amount of NFI as judged in band shift experiments with a NFI consensus oligonucleotide (data not shown). However, the DNA binding domain of NFI has been reported to be sufficient for adenovirus DNA replication [23, 38, 39], suggesting that different regions of NFI are required for transcriptional activation and DNA replication. We conclude that the carboxy-terminal half of NFI, containing the potential transactivation domains [38] is required for the observed transcriptional activity on the MMTV promoter.

The NFI-depleted extract was used to confirm the lack of effect of NFI upon hormone receptor dependent transcription. In the depleted extract the effect of adding purified PR or GR was indistinguishable from that observed in the intact HeLa nuclear extract or after complementation of the depleted extract with recombinant NFI [Fig. 1(C)]. Thus, neither PR nor GR require NFI for *in vitro* activation of MMTV transcription.

Participation of octamer transcription factors in basal transcription

We have previously shown that cell-free transcription from the MMTV promoter is not drastically influenced by mutations in the octamer motifs [14]. The intact nuclear extract can be used to assay recombinant octamer binding factors in terms of their activity as transcription factors. The results of such experiments show that both OTF1 and OTF2 can enhance transcription from the MMTV promoter (Fig. 4). The amount of factor added was calculated in band shift experiments with an octamer consensus oligonucleotide (data not shown). Based on this estimate the transcriptional activity of OTF2 was slightly higher than that of OTF1 (Fig. 4). Contrary to the intact octamer transcription factors, a recombinant protein containing the POU-domain of OTF1 (amino acids 269–440), albeit able to efficiently bind to DNA, does not exhibit transcriptional activity (Fig. 4), indicating that regions of the protein outside of the DNA binding domain are needed to generate a functional protein.

The OTF1 concentration in the intact HeLa extract seems to be limiting for transcription, as addition of purified native or recombinant OTF1 enhances transcription from the MMTV promoter. The fact that not only OTF1 but also OTF2 is able to mediate basal MMTV transcription is interesting, as a promoter selective activity of both octamer transcription factors has been reported for other genes [40]. Moreover, the POU domain of OTF1, that is sufficient to support adenovirus DNA replication [27], is not effective in supporting transcription from the MMTV promoter. Again, these results suggest that different domains of OTF1 are used for stimulation of DNA replication and MMTV transactivation.

The effect of OTF1 or OTF2 on MMTV transcription is of similar magnitude in NFI-depleted extract and in complete extract (Fig. 4). There might be two explanations for this finding: either the two classes of transcription factors bind to the same template but do not synergize, or each factor binds to a separate template molecule.

Our new studies confirm the basic observation that the effect of PR on cell-free transcription of MMTV is at least partly mediated by binding of OTF1 to the octamer motifs in the MMTV promoter [14]. Mutation of these sites reduces the receptor-mediated enhancement of transcription [14]. A similar behaviour is observed with GR (data not shown), suggesting that the PR and GR use similar pathways for *in vitro* transactivation. We have previously found that GR, as well as PR, stabilizes binding of OTF1 to the MMTV promoter [14] and this could explain the role of the octamer motifs in GR-induced MMTV transcription. However, mutation of both octamer motifs and the

NFI binding sites does not abolish induction by PR completely (data not shown), suggesting that on these mutant promoters the receptor can also act by recruiting basic transcription factors to the TATA box. The contribution of this pathway to induction of the wild type promoter remains to be established.

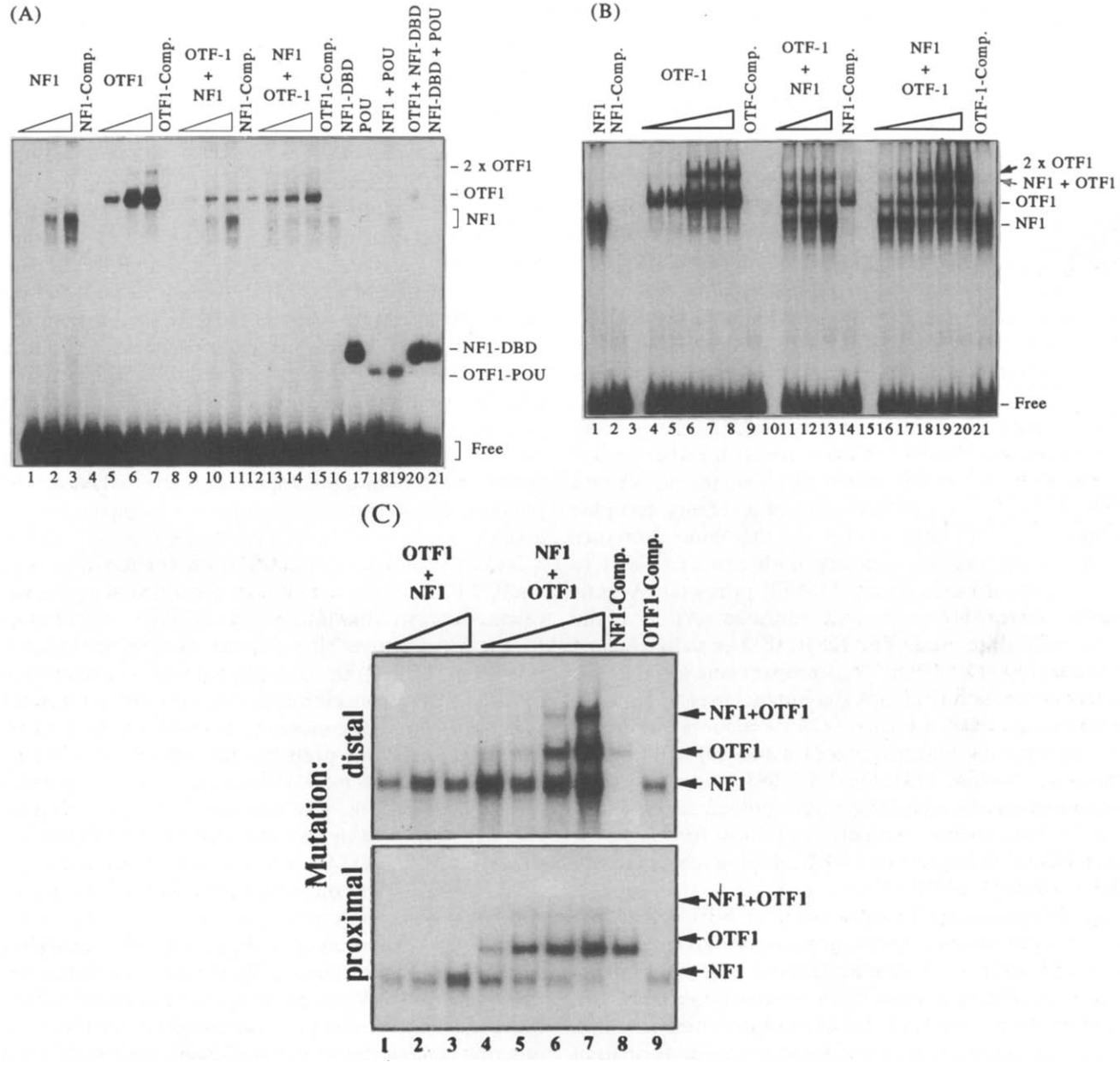

Fig. 5. Binding of NFI and OTF1 to the MMTV promoter. (A) DNA binding experiment with of an excess of promoter fragment. The GNO oligonucleotide (70,000 cpm) was end-labeled and used for band shift assays with recombinant preparations of NFI or OTF1. The amounts of NFI used were: 8 ng (lanes 1 and 9), 16 ng (lanes, 2, 10, 13–16 and 19), 24 ng (lanes 3, 4, 11 and 12). The amounts of OTF1 used were: 6 ng (lanes 5 and 13), 12 ng (lanes 6, 9–12, 14 and 20) and 24 ng (lanes 7, 8, 15 and 16). The amount of the DNA binding domain of NFI (NFI-DBD) was 15 ng (lanes 17, 20 and 21) and the amount of the POU domain of OTF1 (POU) was 4 ng (lanes 18, 19 and 21). When indicated 100 ng of specific competitor DNA containing the consensus sequence of NFI (NFI-Comp., lanes 4 and 12) or the octamer consensus motif (OTF1-Comp., lanes 8 and 16) were added to the reactions. (B) DNA binding experiment with limiting amounts of promoter fragment. The experimental protocol was similar to that described under (A), but using only one fifth the amount of the radioactive GNO oligonucleotide. The amounts of NFI used were: 8 ng (lane 11), 16 ng (lanes 12 and 16–21) or 24 ng (lane 1, 13 and 14). The amounts of OTF1 used were: 6 ng (lanes 4 and 16), 12 ng (lanes 5, 11–14 and 17), 24 ng (lanes 7 and 18), 36 ng (lanes 8 and 19) and 48 ng (lanes 10, 20 and 21). Specific competitor DNA was added as in (A). (C) DNA binding experiment under the same conditions as in (B), but using GNO oligonucleotides carrying mutations in the promoter distal (top) or in the promoter proximal (bottom) octamer motifs. The amounts of NFI used were: 8 ng (lane 1), 16 ng (lanes 2 and 4–9) and 24 ng (lane 3). The amount of OTF1 used were: 6 ng (lane 4), 12 ng (lanes 1–3 and 5), 24 ng (lane 6) and 36 ng (lanes 7–9). When indicated, specific competitor DNA was added as in (A).

Independent binding of NFI and OTF1 to the MMTV promoter

In view of the functional independence of NFI binding site and octamer motifs, we asked whether NFI and OTF1 bind synergistically or independently to the MMTV promoter. We used the recombinant proteins and an excess of a promoter fragment, in band shift assays which reflect the conditions in the *in vitro*-transcription experiments. We observe specific complexes with recombinant NFI or OTF1, that are selectively competed by the appropriate oligonucleotide [Fig. 5(A)]. At low concentrations of OTF1 we see a single retarded complex, the majority of which reflects occupancy of the promoter distal octamer motif that is known to bind OTF1 with high affinity [14]. At higher concentration of OTF1 we detect an additional slower migrating complex than most likely represents the binding of two OTF1 molecules to the two octamer motifs of the MMTV promoter [Fig. 5(A), lanes 6 and 7]. When NFI and OTF1 are added together at low concentrations relative to the DNA fragment, we find no indication for the formation of a ternary complex containing NFI and OTF1 on the same promoter [Fig. 5(A)]. On the contrary both proteins seem to compete for binding to the MMTV promoter. A similar behavior is observed when truncated versions of the two factors are used. The NFI-DBD actually reduces binding of OTF1 [Fig. 5(A), compare lane 6 and 21] or its POU domain [Fig. 5(A), compare lanes 19 and 22], whereas the POU domain of OTF1 reduces binding of NFI [Fig. 5(A), compare lanes 2 and 20] or its DNA binding domain, although NFI-DBD appears to be dominant in our assay [Fig. 5(A), compare lanes 17 and 21]. Thus, under optimal conditions for detecting synergism, there is no DNA binding cooperativity between NFI and OTF1.

We next wanted to know whether NFI and OTFI can bind to the same template molecule. To answer this question we performed similar band shift experiments but using limiting amounts of the promoter fragment. Under these conditions, we see a larger fraction of the DNA molecules participating in complex formation with either NFI or OTF1 and a very clear complex with two OTF1 molecules [Fig. 5(B), lanes 11–13 and 17–20]. This complex seems to contain NFI and OTF1 as it is specifically competed by an NFI oligonucleotide as well as by an oligonucleotide containing the octamer motif [Fig. 5(B), lanes 14 and 21]. The observed intensity of this complex is weak and does not indicate any cooperativity in DNA binding between NFI and OTF1.

The ternary complex with NFI and OTF1 could be formed with an OTF1 molecule bound to the promoter proximal or to the promoter distal octamer motifs. To address this question we analyzed the binding behavior of promoter fragments carrying mutations in one of the two octamer motifs [Fig. 5(C)]. Mutation of the strong promoter distal octamer motif reduces binding of OTF1 at low protein concentration but does not interfere with the appearance of the ternary complex at higher protein concentrations [Fig. 5(C) top]. On the contrary, mutation of the promoter proximal octamer motif, while having little effect on binding of OTF1 alone, drastically reduces the intensity of the band corresponding to the ternary complex with NFI and OTF1 [Fig. 5(C) bottom]. We conclude that the MMTV promoter can accommodate a NFI homodimer and an OTF1 monomer bound to the promoter proximal octamer motif. Quantitative analysis of the retarded complex demonstrate that binding of OTF1 and NF1 to the promoter carrying the octamer distal mutation is synergistic. This is demonstrated by the increment in intensity of the NF1 containing complex observed with increasing concentrations of added OTF1 [Fig. 5(C) top panel, compare lanes 5, 6 and 7] and by the decreased intensity of the NF1 complex when an octamer oligonucleotide is added as competitor (compare lanes 7 and 9). Similarly, the intensity of the OTF1 containing complex decreases when a NF1 oligonucleotide is used as competitor (compare lanes 7 and 8).

Taken together the binding data suggest that NFI and OTF1 have the potential to synergize in a properly oriented array of binding sites but compete for binding to their respective sites in the wild type MMTV promoter. When the amount of DNA fragment was reduced and its concentration was limiting, we detected a ternary complex containing a dimer of NFI and a monomer of OTF1 probably bound to the promoter proximal octamer motif. However, the level of this ternary complex is low compared to the levels of complexes containing only NF1 or OTF1. Therefore, though NFI and OTF1 can physically bind to a single MMTV promoter, this interaction is not synergistic and is not observed under conditions of cell-free transcription on the natural configuration of sites of the MMTV promoter. This suggests that basal transcription of the MMTV promoter *in vitro* can occur via two independent pathways, one mediated by NFI and the other by octamer transcription factors. Only the second pathway is enhanced in the presence of hormone receptors *in vitro*. This is reminiscent of the previously reported independent functions of NFI and OTF1 in mediating adenovirus DNA replication [26]. It remains to be established whether the two pathways for transcriptional activation are independent, or even mutually exclusive, in the intact cells.

CONCLUSIONS

In principle, hormone receptors could activate transcription either by modulating the efficiency of initiation at individual promoters or by recruiting inactive promoters into an active state. The first possibility envisages the existence of several states of activity

for each promoter depending on the number and/or the array of transcription factors. The second possibility postulates the existence of only two functional promoter states, active or inactive. In the second model the function of inducers is to recruit promoters into the active state. This model accounts for most experimental observations in inducible systems and in particular for the hormonal induction of the MMTV promoter [41]. Thus, the hormone receptors would activate the MMTV promoter by recruiting other transcription factors and the general transcriptional machinery. Our results suggest that they could accomplish this function by at least two independent pathways. One pathway, mediated by octamer transcription factors, can be reproduced *in vitro* using free DNA as template. As the receptors facilitate the binding of purified OTF1 to the MMTV promoter [14], we assume that this pathway involves a direct interaction between hormone receptors and octamer transcription factors. The second transactivation pathway, mediated by NFI, cannot be reproduced *in vitro* with free DNA. We postulate that this reflects the requirement for a nucleosomally organized chromatin template and hypothesize that *in vivo* hormonal induction of the MMTV promoter is partly mediated by an alteration in chromatin structure that enables binding of NFI to the promoter [13].

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