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Glucocorticoids and Chromosomal Position Modulate Murine Mammary Tumor Virus Transcription by Affecting Efficiency of Promoter Utilization

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The rate of transcription of murine mammary tumor virus (MTV) sequences in MTV-infected rat hepatoma tissue culture cells is strongly affected by both glucocorticoid hormones and the chromosomal position of provirus integration. We have characterized MTV RNAs produced in J2.17 and M1.54, independent isolates containing, respectively, 1 and 10 proviruses integrated at distinct chromosomal loci. M1.54, but not J2.17, synthesized MTV RNA in the absence of glucocorticoids; the rate of hormone-stimulated viral gene transcription in M1.54 was 50- to 100-fold higher than in J2.17. In each case in which MTV genes were expressed (J2.17 induced, M1.54 basal and induced), the viral RNAs produced were indistinguishable. RNA blotting revealed accumulation of two transcripts, 7.8 and 3.8 kilobases; the latter was likely produced from the former by RNA splicing. Sites used for transcription initiation, polyadenylation, and splicing have been identified from the sizes of end-labeled hybridization probes protected from digestion with mung bean nuclease; the unique initiation and polyadenylation sites were both encoded within the MTV long-terminal-repeat sequence. The efficiencies of splicing and of utilization of the polyadenylation signal did not appear to vary as functions of chromosomal position or hormonal stimulation. Differences in rates of viral gene transcription were reflected in the differential accumulation of the 5'-terminal 136 nucleotides of MTV RNA. Thus, glucocorticoids and chromosomal position appeared to affect solely the efficiency of utilization of the MTV promoter, leaving unchanged the sites of initiation, splicing, and polyadenylation, as well as the efficiencies of the latter two processes.

During retrovirus infection, proviral DNA encoding the viral RNA genomes integrates stably in a unique configuration at apparently random sites in the host genome (see reference 51 for review); proviral elements appear to specify at least one transcriptional promoter region which is recognized by host cell machinery. As such, proviral DNAs inserted at distinct chromosomal loci could be used as position-specific probes of cellular transcriptional regulatory mechanisms. For example, expression of mouse mammary tumor virus (MTV) RNA in mammary tumor cells and in infected cells of heterologous origin (see reference 38 for review) is regulated by glucocorticoid hormones such as dexamethasone. Thus, in infected cultured rat hepatoma (HTC) cells, dexamethasone increases rapidly and selectively the rate of MTV gene transcription (40); this response is mediated by the cell-encoded glucocorticoid receptor protein (25, 41), which binds specifically to pure MTV DNA in vitro (20, 35). The rate of transcription in infected HTC cells is also affected by the chromosomal locus at which the proviral element is integrated (50). This modulatory effect is not a function of the transcriptional activity or hormone responsiveness of the integration region; rather, the chromatin structure of that region appears to specify the structure and the potential for expression of the incoming MTV provirus (16).

By what mechanisms do glucocorticoids and chromosomal position exert these effects? In procaryotes, several distinct molecular strategies for regulating transcription have been identified. Thus, one class of regulatory factors directly increases or decreases the efficiency with which RNA polymerase utilizes a given "basal" promoter (37). In other cases, an entirely new "regulated" promoter is activated (8), thereby initiating expression of the same genetic information from an alternative 5' terminus. Still others modulate the efficiency of transcription termination (19, 22) as a mode for altering tran-

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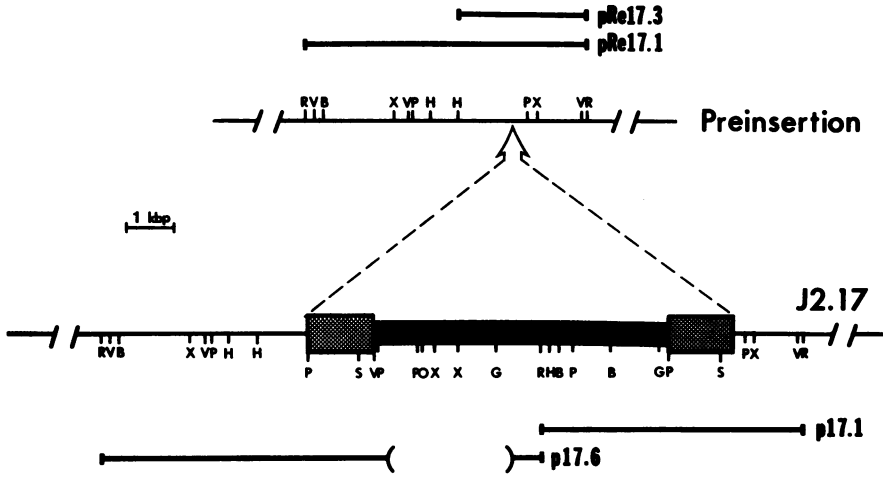

FIG. 1. Restriction maps of the MTV provirus and adjacent cellular DNA in J2.17, and of the corresponding preinsertion region in HTC. Restriction endonuclease sites in cellular (——) and proviral DNA: (B) *Bam*HI, (G) *Bgl*II, (H) *Hin*dIII, (O) *Xho*I, (P) *Pst*I, (R) *Eco*RI, (S) *Sac*I, (V) *Pvu*II, (X) *Xba*I. The 1.3-kb MTV LTRs are respresented as stippled boxes; the remainder of the MTV genome is diagrammed as a solid box. The site of proviral integration is indicated with an arrow; direction of proviral transcription is left to right. The insert portions of recombinant clones (bold lines) are oriented with respect to the genomic sequences they represent. The deletion of MTV sequences in clone p17.6 (see text) is defined by parentheses.

scriptional rates or mRNA stability or both. Characterization of certain regulated eucaryotic RNAs implies that several distinct classes of control processes affect transcription in higher cells as well (5, 49, 55).

The present report describes an analysis of MTV transcripts produced in chronically infected HTC cells under conditions in which effects of hormone treatment and of chromosomal position can be separately assessed. Various cloned hybridization reagents were used to estimate the overall size of the viral RNAs, to map presumptive sites for initiation, polyadenylation, and splicing of MTV RNA, and to measure the relative efficiencies of each of these processes.

MATERIALS AND METHODS

Cells. Rat HTC cells (HTC 4.1), MTV-infected clones (J2.17 and M1.54), and growth conditions were as described previously (41, 53). Presence of the viral genes in the infected lines has no apparent affect on cell growth or viability; no detectable production of MTV virus particles occurs in these lines.

Preparation of cellular DNA and RNA. High-molecular-weight DNA was prepared according to Polsky et al. (36) from log-phase suspension cultures (7×10^5 cells per ml).

Total cellular RNA was isolated from cells grown to a confluent monolayer by a combination of guanidinium thiocyanate denaturation (6) and sedimentation velocity centrifugation (18) as described before (17).

Nuclear RNA was prepared by the same method from purified nuclei isolated as described previously (46) with minor modifications. Confluent cultures were chilled by replacement of medium with ice-cold phosphate-buffered saline; all subsequent manipulations were at 0 to 4°C. Cells were rinsed, harvested by scraping, pelleted (5 min, 1,500 rpm; International PR-6000), resuspended at 2×10^7 cells per ml, and swollen for 5 min in buffer A (20 mM Tris-hydrochloride [pH 8.0], 6 mM $CaCl_2$, 4 mM $MgCl_2$, 0.5 mM dithiothreitol). An equal volume of buffer B (600 mM sucrose, 0.2% Nonidet P-40, 0.5 mM dithiothreitol) was added, and cells were broken with 10 strokes with the tight-fitting pestle in a Dounce homogenizer. Nuclei were harvested by centrifugation (5 min, 2,000 rpm, International PR-6000) and resuspended for RNA isolation.

Polyadenylated RNA was fractionated on polyuridylic acid-Sephadex G-10 essentially as described before (7). Approximately 1 mg of total RNA was applied to a 0.5-ml polyuridylic acid-Sephadex column in binding buffer (200 mM NaCl, 1 mM EDTA, 0.2% sodium dodecyl sulfate, 10 mM Tris-hydrochloride, pH 7.5); after extensive washing, RNA was eluted from the column in 90% formamide–0.2% sodium dodecyl sulfate–1 mM EDTA–10 mM Tris-hydrochloride, pH 7.5 and concentrated by ethanol precipitation.

Molecular cloning. Restriction endonuclease digestion and ligation (New England Biolabs), packaging into phage particles (13), screening of plaques (2), and subcloning in pBR322 (27) were as described previously.

Molecular cloning of an *Eco*RI fragment containing the right-hand portion of the MTV provirus together with flanking cellular sequences from J2.17 DNA and of an *Eco*RI fragment representing the corresponding "preinsertion fragment" from HTC DNA in vector pBR322, designated p17.1 and pRe17.1 (Fig. 1), respectively, has been reported (50). Certain sequences

downstream of the *Sac*I site in the left-hand portion of the MTV provirus apparently preclude cloning the intact element in *Escherichia coli* (see below); therefore, a 5.5-kilobase (kb) fragment extending from the *Sac*I site within the MTV long terminal repeat (LTR) upstream to the cellular *Eco*RI site (Fig. 1) was cloned in λ Charon 16A (10). This fragment was then ligated at the *Sac*I site to a 1.1-kb *Sac*I-*Eco*RI fragment from an independent MTV clone, λ2-1a, of J. Majors (personal communication), which contains contiguous MTV sequences but lacks the so-called poison sequence; the entire 6.6-kb *Eco*RI fragment was then transferred to the *Eco*RI site of pBR322 and designated p17.6 (Fig. 1).

Sequences that are highly reiterated in the HTC genome reside within the *Pst*I-*Pvu*II fragment of pRe17.1 and extend upstream through at least the *Xba*I site (Fig. 1); the *Hin*dIII-*Eco*RI fragment of pRe17.1 was subcloned in pBR322 and denoted as pRe17.3 and contains no highly reiterated sequences (Fig. 1).

Gel electrophoresis, transfer, and hybridization. Procedures for electrophoresis, Southern (45) blotting, and hybridization analysis of DNA were as described before (50). Reiterated sequences were detected in cloned DNA fragments as described by Steinmetz et al. (47).

RNA was electrophoretically fractionated (18 h, 35 V) in 0.8% agarose gels containing 2.2 M formaldehyde; the running buffer consisted of 1 mM EDTA–5 mM sodium acetate–10 mM sodium phosphate, pH 7.0. RNA samples were incubated at 60°C for 5 min in sample buffer (50% formamide, 2.2 M formaldehyde in running buffer); bromophenol blue and Ficoll (3% final concentration) were added before loading. RNA was blotted (48) to nitrocellulose in 20× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate); filters were dried at 80°C for 2 h in vacuo and incubated for 6 to 18 h at 42°C in annealing mix (3× SSC, 50% [vol/vol] formamide, 50 mg of sheared, denaturated salmon sperm DNA per ml, 5× Denhardt [9] solution [1× Denhardt solution is 0.02% (wt/vol) each bovine serum albumin, polyvinyl pyrrolidone, and Ficoll]).

Hybridization with nick-translated probes (see below) was performed by saturating the nitrocellulose filter (1.5 ml/100 cm^2) with annealing mix containing 10% dextran sulfate and 10^6 cpm of the indicated probe per ml; filters were sealed in plastic bags and incubated at 41°C for 18 h. The filters were rinsed briefly at room temperature and then washed three times for 20 min each at 50°C in 0.1× SSC–0.1% sodium dodecyl sulfate. Autoradiograms were exposed at −70°C (Kodak RP-Royal X-Omat film in the presence of a Dupont Cronex Lightning Plus intensifying screen).

Labeled hybridization reagents. DNA restriction fragments were electrophoretically separated in low-melting-temperature agarose gels (Sea Plaque). After staining, desired bands were excised, dissolved in 2 volumes of extraction buffer (300 mM NaCl, 10 mM Tris-hydrochloride [pH 7.4], 1 mM EDTA, 0.5% sodium dodecyl sulfate) at 68°C for 45 min, extracted successively with phenol and chloroform, and precipitated with ethanol; recoveries were 60 to 90%. Nick-translated hybridization probes were prepared from cellular DNA and cloned recombinant fragments as described previously (50).

End-labeling of DNA restriction fragments was accomplished by the kinase exchange reaction (4) to label 5′ ends and by the use of T4 DNA polymerase (32) to label the most proximal 20 nucleotides at 3′ ends. Labeled products were asymmetrically cleaved with a second restriction enzyme, and the desired fragment was electrophoretically purified; 3′ end-labeled products were subjected to further electrophoresis through a thin denaturing polyacrylamide gel (5% in TBE [90 mM Tris-borate, pH 8.2–2 mM EDTA]–8 M urea) (31) to recover the full-length single-stranded fragment. The band was exised, extracted in 1 ml of 150 mM NaCl–10 mM Tris-hydrochloride (pH 7.4)–1 mM EDTA at 37°C for 18 h, and precipitated with ethanol.

Nuclease protection experiments. Nuclease protection experiments were performed by a modification of the procedure of Favaloro et al. (15). RNA samples (25 μg) were mixed with a sequence excess of a given end-labeled DNA fragment, coprecipitated with ethanol, desiccated, and resuspended in 10 μl of hybridization buffer {80% (vol/vol) formamide freshly deionized with AG501-X8(D) mixed bed resin (Bio-Rad), 400 mM NaCl, 40 mM sodium PIPES [piperazine-*N*,*N*′-bis(2-ethane-sulfonic acid)], pH 6.6, 1 mM EDTA}. After denaturation (85°C, 15 min) and hybridization (3 h, 52°C), 250 μl of nuclease buffer (250 mM NaCl, 30 mM sodium acetate [pH 4.8], 1 mM $ZnSO_4$, 5% glycerol, 20 μg of sheared denatured salmon sperm DNA per ml; 0°C) was added together with S1 nuclease or mung bean nuclease as indicated. Digestions were carried out at 45°C for S1 and 37°C for mung bean nuclease and were terminated by the addition of 75 μl of quenching buffer (2 M potassium acetate, 50 mM EDTA, 250 μg of yeast RNA per ml).

Desiccated ethanol precipitates were resuspended in 5 μl of loading buffer (80% deionized formamide, 1 × TBE, bromophenol blue, xylene cyanol) and electrophoresed in thin denaturing 5% polyacrylamide gels (31). Size markers were 5′ end-labeled restriction fragments with 3′ hydroxyl ends; thus, no correction need be made in reading the length of the nuclease-protected fragments. Fragments were visualized by autoradiography as described above.

The two nucleases were compared directly in digestions of J2.17 cell RNA that had been hybridized with an end-labeled DNA probe (see Fig. 3e and h below); whereas a single fragment of 136 nucleotides survived mung bean nuclease digestion, S1 nuclease digestion yielded a mixture of fragments primarily 136 to 140 nucleotides in length. The greater apparent specificity or efficiency of mung bean nuclease has been seen elsewhere (21); this enzyme was used in all subsequent experiments.

RESULTS

MTV transcripts in infected HTC cell lines. Our initial experiments were with J2.17, an infected HTC derivative containing a single stably integrated MTV element per cell, which is transcribed only in the presence of dexamethasone (39, 53) at a relative rate of about 30 ppm (50). MTV sequences and flanking cellular DNA both 5′ (see Materials and Methods) and 3′ to the provirus have been cloned from J2.17; preinsertion fragments bearing the intact corresponding

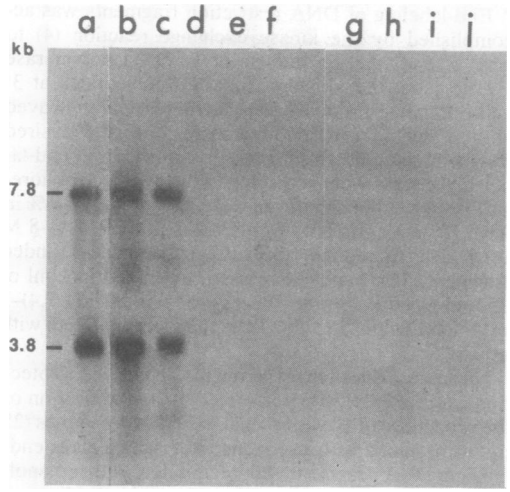

FIG. 2. Blot hybridization of MTV RNA in J2.17 and M1.54. Whole-cell RNA was electrophoretically fractionated, blotted, and hybridized with nick-translated [^{32}P]DNA from inserts of (a to f) pMTV1 and (g to j) pRe17.3. (a) 0.33 μg of RNA from M1.54 treated with dexamethasone for 20 h; (b) 10 μg of RNA from untreated M1.54; (c, g) 10 μg of RNA from J2.17 treated for 20 h with dexamethasone or (d, h) from untreated J2.17; (e, i) 10 μg of RNA from HTC cells treated with dexamethasone for 20 h or (f, j) from untreated HTC.

integration site have been cloned from uninfected HTC cell DNA (50); restriction endonuclease cleavage sites (39, 44) relevant to the present study are shown in Fig. 1 (see also Materials and Methods).

Total cell RNA from hormone-treated and control cultures of J2.17 was electrophoresed in denaturing agarose gels, transferred to nitrocellulose filters, and hybridized with cloned MTV [^{32}P]DNA. Two distinct species of MTV RNA, 7.8 and 3.8 kb, were observed in J2.17 cells treated with dexamethasone for 20 h (Fig. 2c); the 3.8-kb product is likely derived by RNA splicing from the 7.8-kb species (see below). Densitometric scans of the autoradiograms, corrected for the length of homology between the hybridization probe and the RNA species (see below and Materials and Methods), suggest that the 3.8-kb transcripts are roughly fourfold more numerous than the 7.8-kb transcripts. As expected, no MTV RNA was detected in untreated J2.17 cells or in uninfected HTC cells grown either in the presence or the absence of dexamethasone (Fig. 2d to f).

When the same RNAs were hybridized with a cloned preinsertion fragment, pRe17.3 [^{32}P]DNA (Fig. 1), no signal was observed (Fig. 2g to j) under conditions in which one transcript per cell could readily be detected; this result is consistent with a previous analysis (50). Thus, the MTV RNA that accumulates in dexamethasone-stimulated J2.17 cells is not extensively associated with sequences transcribed from flanking cellular DNA, and, in fact, such sequences were not observed in any size class of RNA at steady state.

MTV transcripts were also examined in M1.54, a second line of infected HTC cells; M1.54 contains 10 MTV proviruses stably integrated at chromosomal loci distinct from the region occupied by the J2.17 provirus (39, 50). MTV RNA in M1.54 is transcribed at a relative basal rate of about 80 ppm, yielding a steady-state level of about 50 copies per cell; upon hormone treatment, the relative synthetic rate increases to 5,300 ppm, and 1,800 copies per cell accumulate (39, 50). The differential accumulation of viral RNA is demonstrated by the results shown in Fig. 2, since lane a, containing RNA from induced M1.54, received 30-fold less RNA than the other lanes. Figure 2b reveals that two size species of MTV RNA accumulate in M1.54 in the absence of dexamethasone and that their electrophoretic mobilities are indistinguishable from the two species observed after dexamethasone stimulation of either M1.54 (Fig. 2a) or J2.17 (Fig. 2c). It is apparent that the relative proportion of the two MTV RNA species is not drastically altered by hormone treatment; coordinate induction by dexamethasone of these two species has been reported in other infected cells (43). We conclude from these experiments that dexamethasone effects a striking increase in accumulation of MTV RNA in both J2.17 and M1.54, but that neither hormonal stimulation nor site of provirus integration affects either the apparent size of the transcripts produced or the ratio of spliced to unspliced viral RNA.

Mapping the 5′ terminus of MTV transcripts. The 5′ terminus of the viral transcripts was identified from the size of end-labeled DNA fragments protected from single-strand-specific nuclease digestion after hybridization with cellular RNA (15). Note that sequences corresponding to the 5′ and 3′ ends of mature viral RNA are juxtaposed at each end of proviral DNA to form a 1,332-base pair (bp) LTR sequence (11). Therefore, a subfragment of p17.6 including the left-hand LTR and flanking HTC DNA (Fig. 1) was 5′ end-labeled on the antisense strand at the MTV *Pvu*II site, which is unique in the integrated provirus, thereby yielding a probe that will detect transcripts from the left-hand LTR but not those from the right-hand LTR. When total cell RNA from hormone-treated J2.17 was hybridized with this probe, a single fragment of 136 nucleotides was protected from digestion with mung bean nuclease (Fig. 3e). Consistent with previous results, no protection was observed with RNA isolated from either untreated J2.17

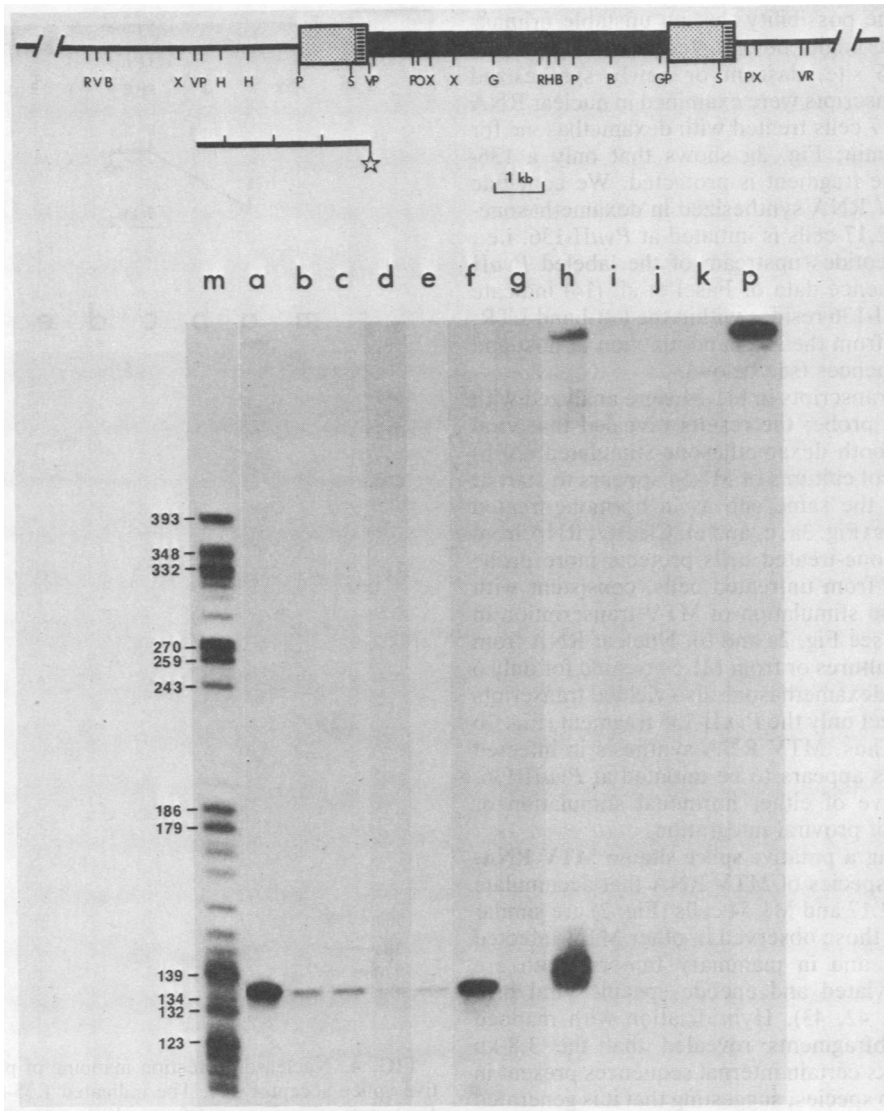

FIG. 3. Nuclease protection mapping of the 5′ terminus of MTV transcripts. The indicated 3.3-kb *Pvu*II restriction fragment from p17.6 (Fig. 1) was 5′ end-labeled as shown (indicated by the star) and annealed with RNA. Hybrids were digested with mung bean nuclease (450 U per reaction, 60 min), except (h), which used S1 nuclease (21 U per reaction, 30 min); probe protected from digestion was visualized by autoradiography after electrophoresis on denaturing 5% sequencing gels. Protection by: (a) whole-cell RNA from M1.54 treated with dexamethasone for 20 h; (b) nuclear RNA from M1.54 treated for 6 min with dexamethasone; (c) whole-cell RNA and (d) nuclear RNA from untreated M1.54; (e, f, and h) total RNA from J2.17 treated with dexamethasone for 20 h; (g) nuclear RNA from J2.17 treated with 10 min with dexamethasone; (i) total RNA from untreated J2.17; (j) HTC treated for 20 h with dexamethasone; (k) untreated HTC. Standards: (m) end-labeled *Hha*I-digested pBR322 restriction fragments as size markers; (p) undigested probe. (f) and (h) were exposed 20-fold longer than the others shown. Cross-hatching denotes the region of the LTR homologous to the 5′ terminus of MTV transcripts.

or uninfected HTC cells (Fig. 3i to k); the sensitivity of these experiments was sufficient to detect MTV transcripts present at 1 to 2% of the level found in dexamethasone-treated J2.17 cells.

Even after 20-fold-longer exposure of the autoradiogram (Fig. 3f), no protected fragments greater than 136 nucleotides were revealed; reconstruction experiments (not shown) indicated that MTV transcripts initiated elsewhere at 3 to 5% of the abundance of the bulk of stable J2.17 MTV RNA would have been detected. To test

further the possibility that an unstable primary transcript might be initiated upstream of the *Pvu*II-136 site, nascent or newly synthesized MTV transcripts were examined in nuclear RNA from J2.17 cells treated with dexamethasone for only 10 min; Fig. 3g shows that only a 136-nucleotide fragment is protected. We conclude that MTV RNA synthesized in dexamethasone-treated J2.17 cells is initiated at *Pvu*II-136, i.e., 136 nucleotides upstream of the labeled *Pvu*II site; sequence data of Fasel et al. (14) indicate that *Pvu*II-136 resides within the left-hand LTR, 1,196 bp from the left-hand junction of host and viral sequences (see below).

MTV transcripts in M1.54 were analyzed with the same probe; the results revealed that viral RNA in both dexamethasone-stimulated (20 h) and control cultures of M1.54 appears to start at precisely the same site as in hormone-treated J2.17 cells (Fig. 3a, c, and e). Clearly, RNA from the hormone-treated cells protects more probe than that from untreated cells, consistent with the 30-fold stimulation of MTV transcription in this line (see Fig. 2a and b). Nuclear RNA from control cultures or from M1.54 treated for only 6 min with dexamethasone also yielded transcripts that protect only the *Pvu*II-136 fragment (Fig. 3b and d). Thus, MTV RNA synthesis in infected HTC cells appears to be initiated at *Pvu*II-136, irrespective of either hormonal stimulation or position of proviral integration.

Mapping a putative splice site on MTV RNA. The two species of MTV RNA that accumulate in both J2.17 and M1.54 cells (Fig. 2) are similar in size to those observed in other MTV-infected cell lines and in mammary tumors; both are polyadenylated and encode specific viral proteins (12, 42, 43). Hybridization with mapped MTV subfragments revealed that the 3.8-kb RNA lacks certain internal sequences present in the 7.8-kb species, suggesting that it is generated from the larger molecule by RNA splicing (12, 43). Thus, MTV gene expression could conceivably be altered by regulation of the efficiency or loci of splicing or both. Although the data in Fig. 2 fail to support this notion, mung bean nuclease mapping was used to determine precisely the splice sites utilized in the presence and absence of dexamethasone.

The 3.8-kb MTV transcript in other cultured cells lacks regions including and upstream of the single proviral *Eco*RI site (Fig. 1), but contains sequences distal to the *Pst*I site that reside 0.9 kb downstream from the *Eco*RI site (12, 43). Consistent with these results, a putative splice acceptor junction was mapped between these sites; the probe, prepared from p17.1 (Fig. 1) as diagrammed in Fig. 4, was 5′ end-labeled at the MTV *Pst*I site described above and contains the 0.9-kb MTV *Eco*RI-*Pst*I fragment fused to the 0.65-kb *Eco*RI-*Sal*I fragment of pBR322. This "tail" of vector sequences allows protection of the MTV segment of the probe by primary transcripts to be readily distinguished from reassociation of the intact probe DNA strands (for

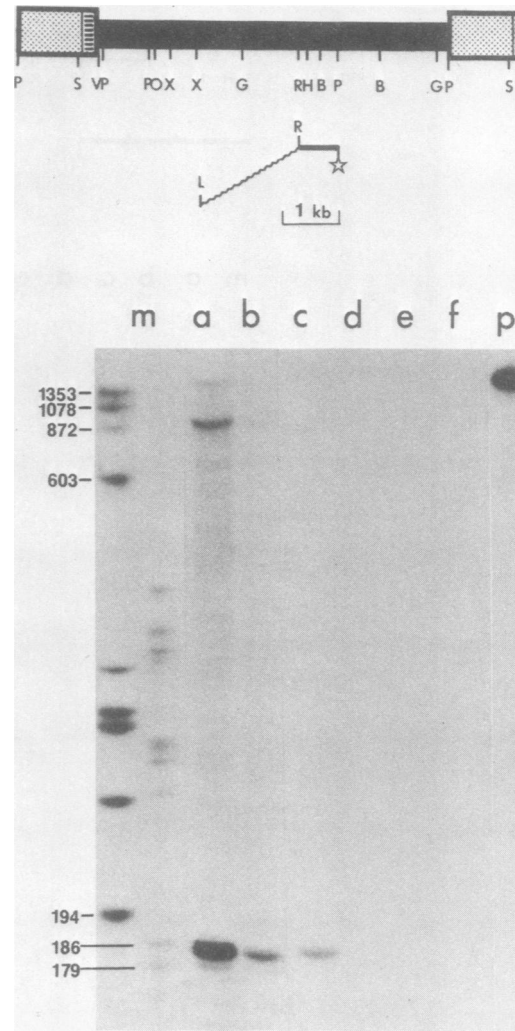

FIG. 4. Nuclease protection mapping of presumptive splice acceptor site. The indicated 1.55-kb *Pst*I-*Sac*I restriction fragment from p17.1, consisting of a 0.9-kb *Pst*I-*Eco*RI MTV segment adjoining 0.65 kb of pBR322 DNA extending from the *Eco*RI (R) to the *Sal*I site (L), was 5′ end-labeled at the *Pst*I site (indicated by the star). Reactions were performed as in the legend to Fig. 3; autoradiogram shows probe fragments protected by RNA from: (a) M1.54 treated with dexamethasone for 20 h or (b) untreated; (c) J2.17 treated for 20 h with dexamethasone or (d) untreated; (e) HTC treated with dexamethasone for 20 h or (f) untreated. Standards: (m, right) *Hha*I-digested pBR322 size markers; (m, left) *Hae*III-digested ϕX174 size markers; (p) undigested probe.

example, see Fig. 4a). Figure 4a to c shows that hybridization of this probe with RNA from hormone-treated J2.17, or from either control or hormone-treated M1.54, results in protection of two predominant fragments in each case. The larger, about 0.9 kb, probably reflects protection by unprocessed MTV transcripts, whereas the 183-nucleotide protected fragment identifies a single putative splice acceptor junction upstream of the *Pst*I site. As expected, more probe is protected by RNA from hormone-stimulated M1.54 than from control M1.54 or induced J2.17 cultures (Fig. 4a to c), whereas no protection is observed with RNA from unstimulated J2.17 or from uninfected HTC cells (Fig. 4d to f).

Using a variety of cloned 3′ end-labeled MTV restriction fragments, we failed to detect any putative splice donor junction (data not shown); it is likely that the splice donor lies within the region of the MTV genome that has been refractory to molecular cloning (11, 23, 30, 50) and is therefore not represented in our probes.

Assuming that the 183-nucleotide fragment indeed denotes a splice acceptor at *Pst*I-183, splicing efficiency can be estimated. Densitometric analysis of various exposures of the autoradiogram shown in Fig. 4a to c reveals, in every case, that the 183-nucleotide fragment is about fourfold more abundant than the 0.9-kb fragment, a ratio similar to that calculated for the relative abundance of the 3.8- and 7.8-kb MTV RNAs (Fig. 2). Thus, these experiments support the idea that splicing of MTV RNA is unaffected by either glucocorticoids or chromosomal sites of proviral insertion.

Mapping a putative polyadenylation site on MTV RNA. If the 7.8-kb MTV RNA corresponds to the unspliced transcript originating at *Pvu*II-136 (Fig. 3), a polyadenylation site is expected to lie within the right-hand LTR close to the sequence at which initiation occurs in the left-hand LTR. The capacity of transcripts to protect a restriction fragment from this region was examined; the probe was prepared from p17.1 by 3′ end-labeling at the *Sac*I site of the right-hand MTV LTR and isolating a 1.8-kb fragment ending at the first *Eco*RI site in the contiguous HTC DNA (Fig. 5). Note that the absence of transcription initiation events upstream of *Pvu*II-136 (Fig. 3) ensures that transcripts that cross the *Sac*I site, which is 102 bp upstream of *Pvu*II-136, represent the 3′ region of an MTV RNA. No protection is observed with RNA from untreated J2.17 or from uninfected HTC cells (Fig. 5j to l), whereas RNA from hormone-treated J2.17 protects a discrete fragment of 133 nucleotides, together with a faint smear of radioactivity at larger fragment sizes (Fig. 5g). Nuclear RNA from J2.17 cells exposed to dexamethasone for only 10 min protects the same 133-nucleotide fragment, but the relative abundance of other fragments is at least fivefold greater (Fig. 5i), as estimated by densitometry, than with total RNA from the steady-state induced cells (Fig. 5g). Conversely, polyadenylated RNA from dexamethasone-stimulated J2.17 cells protects a greater proportion of the 133-nucleotide fragment (Fig. 5h).

Together, these results suggest that a major polyadenylation site for MTV RNA is located within the right-hand MTV LTR at *Sac*I+133 and that polyadenylation reflects 3′ processing of transcripts that extend beyond this site. We assume that the broad distribution of fragments seen in Fig. 5g and i reflects primary nuclear transcripts that are degraded or terminated heterogeneously, mostly downstream of *Sac*I+133. Less than 5% of these transcripts extend as much as 500 nucleotides past *Sac*I+133 since a probe 5′ end-labeled at the *Xba*I site of p17.1, about 250 bp beyond the MTV-HTC junction (Fig. 1), was not protected by J2.17 nuclear RNA (Ucker, unpublished data); these results are consistent with our earlier conclusion (50) that the flanking cellular sequences are not transcribed at rates comparable to proviral sequences.

When similar experiments were carried out with RNA from hormone-treated (20 h) and untreated M1.54 cells, the strong hormonal induction was again apparent, and the same predominant fragment was protected (Fig. 5a and d); minor species a few nucleotides shorter were also observed, suggesting either some heterogeneity in the polyadenylation site or overdigestion of the hybrids. Because the HTC sequences carried on the probe were not homologous to those adjacent to the proviruses in M1.54, transcripts proceeding beyond the right-hand LTR-HTC junction at *Sac*I+237 should protect a discrete probe fragment of about 237 nucleotides, thereby facilitating estimates of polyadenylation efficiency. In fact, a smear ending in a 212-nucleotide fragment was observed (Fig. 5b, c, and f), perhaps reflecting a sequence difference between the right-hand MTV LTR of the M1.54 proviruses and the J2.17-derived probe.

In any case, the majority of MTV transcripts recovered from total, polyadenylic acid-enriched or nuclear fractions of dexamethasone-treated (20 h or 6 min for whole cells and nuclei, respectively) or control cultures of M1.54 (Fig. 5a to f) appear to be polyadenylated at about *Sac*I+133. Densitometric measurements suggest that >90% of the transcripts appear to be processed at this site at steady state, whereas much of the remaining fraction may be polyadenylated beyond the distal junction of the right-hand LTR (Fig. 5a, b, c, and f). As with splicing, the major presumptive polyadenylation site for MTV tran-

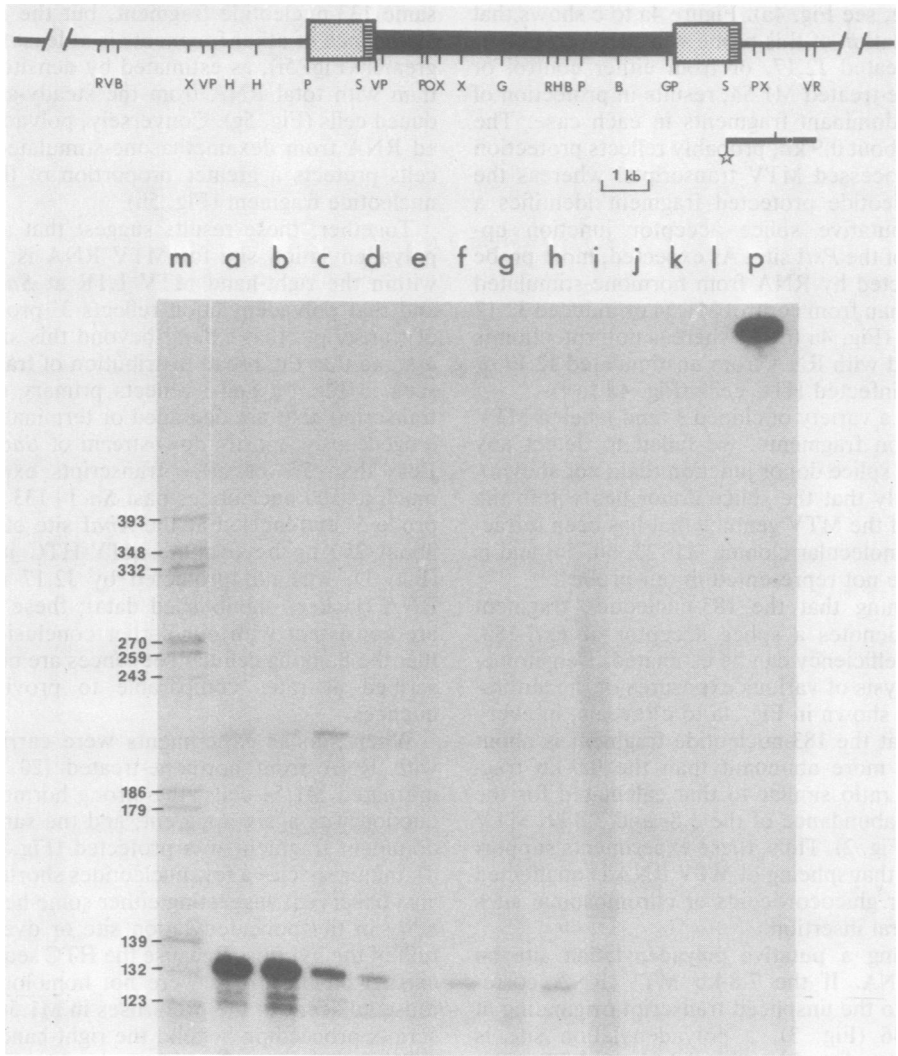

FIG. 5. Nuclease protection mapping of a presumptive polyadenylation site. The indicated 1.6-kb *Sac*I-*Eco*RI restriction fragment from p17.1 (Fig. 1) was 3′ end-labeled at the *Sac*I site as shown (indicated by the star). After annealing with RNA, digestions were performed with mung bean nuclease (900 U per reaction, 2 h), and protected probe was visualized after electrophoresis as before (Fig. 3). Protection afforded by: (a) total and (b) polyadenylated RNA from M1.54 treated for 20 h with dexamethasone; (c) nuclear RNA from M1.54 treated with dexamethasone for 6 min; (d) total, (e) polyadenylated, and (f) nuclear RNA from untreated M1.54; (g) total and (h) polyadenylated RNA from J2.17 treated for 20 h with dexamethasone; (i) nuclear RNA from J2.17 treated with dexamethasone for 10 min; whole-cell RNAs from (j) untreated J2.17, (k) HTC treated for 20 h with dexamethasone, and (l) untreated HTC. (m) Markers as in Fig. 3; (p) undigested probe.

scripts was not detectably affected by the HTC sequences flanking the J2.17 and M1.54 proviruses, and there was no effect of dexamethasone on the apparent site of polyadenylation in M1.54.

DISCUSSION

The present report describes in detail a transcription unit whose expression is strongly affected both by glucocorticoids and by the position of the encoding DNA within the cell genome. We have determined the endpoints and a splice junction of the MTV transcripts produced in infected HTC cells; this analysis was facilitated by previous descriptions of MTV RNAs detected in mammary tumors and other infected cells (12, 42, 43), and by prevailing views of the general features of transcription (for example, see 3, 33, 55) and replication (51) of

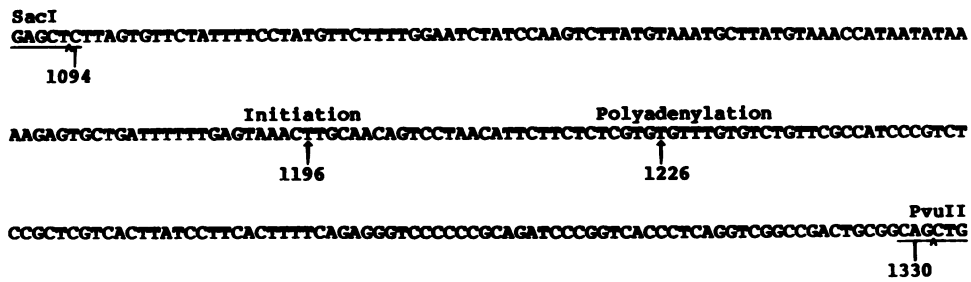

FIG. 6. Nucleotide sequence of the *Sac*I-*Pvu*II fragment of the MTV LTR. Sequence of the sense strand, determined by Fasel et al. (14), is shown; nucleotide 1 is the left-hand end of the 1,332-bp LTR. Carets at 1,093 and 1,331 denote the *Sac*I and *Pvu*II cleavage sites, respectively; loss of the two terminal nucleotides of each LTR upon provirus integration (11, 26) disrupts the *Pvu*II site in the right-hand LTR (Fig. 1). Presumptive sites of transcription initiation and polyadenylation are indicated.

retroviral genomes. Consistent with previous results (50), the MTV transcription unit is encoded virtually entirely within the proviral sequences: sites for initiation and polyadenylation reside within the left and right MTV LTRs, respectively.

Fasel et al. (14) have cloned and sequenced an MTV LTR from a population of HTC cells infected with the same stock of GR strain MTV (40) that generated J2.17 and M1.54. Using their sequence data, our results suggest that transcription initiates in the left-hand LTR at or close to a T residue at position 1,196 and that polyadenylation occurs predominantly near a site corresponding to a T residue at position 1,226 in the right-hand LTR (Fig. 6). The 5′ terminus of MTV RNA from mature virions has been mapped by independent procedures (30) close to the site implicated here. The major polyadenylation site for MTV RNA in infected HTC cells appears to be about 20 nucleotides downstream from that inferred from MTV virion RNA produced in mammary tumor cells (28); the basis of this difference has not been investigated. The donor and acceptor sites for the RNA splicing event that presumably generates the 3.8-kb MTV RNA have not been mapped precisely in other infected cells or tumors. However, DNA sequencing of a defective MTV provirus that appears to have arisen by reverse transcription of a 3.8-kb RNA has revealed a presumptive splicing event at *Pst*I-183 (J. Majors, personal communication), the same site identified in the present study as a putative splice acceptor junction. Thus, these correlations provide strong support for the functional assignments of initiation, splice acceptor, and polyadenylation sites that we have derived from our nuclease mapping experiments.

The major focus of this work was to compare the viral RNAs in two clonal isolates of infected HTC cells that contain regulated proviruses integrated at distinct chromosomal loci. We conclude from our results that the molecular fine structure and relative levels of the two predominant MTV transcripts that accumulate in infected HTC cells are unaffected by either hormonal stimulation or position of proviral integration, whereas their intracellular concentration is highly sensitive to both factors (50). In fact, the hormonal and position effects are readily detected by monitoring the accumulation of the 5′ 136 nucleotides of the MTV transcript (Fig. 3a, c, e, and i); the inference from nuclear RNA analyses (Fig. 3b, d, and g) that these differences reflect distinct rates of synthesis of this 5′ oligonucleotide has been directly confirmed in other experiments (D. S. Ucker and K. R. Yamamoto, manuscript in preparation). Taken together, the simplest interpretation of our results is that both glucocorticoids and chromosomal position modulate rates of MTV transcription solely by affecting the efficiency of initiation at the *Pvu*II-136 position in the left-hand LTR.

Transcription of non-MTV sequences fused downstream of the MTV LTR can be hormone regulated in vivo (24, 26, 29). The structural features on the genome that are recognized by RNA polymerase II and the mechanisms that modulate the capacity of this enzyme to initiate transcription at particular genetic loci are virtually unknown. The MTV LTR contains two sequence elements, CCTAT (centered at −86 from the initiation site) and TATAAA (at −30 from the initiation site), similar to those at comparable locations near the start sites of many genes transcribed by RNA polymerase II (1); the function of these sequences in the initiation process, if any, has not been determined. In addition, purified glucocorticoid receptors have been shown to bind selectively to MTV DNA in vitro (20, 34; K. R. Yamamoto, F. Payvar,

G. L. Firestone, et al., Cold Spring Harbor Symp. Quant. Biol., in press); in particular, specific sites have been detected within two adjacent fragments of MTV DNA that map 110 to 449 bp upstream of the initiation site (34; Yamamoto et al., in press). It is intriguing that a fragment that includes this receptor binding region has been shown to confer hormonal regulation in vivo on a heterologous promoter to which it is fused (Yamamoto et al., in press; V. Chandler, B. Maler and K. R. Yamamoto, submitted for publication); the mechanism and significance of this effect are being investigated.

Feinstein et al. (16) have presented evidence that MTV proviruses acquire the chromatin structure of the genomic region into which they integrate and that these differences in chromatin configuration may account for the chromosomal position effects on MTV gene expression. It has been speculated that multiple steroid receptor binding events might alter chromatin structure across an entire transcriptional unit, thereby affecting its expression (52); interestingly, specific receptor-DNA interactions have been detected in vitro not only proximal to the MTV initiation site, but also at widely separated regions within the transcribed sequences (34; Yamamoto et al., in press). According to this view, the efficiency of promoter utilization could be specified by glucocorticoids and by chromosomal position via a common mechanism involving chromatin structure. Thus, the site of provirus integration would determine its basal chromatin structure and expression, and the competence of the glucocorticoid receptor to alter that structure would in turn determine the "induced" structure and expression of the MTV element. Alternatively, hormonal and position effects on MTV RNA initiation may be unrelated; the receptor protein, for example, might specifically enhance the RNA polymerase-DNA interaction at the promoter, whereas chromatin structures could passively and differentially affect access of the polymerase or specific regulatory proteins or both to their binding sites. Distinguishing these possibilities will likely be of general relevance to understanding the process of transcription initiation, as well as the mechanisms that control it during development and in the course of cellular growth and metabolism.

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LITERATURE CITED

1. **Benoist, C., K. O'Hare, R. Breathnach, and P. Chambon.** 1980. The ovalbumin gene—sequence of putative control regions. Nucleic Acids Res. **8**:127–142.
2. **Benton, W. E., and R. W. Davis.** 1977. Screening λgt recombinant clones by hybridization to single plaques in situ. Science **196**:180–182.
3. **Benz, E. W., R. M. Wydro, B. Nadal-Ginard, and D. Dina.** 1980. Moloney murine sarcoma DNA is a transcription unit. Nature (London) **288**:665–669.
4. **Berkner, K. L., and W. R. Folk.** 1977. Polynucleotide kinase exchange reaction. J. Biol. Chem. **252**:3176–3184.
5. **Carlson, M., and D. Botstein.** 1982. Two differentially regulated mRNAs with different 5′ ends encode secreted and intracellular forms of yeast invertase. Cell **28**:145–154.
6. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
7. **Coffin, J. M., J. T. Parsons, L. Rymo, R. K. Haroz, and C. Weissmann.** 1974. A new approach to the isolation of RNA-DNA hybrids and its application to the quantitative determination of labeled tumor virus RNA. J. Mol. Biol. **86**:373–396.
8. **deCrombrugghe, B., and I. Pastan.** 1978. Cyclic AMP, the cyclic AMP receptor protein, and their dual control of the galactose operon, p. 303–324. *In* J. H. Miller and W. S. Roznikoff (ed.), The operon. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **Denhardt, D. T.** 1966. A membrane-filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Commun. **23**:641–646.
10. **deWet, S. R., D. L. Daniels, J. L. Schroeder, B. G. Williams, K. Denniston-Thompson, D. D. Moore, and F. R. Blattner.** 1980. Restriction maps for twenty-one charon vector phages. J. Virol. **33**:401–410.
11. **Donehower, L. A., A. L. Huang, and G. L. Hager.** 1981. Regulatory and coding potential of the mouse mammary tumor virus long terminal redundancy. J. Virol. **37**:226–238.
12. **Dudley, J. P., and H. E. Varmus.** 1981. Purification and translation of murine mammary tumor virus mRNA's. J. Virol. **39**:207–218.
13. **Enquist, L., and N. Sternberg.** 1979. *In vitro* packaging of λDam vectors and their use in cloning DNA fragments. Methods Enzymol. **68**:281–298.
14. **Fasel, N., K. Pearson, E. Buetti, and H. Diggelmann.** 1982. The region of mouse mammary tumor virus DNA containing the long terminal repeat includes a long coding sequence and signals for hormonally regulated transcription. EMBO J. **1**:3–7.
15. **Favaloro, J., R. Treisman, and R. Kamen.** 1980. Transcription maps of polyoma virus-specific RNA: analysis by two-dimensional nuclease S1 gel mapping. Methods Enzymol. **65**:718–749.
16. **Feinstein, S. C., S. R. Ross, and K. R. Yamamoto.** 1982. Chromosomal position effects determine transcriptional potential of integrated mammary tumor virus DNA. J. Mol. Biol. **156**:549–566.
17. **Firestone, G. L., and E. C. Heath.** 1981. The cyclic AMP-mediated induction of alkaline phosphatase in mouse L-cells. J. Biol. Chem. **256**:1396–1403.
18. **Glisin, V., R. Crkvenjakov, and C. Byus.** 1974. Ribonucleic acid isolated by cesium chloride centrifugation. Biochemistry **13**:2633–2637.
19. **Gottesman, M., A. Oppenheim, and D. Court.** 1982. Retroregulation: control of gene expression from sites distal to the gene. Cell **29**:727–728.
20. **Govindan, M. V., E. Spiess, and J. Majors.** 1982. Purified glucocorticoid receptor-hormone complex from rat liver cytosol binds specifically to cloned mouse mammary tumor virus long terminal repeats *in vitro*. Proc. Natl. Acad. Sci. U.S.A. **79**:5157–5161.
21. **Green, M. R., and R. G. Roeder.** 1980. Definition of a

novel promoter for the major adenovirus-associated virus mRNA. Cell 22:231–242.
22. **Greenblatt, J.** 1981. Regulation of transcription termination by the *N* gene protein of bacteriophage lambda. Cell 24:8–9.
23. **Groner, B., E. Buetti, H. Diggelmann, and N. E. Hynes.** 1980. Characterization of endogenous and exogenous mouse mammary tumor virus proviral DNA with site-specific molecular clones. J. Virol. 36:734–745.
24. **Groner, B., N. Kennedy, U. Rahmsdorf, P. Herrlich, A. B. Van Ooyen, and N. E. Hynes.** 1982. Introduction of a proviral mouse mammary tumor virus gene and a chimeric MMTV-thymidine kinase gene into L cells results in their glucocorticoid responsive expression, p. 217–228. *In* J. E. Dumont, J. Nunez, and G. Schultz (ed.), Hormones and cell regulation, vol. 6. Elsevier/North-Holland, New York.
25. **Grove, J. R., B. S. Dieckmann, T. A. Schroer, and G. M. Ringold.** 1980. Isolation of glucocorticoid-unresponsive rat hepatoma cells by fluorescence-activated cell sorting. Cell 21:47–56.
26. **Huang, A. L., M. C. Ostrowski, D. Berard, and G. L. Hager.** 1981. Glucocorticoid regulation of the Ha-MuSV p21 gene conferred by sequences from mouse mammary tumor virus. Cell 27:245–255.
27. **Ingolia, T. D., E. A. Craig, and B. J. McCarthy.** 1980. Sequence of three copies of the gene for the major Drosophila heat shock induced protein and their flanking regions. Cell 21:669–679.
28. **Klemenz, R., M. Reinhardt, and H. Diggelmann.** 1981. Sequence determination of the 3' end of mouse mammary tumor virus RNA. Mol. Biol. Rep. 7:123–126.
29. **Lee, F., R. Mulligan, P. Berg, and G. Ringold.** 1981. Glucocorticoids regulate expression of dihydrofolate reductase cDNA in mouse mammary tumor virus chimaeric plasmids. Nature (London) 294:228–232.
30. **Majors, J. E., and H. E. Varmus.** 1981. Nucleotide sequences at host-proviral junctions for mouse mammary tumour virus. Nature (London) 289:253–258.
31. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. 65:499–560.
32. **O'Farrell, P. H., E. Kutter, and M. Nakanishi.** 1980. A restriction map of the bacteriophage T4 genome. Mol. Gen. Genet. 179:421–435.
33. **Ostrowski, M. C., D. Berard, and G. L. Hager.** 1981. Specific transcriptional initiation *in vitro* on murine type C retrovirus promoters. Proc. Natl. Acad. Sci. U.S.A. 78:4485–4489.
34. **Payvar, F., G. L. Firestone, S. R. Ross, V. L. Chandler, O. Wrange, J. Carlstedt-Duke, J.-A. Gustafsson, and K. R. Yamamoto.** 1982. Multiple specific binding sites for purified glucocorticoid receptors on mammary tumor virus DNA. J. Cell. Biochem. 19:241–247.
35. **Payvar, F., O. Wrange, J. Carlstedt-Duke, S. Okret, J.-A. Gustafsson, and K. R. Yamamoto.** 1981. Purified glucocorticoid receptors bind selectively *in vitro* to a cloned DNA fragment whose transcription is regulated by glucocorticoids *in vivo*. Proc. Natl. Acad. Sci. U.S.A. 78:6628–6632.
36. **Polsky, F., M. H. Edgell, J. G. Seidman, and P. Leder.** 1978. High capacity gel preparative electrophoresis for purification of fragments of genomic DNA. Anal. Biochem. 87:397–410.
37. **Reznikoff, W. S., and J. N. Abelson.** 1978. The *lac* promoter, p. 221–243. *In* J. H. Miller and W. S. Reznikoff (ed.), The operon. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. **Ringold, G. M.** 1979. Glucocorticoid regulation of mouse mammary tumor virus gene expression. Biochim. Biophys. Acta 560:487–508.
39. **Ringold, G. M., P. R. Shank, H. E. Varmus, J. Ring, and K. R. Yamamoto.** 1979. Integration and transcription of mouse mammary tumor virus DNA in rat hepatoma cells. Proc. Natl. Acad. Sci. U.S.A. 76:665–669.
40. **Ringold, G. M., K. R. Yamamoto, J. M. Bishop, and H. E. Varmus.** 1977. Glucocorticoid-stimulated accumulation of mouse mammary tumor virus RNA: increased rate of synthesis of viral RNA. Proc. Natl. Acad. Sci. U.S.A. 74:2879–2883.
41. **Ringold, G. M., K. R. Yamamoto, G. M. Tomkins, J. M. Bishop, and H. E. Varmus.** 1975. Dexamethasone-mediated induction of mouse mammary tumor virus RNA: a system for studying glucocorticoid action. Cell 6:299–305.
42. **Robertson, D. L., and H. E. Varmus.** 1979. Structural analysis of the intracellular RNAs of murine mammary tumor virus. J. Virol. 30:576–589.
43. **Robertson, D. L., and H. E. Varmus.** 1981. Dexamethasone induction of the intracellular RNAs of mouse mammary tumor virus. J. Virol. 40:673–682.
44. **Shank, P. R., J. C. Cohen, H. E. Varmus, K. R. Yamamoto, and G. M. Ringold.** 1978. Mapping of linear and circular forms of mouse mammary tumor virus DNA with restriction endonucleases: evidence for a large specific deletion occurring at high frequency during circularization. Proc. Natl. Acad. Sci. U.S.A. 75:2112–2116.
45. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503–517.
46. **Stallcup, M. R., J. Ring, and K. R. Yamamoto.** 1978. Synthesis of mouse mammary tumor virus ribonucleic acid in isolated nuclei from cultured mammary tumor cells. Biochemistry 17:1515–1521.
47. **Steinmetz, M., J. Hochtl, H. Schnell, W. Gebhard, and H. G. Zachau.** 1980. Cloning of V region fragments from mouse liver DNA and localization of repetitive DNA sequences in the vicinity of immuno-globulin gene segments. Nucleic Acids Res. 80:1721–1729.
48. **Thomas, P. S.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. U.S.A. 77:5201–5205.
49. **Tjian, R.** 1981. T antigen and the control of SV40 gene expression. Cell 26:1–2.
50. **Ucker, D. S., S. R. Ross, and K. R. Yamamoto.** 1981. Mammary tumor virus DNA contains sequences required for its hormone regulated transcription. Cell 27:257–266.
51. **Varmus, H. E.** 1982. Retroviruses, p. 411–504. *In* J. A. Shapiro (ed.), Mobile genetic elements. Academic Press, Inc., New York.
52. **Yamamoto, K. R., and B. M. Alberts.** 1976. Steroid receptors: elements for modulation of eukaryotic transcription. Annu. Rev. Biochem. 45:721–746.
53. **Yamamoto, K. R., M. R. Stallcup, J. Ring, and G. M. Ringold.** 1978. Mammary tumor virus DNA: a glucocorticoid-responsive transposable element. Cold Spring Harbor Symp. Quant. Biol. 42:625–638.
54. **Yamamoto, T., B. deCrombrugghe, and I. Pastan.** 1980. Identification of a functional promoter in the long terminal repeat of rous sarcoma virus. Cell 22:787–797.
55. **Young, R. A., O. Hagenbuchle, and U. Schibler.** 1981. A single mouse α-amylase gene specifies two different tissue-specific mRNAs. Cell 23:451–458.