Volume 17 Number 8 1989 Nucleic Acids Research

Dual function of a nuclear factor I binding site in MMTV transcription regulation

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Received December 15, 1988; Revised and Accepted March 20, 1989

ABSTRACT

Using linker-scanning mutagenesis we had previously identified four elements within the MMTV LTR which are necessary for transcriptional stimulation by glucocorticoid hormones. Two of them overlapped with regions to which the glucocorticoid receptor binds in vitro. The third element contained a NF-I binding site, and the fourth the TATA box. Here we show that mutations that abolish in vitro binding of NF-I had a negative effect also on the basal activity of the MMTV promoter of LTR-containing plasmids stably integrated in Ltk$^-$ fibroblasts. The analysis of double mutants altered in the NF-I plus either one of the receptor binding elements further demonstrated that the NF-I site functionally cooperated with the proximal (−120) element, which alone was extremely inefficient in stimulation. The stronger distal (−181/−172) element was independent of NF-I and showed functional cooperativity with the proximal hormone-binding element.

INTRODUCTION

Transcription from the proviral DNA of mouse mammary tumor virus (MMTV) is controlled by steroid hormones (1,2) through their binding to receptor proteins and the interaction of hormone−receptor complexes with regulatory DNA sequences, thereby causing an increase of the number of active RNA polymerase II molecules on the MMTV promoter (3). The hormone-responsive sequences are located in the long terminal repeat (LTR) of the provirus (4,5,6,7), in a region of approximately 200 bp upstream of the transcription start site (8,9,10). While steroids like progestins and androgens have recently been shown to also affect MMTV transcription in mammary cells (11,12,13,14,15), it is the response to glucocorticoids in cultured fibroblasts that has been studied in greatest detail (16,17,18,19). In vitro binding of purified glucocorticoid receptor to MMTV DNA has been demonstrated, showing several binding sites within the glucocorticoid regulatory region (20,21,22).

Several reasons make MMTV a particularly suitable model system for studying transcription regulation by glucocorticoid hormones. High stimulation factors (200-fold and more; ref.19) are observed after addition of dexamethasone, a synthetic glucocorticoid, to cells containing the natural MMTV promoter plus the regulatory region. Stimulation occurs over a low basal promoter activity which is independent of the presence of hormone or of hormone-responsive sequences (8,9). These sequences are located relatively close to the promoter and contain several regulatory elements which act in a cooperative fashion to achieve an optimal level of transcription (18). In a previous study using linker-insertion mutants (18) we have identified four distinct control elements for glucocorticoid regulation (Fig. 1A). Two of them (a distal one, between −181 and −172, and a proximal one,

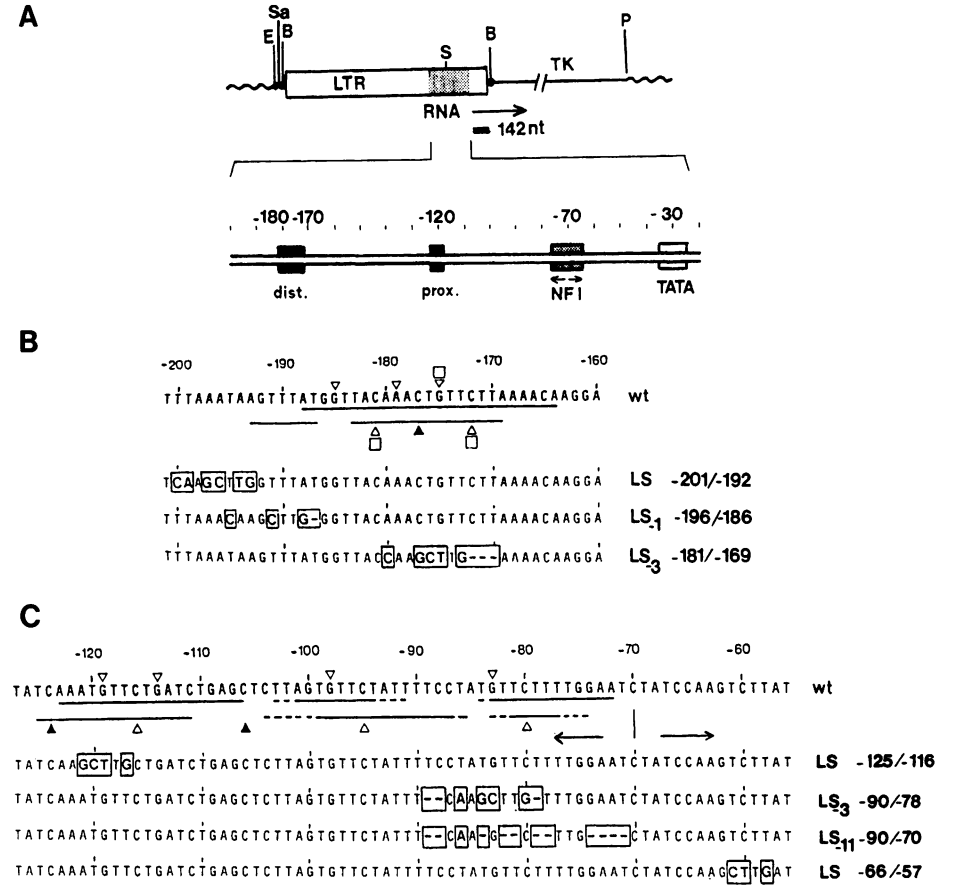

Figure 1. Scheme of plasmids.
A. The MMTV LTR is joined to the coding region of the herpes virus TK gene via a Bam HI linker. The wavy line denotes pBR322 sequences. Restriction sites are: E, Eco RI; Sa, Sal I; B, Bam HI; S, Sst I; P, Pvu II. The position of the 142-nt S1-nuclease protected fragment is indicated. The GRE-containing segment of the LTR is enlarged, the numbers being the distance in bp from the transcription initiation site. Previously defined glucocorticoid regulatory elements (18) are boxed: those contained in receptor binding sites (distal and proximal), the NF-I binding site, and the TATA box. **B** (distal) and **C** (proximal): sequence of regulatory regions and mutants therein (coding strand). Below the wild-type sequence (7), two lines indicate the DNase I footprints (on both strands) of purified glucocorticoid receptor (20). Triangles (open for protection, filled for enhancement) point to residues involved in methylation protection experiments, and squares to residues involved in methylation interference (22). In the sequence of linker scanning mutants (18), mutated nucleotides are boxed, and hyphens indicate deletions.

around −120) overlapped with sequences that interact in vitro with purified receptor (Fig. 1B,C). The other two were more proximally located: One contained the TATA-box (23) and its deletion resulted in both reduction of hormone response and generation of low levels of RNA with heterogeneous start sites. The other overlapped with the binding site for the so-called 'TGGCA-binding protein' (24,25,26,27), a functional equivalent of the ubiquitous nuclear factor I (NF-I; 28,29), which was shown to increase the rate of initiation

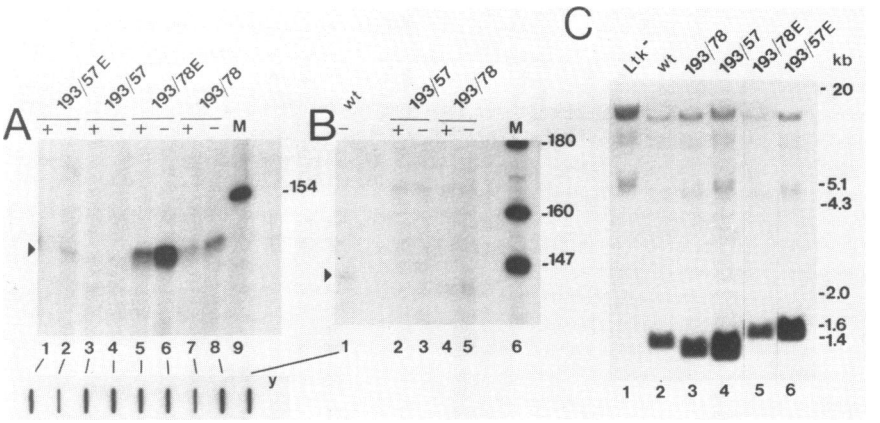

Figure 2. A,B. S1 nuclease protection assay of RNA transcribed from the GRE deletion mutants including or not the NF-I binding site, and from their derivatives containing the SV40 enhancer (E).
Stably transfected Ltk^+ cells were treated (+) or not (−) with 1 μM dexamethasone for 16 hr, and the cytoplasmic RNA was extracted. 20 μg of RNA were annealed to an excess of 5′-labeled, MMTV-LTR containing Bam HI fragment (Fig.1A) and digested with S1 nuclease. The 142-nt fragment protected by correctly initiated RNA is indicated by an arrowhead. Numbers to the right give the size in nucleotides of marker DNA fragments (lane M). **A** (bottom panel): slot-blot analysis with 2 μg of RNA hybridized to a β-actin probe. Slot y contains yeast RNA as a control. **C.** Southern blot analysis of Bam HI-digested DNA from the same transfected cells (lanes 2−6) shown in A and B, and from untransfected Ltk^- cells (lane 1). The filter was hybridized to a ^{32}P-labeled LTR probe. Weak bands present in all lanes are due to MMTV copies endogenous to L cells and constitute an internal standard for DNA amounts in each lane.

of adenovirus replication (28,30,31,32) via the recognition of binding sites in the viral DNA (33,31,32,29). The fact that both TATA and NF-I elements map in a region that by itself does not provide any hormone response (8,9) suggests that they act in concert with receptor binding sites in the process of transcriptional stimulation by the hormone. While the role of the TATA box in promoter activity is well established (23), it was unclear from the previous studies (18,27) whether the NF-I binding site affected only the stimulation of transcription or the basal level as well. Here we investigate the role of NF-I in hormone-independent transcription, and its functional interactions with the other control elements.

MATERIALS AND METHODS

Plasmid construction, transfection and nucleic acids analysis.

Construction of the linker scanning mutants has been described previously (19). The plasmids 193/78E and 193/57E contain the simian virus 40 (SV40) 72-bp repeat enhancer (present as an Xho I fragment in the rabbit β-globin-containing plasmid H514, provided by W. Schaffner) inserted into their unique Sal I site (Fig. 1A). Double mutants were assembled by ligation of three gel-purified DNA fragments: 1) the 3.5-kb Eco RI/Bam HI fragment containing pBR322 and thymidine kinase sequences (Fig. 1A) as a vector; 2) a 1-kb Eco RI/Sst I fragment from the upstream mutant; 3) a 0.25-kb Sst I/Bam HI fragment from the downstream mutant. After cloning in E. coli HB 101, recombinants were recognized by the presence and location of the two synthetic Hind III restriction sites that constitute the mutations. The sequence of the 123-bp spacer fragment of mouse DNA has been determined (19). Stable and transient transfection assays by calcium phosphate precipitation (34), isolation of nucleic acids, DNA and RNA blot analysis, and nuclease S1 mapping (35) were performed as described previously (18,19).

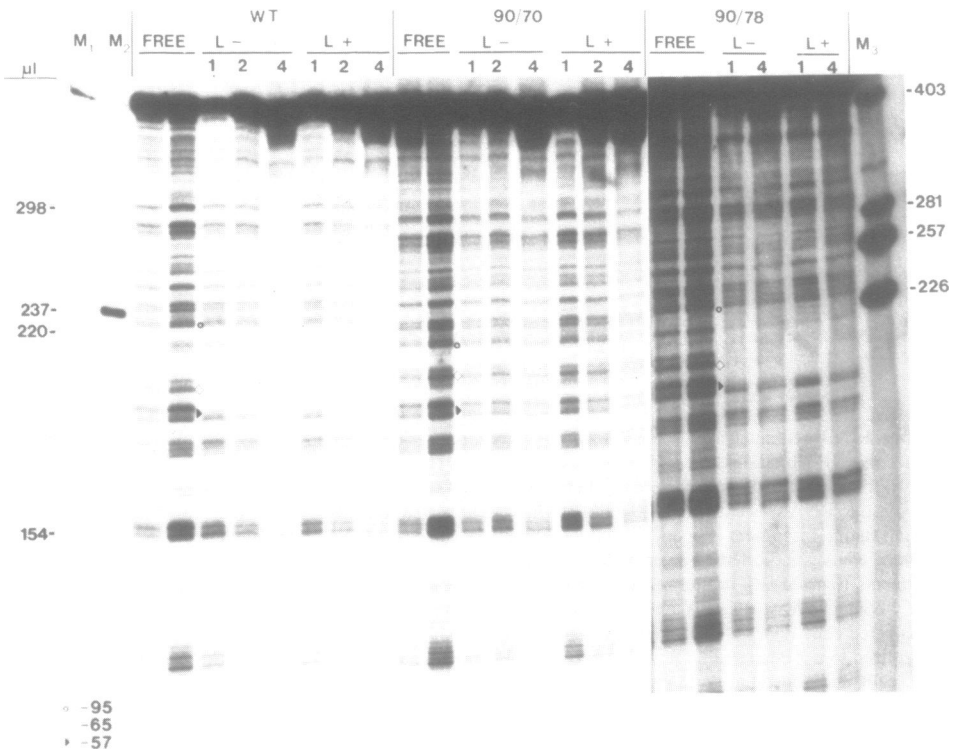

Figure 3. DNase I footprinting analysis of NF-I on wild-type and mutated MMTV LTR DNA.
A 445-bp Sty I-Bam HI fragment from −306 to the 3′ end of the LTR (3′-^{32}P-end-labeled in the coding strand at the Bam HI site of the linker at +139) was incubated either with no extract (*FREE*) or with increasing amounts of nuclear extracts from Ltk$^-$ cells, either untreated (L−) or treated for 16 h with 1µM dexamethasone (L+). After limited digestion with DNase I, the deproteinized DNAs were electrophoresed on a denaturing 6% polyacrylamide gel with radioactive size markers (M). Numbers on both sides of the autoradiogram denote fragment lengths in nucleotides. Numbers at the bottom refer to positions with respect to the RNA start site, as determined by sequencing of the probe.

DNase I footprinting

Ltk$^-$ cells were lysed with 0.5% Triton X-100 and nuclear extracts were prepared according to ref. 36. DNase I protection experiments (37) were performed according to ref. 38 using a DNA probe asymmetrically labeled with α^{32}P-dGTP and Klenow DNA polymerase. The probe was incubated for 15 min at 0 C with or without nuclear extract, in the presence of 50 µg/ml of poly (dI:dC).

RESULTS

NF-I contributes to the basal level of transcription from the MMTV promoter.

Previous data (18,27) demonstrated that the NF-I binding site in the MMTV LTR is required for maximal transcriptional stimulation by glucocorticoid hormones. Results obtained with deletion mutants of the proximal receptor binding site including or not the NF-I element (19) suggested that the latter may be important for the basal level of transcription as well. To examine this possibility, deletion mutants were constructed in which both glucocorticoid response elements were deleted, including or not the NF-I binding site. Their structure

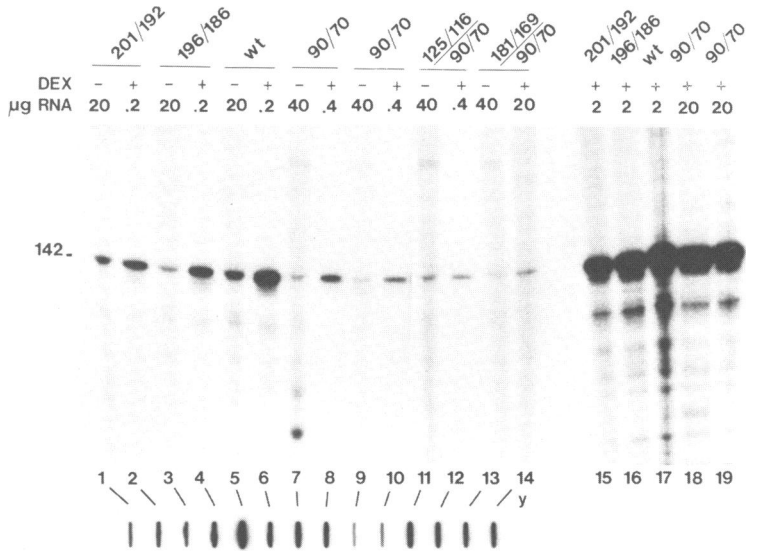

Figure 4. S1 nuclease protection assay of cytoplasmic RNA from Ltk$^+$ cells stably transfected with LTR-TK plasmids (as indicated above the lanes).
For quantitative comparisons, aliquots of the S1-digested samples were applied to the gel, corresponding to the amounts of RNA given above each lane, while the slot-blot at the bottom contains equal amounts (2 µg) of RNA hybridized to a labeled β-actin probe. Pools of Ltk$^+$ cells were grown for 16 h in the absence (−) or presence (+) of 1µM dexamethasone in the culture medium before RNA extraction. For S1 analysis, the (5′-^{32}P)-labeled DNA probe was an LTR fragment from −204 to + 134 with respect to the RNA start site (0.35-kb Eco RI−Bam HI fragment of the plasmid Δ47B—ref. 19). Correctly initiated RNA protects from S1 digestion a fragment of 142 nt. Mutants 201/192 and 196/186 were shown previously to behave similarly to wild type (18) and are included for comparison. The data for two independent pools of transfectants with the mutant 90/70 are shown. For double-linker scanning mutants, the RNAs used in lanes 11/12 and 13/14 are those of samples e and h, respectively, of Fig. 5.

and sequence can be deduced from Figure 1, by inserting a Hind III linker between positions −193 and −78 (for the mutant that retains the NF-I binding site) or between −193 and −57 (for the mutant without NF-I binding site). Another pair of plasmids, 193/78E and 193/57E, was derived from the previous ones by inserting the 72-bp repeat enhancer of SV40 DNA in the Sal I site of the linker upstream of the LTR (Fig. 1A), to increase the overall transcription. Mouse Ltk$^-$ cells were transfected with the plasmid DNA and pools of HAT-resistant clones, selected and grown in the absence of dexamethasone, were analyzed. Figure 2 (A and B) shows S1 nuclease protection assays of cytoplasmic RNA of cells transfected with the deletion mutants. Correctly initiated RNA protects a fragment of 142 nucleotides (arrow), from the cap site to the 3′ end of the LTR, as indicated in Figure 1A. The mRNA content of each RNA preparation was verified by slot-blot hybridization to a mouse β-actin probe (Fig. 1A, bottom) and shown to be constant, within the limits of experimental variability. Deletion of both glucocorticoid responsive elements from −193 to −78 resulted, as expected, in the loss of dexamethasone inducibility (Fig. 2B, lanes 4 and 5; and 2A, lanes 7 and 8) but the basal level of transcription was unaffected when compared to cells stably transfected with the wild-type plasmid (Fig. 2B, lanes 4−5 vs. 1). Additional deletion of the NF-I binding site, in mutant 193/57, resulted in a decrease of transcription to undetectable levels (Fig. 2B, lanes 2 and 3; and 2A, lanes 3 and 4).

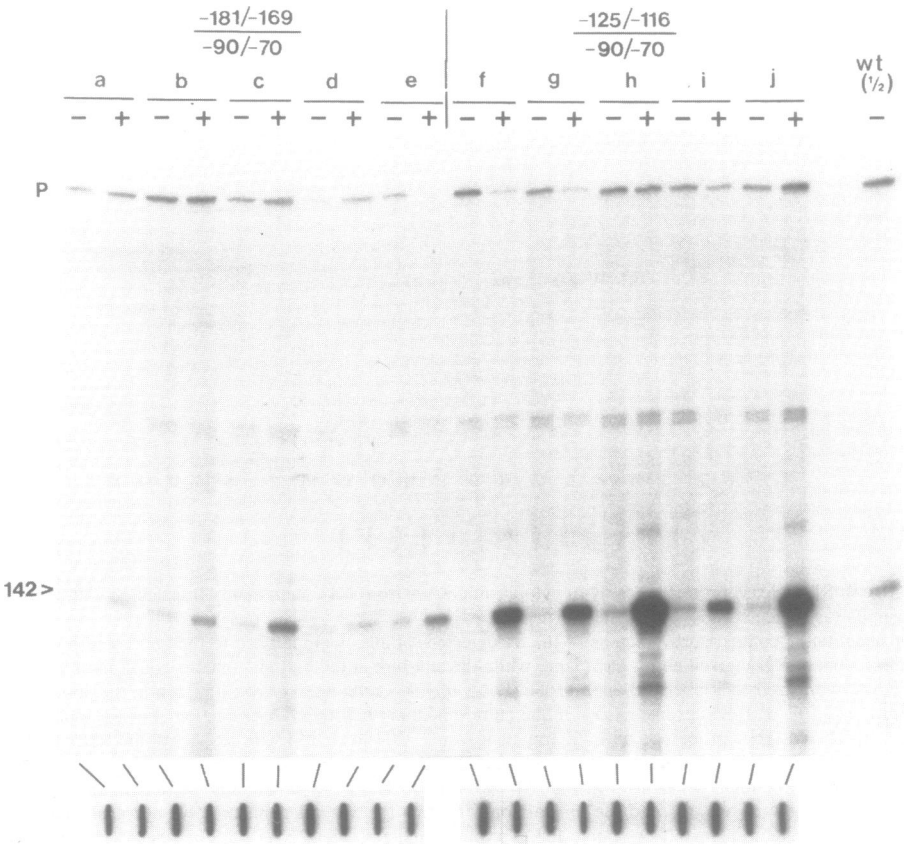

Figure 5. S1 nuclease protection assay of RNA transcribed from double-linker scanning mutants in stably transfected cells.
50 μg of cytoplasmic RNA from independent pools of Ltk$^+$ transfectants (*a* to *j*) and 25 μg of wild-type control RNA were used. Further details are as in Fig. 4. The panel at the bottom shows a slot-blot analysis with 2 μg of RNA hybridized to a β-actin probe.

A reduction in the basal level of mRNA upon deletion of the NF-I binding site was apparent also in the pair of mutants that contain the SV40 enhancer to augment the overall signal (Fig. 2A, lanes 5 and 6 compared to lanes 1 and 2). The weaker signal observed with the same transfectants after treatment with dexamethasone may reflect a toxic effect of this hormone for L cells (9). A Southern blot analysis of the DNA of the transfectants is depicted in Figure 1C. It revealed the presence of comparable amounts of integrated plasmids, as compared to the intensity of the endogenous bands of Ltk$^-$ cells (in lane 1). Moreover, the size of the fragment corresponding to the transfected LTR (between about 1.3 and 1.6 kb) was in accordance with that of the respective plasmids, and no major rearrangements were detected that could account for the differences in transcription efficiency of the two mutants.

We had previously observed (18) that Ltk$^-$ cells transfected with the mutant 90/70, where half of the NF-I binding site is deleted, had levels of dexamethasone-induced RNA

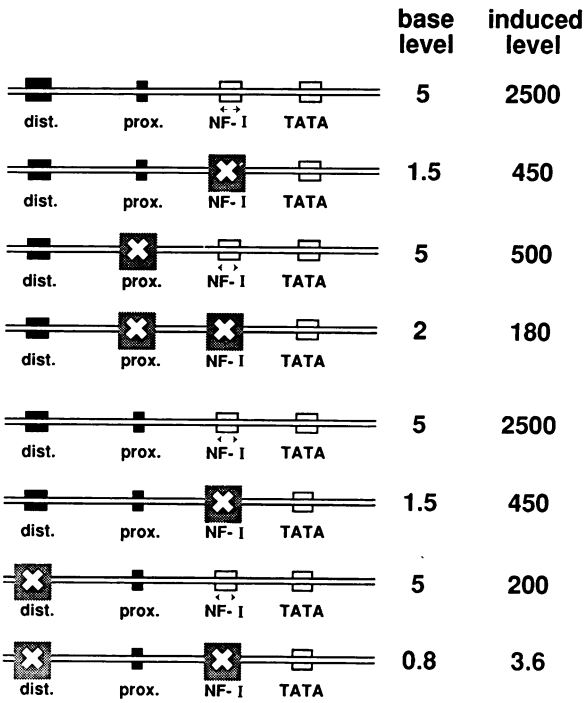

Figure 6. Quantitation of the transcriptional activity of mutant plasmids, based on densitometric scanning of the 142-nt S1 nuclease-protected fragments on autoradiograms. The values, expressed in arbitrary units, are based on data from 3 to 5 independent pools of stable transfectants (see Fig. 4 and 5). The top line of each panel depicts the relevant portion of MMTV LTR with the glucocorticoid response elements defined previously (Fig.1A). A cross over a box denotes a mutation: for the NF-I element, 90/70 (Fig. 1C); for the proximal element, 125/116 (Fig.1C); for the distal element, 181/169 (Fig. 1B).

five- to ten-fold lower than cells containing the mutant 90/78, in which the NF-I recognition sequence is intact (Fig. 1C). These and other results implied a role for NF-I in glucocorticoid stimulation of MMTV transcription, in conjunction with hormone-responsive sequence elements (18,27). We wanted to know if the 90/70 mutation also had an effect on the basal, uninduced transcription level. First, the in vitro interactions of wild-type and mutant DNA with NF-I-containing nuclear extracts from Ltk^- cells were investigated in DNase I footprinting experiments (37). As shown in Figure 3, Ltk^- cells contain a DNA binding activity that protected the NF-I recognition sequence (between approximately -80 and -58; ref.26) of the wild-type DNA, while it did not give any protection, even at the highest concentration of extract, on the DNA with the 90/70 mutation. In contrast, a footprint was observed in the same area on the 90/78 mutant DNA. No difference in the patterns was observed whether the extracts were from untreated or dexamethasone-treated Ltk^- cells. These data suggest that the deletion of half of the NF-I recognition sequence in the mutant 90/70 strongly reduces its affinity for NF-I in vitro, whereas NF-I binding does occur when the complete site is preserved, as in the mutant 90/78. Since no further footprints were observed on these DNAs over the stretch analyzed, it is likely that the NF-I binding in vitro occurred independently, and that the effect on the base level of transcription observed

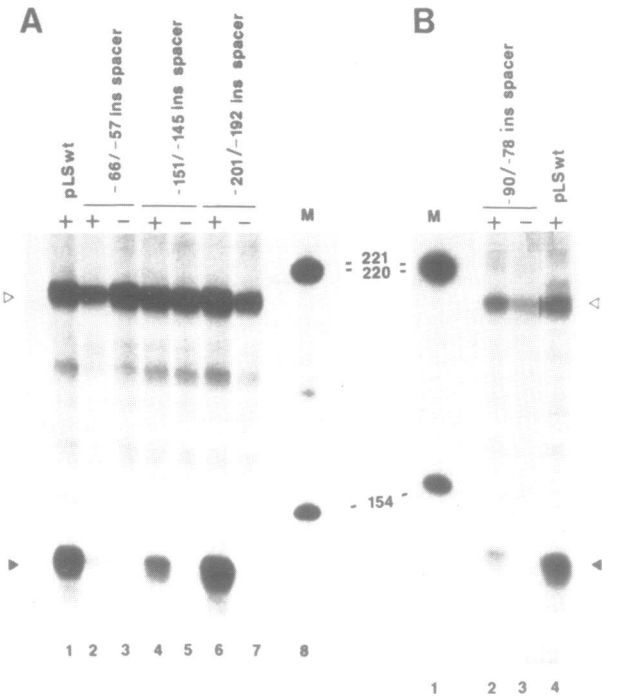

Figure 7. Spacer insertion mutants.
S1 nuclease protection assays were performed with 25 μg of cytoplasmic RNA of cells transiently transfected with 20 μg of MMTV plasmids plus 1 μg of β-globin plasmid H514. Correctly initiated RNAs protect a fragment of 142 nt of the MMTV probe (as in Fig.2) and of 212 nt of the globin probe (Bam HI-digested pH514) (arrows). When indicated (+), 1 μM dexamethasone was added to the cells 16 h prior to RNA isolation. End-labeled Hinf I fragments of pBR322 were used as size markers (lane M).

in the mutant 193/78, as opposed to 193/57 (Fig. 2) is due to NF-I binding.

Additional evidence for a functional role of NF-I in the uninduced activity of the MMTV promoter was obtained in quantitative S1 nuclease protection assays with RNA from cells stably transfected with plasmids containing the 90/70 mutation. With respect to wild type, a reduction in the order of 3- to 5-fold of the level of specific transcripts in the absence of dexamethasone was observed with the mutant 90/70, either alone (in three independent pools of transfectants) or in combination with other mutations (see below). A representative set of data is shown in Figure 4: whenever the 90/70 deletion was present in the transfected plasmid, the specific band protected by 40 μg of RNA (lanes 7,9,11,13) was weaker than the band protected by only 20 μg of RNA of transfectants with a wild-type phenotype (lanes 1,3,5). Correction for the mRNA content of the different preparations, as detected with the β-actin probe (Fig. 4, bottom) did not alter the conclusions. As a control, the stimulation factors by dexamethasone were also determined, by comparing with the S1-protected signals obtained with 100-fold less RNA from hormone-induced cells (Fig. 4, + DEX lanes). In agreement with our previous results (18), the linker-scanning mutants 201/192 and 196/186 (Fig. 1B) had an induction factor similar to the wild-type plasmid, here of approximately 500-fold. For the 90/70 mutant, an induction factor of about 300-fold was measured, while the absolute level of induced RNA was several fold less than the

Table 1. Effect of spacer insertions on the relative amounts of dexamethasone induced mRNA

	pLS stable[a]/transient[b]	pLS ins spacer stable[a]/transient[b]	ratio $\frac{+\text{spacer}}{-\text{spacer}}$
wild type	100/100		
−201/−192	100/180	100/120	1.0
−151/−145	100/n.d.	11/30	0.1−0.3
−90/−78	90/n.d.	n.d./15	0.16
−66/−57	40/n.d.	3/4	0.1

[a] Induced mRNA levels are given as a percentage of wild type
[b] MMTV signals from induced cells were normalized to the β-globin signals and expressed as percentage of wild type (included in the same experiment)
n.d. Not determined

wild type (Fig. 6), in agreement with previous observations (18). We conclude that the contribution of the NF-I binding site to the glucocorticoid-stimulated MMTV transcription involves a component that affects the basal promoter activity.

Functional cooperation of NF-I- and proximal glucocorticoid receptor binding sites. In a previous study using linker-scanning mutants (18) we had identified two glucocorticoid regulatory elements that mapped in regions of the MMTV DNA that bind hormone/receptor complexes in vitro (20,21). Mutations in the distal element (between −172 and −181) reduced the hormone response by ten-to twenty-fold, while a 4-bp mutation in the proximal element (between −121 and −117) reduced it by approximately five-fold. To investigate possible functional interactions between either of these elements and the NF-I-binding site, two double mutants were constructed by recombining single mutants in vitro at a Sst I restriction site located at position −106 from the RNA start site (Fig. 1A). Their structure can be deduced from Figure 1 B and C: Mutant 181/169//90/70 is simultaneously altered in the distal and the NF-I regulatory elements, and mutant 125/116//90/70 is simultaneously altered in the proximal and the NF-I elements. Their transcriptional activity was determined in stable transfection assays. Five independent pools of Ltk^+ cell clones were analyzed for each mutant. Figures 4 (lanes 11 to 14) and 5 show autoradiograms of S1 nuclease protection assays, along with control β-actin hybridization below each lane. It is immediately apparent that the mutant 181/169//90/70 responds only weakly to dexamethasone, while the mutant 125/116//90/70 shows a more substantial response. This was quantitated more accurately in titration experiments where dilutions of (+) samples were applied to the gel next to the undiluted (−) samples, and densitometric values of the 142-nt protected band were plotted as a function of RNA amount. The quantitative results are summarized in Figure 6, along with those of identical analyses on the parental single mutants. As shown in the top panel of Figure 6, removal of the NF-I binding site from an LTR already mutated in the proximal glucocorticoid responsive element caused a reduction in hormonal stimulation, with respect to the proximal site single mutant, by approximately the same factor as for the basal level of transcription (from 500 to 180, while the base level dropped from 5 to 2). On the other hand (Fig. 6, lower panel), cumulating the NF-I mutation with a distal-element mutation resulted in a much lower hormonal stimulation than expected from the drop observed in the basal level: Instead of about 6-fold (from 5 to 0.8 in base level), the induced level was reduced by almost 60-fold (from 200 to 3.6). These results show that the distal element alone, in the double mutant 125/116//90/70, was able to provide a dexamethasone induction in the order of 100-fold, whereas the proximal element alone,

in the double mutant 181/169//90/70, mediated only a very weak stimulation by dexamethasone (of about 4-fold over base level). This unexpected weakness of the element located around position −120 suggests a requirement for a functional cooperation, in the natural context of the MMTV regulatory region, between this site and sequences between −90 and −70, possibly the NF-I binding site.

Construction and analysis of spacer mutants.

The results of the mutational analyses suggested a functional cooperation between the different regulatory sequences to produce a maximal transcriptional stimulation by glucocorticoid hormones (18,19, and this study). In an attempt to disrupt such cooperation, spacer mutants were constructed by inserting a long stretch of DNA between any of the regulatory elements defined earlier. As a spacer we used a 123-bp Hind III fragment randomly cloned from mouse DNA for a previous study (19), that does not contain any transcriptional signals, nor consensus GRE sequences (19). It was chosen in a size range that should not produce any effects of stereospecificity in the alignment of the regulatory elements involved (39), but the precise number of base pairs is irrelevant. Via its Hind III ends, it was inserted into the DNA of four different linker scanning mutants (18). The approximate location of the spacer insertions with respect to the regulatory elements in the MMTV DNA can be deduced from the scheme in Figure 1A. The mutant 201/192 INS contained the spacer sequence upstream of the glucocorticoid regulatory region and was designed to determine whether the spacer had an inhibitory effect on transcription. The spacer DNA was inserted between the distal and the proximal receptor binding sites in the plasmid 151/145 INS, between the proximal element and the NF-I binding site in 90/78 INS, and between the NF-I binding site and the TATA box in 66/57 INS. In the latter mutant, the palindromic sequence of the NF-I binding site was reconstituted by the Hind III linker sequence and only the bases at positions −58, −60 and −61 are mutated (Fig. 1C). The transcriptional activity of the spacer mutants was determined in transfection assays by quantitative S1 nuclease mapping. Figure 7 shows the autoradiogram of a transient expression assay where the MMTV mutants were cotransfected with a plasmid expressing the genomic rabbit β-globin gene as an internal standard not affected by dexamethasone. Globin-specific mRNA protected a 212-nt fragment of a probe 5′-end-labeled at a Bam HI site in the second exon (40). MMTV-specific transcripts protected a 142-nt fragment. It can be seen from Figure 7 that their relative abundance in dexamethasone-treated cells was strongly reduced, compared to wild type, with the spacer insertion mutants 66/57 INS, 90/78 INS, and 151/145 INS, but not with 201/192 INS. Quantitative data from transient and stable transfection assays are given in Table 1, where the spacer insertion derivatives are compared with the correspondent linker scanning mutants. Insertion of the spacer DNA at any of the three positions between regulatory elements decreased the glucocorticoid response 5 to 10-fold. No decrease was observed when the spacer was inserted immediately upstream of the GRE at −192, indicating that the inserted sequence did not exert any inhibitory effect on the promoter.

DISCUSSION

The naturally occurring binding site for NF-I upstream of the MMTV promoter (26,41) had been previously shown to participate in the MMTV transcriptional stimulation by glucocorticoid hormones (18,27). Here we show that its function is a dual one: on one hand as an upstream element for the basal, hormone-independent promoter activity, on

the other hand as an adjuvant in the hormone response via a synergistic interaction with the proximal glucocorticoid control element.

Several lines of evidence demonstrate the existence of a basal transcriptional activity of the MMTV promoter: 5'-deletions of the whole LTR to −105 (8) or −80 (9), and internal deletions of all glucocorticoid-responsive sequences in an otherwise complete LTR (−193/78; Fig. 2B) allowed to experimentally dissociate hormonally stimulated from basal transcriptional activity. This residual RNA synthesis in stably transfected cells occurs at the same level as from a wild-type LTR in the absence of glucocorticoids (8,9; Fig. 2B) and can be detected in S1 nuclease protection assays, which allow to discriminate between correctly initiated and 'read-through' transcripts. It is this basal level of RNA synthesis that was decreased when the binding of NF-I was prevented (Fig. 3) by removal of the whole dyad-symmetrical site to −57 (Fig. 2) or of the 5' half of it to −70 (Figs. 4 and 5). We can not exclude a partial contribution of one half-site to the basic promoter activity, since the deletion 193/57 (both with and without an SV40 enhancer; Fig. 2A) seems to give a stronger reduction than deletions to only −70 (90/70: Fig. 6; 193/70: ref.18). The NF-I binding site can not be replaced by the distal glucocorticoid regulatory element, since no correct RNA was produced from the mutant 161/57 (19).

It has been reported (42) that NF-I proteins, which constitute a heterogeneous family with molecular weights between 52 and 66 kd, are biochemically and functionally indistinguishable from a transcriptional activator (CTF) that recognizes the sequence CCAAT present in some promoters, e.g. those of the herpes virus TK (43,44), of the heat-shock hsp70 (45) or of the globin genes (46,47,48,49). Recently, a molecular analysis of human CTF/NF-I complementary DNA clones (50) pointed to differential splicing as a source of heterogeneity among members of the family. Still, all individual gene products analyzed possessed both functions, of transcriptional activators and of initiation factors for DNA replication (50). A sequence resembling the CCAA(T) consensus AGCCAA (42) is present in each half of the MMTV NF-I binding site. In vitro, binding affinities are influenced by the extent of homology to the consensus sequence and by the presence or absence of a dyad symmetry, while for in vivo function, the position of the CCAAT site relative to a promoter is also determinant (42). In the case of MMTV, the complete symmetrical site is clearly required for the participation in glucocorticoid stimulation, since the half site of the mutant 90/70 was unable to provide a cooperation in Ltk^- cells (Fig. 6). In vitro, this mutant was also unable to bind NF-I from Ltk^- nuclear extracts (Fig. 3). In human mammary MCF-7 cells as well, glucocorticoid stimulation was destroyed by the mere alteration of the distance betweeen the two halves (27). Such sensitivity may reflect a low affinity of the factor for the MMTV site, which shows a rather poor homology to the consensus sequence (42), as well as different abundances of the factor(s) according to cell types (50).

From a previous mutational analysis (18) we had deduced that distal and proximal regions act cooperatively in the glucocorticoid response. The results with spacer- and double-linker insertion mutants allowed to define in more detail the functional relationships between elements. At every position where a long DNA segment was inserted to increase the spacing between elements, the glucocorticoid response dropped approximately ten-fold, to levels comparable to, or higher than those described for genes where the GREs are located distantly from the promoter, like the tryptophan oxygenase (TO) gene (51) or the tyrosine aminotransferase (TAT) gene (52). In TAT, cooperativity between two GREs has been observed (52). That the high induction factor of MMTV is the combined result of multiple,

mutually enhancing elements is apparent from the analysis of double mutants, as compared to single-site mutants (Fig. 6). We had previously shown that the two short receptor binding sites between −100 and −78 (20) did not contribute to the glucocorticoid response in Ltk^- cells (53) and therefore the mutant 90/70 is functionally altered in the NF-I binding site, as reflected in its in vitro binding behaviour (Fig. 3). The distal element, which is functionally the strongest one (18,19,54) displayed cooperativity with the proximal element, not with the NF-I site (Fig. 6, Top). The proximal element, by itself exceedingly weak, displayed a strong cooperativity with the NF-I site (Fig. 6, Bottom). In turn, because of its effect on the basal level of transcription discussed above, the NF-I site may be considered as having an interaction with the promoter itself, or with the NF-III consensus site (55,56) located 5′ of the TATA box, and whose function needs to be elucidated. An enhancement of the effect of a GRE by the juxtaposition of another element (in this case, the CACCC box; 49) was observed in the TO gene, both at its natural location at −450 (51) and when placed closely upstream of the TK promoter in heterologous constructs (57). In intact cells, an altered reactivity to dimethylsulfoxide following glucocorticoid induction was observed at a TGT sequence close to the GREs at −2.5 kb of the TAT gene (58). In vitro, no cooperative binding of the purified glucocorticoid receptor to the MMTV DNA could be demonstrated (22), nor of NF-I and receptors (26,27). That the NF-I binding site of MMTV DNA, in addition to the promoter/cap site region, is involved in transcriptional activity within the cell was suggested by results of in vivo exonuclease III footprinting, where boundaries of protection were observed at −82 and +12 in glucocorticoid-treated cells (41). The fact that no −82 (NF-I) boundary was observed in the absence of dexamethasone (41) probably reflects the inability of exo III to be arrested by factors interacting only weakly with the DNA. In fact, in this study (41) no hormone-receptor complexes bound at the GRE in hormone-treated cells were detected, either. Taken together, these observations suggest that the hormone-receptor complex might stabilize the interactions of NF-I and other transcription factors (e.g. TATA-box binding factors, NF-III) with the promoter. This may occur by cooperative binding, as it was shown for the purified factors TF-IID and USF on the adenovirus major late promoter (59), or more indirectly by displacing or altering the interactions with a nucleosome (60). The use of nuclear extracts for in vitro footprinting experiments and of more sensitive assays in vivo may help clarify this point. Support for the notion of cooperativity between GRE-bound receptor and downstream DNA-bound factors may be found in a report (61) showing that the insertion at −104 of a yeast regulatory element recognized by the GAL4 protein inhibits glucocorticoid induction in vivo, unless the GAL4 protein is present. Whether the synergistic effects require direct protein−protein contacts, and whether any of the domains of the glucocorticoid receptor (in particular the 'modulatory' N-terminal half of it—62,63) are involved, will need further studies, e.g. using mutated receptor molecules in combination with wild-type and altered recognition sequences.

ACKNOWLEDGMENTS

We thank F.A van der Hoorn for discussions, and Pascal Cousin for technical assistance. This work was supported by the Swiss National Science Fund. B.K. was a recipient of an ISREC fellowship.

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