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Biochemistry

Subfragments of the large terminal repeat cause glucocorticoid-responsive expression of mouse mammary tumor virus and of an adjacent gene

(mouse mammary tumor virus large terminal repeat–thymidine kinase chimeric gene/DNA-mediated gene transfer/deletion mutants/hormone induction)

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ABSTRACT **After transfection of mouse mammary tumor virus (MMTV) proviral DNA into cultured cells, the DNA is transcribed in a glucocorticoid-sensitive fashion. The large terminal repeat (LTR) region of MMTV is 1,328 nucleotides long and contains the regulatory information necessary for the hormonal response. We have constructed a MMTV LTR–thymidine kinase (*tk*) chimeric gene and have tested the biological activity of molecules containing various deletions in the LTR after transformation of LTK$^-$ APRT$^-$ mouse cells. In the TK$^+$ transformants, both a LTR–*tk* chimeric RNA and an authentic *tk* RNA are correctly initiated and transcribed. The synthesis of the chimeric RNA as well as that of the *tk* RNA is hormonally regulated. A plasmid containing 202 nucleotides of LTR DNA 5′ to the RNA initiation site is fully sensitive to glucocorticoids; 50 nucleotides still cause a residual inducibility.**

The expression of mouse mammary tumor virus (MMTV) in cultured cells has been recognized as an important model system for studying the mechanism of action of glucocorticoid hormones (1, 2). Gene cloning and DNA-mediated proviral gene transfer experiments have shown that a DNA sequence responsible for the hormonal induction of MMTV is present within the provirus (3–5). Chimeric molecules containing the large terminal repeat (LTR) region of MMTV ligated with different genes have been constructed and used to show that hormone inducibility can be conferred to genes located 3′ of the MMTV LTR (6–8). The LTR sequence of MMTV comprises 1,328 nucleotides (9–11) and a RNA initiation site has been detected close to its 3′ end. Sequences located 5′ of a RNA initiation site are required for maximal and accurate initiation of transcription of eukaryotic genes (12–14). Assuming that regulatory signals involved in the hormonal response are located 5′ of the LTR RNA cap site, we have investigated the biological function of these sequences by the construction of specific deletion mutants of a LTR–thymidine kinase (*tk*) chimeric gene. These deletion mutants were introduced into cultured cells and stable transformants were isolated. Analysis of the transcripts made from this series of deletion molecules has shown that a plasmid containing 202 nucleotides 5′ to the LTR RNA initiation is capable of transcribing RNA in a hormone-sensitive fashion. Deletions in which 137 or 50 nucleotides remain resulted in a decreased inducibility. We have also observed hormone-sensitive transcripts initiating correctly in the *tk* gene. This suggests that the glucocorticoid regulatory region in the MMTV LTR can influence the transcription of another gene located in its vicinity.

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MATERIALS AND METHODS

Construction of a LTR–*tk* Chimeric Molecule and the Deletion Plasmids. The top portion of Fig. 1 shows the LTR–*tk* chimeric gene, plasmid 2.6, whose construction has been described (8). Briefly, a 4.2-kilobase (kb) *Eco*RI fragment containing the envelope region and the 3′ LTR of MMTV and 222 nucleotides of flanking mouse DNA (see ref. 15 for its isolation) was ligated to another 4.2-kb *Eco*RI fragment containing the herpes simplex virus (HSV) *tk* gene and nucleotides 2,065–4,360 of pBR322 (16). In this construction the *tk* gene is contained within a 2.0-kb *Eco*RI–*Pvu* II fragment (17) and 77 base pairs (bp) of HSV DNA precede the *tk* cap site. Both the LTR and the *tk* are oriented in the same transcriptional direction.

To introduce specific deletions in the LTR region of plasmid 2.6 two different methods were used. By using the method shown schematically in the top portion of Fig. 1, *Bam*HI linkers were ligated onto plasmid 2.6 molecules that had been randomly linearized with DNase I (18, 19). The molecules were digested with *Bam*HI sized on a low-gelling agarose gel and those ranging in size from 5.0 to 7.0 kb were isolated from the gel, ligated, and used to transform *Escherichia coli* cells. Seven plasmids were chosen for further study. Each plasmid contains a 0.5-kb *Eco*RI–*Bam*HI fragment of the MMTV *env* gene, various lengths of the 3′ LTR, and the 222 bp of mouse genomic DNA. The bottom portion of Fig. 1 shows how additional deletion mutants were constructed. Deletion plasmid −451 was linearized with *Bam*HI and then was incubated with BAL-31 nuclease (20) and molecules of the desired length were selected by preparative gel electrophoresis. The DNA was recircularized with T4 DNA ligase, bacteria were transformed, and four different plasmids were selected. Each of these plasmids contains different lengths of the original 0.5-kb *Eco*RI–*Bam*HI MMTV DNA fragment (≈0.4 kb in plasmid −202 and ≈0.15 kb in plasmids −137, −50, and −37) ligated to a deleted LTR and the 222 bp of flanking mouse DNA.

The extent of the deletion was determined by sequence analysis (21) of the LTR region remaining in each plasmid and comparison of it to the sequence of the intact LTR (11). The LTR RNA initiation site is 134 nucleotides from the 3′ end of the LTR (unpublished data). The name of the deletion plasmids refers to the base pairs of LTR remaining upstream (−) or downstream (+) from the RNA initiation site.

Transformation of LTK$^-$ Cells. Plasmid DNA was introduced into LTK$^-$ APRT$^-$ cells by using the calcium phosphate

Abbreviations: MMTV, mouse mammary tumor virus; LTR, large terminal repeat; *tk*, thymidine kinase; bp, base pair(s); kb, kilobase(s); HSV, herpes simplex virus.

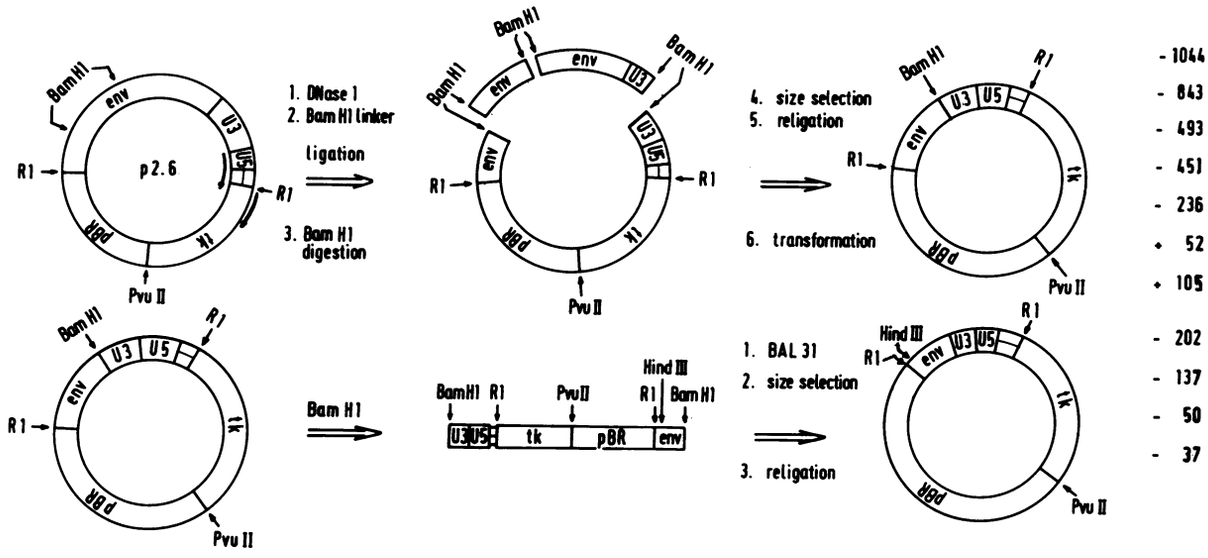

FIG. 1. Construction of a MMTV LTR–*tk* chimeric plasmid and deletion mutants. The top portion shows the MMTV LTR–*tk* chimeric plasmid 2.6 (8) used in the construction of the different deletion plasmids. The envelope (*env*) gene of MMTV along with the 3′ LTR (U3/U5) are separated from the *tk* gene by 222 bp of mouse genomic DNA (⊟). The arrow on the inside of the circle indicates the direction of transcription from the LTR cap site. The arrow on the outside indicates transcription from the *tk* cap site. Two different sets of deletion plasmids were studied. Their construction is described in the text and is diagrammatically shown here. The name of each deletion plasmid is listed on the right and refers to the nucleotides of LTR DNA remaining upstream (−) or downstream (+) from the LTR RNA initiation site.

precipitation technique (22) as described (3, 4). LTK^+ transformants were selected and grown to mass culture. The transformation efficiency was ≈0.2–0.3 TK^+ colonies per ng of *tk* gene per 5×10^5 cells. This efficiency is ≈1/10th of that observed with plasmids containing an intact *tk* gene. This has also been observed by McKnight *et al.* (14). In some cases the cells were grown in 1 μM dexamethasone for 24 hr before harvesting.

Quantitation of Specific RNA Transcripts in the Transformed Cells. Total poly(A)$^+$ RNA was prepared from LTK^+ cell clones (4). *tk*- and LTR-specific transcripts were quantitated by using a RNA "dotting" procedure (23). Duplicate filters were prepared and were pretreated and hybridized (4, 24) to a LTR- or a *tk*-specific probe. After autoradiography, the hybridization signals were cut from the nitrocellulose filters and eluted, and the radioactivity was counted. The *tk* probe was a ^{32}P-labeled nick-translated 2.0-kb *Pvu*II fragment isolated from plasmid M2 (17). The LTR probe consisted of a 0.53-kb *Pst* I fragment subcloned from the λ recombinant GR-40 (3).

Analysis of the 5′ Ends of the RNA Transcripts. The 5′ ends of the RNA transcripts were analyzed by using the nuclease S1 mapping technique (25, 26). Two different DNA fragments, described in Fig. 2, were prepared and were ^{32}P-labeled at their 5′ ends (21). Poly(A)$^+$ RNA and the ^{32}P-end-labeled DNA fragment were hybridized (in 80% formamide/0.4 M NaCl/40 mM Pipes, pH 6.4/5 mM EDTA) and treated with nuclease S1 as described (13). The nuclease S1-resistant DNA was isolated and displayed by electrophoresis on a 6.5% polyacrylamide sequencing gel (13, 21).

RESULTS

Expression of LTR–*tk* Chimeric Plasmids in L Cells. To test the biological activity of the chimeric LTR–*tk* plasmids they were introduced into mouse LTK^- $APRT^-$ cells and stable TK^+ transformants were isolated. This selection is possible without the functional assistance of the promoter located in the LTR because the *Eco*RI site used to construct plasmid 2.6 is located 77 bp upstream from the *tk* RNA cap site (14). McKnight *et al.* (14) have shown that when the *tk* gene is preceded by only 77 nucleotides it has a transformation efficiency that is 1/20th that of an intact *tk* gene and transcribes less *tk* mRNA than an intact *tk* gene, but the TK^+ transformants that we selected contain an authentic 1.3-kb *tk* mRNA (8). Plasmid 2.6 also contains a pro-

Table 1. Hormone sensitivity of LTR and *tk* transcripts in the transfected cells

Plasmid name	Nucleotides of LTR present	Fold induction of RNA	
		tk	LTR
2.6	1,328	4.2	5.6
−1,044	1,178	3.1	4.1
−843	977	3.6	5.3
−493	627	3.5	4.3
−451	585	3.0	5.9
−236	370	3.3	2.6
−202	336	1.6	2.0
−137	271	1.6	1.6
−50	184	1.0	1.1
−37	171	1.0	ND*
+52	82	1.0	ND*
+105	29	1.0	ND*

tk- and LTR-specific RNA was quantitated by using a RNA dot hybridization technique (23). The numbers listed for the induction represent the ratio of the slope of the line obtained with RNA from cells grown with hormone over that obtained with RNA from cells grown without hormone. The results are the average of at least two experiments. As an example of the data obtained: 0.1, 0.5, 1.0, and 2.0 μg of poly(A)$^+$ RNA from plasmid −451-transfected cells hybridized 18, 80, 240, and 318 cpm, respectively, of *tk* probe. The same amount of RNA from cells grown with hormone hybridized 103, 507, 952, and 1,508 cpm, respectively. When the same amounts of RNA from plasmid −451-transfected cells were hybridized against a LTR probe, 15, 61, 106, and 154 cpm hybridized without hormone and 100, 275, 528, and 925 cpm hybridized with hormone. In a control experiment nonmuscle actin mRNA (27) was quantitated. RNA from cells transformed with plasmid 2.6 grown in the presence or absence of hormone was dotted and hybridized with ^{32}P-labeled actin cDNA plasmid. Identical signals were observed with both RNA preparations (data not shown).
*LTR RNA was not detectable above the background.

moter in the LTR. We have previously shown (8) that cells transformed with plasmid 2.6 contain a dexamethasone-sensitive LTR–*tk* chimeric RNA of ≈1.8 kb. This is the size molecule expected if transcripts initiate in the LTR and terminate in the *tk* gene. The hormone sensitivity of each deletion plasmid was analyzed by isolating poly(A)$^+$ RNA from the transformants grown with or without dexamethasone.

The amount of LTR- and *tk*-specific RNA was quantitated by dotting RNA onto a nitrocellulose filter and hybridizing with specific probes. Comparison of the slopes of the curves obtained with RNA isolated from cells grown in the absence and in the presence of dexamethasone was used as an approximate measure of hormonal induction. The results are presented in Table 1. In cells transformed with plasmid 2.6, as well as deletion plasmids −1,044 to −50, we could detect LTR-specific RNA. In each case, the amount of RNA was enhanced in cells grown in dexamethasone, although the induction factor for RNA from plasmid −137- and plasmid −50-transformed cells was low. In cells transformed with plasmids −37, +52, and +105 we could not detect LTR-specific RNA using this technique. Cells transformed with each of the plasmids contain *tk* RNA. In plasmid 2.6-transformed cells and in deletion plasmid −1,044 to −137-transformed cells the *tk* RNA is hormone inducible. All RNA molecules containing LTR and *tk* sequences, regardless of the site of initiation and polarity of transcription, hybridize in this analysis. Therefore, we have used these results only as a first approximation to locate the hormone-sensitive region of the LTR, and we conclude that the presence of a few hundred nucleotides 5′ to the LTR RNA initiation site is sufficient to cause hormone-sensitive RNA expression.

RNA Initiates Correctly at the LTR and at the *tk* Cap Site. To study the hormone inducibility of transcripts that initiate correctly in the LTR or *tk* gene we have analyzed the 5′ end of the RNA using the nuclease S1 mapping technique (25, 26). To investigate transcripts that initiate in the LTR, a *Cla* I–*Hpa* II DNA fragment (Fig. 2, top portion) was used. The *Hpa* II site is located 106 nucleotides downstream from the LTR RNA initiation site. The results of the nuclease S1 analysis are presented in Fig. 3. Poly(A)$^+$ RNA isolated from a MMTV-induced mammary tumor was used as a positive control. Mammary tumor RNA as well as RNA from cells transformed with plasmid 2.6 and the deletion plasmids −451, −236, −202, −137, and −50 were able to protect the expected 106-nucleotide DNA fragment from nuclease S1 digestion. In each case the 106-nucleotide fragment is only visible when RNA from cells grown in the presence of hormone was used in the analysis. We con-

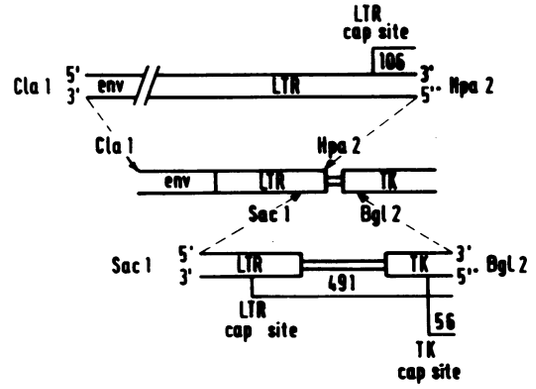

FIG. 2. Restriction enzyme fragments of plasmid 2.6 used in nuclease S1 mapping. The region of plasmid 2.6 containing the two fragments used in the nuclease S1 analysis is shown in the middle portion. A *Cla* I–*Hpa* II fragment of 2.1 kb was used to map transcripts initiating in the LTR. The LTR cap site is located 134 nucleotides from the 3′ end of the LTR (unpublished data). The LTR cap site is 106 nucleotides from the *Hpa* II site. A *Sac* I–*Bgl* II fragment of 587 bp was used to map transcripts initiating at the LTR cap site and at the *tk* cap site. The *Bgl* II site is located 56 nucleotides downstream from the *tk* RNA initiation site (14) and 491 nucleotides downstream from the LTR cap site (unpublished data).

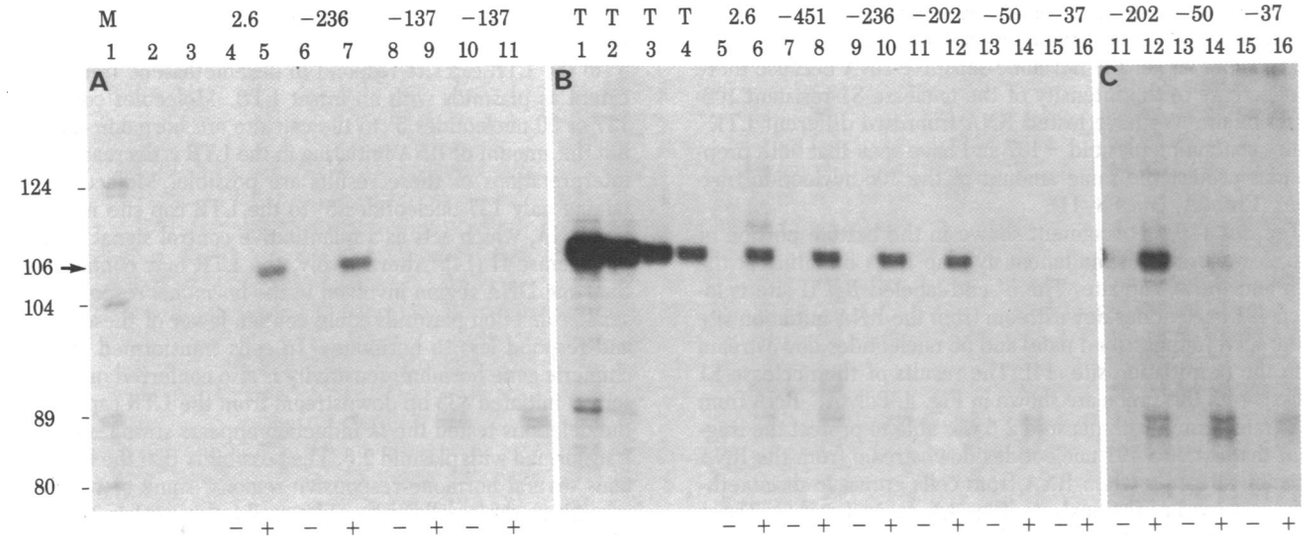

FIG. 3. Nuclease S1 mapping with the *Cla* I–*Hpa* II fragment. Ten micrograms of poly(A)$^+$ RNA from the transfected cells was hybridized at 45°C with 1.0×10^{-2} pmol of the ^{32}P-end-labeled DNA probe and then was treated with nuclease S1, and the nuclease-resistant DNA was sized on a gel (13, 26, 28). The specific activity of the labeled probe used in *A* was 3.5×10^5 cpm/pmol and in *B*, 2.5×10^6 cpm/pmol. (*A*) Lane 1, *Hae* III-digested pBR322 ^{32}P-end-labeled marker DNA (M); lane 2, 2.0×10^{-5} pmol of the DNA probe; lane 3, 1.0×10^{-2} pmol of the probe hybridized with yeast RNA and nuclease S1-treated; lanes 4–11, results obtained with RNA isolated from cells transfected with plasmids 2.6, −236, −137, and −137, respectively (two different cell clones). The cells were grown, as indicated, in the absence (−) or presence (+) of 1 μM dexamethasone. The arrow at 106 nucleotides indicates the size of the expected nuclease S1-resistant fragment. (*B*) Lanes 1–4, the DNA probe was hybridized with 1.0, 0.4, 0.1, and 0.04 μg, respectively, of poly(A)$^+$ RNA isolated from a MMTV-induced GR mouse mammary tumor (T) (≈3% is MMTV specific). The tumor RNA was kindly provided by R. Michalides (Netherlands Cancer Institute). Lanes 5–16, RNA was isolated from cells transfected with plasmids 2.6, −451, −236, −202, −50, and −37. (*C*) Lanes 11–16 of the gel shown in *B* exposed three times longer than in *B*.

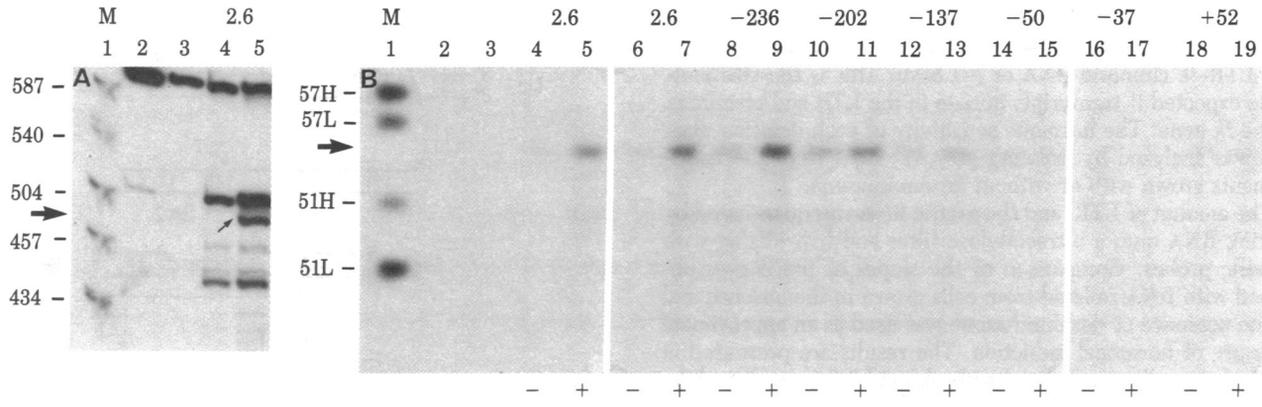

FIG. 4. Nuclease S1 mapping with the *Sac* I–*Bgl* II probe. Six micrograms of poly(A)$^+$ RNA from the transfected cells was hybridized at 41°C with 1.0×10^{-2} pmol of the ^{32}P-end-labeled probe (specific activity = 2.3×10^6 cpm/pmol) and then was treated with nuclease S1, and the resistant products were sized on a gel. (*A*) Lane 1, *Hae* III-digested pBR322 ^{32}P-end-labeled marker DNA (M); lane 2, 1.0×10^{-4} pmol of the probe; lane 3, 1.0×10^{-2} pmol of the probe hybridized with yeast RNA and nuclease S1-treated; lanes 4 and 5, poly(A)$^+$ RNA from plasmid 2.6-transfected cells grown in the absence (lane 4) or presence (lane 5) of hormone. The arrow at 491 bp indicates the size of the expected nuclease S1-resistant fragment. The band at 504 bp represents a DNA fragment that contaminated the electrophoretically purified 587-bp probe. The other fragments are probably nuclease S1 degradation products. (*B*) Only the area of the gel containing the 56-bp fragment is shown. Lanes 1–3, same as lanes 1–3 in *A* (H, heavy; L, light). Lanes 4–19, results obtained with RNA from cells transfected with plasmids 2.6 (two different cell clones), −236, −202, −137, −50, −37, and +52, respectively. The cells were grown in the absence (−) or presence (+) of hormone. The arrow at 56 indicates the size of the expected nuclease S1-resistant fragment. RNA from cells transfected with a plasmid containing only the *tk* gene also protects this 56-bp fragment from nuclease S1 (data not shown).

clude that each of the deletion molecules containing from 450 to 50 nucleotides of LTR sequence upstream from the RNA initiation site is sensitive to glucocorticoid hormones. Fig. 3*C* represents a longer exposure of lanes 11–16 of Fig. 3*B*. Here it can clearly be seen that cells transformed with plasmid −50 and grown in dexamethasone contain a RNA species able to protect the 106-bp fragment. RNA from cells transformed with plasmid −37 (Fig. 3 *B* and *C*, lanes 15 and 16) is able to protect little, if any, of this DNA fragment from nuclease S1 digestion.

The amount of RNA initiating in the LTR in the presence of hormone appears approximately the same in plasmid 2.6-transformed cells and in cells containing deletion plasmids −451, −236, and −202. Cells containing deletion plasmids −137 and −50 contain less of the hormone-sensitive RNA because there is a decrease in the intensity of the nuclease S1-resistant 106-bp fragment. We have tested RNA from two different LTK$^+$ clones containing plasmid −137 and have seen that both preparations protect the same amount of the 106-nucleotide fragment (Fig. 3*A*, lanes 8–11).

The *Sac* I–*Bgl* II fragment shown in the bottom portion of Fig. 2 was used to simultaneously map RNA initiating at the LTR and the *tk* cap site. The 5′-end-labeled *Bgl* II site is located 491 nucleotides downstream from the RNA initiation site in the LTR (unpublished data) and 56 nucleotides downstream from the *tk* initiation site (14). The results of the nuclease S1 analysis with this probe are shown in Fig. 4. Poly(A)$^+$ RNA from cells transformed with plasmid 2.6 was able to protect the fragment that extends 491 nucleotides downstream from the RNA initiation site only when RNA from cells grown in dexamethasone was used in the analysis (Fig. 4*A*, lanes 4 and 5). These results show that a hormone-sensitive RNA initiates correctly in the LTR and that transcription continues into the *tk* gene. Fig. 4*B* shows that RNA isolated from cells transformed with plasmid 2.6 and with various deletion plasmids can protect the expected 56-bp fragment from nuclease S1 digestion. RNA isolated from cells grown with dexamethasone containing plasmids 2.6, −236, −202, and −137 was able to protect more of the 56-nucleotide fragment from nuclease S1 digestion (Fig. 4*B*, lanes 4–13). Thus, transcripts initiating at the *tk* cap site also appear to be dexamethasone sensitive. The induction, based on a densitometry analysis of the 56-nucleotide band shown in Fig. 4, was 5.4-, 3.4-, 1.4-, and 1.5-fold for plasmid 2.6-, plasmid −236, plasmid −202, and plasmid −137-transformed cells, respectively. RNA transcripts that initiate at the *tk* promoter in cells containing plasmids −50, −37, and +52 appear insensitive to glucocorticoids (Fig. 4*B*, lanes 14–19).

DISCUSSION

We have tested the glucocorticoid response of a series of plasmids containing a MMTV LTR–*tk* chimeric gene with increasing deletions in the 5′ region of the MMTV LTR. We have observed that plasmids containing at least 202 nucleotides of DNA 5′ to the LTR cap site respond to dexamethasone to the same extent as plasmids with an intact LTR. Molecules containing 137 or 50 nucleotides 5′ to the cap site are hormone sensitive but the amount of RNA initiating in the LTR is decreased. Two interpretations of these results are possible. Molecules containing only 137 nucleotides 5′ to the LTR cap site may have lost DNA, which acts as a quantitative control signal for RNA polymerase II (14). Alternatively, the LTR may contain more than one DNA region involved in the hormonal response. The smaller deletion plasmids could contain fewer of these regions and respond less to hormones. In cells transformed with the chimeric gene hormone sensitivity is also conferred on *tk* transcripts initiated 433 bp downstream from the LTR cap site. Of the deletions tested the *tk* induction appears strongest in cells transformed with plasmid 2.6. The possibility that the LTR contains several hormone-responsive regions, some of which are missing in the smaller plasmids, could also explain these results.

Each of the deletion plasmids contains a small region (150–500 bp) of MMTV envelope DNA bordering the LTR. It seems unlikely that this piece of DNA, which originates from the center of the proviral genome, confers hormone responsiveness on the LTR-initiated transcripts but in these molecules we cannot rule out this possibility. We have constructed other LTR–*tk* chimeras that do not contain this region of the provirus and the LTR–initiated transcripts are hormone inducible (data not shown). It is clear that the induction of the *tk* transcripts is due

neither to the MMTV envelope DNA nor to the 222 bp of mouse DNA separating the LTR and the *tk* gene. Both of these regions are present in plasmids +52 and +105 and their *tk* RNA is not hormone inducible. We favor the hypothesis that the 202 nucleotides of LTR DNA 5′ to the RNA initiation site confer hormone inducibility on the LTR transcripts as well as on the downstream-initiated *tk* transcripts.

The results of the nuclease S1 analysis reveal that cells transformed with the LTR–*tk* plasmids do not initiate transcripts at the LTR cap site in the absence of hormones. This result was not expected because we can detect correctly initiated transcripts in cells grown without hormone and transformed with both an intact MMTV provirus (3, 4) and a provirus from which the first 516 bp of the 5′ LTR have been deleted (23). By using a RNA dotting technique (Table 1) LTR transcripts were detected in the RNA isolated from cells grown without hormone. With this technique all LTR-containing transcripts, regardless of their site of initiation or polarity of transcription, will be detected. In view of the results of the nuclease S1 mapping analysis, only these incorrect transcripts were detected in the cells grown without hormone.

The DNA sequence preceding two other glucocorticoid-regulated genes has been compared to the MMTV LTR sequence. A similarity in a 21-bp region located 370–480 nucleotides upstream from the RNA initiation site of MMTV, rat growth hormone, and human proopiomelanocortin has been observed (28). At least for MMTV, the deletion of this region does not inhibit dexamethasone-sensitive expression. The DNA sequence analysis of the LTR has also revealed that the first 959 nucleotides of its U3 region contain an open reading frame capable of coding for a 37-kd protein (29). Because plasmids containing a deletion of the entire open reading frame are still capable of hormone-sensitive transcription, the open reading frame protein appears to play no role in this process.

It has recently been shown that the purified glucocorticoid hormone receptor can bind to MMTV DNA (30–33). Geisse *et al.* (32) have shown that, in comparison to deletion plasmid −451, deletion plasmid +105 shows a decreased ability to bind to the glucocorticoid receptor complex in an *in vitro* binding assay. These data indicate that the biological activity, to respond to glucocorticoid hormones, and the ability to preferentially bind the receptor protein can be localized to the same region of the MMTV genome.

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