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Modulation of enhancer activity by the hormone responsive regulatory element from mouse mammary tumor virus

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Addition of the transcriptional enhancers present in the U3 region of the Harvey murine sarcoma virus (HaMuSV) long terminal repeat (LTR) to recombinant chimeras in which the HaMuSV transforming gene (Ha-v-*ras*) is expressed from the mouse mammary tumor virus (MMTV) promoter increases the ability of the MMTV v-*ras* chimeras to transform mouse fibroblasts in culture 50- to 100-fold. Significant stimulation of transfection efficiency occurs only when glucocorticoids are present in the culture medium. Glucocorticoids also elevate the steady-state concentration of MMTV-initiated v-*ras* mRNA in cell lines isolated from these transfections, and MMTV-v-*ras* fusion transcripts are initiated at the normal MMTV cap site; potential cryptic initiation events associated with the enhancer could not be detected. The ability of the enhancer to increase the transcriptional activity of the MMTV promoter was also studied in acute transfection assays where expression of the chloramphenical acetyl transferase (CAT) gene is driven by the MMTV promoter. In this system the strong positive effect on MMTV transcription is again obtained only when the cells are hormone treated. These experiments indicate that the hormone-regulatory region is capable of modulating the function of an exogenously introduced enhancer element.

Key words: enhancers/glucocorticoid regulation/MMTV LTR hormone responsiveness assay

Introduction

The long terminal repeat (LTR) of mouse mammary tumor virus (MMTV) contains regulatory sequences responsible for glucocorticoid hormone-mediated induction of MMTV transcription. This was first established by experiments in which MMTV LTR was fused by recombinant DNA techniques to structural genes not normally regulated by glucocorticoids and whose functional product provides a positive selection in tissue culture cells, either the v-*ras* transforming gene of Harvey murine sarcoma virus (HaMuSV) (Huang *et al.*, 1981), or the dihydrofolate reductase gene (*dhfr*) (Lee *et al.*, 1981). After introduction of these chimeric molecules into eukaryotic cells *via* calcium phosphate transfection, a fusion transcript for the particular structural gene used was initiated at the normal MMTV cap site and could be regulated by glucocorticoid hormones. It was independently found that the glucocorticoid receptor selectively binds to cloned MMTV LTR DNA fragments *in vitro* (Payvar *et al.*, 1982; Govindin *et al.*, 1982; Pfahl, 1982). High affinity binding sites have been mapped immediately upstream from the LTR promoter by nuclease protection or 'footprinting' experiments (Scheidereit *et al.*, 1983; Payvar *et al.*, 1983) which show that the receptor can interact with specific sequences within the LTR located upstream from the MMTV transcription initiation site.

A surprising observation from the early gene transfer experiments was that the MMTV promoter, even in the presence of hormone, was very weak in driving expression for the selectable marker. This property was especially evident in the low frequency with which these recombinants generated transformed foci of cells (Huang *et al.*, 1981; Lee *et al.*, 1981). In contrast, the viral promoter has been observed to be very efficient in murine mammary epithelial host cells (Young *et al.*, 1977). A number of laboratories have recently characterized 'enhancer' or 'activator' elements from various viral genomes that can markedly increase the transcriptional activity of a promoter (Banerji *et al.*, 1081; Benoist and Chambon, 1981; Sekikawa and Levine, 1981; Levinson *et al.*, 1982). The low transfection efficiency of selection markers driven from the MMTV LTR led us to test the possible susceptibility of the MMTV promoter to stimulation by one of these elements. Lee *et al.* (1981) had observed that incorporation of the SV40 *ori* region (which contains a strong enhancer element) in MMTV LTR-*dhfr* fusions resulted in a higher transfection efficiency for the chimeric DNA.

Here we show that the efficiency of focus formation for MMTV LTR v-*ras* chimeras, tested on murine fibroblasts, is markedly enhanced by the addition of an activator element isolated from a type C murine retroviral LTR. High efficiency transfection is only observed in the presence of glucocorticoid hormones. This indicates that the hormone regulatory element is still operative in the presence of the enhancer. Furthermore, the strong dependence of the transfection signal on the presence of hormone (100-fold stimulation) provides a rapid and sensitive assay system for high-resolution mapping of the hormone response regulatory sequences. Finally, the enhancement effect is also seen in a transient expression assay with chimeras between the MMTV LTR and the chloramphenicol acetyl transferase (CAT) gene (Gorman *et al.*, 1982). This argues that activation of the LTR promoter is a direct effect on MMTV-driven expression, not a secondary effect such as increased efficiency of integration.

Results

Construction and transfection of chimeric plasmids

To investigate whether the low number of transformed foci obtained on transfection of NIH 3T3 cells with MMTV LTR v-*ras* recombinants (Huang *et al.*, 1981) was caused by low transcriptional activity from the MMTV promoter, a transcription 'enhancer' was added to these chimeras. The enhancer element was isolated from a molecular clone of the HaMuSV-defective transforming retrovirus (Hager *et al.*, 1979). Plasmid pM13 was constructed as described in Figure 1. It contains ~500 bp of HaMuSV information, including 250 bases of 3′ unique information and 250 bases of U3 LTR information. The U3 sequences included in the chimera begin at position −160 relative to the viral cap site (Ostrowski *et*

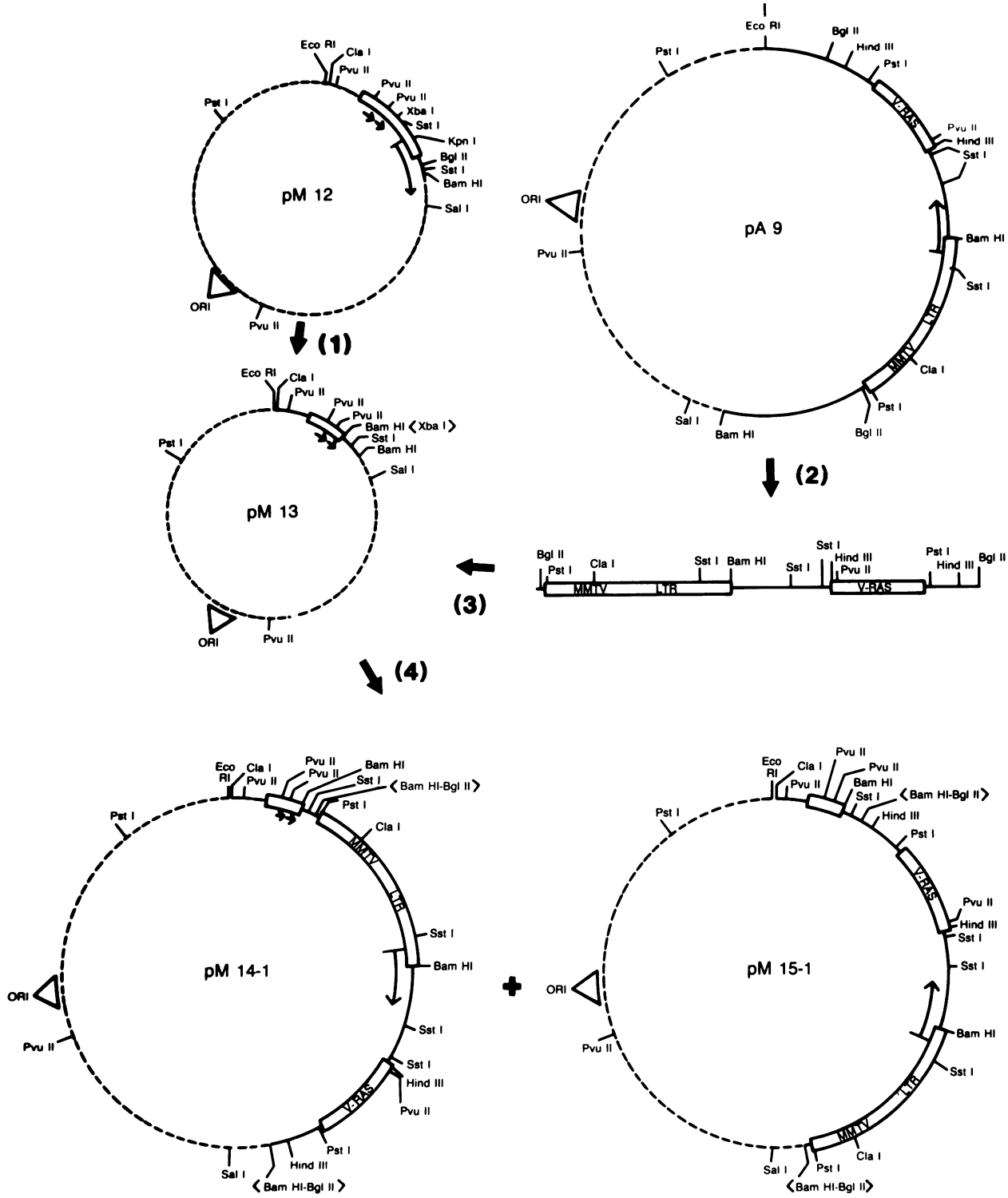

Fig. 1. Construction of recombinant plasmids which contain the MMTV LTR and the HaMuSV enhancer. Plasmids pM14-1 and pM15-1 were generated by the following sequence of manipulations: **(1)** pM12 was digested with restriction endonucleases *Xba*I and *Bgl*II, treated with Klenow fragment in the presence of dNTPs, and recircularized with T4 DNA ligase in the presence of *Bam*HI linkers, thus generating pM13. **(2)** pA9 was digested with *Bgl*II, and the 2.9-kb restriction fragment that contains the MMTV LTR-v-*ras* fusion was purified from an agarose gel. **(3)** pM13 was subjected to partial restriction digestion with *Bam*HI, and then **(4)** ligated with MMTV-v-*ras Bgl*II fragment, thus yielding pM14-1 (which has the HaMuSV enhancer aligned 5′ to the MMTV LTR and in the same transcriptional orientation), and pM15-1 (which has the enhancer located 3′ to MMTV LTR and in the opposite orientation). Dotted lines represent pBR322 sequences, open boxes HaMuSV LTR sequences, MMTV LTR or v-*ras* sequences (as indicated). Large arrows show the direction of transcription from viral promoters, and small arrows indicate the direct-repeat enhancer element.

al., 1981) and extend to the 5′ end of the LTR. Thus, they do not include the conventional elements associated with eukaryotic promoters, such as the TATA or CAT sequences (Corden *et al.*, 1980). Sequence analysis of this U3 information reveals the presence of near perfect 75-bp direct repeats (Figure 2). These repeats are almost indistinguishable

Fig. 2. Sequence of the 75-bp direct-repeat enhancer region of HaMuSV LTR. Sequence of the sense DNA strand is given. Numbers refer to the distance in nucleotides from the viral cap site. The sequence was determined by the method of Maxam and Gilbert (1977; see Materials and methods). Small letters represent the sequence from −181 to −254 repeated in order to compare it with the sequence of the direct repeat located at −255 to −330.

from those found in the murine sarcoma virus (MSV) LTR (Dhar *et al.*, 1980), and therefore should be expected to exert a transcriptional effect analagous to that reported for the MSV repeats (Levinson *et al.*, 1982).

The MMTV v-*ras* fusion was cloned into the unique *Bam*HI restriction site of pM13, which is located 30 bp downstream of the direct repeats (Figure 2), such that molecules containing both orientations of the LTR v-*ras* chimera with respect to the repeats were obtained. The first, designated pM14-1, has the HaMuSV repeats located 5′ to the LTR and in the same orientation as the MMTV promoter. In the second, pM15-1, the repeats are located 3′ to v-*ras* and in the reverse orientation relative to the MMTV promoter.

Both of these molecules were introduced into NIH 3T3 cells *via* calcium phosphate precipitation, and their ability to form transformed foci was compared with the parent molecules. The results of several such transfection experiments are presented in Table I. Inspection of the data clearly indicates that addition of the direct repeat enhancer sequences to the MMTV-v-*ras* chimeras increased 2- to 3-fold the ability of the chimeras to transform 3T3 cells when compared with molecules that do not contain an enhancer element (such as pA12 and pA15). This level of transformation is approximately equivalent to that obtained with molecularly cloned HaMuSV. The increased transformation activity is dependent on two important variables; (i) the presence of glucocorticoid hormones during the transfection assay, and (ii) the proper orientation of the enhancer with respect to the MMTV LTR (i.e., pM14-1 forms foci efficiently when steroid is present, while pM15-1 does not).

Analysis of newly acquired MMTV-v-ras sequences in transformed cells

To confirm the presence of the chimeric molecules in the transformed cells, total cellular DNA was prepared and analyzed by restriction digestion and Southern blotting. Autoradiograms derived from these hybridized blots are presented in Figure 3. ^{32}P-Labeled restriction fragments containing MMTV LTR (Figure 3a), v-*ras* (Figure 3b) or HaMuSV (Figure 3c) were used as probes. These data clearly indicate that the chimeric plasmid pM14-1 is present in transformed cells. For example, DNA from pM14-1 transformed cells restricted with the endonuclease *Pst*I, or with a combination of the enzymes *Eco*RI and *Xba*I should contain fragments predicted to be 2661 bp or 3640 bp in length, respectively. The sizes actually found (Figure 3a and b, lanes 3 and 7 for *Pst*I; lanes 4 and 8 for *Eco*RI-*Xba*I) are in excellent agreement with these predictions. Similar results were found for *Hind*III digestion (Figure 3a, lanes 5 and 9, predicted size 2625 bp; Figure 3b, lanes 5 and 9, predicted size 890 bp) and *Cla*I digestion (Figure 3a, lanes 2 and 6, Figure 3c, lanes 2 and 3, predicted size 990 bp), when blots are hybridized with MMTV LTR or HaMuSV LTR probes. The copy number of the intact chimeric plasmid in various cell lines varied between five and thirty per cell. The arrangement of the multiple copy intact chimeras was deduced as follows. High mol. wt. DNA from lines 835 or 837 was restricted with an enzyme known to cut pM14-1 once (i.e., *Eco*RI). Part of the restricted sample was diluted and incubated with T4 DNA ligase. The ligated DNA was used to transform *Escherichia coli* LE392 (see Materials and methods). DNA that was restricted and ligated produced ~40 ampicillin-resistant colones per gram of input DNA, while no colonies were obtained when either unrestricted DNA or restricted, unligated DNA were used. Approximately one-half of the plasmids rescued in this manner were found to be identical to pM14-1 both in their restriction map and in their ability to transform NIH 3T3 cells following transfection (data not shown). This data is consistent with the interpretation that the recombinant plasmid that is acquired following transfection is present as an amplified, tandemly repeated unit within the host genome (Perucho *et al.*, 1980).

S1 mapping of MMTV-initiated p21 mRNA

Transformed foci produced by transfection of pM14-1 in dexamethasone-containing media were subsequently grown either in the presence or absence of added hormone. MMTV v-*ras* fusion transcripts were characterized by S1 nuclease mapping using end-labeled probes (Berk and Sharp, 1977). Figure 4 presents representative results obtained using total cellular RNA isolated from transformed cells. The probe used in these experiments is derived from pM14-1 and is 5′ end-labeled on the minus-DNA strand at the *Hind*III site that lies within v-*ras* protein-coding region (Dhar *et al.*, 1982). This probe would detect the 5′ end of any RNA initiated within either the MMTV LTR or the enhancer region. A v-*ras* RNA species initiated at the MMTV cap site (Huang *et al.*, 1981) should protect a labeled fragment of this probe of 780 bp in length. As seen in Figure 4, the experimentally determined size (770 bp) of the major nuclease-resistant band found in several different cell lines was in close agreement with the prediction. No larger bands that would indicate initiation of transcription from the region of the 75-bp repeats were found. However, several smaller bands, which originate from the 30S portion of HaMuSV, were detected in S1 mapping of transcripts from cells transformed by MMTV-*ras* fusions that do not contain enhancers, and in cells transformed by clones of HaMuSV (see Huang *et al.*, 1981). While uncertain of the origin of these minor bands, we feel that they probably reflect internal cutting of hybrids by S1 rather than authentic 5′ ends, since this region is not capable of driving expression of v-*ras* in 3T3 cells, even when enhancers are linked to it (unpublished observation). The amount of 770-bp RNA increased when cells were grown in media containing glucocorticoid hormone. Densitometric scans of autoradiographs such as those shown in Figure 4 reveal that the extent of induction by hormone is 3- to 7-fold.

Table I. Addition of HaMuSv 75-bp tandem repeats to MMTV LTR-v-*ras* chimeras enhances focus-forming ability when cells are grown in hormone-containing medium

	DNA transfected	Exp. no.	Amount of DNA	−Dex foci formed (foci/μg DNA)		+Dex foci formed (foci/μg DNA)	
1.	HaMuSV (pPst 8)[a]	1	0.1	20	(200)	84	(840)
2.	HaMuSV (pPst 8)[a]	2	0.1	56	(560)	46	(460)
3.	HaMuSV (pPst 8)[a]	3	0.1	78	(780)	73	(730)
4.	HaMuSV (pPst 8)[a]	4	0.1	170	(1700)	107	(1070)
5.	pA9	1	0.5	0	–	1	–
6.	pA9	5	0.1	0	–	0	–
	pA9		0.3	0	–	0	–
7.	pA13[b]	4	0.1	2	–	0	–
	pA13[b]		0.3	0	–	0	–
8.	pA15-1[b]	4	0.1	0	–	0	–
	pA15-1[b]		0.3	0	–	0	–
9.	pM14-1	1	0.1	0	–	22	(220)
	pM14-1		1.0	0	–	184	(184)
10.	pM14-1	2	0.1	0	–	37	(370)
	pM14-1		1.0	1	–	167	(167)
11.	pM14-1	4	0.3	1	–	321	(1070)
12.	pM14-1	5	0.2	4	–	176	(880)
13.	pM14-2[c]	3	0.1	1	–	121	(1210)
	pM14-2[c]		0.3	5	–	500	(1667)
	pM14-2[c]		0.5	2	–	500	(1000)
14.	pM15-1	1	0.1	0	–	1	–
	pM15-1		1.0	0	–	0	–
15.	pM15-1	2	0.1	0	–	0	–
	pM15-1		1.0	0	–	0	–
16.	pM15-3[d]	4	0.1	1	–	0	–
	pM15-3[d]		0.3	0	–	0	–
17.	pM15-4[d]	4	0.1	0	–	0	–
	pM15-4[d]		0.3	0	–	0	–
18.	pM15-5[d]	4	0.1	0	–	0	–
	pM15-5[d]		0.3	0	–	0	–

[a]see Hager *et al.* (1979) for structure of pPst 8.
[b]pA13 and pA15-1 are MMTV-v-*ras* fusions that do not contain MMTV envelope sequences, in contrast to pA9.
[c]pM14-2 is an independently derived clone identical to pM14-1, except it lacks the *Bam*HI and *Sst*I-containing DNA fragment located between MMTV LTR and enhancers in pM14-1.
[d]pM15-3, 4 and 5 and independently derived recombinants identical to pM15-1.

Transient expression assay using MMTV-CAT fusions

The above experiments indicate that an element derived from HaMuSV LTR is capable of dramatically increasing the focus-forming ability of MMTV LTR v-*ras* fusions. Because of the marked effect of glucocorticoid hormone (primarily a transcriptional effect in the MMTV system), an increased efficiency of transcription from the MMTV promoter is the most likely mechanism for the effect exerted by the 75-bp repeats. In an attempt to define more precisely the nature of the enhancement, we utilized the chloramphenicol acetyl transferase (CAT) system of Gorman *et al.* (1982) to study the response to hormone in transient expression of the enhancer-LTR recombinants before stable integration of acquired DNA can occur.

Recombinants were constructed that have the CAT structural gene inserted either into the *Bam*HI site of pM14-1 (see Figure 1) between the MMTV LTR and v-*ras* sequences (pMK-1), or that have only the MMTV LTR fused to CAT (pMK-2). Forty-eight hours after these plasmids were transfected *via* calcium-phosphate precipitation, cells were harvested and extracted (Gorman *et al.*, 1982). Under the conditions used to assay enzyme activity in extracts prepared from cells transfected with pMK1 (either in the presence or absence of hormone), enzyme activity was linear with time of incubation (data not shown). Furthermore, if dexamethasone was included in the tissue culture medium, the assayable CAT activity was increased 10-fold when compared with equivalent amounts of extract made from cells that had no hormone added to their medium (Figure 5, lanes 3 and 4). In contrast, cells transfected with pMK2 (no enhancer present) contain very low CAT activity in the presence or absence of hormone (Figure 5, lanes 1 and 2), although hormone treatment still results in increased CAT activity. Hormone treatment of transfected cells had no effect on CAT activity when expression was driven by the SV40 early promoter instead of MMTV LTR (Figure 5, lanes 5 and 6). The results presented in Figure 5 were obtained with mouse 34i cl-101 cells as recipients, but similar findings were obtained using mouse L-cells (data not shown). In these experiments, hormone-treated cells transfected with pMK1 (the enhancer-containing recombinant) have ~25-fold more CAT activity than hormone-treated cells transfected with pMK2, the recombinant containing no exogenous enhancer (95% conversion to acetylated form *versus* 4% conversion to acetylated form, respectively; see legend to Figure 5).

S1 mapping of transiently expressed RNAs

To demonstrate rigorously that increased CAT activity reflects increased transcriptional activity of the MMTV promoter, we performed S1 mapping on RNA species present in cells 48 h after transfection (Figure 6). For these studies, we used NIH 3T3 cells as the transfection target, and pM14-1

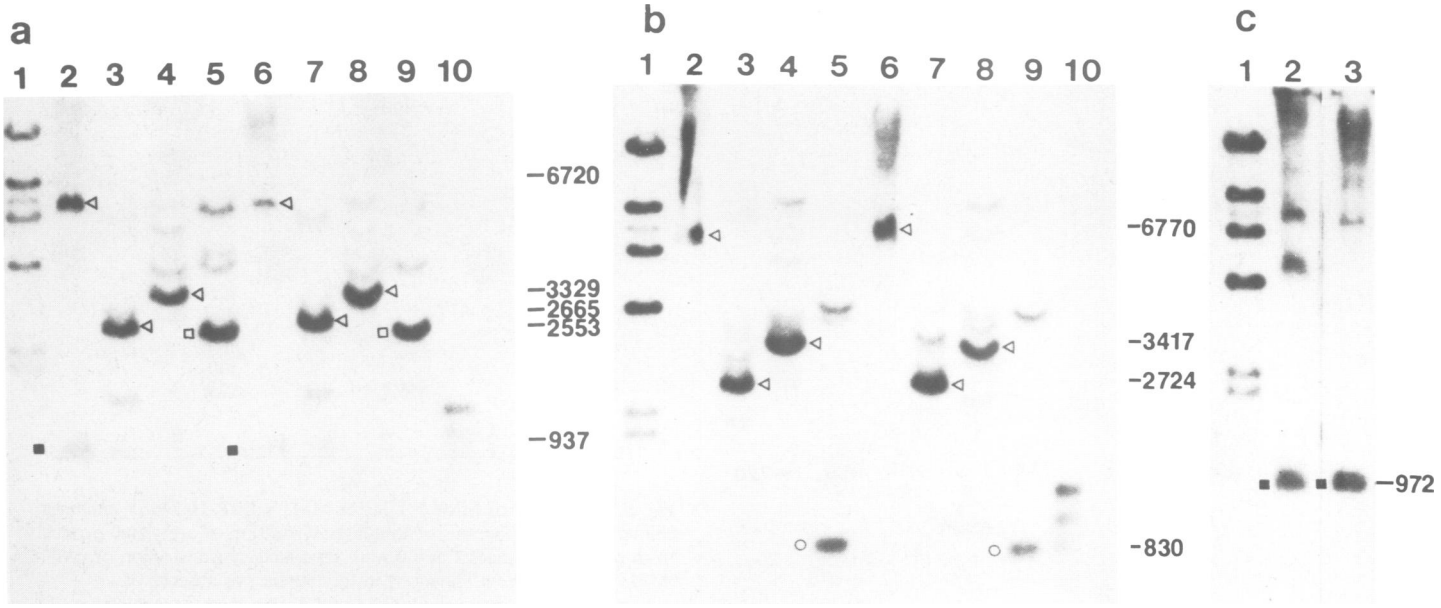

Fig. 3. Mapping of DNA sequences acquired *via* transfection and contained within transformed NIH 3T3 cells. Autoradiograms of Southern blots of restriction endonuclease-cleaved total cellular DNA from cells transformed by pM14-1. Blots were hybridized with ^{32}P-labeled MMTV LTR **(a)**, v-*ras* **(b)** or HaMuSV LTR **(c)**. Source of the DNA was either cell line 835 (**a** and **b**, **lanes 2–5**; **c**, **lane 2**) or 837 (**a** and **b**, **lanes 6–9**; **c**, **lane 3**). Enzymes used to cleave DNA were *Cla*I (**a** and **b**, **lanes 2** and **6**; **c**, **lanes 2** and **3**), *Pst*I (**a** and **b**, **lanes 3** and **7**), *Eco*RI/*Xba*I double digest (**a** and **b**, **lanes 5** and **8**) and *Hind*III (**lanes 5** and **9**). **Lane 1** in each panel contains λ-*Hind*III digested DNA markers and **lanes 10** contain φX-*Hae*III digested DNA markers. The symbols beside the bands indicate their specific hybridization pattern; ◁, fragments that hybridized to both MMTV and v-*ras* probe; □, fragments that hybridized to only MMTV LTR probe; ○, fragments that hybridize only to v-*ras* probe; ■, fragments that hybridize to MMTV LTR and HaMuSV LTR probes. Numbers on the side indicate the size in nucleotides of these fragments.

and pA13 as the DNA molecules transfected. The probe used for these experiments was a 225-bp MMTV LTR fragment end-labeled on the (−) DNA strand at the unique *Bam*HI site (see Figure 1 and legend to Figure 6). MMTV-initiated RNA would be expected to protect a fragment of this probe 115 bp in length.

Figure 6 shows that RNA from a cell line transformed by pM14-1 (lane 7) protects a fragment 118 bp in length, in good agreement with the predicted size. Among the RNAs from transiently transfected cells, only RNA from those transfected with pM14-1 in the presence of hormone contains a fragment of this size (lane 2). While it is apparent that other bands are present following the S1 analysis, the only band affected by hormone treatment is the 118-bp fragment in lane 2; all bands except the 118-bp band are also seen in the mock transfection control (lanes 5 and 6). We conclude that these other bands reflect artifacts of the S1 technique and not RNA transcripts present within cells. From this analysis it is difficult to determine the extent to which either enhancer or hormone increase the concentration of MMTV-initiated RNA, since it is not possible to detect this RNA in cells transfected with pA13 (no enhancer, lanes 3 and 4) or pM14-1 in the absence of hormone; over-exposure of the autoradiograms (Figure 6) indicates the hormone induction effect must be at least 20-fold. Most importantly, these experiments indicate that increased CAT activity in transient assays and increased focus formation, both driven by the enhancer element, are due primarily to increased transcription of MMTV-initiated RNA.

Discussion

Assays with enhancer-containing chimeras

Sequences sufficient for hormone-modulated expression of MMTV-initiated RNA reside within the LTR. Assays currently utilized for testing mutant LTRs depend on analyzing the effect of hormone treatment on LTR-initiated mRNA (or the resultant gene product) present within stably transformed cell lines (Hynes *et al.*, 1983; Buetti and Diggelmann, 1983; Majors and Varmus, 1983). The existence of 'position effects', differences in expression of MMTV-initiated information that apparently arise from association between MMTV proviral sequences and random chromosomal information, is well documented (Parks *et al.*, 1976; Ringold *et al.*, 1979; Thompson *et al.*, 1979). Since the chromosomal location of MMTV chimeric DNA acquired by transfection also varies in individual cell lines, position effects are a potential complication in determining an accurate phenotype for a particular mutant. The MMTV promoter is also remarkably inefficient when tested in fibroblastic cell lines, raising the potential concern of interaction with cellular sequences when selection pressure is applied during the generation of stable transformants.

Both of the assays described here, the focus-forming assay using LTR v-*ras* fusion and the CAT transient expression assay, represent an approach in which the responsiveness of LTR fusions is averaged over a large number of events, rather than measured in independently selected cell lines. Furthermore, both assays are dependent on MMTV-initiated expression shortly after transfection before stable integration has occurred; potential position effects are thus minimized. These assays, especially the transfection assay, also allow a large number of candidate mutant LTRs to be screened in a simple, straightforward and sensitive procedure.

The focus formation assay and the CAT acute expression system both require the addition of a heterologous transcriptional enhancer element to the MMTV LTR for easily quantifiable levels of expression. Lee *et al.* (1981) have previously

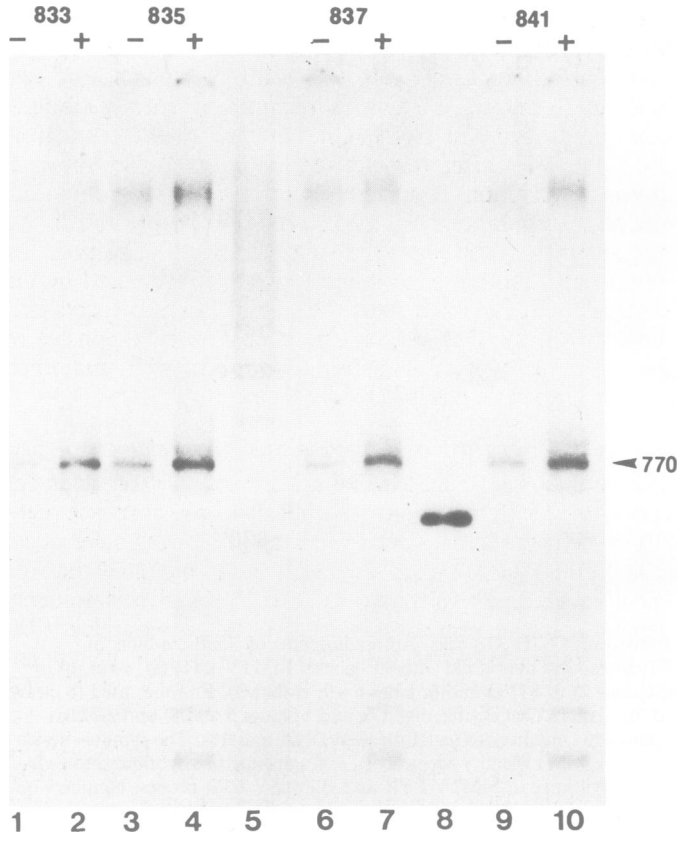

Fig. 4. S1 mapping of total cellular RNA from transformed cells. Autoradiogram of native acrylamide gel electrophoresis of S1 nuclease-resistant hybridization products. The probe used was end-labeled on the anti-sense DNA strand at the *Hind*III site located within v-*ras* coding sequences (Dhar *et al.*, 1982). The 3′ end of the probe was coincident with *Eco*RI site of pM14-1 (see Figure 1), and was 2.3 kb in length. The identity of cell lines used for preparation of RNA and hormonal status of the growth media prior to RNA isolation are indicated at the top of the lanes. **Lane 5** contained ϕX-*Hae*III digested size markers. **Lane 8** contained the probe further digested with *Bam*HI, thus producing a fragment 100 bp shorter than hybrids produced by RNA initiated at the MMTV cap site.

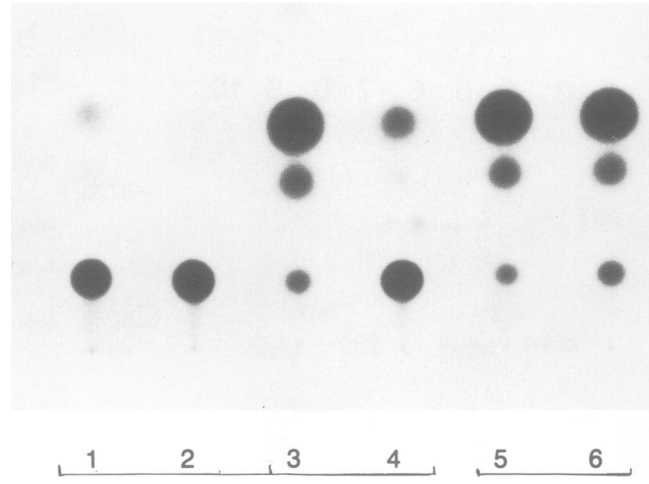

Fig. 5. CAT assay of MMTV LTR or MMTV LTR-HaMuSV enhancer chimeras. Autoradiograms of thin-layer separation of acetylated products from precursors. **Lanes 1** and **2** were generated by transfection of pMK-2 (MMTV LTR without enhancer) in the presence or absence of dexamethasone, respectively; **lanes 3** and **4** were from a transfection of pMK-1 (MMTV LTR with enhancer) in the presence or absence of hormone; **lanes 5** and **6** were from a transfection of pSV2-CAT (Gorman *et al.*, 1982) in the presence or absence of hormone, respectively. Conversion of precursor to product (expressed as percent of precursor converted) was 4% in **lane 1**, 0.2% in **lane 2**, 95% in **lane 3**, 8% in **lane 4**, 96% in **lane 5** and 98% in **lane 6**.

reported a similar phenomenon. In their experiments, however, the stimulation in recovery of foci was unaffected by the presence or absence of hormone in the transfection protocol. In contrast, we detect significant enhancement of focus formation by the HaMuSV 75-bp repeat only when glucocorticoids are added to the cell culture medium. One possible explanation for this disparity might be that considerable differences in intracellular concentration of the two gene products are required to reach the threshold for transformation. Thus, the amount of v-*ras* protein expressed from the chimeras early after DNA uptake could be insufficient for transformation unless stimulated by hormone, whereas concentrations of dihydrofolate reductase sufficient to commit the cell to low level methotrexate resistance might be expressed from the uninduced promoter. It is noteworthy in this regard that we have identified clonal cell lines obtained with LTR-v-*ras* fusions in which the transformed phenotype itself is dependent on hormone, and is reversible upon removal of hormone (Huang *et al.*, 1981; Hager *et al.*, 1982).

Although a number of events are clearly involved in the generation of a scorable focus of transformed cells, two lines of evidence argue that the rate of transcription is the dominant factor in the increased recovery of v-*ras*-induced foci in the presence of hormone. Firstly, when hormone is present in the medium, foci appear between 3 and 5 days after transfection. Thus, most of the p21 expression that eventually leads to a focus must occur very early after DNA uptake, and prior to stable integration. We normally wait 10−12 days to score the experiment because foci that develop in the absence of hormone appear more slowly. Secondly, when so-called 'transient' RNA levels were examined by S1 analysis shortly (48 h) after transfection, we found that the accumulation of transcripts was markedly stimulated in the hormone-treated cells, and that initiation events were only detectable at the normal cap site (Figure 6). Transcription is therefore proceeding at a much higher rate in hormone-treated cells during a major portion of the period required for development of the focus.

Utilization of normal cap site

When an exogenous activator is added to a eukaryotic promoter, it must be confirmed that enhanced transcription is still initiated at the normal cap site. Wasylyk *et al.* (1983) have reported the ability of enhancers to elicit the utilization of cryptic promoters, even within prokaryotic sequences. S1 mapping of RNAs derived from cells transformed with enhancer containing LTR v-*ras* fusions (Figure 4) shows that the only detectable transcripts that accumulate in these cells have 5′ termini that correspond to the predicted MMTV cap site (Donehower *et al.*, 1981; Ucker *et al.*, 1981). The 5′ ends measured in transient expression with the enhancer-containing CAT plasmid also map to the normal cap site (Figure 6). We are therefore dealing with *cis*-activation of transcription at the normal MMTV initiation site, not artifactual activation of new promoter sites.

Transfection signal reflects transcriptional effect

Comparison of the S1 mapping data obtained with stable

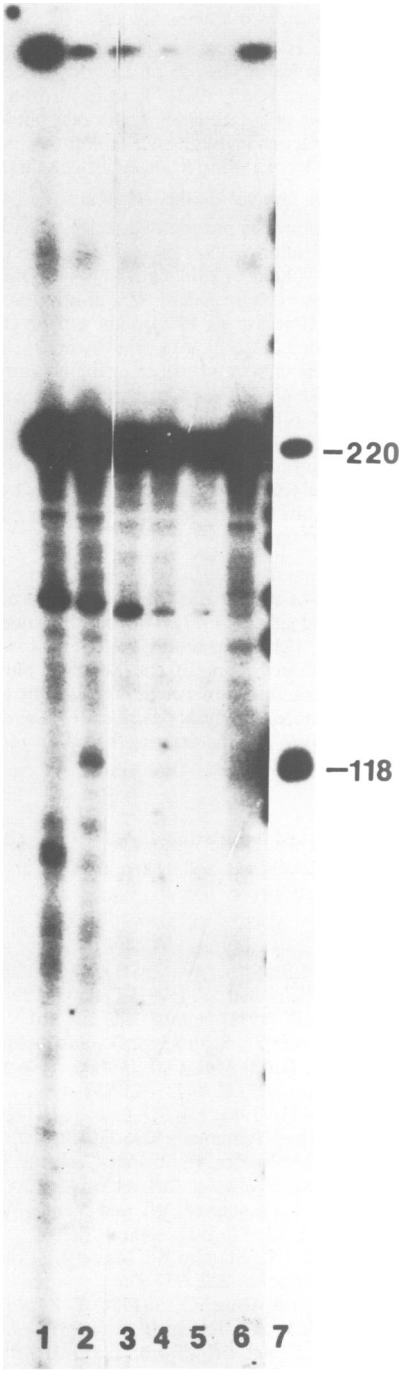

Fig. 6. S1 mapping of transcripts following transient expression. The probe used was a 225-bp fragment bounded by the *Sst*I site present in the MMTV LTR at position −107 and by the *Bam*HI site that demarcates the MMTV LTR from HaMuSV 30S sequences. This fragment was ^{32}P end-labeled at the *Bam*HI site on the anti-sense DNA strand. RNA initiated at the MMTV cap site should protect a fragment of this probe 115 bp in length. Hybridization and S1 treatment were carried out in the usual manner (see Materials and methods), using 10 μg of RNA per reaction. Ethanol-precipitated samples were resuspended in sample buffer (80% formamide, 0.01% bromophenol blue, 0.01% xylene cylanol), boiled 1 min, and electrophoresed on denaturing 8% acrylamide gels (20:1 acrylamide:bis-acrylamide). After electrophoresis, gels were fixed, dried down and autoradiographed. Exposure of the gel was 16 h for **lanes 1–6** and 4 h for **lane 7**. Identity of RNAs used in these experiments: **lanes 1** and **2**, RNA from pM14-1 transfected cells grown either in the presence or absence of 10^{-6} M dexamethasone, respectively; **lanes 3** and **4**, RNA from pA13 transfected cells grown in the presence or absence of hormone, respectively; **lanes 5** and **6**, mock-transfected cells, grown in the presence or absence of hormone; **lane 7**, RNA from a cell line transformed by pM14-1, grown in the presence of hormone.

transfectants (Figure 4) with the results from transient expression assays (Figure 6) indicates that induction of the MMTV promoter is less pronounced in the stable transformants. This discrepancy again emphasizes the position effects inherent in studies utilizing individual cell lines containing randomly integrated DNA. Quantitation of the transcripts present shortly after transfection by S1 mapping shows a dramatic induction, consistent with the 50- to 100-fold effect on focus stimulation. Since foci produced by pM14-1 in the presence of hormone appear 3–5 days after transfection, the number of foci recovered is most strongly influenced by the extent of expression early in the transfection protocol, i.e., the focus-formation assay is apparently most responsive to the extent of expression before association with undefined cellular sequences has occurred.

Mechanism of action of hormone regulatory element

The interaction of the MMTV LTR with an exogenous enhancer may lend insight into the nature of the hormone regulatory element that resides within the LTR. Chandler *et al.* (1983) and Yamamoto *et al.* (1983) have suggested that the hormone regulatory sequence is an enhancer that is uniquely dependent on an activated steroid hormone complex. One would, therefore, expect addition of a non-regulated enhancer to plasmid constructions harboring the MMTV LTR to result in an additive effect on the cumulative level of expression obtained, as was reported for chimeras containing both the SV40 and HaMuSV enhancers (Berg *et al.*, 1983). Our data is not consistent with this expectation. The most pronounced effect we observe is the inability of the HaMuSV enhancer to activate expression from MMTV LTR in the absence of hormone (compare lines 1–4 with 9–13 in Table I, and lanes 3 and 4 with lanes 5 and 6 in Figure 5). In Figure 5, the effect of the HaMuSV enhancer on a heterologous promoter is compared with the effect of the SV40 enhancer on its homologous promoter. The MuLV retrovirus enhancer is a more effective activator of the SV40 promoter in murine cells than the SV40 enhancer itself (Laimins *et al.*, 1982). Thus, it would be expected that the signal in lane 4, Figure 5 (MMTV LTR plus HaMuSV enhancer), should be at least as high as that in lanes 5 and 6 (SV40 enhancer). Such was the case only after hormone was added to the assay.

It might be suggested that an 'inactive' enhancer could confer a blocking effect on an active enhancer in the absence of a putative effector molecule (i.e., hormone regulatory element in the absence of activated hormone-receptor complex). An interesting example of a presumably inactive enhancer is the element identified in the immunoglobulin heavy chain locus (Banerji *et al.*, 1983; Gillies *et al.*, 1983). This enhancer is highly cell-specific, demonstrating strong activation in lymphocytes, but showing no effect in fibroblasts. This element has been inserted between the SV40 enhancer and the β-globin promoter with no observed blocking effect when assayed by transient expression in fibroblasts (W.Schaffner, personal communication). Thus, insofar as it has been tested, the concept of a blocking effect by an inactive enhancer remains unsupported.

Our data suggests that the MMTV LTR is capable of regulating the function of the exogenous enhancer; these observations led us to postulate (Hager *et al.*, 1983) that the MMTV regulatory element modulates transcription by a fundamentally different mechanism from enhancers already described. A precedent for regulation of enhancer function exists in early-late control of SV40 transcription. In this case,

a strong enhancer (located immediately adjacent to both the early and late promoters) activates expression from the early promoter at early times, but T-antigen binding antagonizes this activation at late times (Myers *et al.*, 1981). The most straightforward interpretation of our data is that the hormone regulatory region is a *cis*-acting, dominant, negative element in the absence of activated hormone receptor complex. The binding of activated receptor relieves this negative effect. Whether the induced regulatory element provides a net positive effect itself, irrespective of exogenous elements, remains to be seen.

Implications for MMTV expression in virally-infected mammary cells

Our experiments indicate that the transcriptional efficiency of the MMTV hormone-regulated promoter can be dramatically increased by a *cis*-acting element in murine cell types that are not normal hosts for MMTV. The relevance of this finding with respect to the expression of MMTV information in virally-infected host tissues remains uncertain. The potential association of viral sequences with cellular enhancers through random integration is one possible explanation of the observed 'position' effects. However, we can draw no conclusions on this point. Other mechanisms could be advanced to explain the apparently 'weak' nature of the MMTV promoter in our experiments. A transcriptional enhancer could be present in a part of the MMTV genome other than the LTR; such is apparently the case for certain strains of Rous sarcoma virus (Luciw *et al.*, 1983; L.Laimins, personal communication). Alternatively, the MMTV LTR might contain a transcriptional activator that is highly tissue specific, analogous to the immunoglobulin heavy chain locus enhancer; the LTR promoter would then function efficiently only in mammary epithelial cells, and not in fibroblasts. In the experiment presented in Figure 5, transient expression of the CAT gene from the LTR in the absence of enhancers was examined in a cell line (34i cl-101) derived from a MMTV-induced mammary tumor. This cell was chosen as a potential biologically relevant host cell for MMTV infection. The low efficiency of the MMTV promoter in this cell does not support the host cell specificity hypothesis, but the difficulty in identifying this (or any) immortalized cell as an authentic epithelial host prevents a conclusive interpretation.

The enhancer-driven assay systems described here should prove useful in describing the relationship between the primary DNA sequence of MMTV LTR and the ability of glucocorticoids to regulate transcription from the viral promoter. Finally, further study of the interactions observed between the hormone regulatory element and transcriptional enhancers may provide fundamental insights into the mechanisms by which these regulatory elements operate.

Materials and methods

Mammalian cell lines

NIH 3T3 cl-17 was a subline of NIH 3T3 mouse embryo fibroblasts (Jainchill *et al.*, 1969) selected for flat morphology. Cells were maintained in Dulbecco's MEM medium (DMEM), containing 2 mM L-glutamine, 10 IU/ml penicillin and 10 μg/ml streptomycin. The medium was supplemented with 10% fetal calf serum (Meloy Laboratories). Dexamethasone (2×10^{-6} M) was added to the medium as described below.

Bacterial strains

Plasmid pBR322 was obtained from Bethesda Research Laboratories. *E. coli* strain LES392 was a gift from Lynn Enquist. Procedures for bacterial growth and transformation, and for plasmid preparation have been previously described (Donehower *et al.*, 1981).

DNA-mediated transformation of mammalian cells

Transfection of NIH 3T3 cells *via* calcium phosphate precipitation was performed either in the presence or absence of 2×10^{-6} M dexamethasone as previously described (Huang *et al.*, 1981). Transformed foci were apparent 3–5 days after transfection in the presence of hormone, but were usually seen at 10–14 days after transfection in the absence of hormone. Scoring of results was done 10–14 days following transfection to include these events.

Transient expression assay using chloramphenicol acetyl transferase

The CAT assay was performed as previously described (Gorman *et al.*, 1982) with the following modifications. DNA (50 μg) was co-precipitated with calcium phosphate in a final volume of 2 ml. One-half of this mixture was added to each of two 10 cm tissue culture dishes; each dish containing either 1.5×10^6 mouse 34i cl-101 cells (Parks *et al.*, 1976), a line actively expressing C3H-S MMTV viral sequences, or Ltk$^-$ cells. After this treatment, cells were grown in DMEM with 10% fetal calf serum either in the presence or absence of 10^{-6} M dexamethasone for 48 h. Cell extracts were prepared (Gorman *et al.*, 1982) and analyzed for CAT enzymatic activity by incubating with 4 mM acetyl-CoA and 1 μCi [^{14}C]chloramphenicol in buffer containing 250 mM Tris pH 7.8 for varying times. Subsequently, the acetylated forms of chloramphenicol were separated from non-acetylated precursors by ascending t.l.c. Acetylated radioactive bands were excised from thin-layer plates and quantitated by liquid scintillation counting.

Other methods

Quantitative S1 mapping of cellular transcripts was performed according to the protocol of Berk and Sharp (1977), with modifications as previously detailed (Ostrowski *et al.*, 1981). Preparation of high mol. wt. DNA (Gross-Bellard *et al.*, 1973), gel electrophoresis and Southern blotting (Southern, 1975) of DNA, and nick translation of cloned probe (Rigby *et al.*, 1977) were performed with modifications previously described (Huang *et al.*, 1981). The U3 region of the HaMuSV LTR was sequenced according to the method of Maxam and Gilbert (1977).

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