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Glucocorticoid regulation of mouse mammary tumor virus: identification of a short essential DNA region

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Transcription of mouse mammary tumor virus (MMTV) DNA is stimulated by steroid hormones. To determine the DNA sequences involved in this regulation, we constructed a plasmid containing the MMTV long terminal repeat (LTR) in front of the coding region of the herpes simplex thymidine kinase gene, from which the promoter had been removed. Portions of the LTR were removed by the nuclease *Bal*31, and the deleted molecules were recloned and tested for transcriptional activity in transfections of Ltk$^-$aprt$^-$ cells. Stably transfected cell clones were selected and hormone-dependent transcription from the MMTV promoter was studied by the S1 nuclease mapping method. The results show that DNA sequences between −105 and −204 base pairs upstream from the initiation site of viral transcription are required for glucocorticoid stimulation.

Key words: deletion mutants/glucocorticoid response/ mouse mammary tumor virus/S1 mapping/transfection

Introduction

Transcription of proviral DNA of mouse mammary tumor virus (MMTV) is stimulated by glucocorticoid hormones in cultured cells, whether mammary tumor cell lines (Parks *et al.*, 1974; Ringold *et al.*, 1975; Scolnick *et al.*, 1976), mouse mammary gland cells (Vaidya *et al.*, 1978) or heterologous infected epithelial cells (Vaidya *et al.*, 1976; Ringold *et al.*, 1977a). Since the stimulation is largely of the rate of transcription (Young *et al.*, 1977; Ringold *et al.*, 1977b; Ucker *et al.*, 1981), MMTV has become a model system for studying steroid hormone action. Hormonal regulation was also observed in cells transfected with molecularly cloned forms of MMTV DNA, both of exogenous (Buetti and Diggelmann, 1981; Owen and Diggelmann, 1983) and endogenous origin (Hynes *et al.*, 1981; Diggelmann *et al.*, 1982). Transfections with subgenomic fragments (Fasel *et al.*, 1982; Payvar *et al.*, 1982) or with recombinant DNAs between MMTV fragments and a marker gene (Lee *et al.*, 1981; Huang *et al.*, 1981; Groner *et al.*, 1981) indicated that hormone-responsive sequences are located within the viral long terminal repeat (LTR). Through DNA sequence analysis, the signals for the initiation of viral transcription had been localized also in the LTR (Klemenz *et al.*, 1981; Donehower *et al.*, 1981, 1983; Fasel *et al.*, 1982; Kennedy *et al.*, 1982). We wanted to determine what part of the 1328 bp long LTR is involved in the hormonal control. We therefore removed increasing portions upstream of the transcription initiation site in a chimaeric plasmid where the MMTV LTR had been linked to the coding region of the herpes simplex (HSV) thymidine kinase (*tk*) gene. Plasmids with deletions were then introduced into Ltk$^-$ cells, and stably transfected Ltk$^+$ cell clones were isolated. The initiation of transcription at the MMTV promoter and its regulation by the glucocorticoid hormone, dexamethasone, were studied in several of these cell lines. The results show that a short region, between −105 and −204 bp from the cap site, is required for glucocorticoid stimulation of MMTV transcription.

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Results

Construction of a MMTV-tk recombinant plasmid

The MMTV DNA portion was derived from a lambda recombinant phage containing an insert of 9 kb (lambda.MMTV-GR clone H; Buetti and Diggelmann, 1981), comprising the complete genetic information of MMTV and one LTR in a permuted arrangement, as shown in the top part of Figure 1. A 3.8-kb *Bam*HI-*Xho*I DNA fragment that contains ~1 kb of 3′ unique viral sequences, a complete

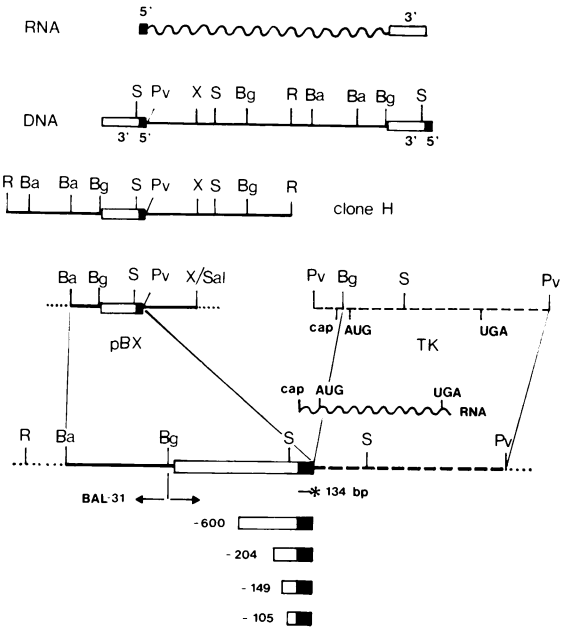

Fig. 1. Structure of plasmids and their origin. From the top: MMTV RNA, with boxes representing unique 5′ and 3′ sequences present in the LTRs of linear and proviral DNA (second line). MMTV clone H insert of a recombinant λ phage (Buetti and Diggelmann, 1981) is permuted around the *Eco*RI site with respect to MMTV linear DNA. pBX is a plasmid containing the 3.8-kb *Bam*HI-*Xho*I fragment of clone H subcloned in the *Bam*HI-*Sal*I sites of pBR322. Plasmids are shown linearized arbitrarily in the vector sequences (flanking broken line). MMTV sequences from the *Bam*HI to the *Pvu*II site at the end of the LTR are conserved in the MMTV-*tk* plasmid in the line below. On the right side is shown the *Pvu*II fragment of HSV-1 DNA that contains the *tk* gene (as part of a larger *Bam*HI fragment in the M2 plasmid of Wilkie *et al.*, 1979). Its *Bgl*II-*Pvu*II *tk*-coding fragment is conserved in the MMTV-*tk* construction, from which a chimaeric MMTV-*tk* RNA (wavy line) is transcribed. The 134-nucleotide S1 nuclease-protected fragment with the 5′ end-labelling (*) is drawn positioned with respect to pMMTV-*tk*. For deleted plasmids (bottom) only the retained portion of the LTR is drawn. Downstream sequences are the same as in pMMTV-*tk*. For upstream junction sequences see Figure 6. S, *Sst*I; Pv, *Pvu*II; X, *Xho*I; Bg, *Bgl*II; R, *Eco*RI; Ba, *Bam*HI; Sal, *Sal*I.

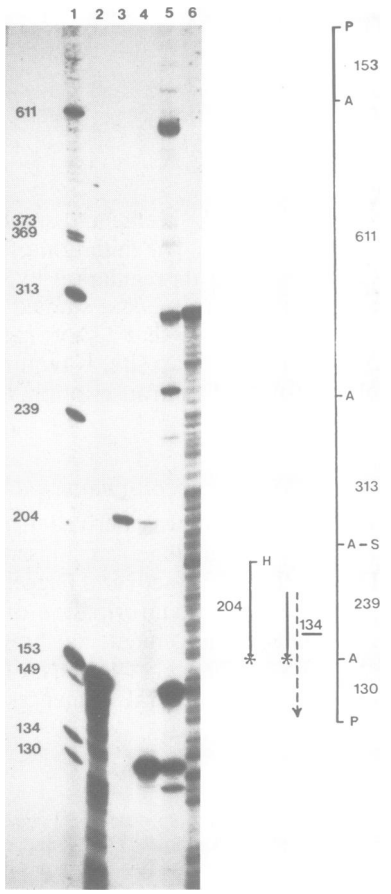

Fig. 2. Determination of the 5′ end of MMTV-GR RNA by S1 nuclease mapping. 5′ ^{32}P-labeled probes were annealed to total RNA of GR cells in culture grown in medium containing 10^{-6} M dexamethasone. Hybrids were digested with S1 nuclease and analyzed on a sequencing gel as described in Materials and methods. The scheme shows a map of the 1.45-kb, LTR-containing *Pst*I fragment of MMTV. P, *Pst*I; A, *Alu*I; S, *Sst*I; H, *Hinf*I. The gel-purified 204-bp probe (**lane 3**) asymmetrically labeled (*) in the minus strand was prepared by *Hinf*I digestion of the 5′ end-labeled 239-bp *Alu*I fragment. Protection of the 204-bp probe by 40 μg of GR RNA is shown in **lane 4**, and schematically on the right. **Lane 1** shows the mixture of 5′ end-labeled *Alu*I fragments. Due to the staggered cut by *Pst*I, the end fragments have different lengths in the two strands: 153(−)/149(+), and 130(−)/134(+); the doublet 369/373 is a partial digestion product containing the sum of the 130- and 239-bp fragments. Protection of this probe by 20 μg of GR RNA is shown in **lane 5**. The broken line in the scheme indicates the protected fragment of 264 nucleotides derived from the partial digestion product (also visible in **lane 5**). **Lanes 2** and **6** are reference sequence ladders (Maxam and Gilbert, 1980): **lane 2** is the T + C sequence of the fragment of 153 nucleotides.

LTR, plus ~1.4 kb of 5′ unique sequences was isolated and recloned in the plasmid pBR322 cut with *Bam*HI and *Sal*I (plasmid pBX in Figure 1). After digestion of pBX with *Pvu*II, the smaller fragment, containing all 5′ unique MMTV DNA plus 1.4 kb of pBR322 DNA, was removed and replaced by a fragment of HSV DNA coding for the viral *tk* gene. The source of *tk* DNA was the plasmid M2 of Wilkie *et al.* (1979), which harbors the HSV-1 *tk* gene. This same plasmid had been used in all our previous co-transfection experiments, where we had verified its biological activity (Buetti and Diggelmann, 1981). Figure 1 shows a map of the *Pvu*II fragment that comprises the complete *tk* gene, and the transcriptional and translational landmarks determined by McKnight (1980). For constructing the chimaeric plasmid, we used the 1.8-kb *Bgl*II-*Pvu*II fragment, in which the *tk* regulatory sequences, the cap site, and 52 bp of the 5′-non-translated portion of *tk* are absent. The *Bgl*II-generated ends were made flush with the Klenow polymerase, and this fragment was linked to the MMTV LTR in the orientation shown in Figure 1. In this MMTV-*tk* recombinant plasmid (pMMTV-*tk*), the *tk* gene is under the control of the MMTV promoter; the resulting chimaeric mRNA drawn in Figure 1 contains a 5′-non-translated region which is 82 nucleotides longer than the normal HSV-1 *tk* transcript described by McKnight (1980).

Biological activity of the MMTV-tk recombinant plasmid

Transfections of Ltk$^-$aprt$^-$ cells with the recombinant plasmid in the form of calcium phosphate precipitate were performed as described in Materials and methods. Ltk$^+$ cell clones growing in 'HAT' medium (Littlefield, 1964) were seen within ~2 weeks and could be expanded as cell lines in selective medium. The efficiency of stable transfection was low (1−3 clones per μg of purified plasmid) as compared with 100−200/μg for the HSV-1 *tk* plasmid. Addition of 10^{-6} M dexamethasone to the medium during the selection did not increase the number of HAT-resistant clones; rather, it made the average size of the individual clones smaller, as compared with clones kept without hormone. This could be due to a general toxic effect of dexamethasone on L-cell growth in the case of long-term exposure to the hormone. The early appearance of the phenotypic conversion of transfected cells and the structure of the recombinant plasmid made it likely that the *tk* gene was expressed in Ltk$^+$ clones from the MMTV promoter and not from cellular promoters rearranged in conjunction with the *tk*-coding sequences (Roberts and Axel, 1982). However, it was important to establish the activity of the MMTV promoter and the accuracy of initiation of transcription, both in the absence and in the presence of glucocorticoid hormone.

Hormonal regulation and initiation of transcription in transfected cells − comparison with virus-infected cells

Transcripts starting in the MMTV portion of the chimaeric plasmid were detected by the S1 nuclease mapping procedure (Berk and Sharp, 1977) using an end-labelled DNA probe (Weaver and Weissmann, 1979). The LTR-containing *Pvu*II fragment of the plasmid pBX (Figure 1) was 5′ end-labelled with ^{32}P and annealed with an excess of total cellular RNA extracted from an Ltk$^+$ cell line resulting from transfection with the MMTV-*tk* plasmid. After digestion of the hybrids with the single-strand-specific S1 nuclease, the products were analyzed by electrophoresis on denaturing polyacrylamide gels and autoradiography. One protected fragment of 134 nucleotides (see below) was detected in the dexamethasone-treated sample (Figure 4A, lane 9; 4B, lane 12). It co-migrated with the fragment protected by RNA from cells transfected with the complete 9-kb MMTV DNA insert (Buetti and Diggelmann, 1981; 4A, lanes 11 and 12), and with the fragment protected by RNA from the GR tumor cell line (4A, lane 2; 4B, lane 13). No bands were observed with RNA from Ltk$^-$ cells (4A, lane 1; 4B, lane 14). This result showed that the pMMTV-*tk* plasmid was transcribed in transfected cells and RNA synthesis was accurately initiated at the proper site. Moreover, it was glucocorticoid-dependent (4A, lanes 9 and 10; 4B, lanes 11 and 12) to an extent comparable with cells transfected with the whole MMTV DNA (4A, lanes 11 and 12) or tumor cells (not shown), where the stimulation factor was of the order of 10- to 100-fold.

The 5′ terminus of MMTV RNA in GR cells was mapped in separate experiments to 134 nucleotides from the *Pvu*II (and *Alu*I) site located 1 bp outside the end of the LTR, i.e., to 133 nucleotides from the right end of the LTR. Evidence from one such experiment is presented in Figure 2. 5′ end-labeled probes were either a mixture of *Alu*I fragments produced by digestion of the purified 1.45-kb *Pst*I fragment that contains approximately the LTR (lane 1), or a 204-bp *Alu*I-*Hinf*I fragment, labeled only in the non-coding strand, encompassing the region where RNA synthesis starts (lane 3), as indicated schematically in Figure 2. With both probes, the protected fragment was 134 nucleotides in length (lanes 4 and 5). The use of the mixture of *Alu*I fragments is convenient in that it provides an internal size marker of 130 nucleotides: the minus-strand of the 3′-terminal *Alu*I-*Pst*I fragment, which was also protected by GR RNA. Due to the staggered cut of the *Pst*I enzyme, the plus-strand of the same end fragment is 134 nucleotides long and can be used as a reference size marker in a neighboring lane (lane 1). Since these fragments all have the same terminal groups, they can be directly compared with respect to their migration in the gel. In contrast, the sequence ladder of lane 2 is expected to be displaced by a distance corresponding to 1.5 nucleotides (Sollner-Webb and Reeder, 1979). The observed difference is ~2 nucleotides, which would again point to a size of 134 nucleotides for the protected fragment. The same conclusion was reached when the sequence ladder produced by chemical degradation of the 204-bp fragment was used for comparison (data not shown). We therefore designated the cap nucleotide of GR RNA as the G in position −133 from the 3′ end of the LTR (Figure 6) also in view of the observation that the majority of viral mRNAs start with a capped purine (Shatkin, 1976). Other laboratories have reported the T in position −135 (Huang *et al.*, 1981; Ucker *et al.*, 1981, 1983), also on the basis of S1 protection experiments.

Construction of deleted plasmids

To define the portion of DNA necessary for the glucocorticoid regulation of the MMTV promoter, deletions were introduced in the LTR of the chimaeric plasmid starting from the unique *Bgl*II site located 42 bp outside the LTR (Figure 1). Linearized molecules were digested to a variable extent with the nuclease *Bal*31 and separated on a preparative agarose gel. Pools of different-sized molecules were extracted and resealed with T4 ligase, with or without the insertion of *Xho*I molecular linkers. After recloning, the deleted plasmids were screened by restriction analysis; the chosen ones were amplified, density-gradient purified and assayed for biological activity in stable transfection experiments (see below). Four plasmids with deletions of 1.15−1.3 kb were characterized only by the distance between the *Sst*I site in the LTR and the *Eco*RI site in the pBR322 portion. Assuming an approximately symmetrical digestion by *Bal*31 on both sides of the *Bgl*II site, the deletion breakpoint should lie between −550 and −650 bp from the RNA initiation site. In view of the results obtained with plasmids having larger deletions (see below), these were not further analyzed and are designated as approximately −600 deletions. For the plasmids with deletions extending to position −204 and −149 (Figure 1), the deletion breakpoint was determined by sequencing after subcloning in phage M13 (Vieira and Messing, 1982; Sanger *et al.*, 1980) as described in Materials and methods. The plasmid with a deletion to position −105 was produced by partial *Sst*I digestion of *Eco*RI-linearized pMMTV-*tk* (Figure 1); after gel purification, the fragment was made flush-ended, circularized and recloned. The sequence of the deleted plasmids in the relevant region is shown in Figure 6.

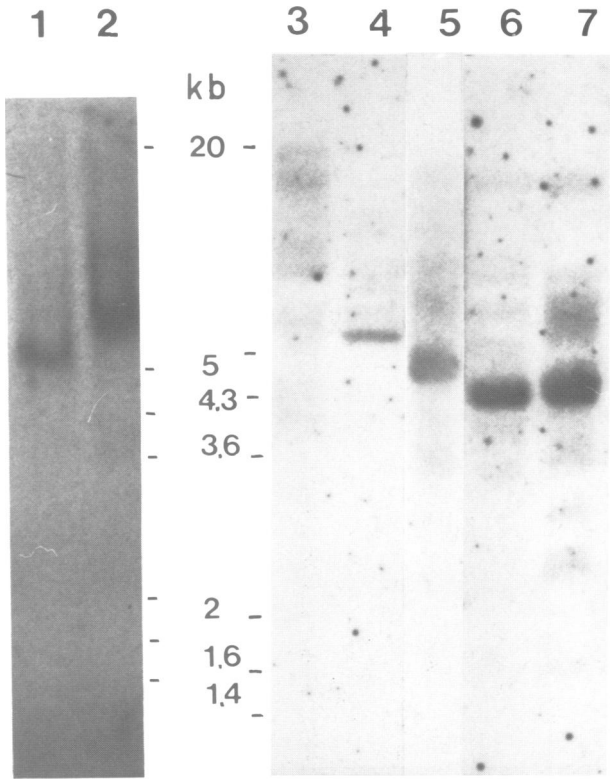

Fig. 3. Southern blot analysis of DNA from Ltk$^+$ cell clones transfected with deleted MMTV-*tk* plasmids. 10−15 μg of DNA were digested with *Eco*RI, electrophoresed on a 0.8% agarose gel in Tris-acetate buffer, and transferred to a nitrocellulose filter. Hybridization was with a [^{32}P]nick-translated plasmid containing the 1.45-kb *Pst*I fragment of MMTV (Fasel *et al.*, 1982). Size marker was λ wt DNA cut with *Eco*RI and *Hind*III. **Lanes 1** and **2**: −204 deletion. **Lanes 3** and **4**: two different approximately −600 deletions. **Lane 5**: −149 deletion. **Lanes 6** and **7**: −105 deletion. Transfectants shown in **lanes 2** and **3** had been selected in the presence of dexamethasone; all others in the absence of the hormone.

Transfection with deleted plasmids

Gradient-purified plasmids were introduced in Ltk$^-$aprt$^-$ cells as described above; HAT-resistant cell colonies appeared after ~2 weeks, whether dexamethasone had been added or not to the medium during selection. In general, we chose for expansion and analysis independent Ltk$^+$ cell clones selected in the absence of hormone. Some results obtained with cells selected in the presence of dexamethasone, then expanded in its absence, are also included in the following description, and specifically mentioned in the Figure legends. We reasoned that applying a selective pressure on the gene that was a marker for the MMTV promoter activity would yield cell clones with mainly intact MMTV-*tk* regions; and that absence of hormone during selection, if possible, would favor the isolation of clones with a sufficiently high number of integrated plasmids to provide a base line of *tk* gene activity and thus facilitate the analysis of the transcripts with or without hormone stimulation. In fact, when we analyzed cellular DNAs from Ltk$^+$ transfectants by the Southern blot procedure (Southern, 1975), we observed that amplification of integrated sequences had occurred in the majority of cases. Figure 3 shows an analysis of cellular DNAs restricted with

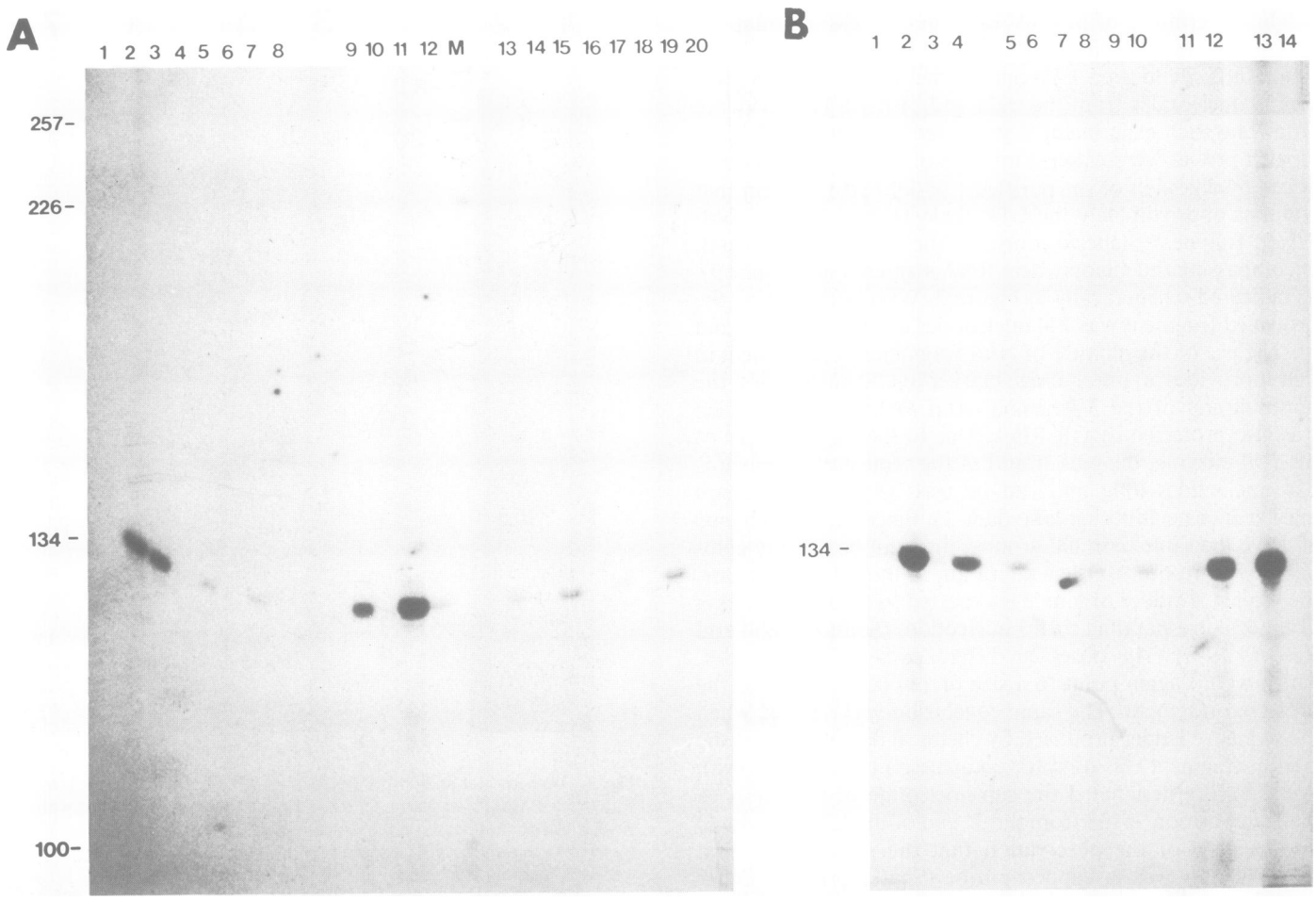

Fig. 4. S1 nuclease mapping of MMTV-initiated transcripts in transfected cells. 5′-^{32}P-labeled probe was the *Pvu*II-digested plasmid pBX, i.e., the LTR-containing 5.1-kb fragment plus the 2.7-kb fragment; the latter is absent from the MMTV-*tk* chimaeric plasmid, and its non-coding strand ends in the plasmid vector, therefore it cannot yield any labeled protected fragments with the RNAs used. This was verified in S1 experiments with RNA from GR cells and cells transfected with the 9-kb MMTV DNA, using either both fragments or gel-purified 5.1-kb fragment as end-labeled probes. Hybrids were digested with S1 nuclease and analyzed on sequencing gels as described in Materials and methods. In the following list, (+) or (−) after the lane number refers to the presence or absence of 10^{-6} M dexamethasone in the culture medium added 17−20 h before RNA extraction. Lane designations separated by a slash (/) are parallel samples of the same transfectant. Control RNAs were from Ltk$^-$ cells (**A1**, +; **B14**, +); the GR tumor cell line (**A2**, +; **B13**, +); Ltk$^+$ cells co-transfected with the *tk* plasmid and a 9-kb MMTV clone H DNA (**A11**, +/**A12**, −). RNA from a cell clone transfected with intact pMMTV-*tk* was used in lane **A9**(+)/**A10**(−), and **B11**(−)/**B12**(+). RNAs from cells transfected with −600 deletion mutants: **A3**(+)/**A4**(−); **A7**(+)/**A8**(−); **B1**(−)/**B2**(+); **B3**(−)/**B4**(+). RNAs from cells transfected with −204 deletion mutants: **A5**(+)/**A6**(−); **A13**(+)/**A14**(−); **A15**(+)/**A16**(−); **A17**(+)/**A18**(−); **A19**(+)/**A20**(−). RNAs from cells transfected with −149 deletion mutants: **B5**(−)/**B6**(+) (20 μg of cytoplasmic RNA); **B7**(−)/**B8**(+) (1.5 μg of poly(A)-containing RNA); **B9**(−)/**B10**(+) (3 μg of the same poly(A)-containing RNA as in **B7**/**B8**). In general, 20 μg of cytoplasmic RNA was used, except in lanes **A9**/**A10** (40 μg) and **B13** (10 μg). Independently isolated transfectants were analyzed. Those shown in **lanes A13**−**A18** had been selected in the presence of dexamethasone and expanded in its absence. **Lane M** contains 5′-labeled marker fragments (*Alu*I-digested pBR322).

*Eco*RI, an enzyme that linearizes the deleted plasmids close to the pBR322-MMTV junction. After agarose gel electrophoresis and transfer onto a nitrocellulose membrane, hybridization was performed with MMTV LTR DNA (1.45 *Pst*I fragment) inserted in pBR322. The whole plasmid, nick-translated with ^{32}P, served as a probe, therefore revealing both MMTV- and pBR322-containing bands. In 18 out of 19 DNAs analyzed, one band of variable size did show a very strong hybridization, see Figure 3 (lane 1, 2, 4−7). On longer exposure of the autoradiograph, numerous faint bands were detected, including the known pattern of endogenous MMTV sequences of L cells, suggesting the presence of many different non-amplified plasmid fragments. In one transfectant (lane 3) no major band was seen, but only an array of differently-sized ones, indicating mere integration of many copies of the plasmid.

DNA sequences between −105 and −204 are required for the glucocorticoid regulation

RNA was extracted from Ltk$^+$ cell clones resulting from transfection with deleted plasmids, and transcripts from the MMTV promoter were mapped by the S1 procedure as described above. Representative results are shown in Figures 4 and 5, where RNAs from cells grown with or without dexamethasone are analyzed in pairs. Comparison of the intensity of the signals in the 134-nucleotide protected cap fragment shows first of all that a full stimulation by the hormone is still present with deletions to approximately −600 (Figure 4B, lanes 1 and 2, compared with lanes 11 and 12). In different transfectants we observed different levels of transcription, but the signal was always stronger in the hormone-treated sample, and mostly below detection in the untreated sample (Figure 4B, lanes 3 and 4; 4A, lanes 3 and 4, 7 and 8). These

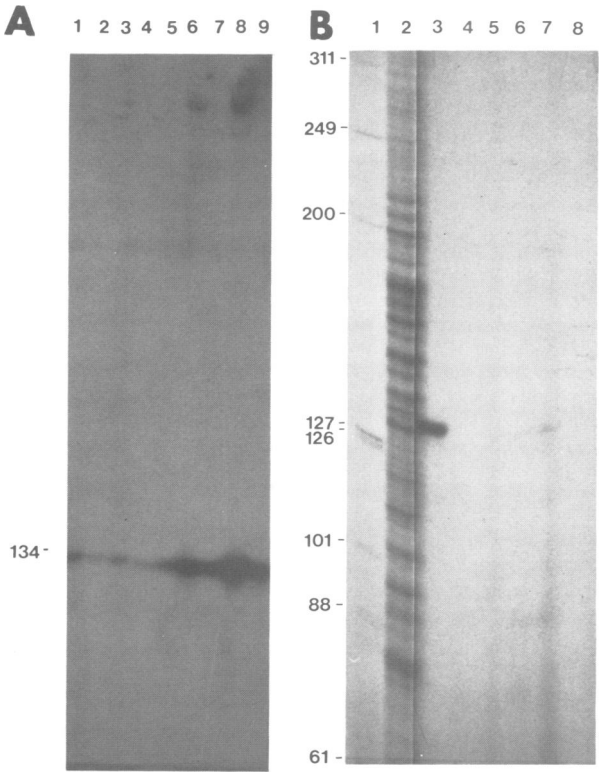

Fig. 5. S1 nuclease mapping of MMTV-initiated transcripts in transfected cells. 5′-^{32}P-labeled probe was *Pvu*II-digested pBX. Explanations are given in the legend to Figure 4. Cytoplasmic RNAs (50 or 60 μg) used in the hybridizations were as follows: from transfectants with −105 deletions, **lanes A1(−)/A2(+)** and **A3(−)/A4(+)**; from transfectants with −149 deletions, **lanes B4(−)/B5(+)** and **B6(−)/B7(+)**; from transfectants with −204 deletions, **A5(−)/A6(+)** (same RNA as shown in Figure 4A, 19/20) and **A7(−)/A8(+)**. Ltk$^-$ RNA (18 μg), **lane B8(+)**. GR RNA (6 μg) in **lane A9(+)** and **B3(+)**. Size markers in **lane B1** are 5′-labeled fragments of *Eco*RII-digested SV40 DNA. **Lane B2** contains a Sanger sequencing T-ladder as a reference (*Xho*I/*Sma*I fragment of −149 plasmid in M13mp8).

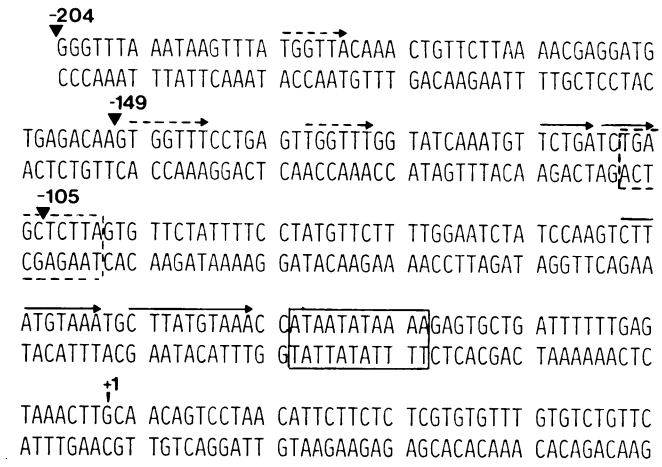

Fig. 6. Sequence of the region of the GR LTR with deletion breakpoints. Both DNA strands are shown. +1 indicates the cap nucleotide (see Results for explanations); the T upstream of it is defined as −1; triangles mark the break in the MMTV sequence in different deleted plasmids. Vector sequences upstream of the remaining MMTV sequences are: in the −204 deletions: pBR322 sequences upstream of position 41 (taking as 0 the *Eco*RI site and counting in the direction of the tetracycline-resistance gene); i.e., from the *Hind*III site: (AAGCTT)TAATGCG(−204). In the −149 deletions: pBR322 sequences upstream of position 223 plus *Xho*I linker: ...GCATCGCCAGTCACTATGGCTCGAG(−149). In the −105 deletions: filled-in *Eco*RI site of pBR322 and upstream sequences: ...GGCCC-TTTCGTCTTCAAGAAT(−105). Arrows indicate repeats (see Discussion). A box with a continuous line indicates the 'TATA' box. A box with a broken line encloses the consensus sequence among different glucocorticoid regulated genes (see Discussion).

variations are probably due to variations in the number of active plasmids in different transfectants. When the signal was detectable in the sample without dexamethasone treatment, a stimulation factor of 10- to 100-fold was found, comparable with that observed with the non-deleted recombinant plasmid or with the intact MMTV DNA clone (see above). Deletions to −204 retained the ability to respond to the hormonal treatment (Figure 4A, lanes 5 and 6; lanes 13−20; Figure 5A, lanes 5−8). The observed stimulation factor was 10- to 20-fold, therefore still in the range of other experimental observations (Varmus *et al.*, 1979; Buetti and Diggelmann, 1981; Hynes *et al.*, 1981; Diggelmann *et al.*, 1982). Although sequences between −204 and −600 may affect its amplitude, the glucocorticoid response itself was still present in all transfectants with deletions to position −204. In the two samples shown in Figure 5A (lanes 5−8) a new, minor protected fragment of ~300 nucleotides is clearly visible, which is also dexamethasone-dependent. Although a more precise mapping of this transcript has not been done, it is tempting to associate it with the presence of a short A+T-rich region 20−30 bp upstream, immediately following the deletion breakpoint (see Figure 6). Further investigation is needed to assess the relevance of this finding with respect to the mechanism of hormone action. When the deletion extended to −149 bp, the results were mixed, although they mainly pointed to a greatly reduced, if not completely abolished, response to the glucocorticoid. A slight stimulation (3-fold) was seen in one transfectant when poly(A)-containing RNA was analyzed (Figure 4B, lanes 7−10). Total cytoplasmic RNA from a second transfectant showed a similar behavior (1.7-fold; Figure 5B, lanes 6 and 7), while in two others the 134-nucleotide band was visible in the sample without hormone, and undetectable in the sample with hormone (one is shown in Figure 4B, lanes 5 and 6); this situation is similar to that found with larger deletions (see below). Removal of all LTR sequences upstream of −105 from the cap (i.e., to the *Sst*I site, see Figure 1) resulted in a complete abolition of the glucocorticoid regulation, while a base level of transcription starting at the correct position was maintained. The results are shown in Figure 5A (lanes 1−4) for two out of the six Ltk$^+$ clones analyzed. The intensity of the 134-nucleotide cap fragment is clearly not increased upon dexamethasone addition but rather slightly reduced, 1.5- to 4-fold; this may be significant, but it is probably within the limits of experimental variation. Thus, we conclude that glucocorticoid regulation of the MMTV promoter requires an upstream DNA region between −105 and −204 bp.

Discussion

The results presented here corroborate the hypothesis that all of the information necessary for the effect of glucocorticoids on MMTV transcription is encoded within a small region of viral DNA. The hormone-responsive sequences are assigned by the present analysis to 100 bp between the positions −105 and −204 upstream of the transcription start site. The remaining ~1000 bp that comprise the upstream sequences in the LTR are shown to be dispensable, but might, to a certain extent, affect the amplitude of the response.

These data also exclude the possibility that the protein coded for by the first 959 nucleotides of the LTR is required for the glucocorticoid regulation (Fasel *et al.*, 1982; Kennedy *et al.*, 1982; Donehower *et al.*, 1983). An effect exerted by plasmid sequences brought closer to the MMTV promoter can not be rigorously excluded, but seems unlikely, due to their similarity (Figure 6) in different constructions that displayed very different biological activities. The chromosomal environment of the newly integrated DNA molecules might introduce an additional variability of the response.

Our data show that the glucocorticoid effect can be separated experimentally from transcription: the basal level of correctly initiated transcripts found with −105 deleted mutants is comparable with the level observed with the entire MMTV DNA in the absence of hormone, indicating no major overlap between hormone-responsive sequences and sequences needed for the correct initiation of transcription.

Whether MMTV sequences between −105 and −204 can transfer hormonal responsiveness onto another promoter not normally regulated by glucocorticoids is now amenable to experimental investigation. Inspection of the DNA sequence between −1 and −204 (Fasel *et al.*, 1982; Figure 6) reveals a 10-bp direct repeat separated by 2 bp just before the 'TATA' box, a 5-bp direct repeat (TCTGA) between −108 and −117, a sequence of 6 bp (TGGTTT) directly repeated at positions −143 to −148 and −130 to −135, and incompletely (TGGTTA) at position −182 to −187. In addition, we compared upstream sequences of different glucocorticoid-regulated genes. Schmid *et al.* (1982) found by computer analysis a 7/10 consensus sequence (TXAGXTCTXA) at position −101 to −111 between the rat tryptophane oxygenase (TO) gene and MMTV LTRs of three different strains. Fasel (1983) found 1-bp variants of this sequence at additional locations in MMTV DNA (−252 and −1230), and at −225/−226 in two other glucocorticoid-regulated genes, the rat growth hormone gene (Barta *et al.*, 1981) and the human proopiomelanocortin gene (Cochet *et al.*, 1982). We noticed that the TO sequence forms a 5-bp inverted repeat, while the other genes show divergence from a dyad symmetry. Repeats and dyad elements are present in essential regions of other regulated genes (Mirault *et al.*, 1982); their significance is at present unknown. In our case, the −105 deleted mutant would have lost half of the consensus sequence at −101, and all those at −252 and −1230, while −149 and −204 mutants would have retained the block at −101, and lost the others. More specific local mutagenesis experiments will help in clarifying the functions of these, or other, DNA structural features. A simple hypothesis, based on analogies with procaryotic systems (Ptashne *et al.*, 1980) would predict that the region −105 to −204 contains sequences that are recognized by the hormone-receptor complex, which through binding to this region would increase the efficiency of the MMTV promoter. Using different techniques, recently several groups have reported that partially purified hormone-receptor complexes preferentially bind *in vitro* to certain cloned MMTV DNA fragments (Payvar *et al.*, 1981, 1982; Govindan *et al.*, 1982; Geisse *et al.*, 1982; Pfahl, 1982). Although binding sites have been described in different regions of MMTV DNA and of mouse flanking DNA, all authors agree on the presence of at least one binding site in the LTR. Finer mapping placed it in the 400 bp at the 3′ end of the LTR. It is noteworthy that these results are compatible with our deletion data.

Materials and methods

Preparation and characterization of deleted plasmids

All plasmids were grown in *Escherichia coli* HB 101. The MMTV-*tk* plasmid shown in Figure 1 was linearized with *Bgl*II; 8 μg were digested at 30°C with 1 unit of *Bal*31 (BRL) in the recommended buffer in a volume of 200 μl. Aliquots were removed between 14 and 25 min of incubation, pooled, and phenol-extracted, then ligated to octameric *Xho*I linkers (Amersham) and digested with *Xho*I. Molecules of the wanted sizes were isolated on a low-melting point agarose gel, circularized with T4 ligase (New England Biolabs, Inc.) and cloned. Small-size plasmid samples were prepared by a Triton lysis procedure (Shalit *et al.*, 1981). Restriction analysis revealed a significant amount of deletions due to a contaminating endonucleolytic activity; we subsequently obtained better results when using shorter digestion times and repeated additions of *Bal*31. To determine the boundaries of large deletions, the fragments between the *Eco*RI site in pBR322 and the *Sma*I site in the *tk* gene were subcloned in the M13 phage mp9 (Vieira and Messing, 1982); for a plasmid with inserted *Xho*I linker, the *Xho*I-*Sma*I fragment was also subcloned in M13mp8 cut with *Sal*I and *Sma*I. DNA sequences were determined by the method of Sanger (Sanger *et al.*, 1980). The plasmid deleted to −105 was prepared by total *Eco*RI and partial *Sst*I digestion of pMMTV-*tk*; the gel-purified 3.8-kb fragment was treated with Klenow polymerase (Boehringer), then circularized with T4 ligase and recloned. The T at position −105 is derived from the filled-in *Eco*RI site of pBR322.

Transfection of L cells and extraction of nucleic acids

Plasmid DNA was purified by CsCl density gradient centrifugation in the presence of ethidium bromide. Partially purified plasmids prepared by the Triton procedure (Shalit *et al.*, 1981) were also used in early experiments with a lower transfection efficiency. Ltk^-aprt^- cells (from M. Perucho and M. Wigler, through N. Hynes) grown in Dulbecco modified Eagle's medium with 10% fetal calf serum were transfected with 15 μg of plasmid DNA per 60 mm Petri dish (~10^6 cells) using the calcium phosphate precipitation method previously described (Graham *et al.*, 1980; Buetti and Diggelmann, 1981). DNA and cytoplasmic RNA were extracted from Ltk^+ cell clones expanded in HAT medium to 3–6 10-cm Petri dishes and grown for the last 17–20 h either in the absence or presence of 10^{-6} M dexamethasone. Cells were lysed on ice with 0.2% Nonidet P-40 containing homogenization buffer, and DNA extracted from the nuclear pellet (Groner and Hynes, 1980). Cytoplasmic RNA was extracted from the supernatant with phenol-chloroform in the presence of 1% SDS at room temperature. In some experiments, total cell RNA was extracted by the hot-phenol procedure (Scherrer, 1969). Poly(A)-containing RNA was isolated by oligo(dT)-cellulose column chromatography. DNA blot analysis was performed according to Southern (1975) as previously described (Buetti and Diggelmann, 1981).

S1 mapping

Restriction fragments were labeled at their 5′ ends with [γ-^{32}P]ATP (Radiochemical Centre, Amersham) and T4 polynucleotide kinase (Boehringer), mixed in excess with RNA, and co-precipitated. The dry pellet was resuspended in hybridization buffer containing 80% (v/v) twice-recrystallized formamide as described by Favaloro *et al.* (1980). The hybridization mixture (10 or 20 μl) was sealed in glass capillaries, denatured (85°C, 15 min) and annealed at 52°C for 4–15 h. The content of the capillaries was rapidly diluted into 0.3 ml of cold S1 buffer (Favaloro *et al.*, 1980) containing 1000 units/ml of S1 nuclease (Sigma) and incubated at 37°C for 30 min. After addition (on ice) of 75 μl of 2.5 M ammonium acetate-50 mM EDTA and 5 μg carrier yeast RNA, nucleic acids were precipitated with 1 volume of isopropanol. The desiccated pellet was resuspended in 3 μl gel sample buffer and electrophoresed in 6% polyacrylamide sequencing gels (Maxam and Gilbert, 1980). Bands were visualized by autoradiography at −70°C with Kodak X-Omat AR films with intensifying screens. The intensity of the bands was quantitated by densitometric scanning of films.

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