JOURNAL OF VIROLOGY, Jan. 1983, p. 148–154
0022-538X/83/010148-07$02.00/0

Vol. 45, No. 1

Cloned Mouse Mammary Tumor Virus DNA Exhibits Glucocorticoid-Dependent Expression in Simian Virus 40-Transformed Mink Cells

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Received 18 May 1982/Accepted 8 September 1982

Mink lung epithelial cells were transfected with two cloned mouse mammary tumor virus (MMTV) DNAs, a 9-kilobase clone derived from an unintegrated exogenous viral genome and a 14-kilobase clone containing an integrated endogenous provirus along with cellular flanking sequences. Mink lung cells were chosen because they do not contain endogenous MMTV sequences. On the basis of our observation that simian virus 40 DNA efficiently transforms these cells, we isolated cell clones containing MMTV DNA by using transformation with simian virus 40 DNA as a selective marker in cotransfection experiments. Levels of the 9-kilobase MMTV mRNA representing the entire viral genome and of the spliced 4.4-kilobase mRNA which codes for the viral envelope proteins were glucocorticoid dependent in transformed cells. Expression of low levels of $Pr77^{gag}$, the precursor of the group-specific viral core proteins, and of $gPr73^{env}$, the precursor of the viral envelope proteins, was also hormone dependent. We conclude that these cloned MMTV DNAs contain all the information necessary for the synthesis of normal viral RNAs and proteins. These findings also provide further evidence that the DNA sequences involved in the hormone responsiveness of MMTV expression are contained within the viral genome.

All mouse strains carry several endogenous mouse mammary tumor virus (MMTV) genomes. These normally are not expressed or are associated only with a low incidence of tumors appearing late in life (22, 24, 26, 27). In contrast, proviral DNA copies arising from horizontally transmitted exogenous MMTV, as well as proviruses transmitted in the germ line of some mouse strains (e.g., GR), are correlated with a high incidence of mammary carcinomas occurring early in the life of the animal (1, 7, 23). Both tumor induction and viral gene expression are regulated by hormones in vivo (2, 3). In cultured mammary tumor cells, and in mouse or other cells infected in culture, expression of viral genes is stimulated by glucocorticoid hormones (30, 34, 38).

As yet there is no in vitro assay for transformation by MMTV; this has prevented a classical approach to the study of MMTV genetics with purified virus strains. This difficulty has been partially overcome by the use of recombinant DNA techniques to isolate defined MMTV DNA sequences. Recent reports have described the molecular cloning and subsequent expression in transfected mouse cells of an exogenous MMTV genome (6) and of endogenous MMTV proviruses flanked by cellular sequences (16, 17; H. Diggelmann, A. L. Vessa, and E. Buetti, Virology, in press). To exclude the possibility that the observed expression of viral products in MMTV-transfected mouse cells resulted from recombination of the transfecting DNA with cellular sequences or from activation of cellular MMTV copies, it was necessary to demonstrate the biological activity of these MMTV clones in cells which do not contain MMTV-related sequences. In this report we describe the transfection of cloned exogenous and endogenous MMTV DNA into mink lung epithelial cells (strain CCL64). To isolate transfected cells containing MMTV DNA, transformation by simian virus 40 (SV40) DNA was used as a selective marker in cotransfection experiments. Nearly all transformed cell clones had acquired multiple copies of both MMTV and SV40 DNA. Hormone-dependent expression of virus-specific RNA, as well as low levels of viral core proteins and envelope proteins, were demonstrated in cells transfected with either exogenous or endogenous MMTV DNA clones. The cloned MMTV DNAs thus contain sufficient information to code for the MMTV RNA species from which viral envelope and core proteins are trans-

lated. Furthermore, these experiments strongly suggest that the site of hormone regulation is carried on the viral genome.

MATERIALS AND METHODS

Transfection. CCL64 cells obtained from J. Schlom were grown in Eagle medium with 10% fetal calf serum. Purified inserts of MMTV DNA clone H were prepared and ligated before transfection as previously described (6). Total Charon 4A-MMTV recombinant phage DNA clone GR-40 was kindly provided by N. E. Hynes (16). Ligated clone H inserts (1 μg) with 9 μg of salmon sperm carrier DNA, or GR-40 DNA (10 μg) without carrier DNA, were cotransfected with 0.1 μg of SV40 DNA (provided by P. Beard) by calcium phosphate precipitation (12) onto about 0.5×10^6 CCL64 cells in 60-mm plates containing 5 ml of medium. The cells were incubated for 20 h with the precipitate and then fed with fresh medium. One day later the cells were trypsinized and reseeded into 100-mm petri dishes. At 24 h after trypsinization, the cells were overlayed with 0.5% soft agar medium. At 3 weeks after transfection, large foci of morphologically transformed cells were picked with cloning pipettes and grown in Eagle medium with 10% fetal calf serum.

Extraction of nucleic acids and filter hybridization. DNA was extracted from transformed cells 7 to 9 weeks after transfection according to the procedure of Groner and Hynes (13). The DNA was transferred to nitrocellulose filters by the method of Southern as previously described (6). Total cellular RNA was extracted with hot phenol (32). Polyadenylate [poly(A)]-containing RNA was isolated by oligodeoxythymidylate-cellulose column chromatography, electrophoresed in agarose gels under denaturing conditions (21), and transferred to nitrocellulose as described by Thomas (36). RNA transferred to filters was hybridized with ^{32}P-labeled MMTV cDNA in 50% formamide as previously described (6). Hybridization of DNA on filters with ^{32}P-labeled MMTV cDNA was performed as described by Groner and Hynes (13).

Protein labeling, immunoprecipitation, and SDS-polyacrylamide gel electrophoresis. Tissue culture cells were labeled with 40 μCi of [^{35}S]methionine per 100-mm plate in 1.5 ml of Earle saline for 1.5 h, and then 1.5 ml of normal medium was added for 2 h. The cells were washed with cold phosphate-buffered saline (PBS) and scraped into 1 ml of PBS–0.5% NonidetP-40–0.5% deoxycholate, and the lysate was centrifuged at 13,000 × *g* for 10 min. The supernatant was brought to 0.1% sodium dodecyl sulfate (SDS), and 0.5-ml aliquots were incubated first for 30 min at 37°C with 5 μl of rabbit anti-MMTV p28 or anti-MMTV gp52 serum and then for 30 min at 4°C with 20 μl of 10% Sepharose-protein A suspension. After being washed three times in PBS–0.1% SDS, the protein was eluted from Sepharose-protein A for 2 min at 95°C in gel sample buffer containing 2% SDS, 5% mercaptoethanol, 7 M urea, and 100 mM Tris-hydrochloride (pH 6.8). After electrophoresis in an SDS-polyacrylamide gel (15%) by the method of Laemmli modified by Blattler et al. (4), the gels were analyzed by autoradiography with Kodak X-Omat R films with Dupont Cronex Lightning Plus intensifying screens. Rabbit antiserum against gp52 was a gift of P. Osterrieth, Liège, Belgium. Rabbit antiserum against p28 purified by agarose gel filtration was prepared as previously described (18, 39).

Immunofluorescence. For nuclear immunofluorescence of SV40 T-antigen, cells grown on cover slips were fixed in a 1:2 methanol-acetone solution for 10 min at −20°C. Fixed cells were incubated for 30 min at 37°C with a 1:20 dilution of hamster antiserum against with a 1:20 dilution of hamster antiserum against T-antigen, washed extensively in PBS, and then incubated for 30 min at 37°C with the fluorescein isothiocyanate-conjugated rabbit antibody to hamster immunoglobulin G. Cover slips were mounted in glycerol buffer (pH 7.5), and photographs were taken on Kodak Tri-X Pan film. Routine controls were done with preimmune serum and untransfected CCL64 cells and by omission of the first antibody.

RESULTS

Transfection of mink cells with cloned MMTV DNA. In experiments in which cell cultures are transfected with DNA by the calcium phosphate precipitation technique (12), there must be some means to select for the small fraction of cells in the total population which stably acquire new DNA sequences (40). In initial experiments we observed no morphologically distinguishable foci in mink cell cultures transfected with cloned MMTV DNA alone. MMTV-specific DNA sequences could not be detected by Southern blot hybridization of total DNA extracted from cultures of transfected cells several weeks after transfection with MMTV DNA alone (data not shown). To select cells which had acquired new DNA, cloned MMTV DNA was cotransfected with SV40 DNA. We had found previously that SV40 DNA transformed mink cells with a high efficiency (ca. 70 foci per 0.1 μg of DNA per 10^6 cells), giving rise to dense foci which could easily be isolated from the surrounding cell monolayer by trypsinization with cloning cylinders or by picking foci grown under soft agar. These SV40-transformed mink cells produced nuclear SV40 T-antigens as shown by immunofluorescence (Fig. 1). When cloned MMTV DNA was cotransfected in a 10- to 20-fold molar excess with respect to SV40 DNA, 25 of 32 SV40-transformed cell clones also contained MMTV DNA.

The exogenous 9-kilobase (kb) MMTV DNA (clone H) derived from the 9-kb circular viral DNA found in infected rat hepatoma cells is present in the λgt · WES bacteriophage in permuted form (6, 31). Therefore, before transfection, inserts were isolated and ligated to form concatamers which should contain viral genes in the original order. Figure 2, lanes 1 and 2, shows the restriction enzyme analysis of DNA isolated from cell clones 7 weeks after transfection with the ligated MMTV clone H and SV40 DNA. The predominant MMTV *Eco*RI restriction frag-

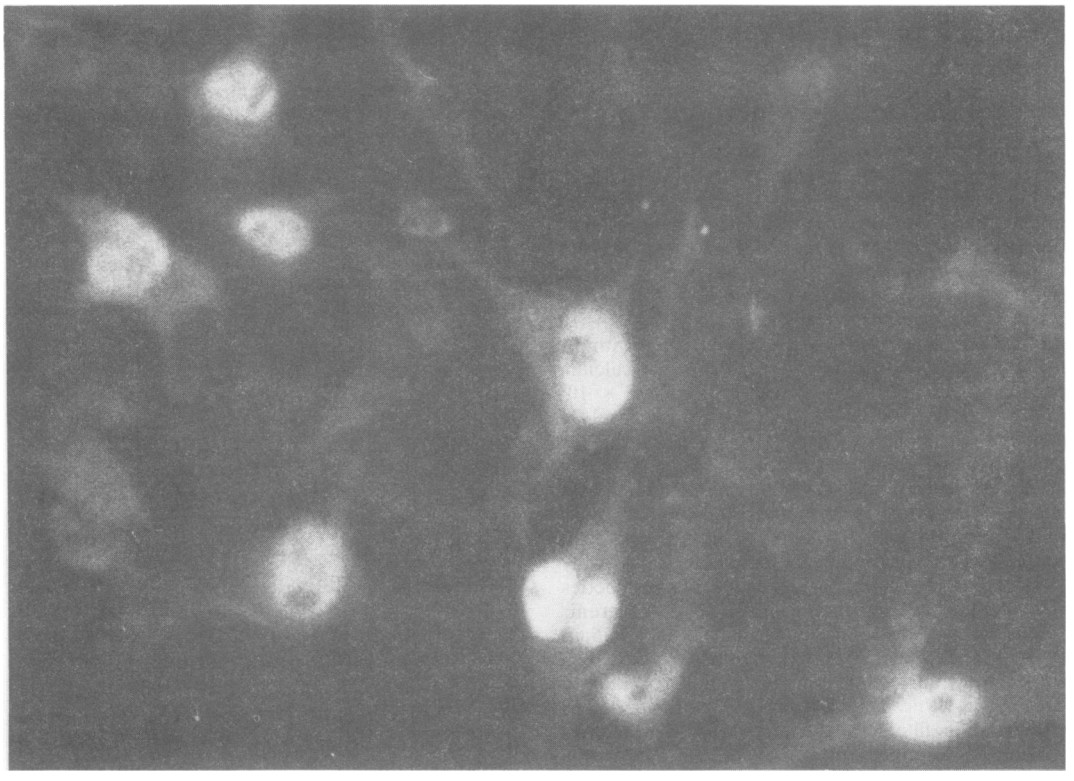

FIG. 1. Indirect immunofluorescent staining showing nuclear localization of large T-antigen in CCL64 cells transformed by transfection with SV40 DNA.

ments found in the cellular DNA comigrated with the 9-kb MMTV insert from an *Eco*RI digest of the recombinant clone (Fig. 2, lane 5). The estimated copy number as determined by comparison of band intensities was one to two intact 9-kb MMTV copies per cell (30 ng of clone H DNA representing 15 gene equivalents per cell). Since the *Eco*RI restriction site was conserved, these copies appeared to be tandemly integrated. The additional MMTV-specific bands may have arisen from partial digestion of concatenated 9-kb DNA or from integration events in which a terminal *Eco*RI site was destroyed.

Total λ phage DNA containing the endogenous MMTV DNA plus cellular flanking sequences was also used in cotransfection with SV40 DNA. The endogenous MMTV DNA (clone GR-40) isolated from GR mouse liver DNA (16) (Fig. 2, lanes 3 and 4) was present in DNA isolated from cell clones more than 8 weeks after transfection. The predominant *Eco*RI fragments comigrated with the MMTV-containing *Eco*RI bands of the recombinant phage DNA (Fig. 2, lane 6). These cells contained approximately three MMTV copies per cell.

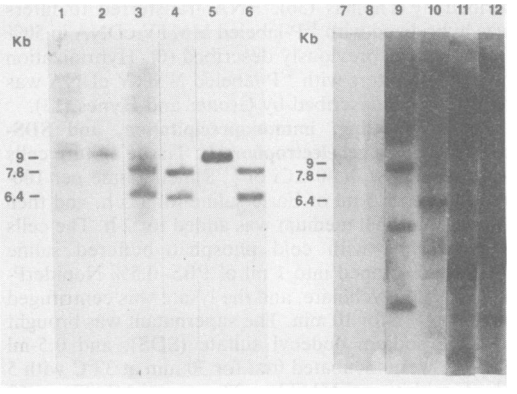

FIG. 2. Southern blot analysis of DNA from CCL64 cells cotransfected with MMTV DNA and SV40 DNA. Cellular DNA (20 μg) was digested with *Eco*RI, electrophoresed on agarose gels, blotted onto nitrocellulose filters and hybridized to ^{32}P-labeled MMTV cDNA (lanes 1 through 8) or ^{32}P-labeled SV40 DNA (lanes 9 through 12). Lanes: 1, 2, and 11, DNA from cells transfected with exogenous MMTV clone H; 3, 4, and 12, DNA from cells transfected with endogenous MMTV DNA clone GR-40; 5 and 6, *Eco*RI digests of recombinant clone H and clone GR-40 DNA, respectively; 7–10, DNA from cells transfected with SV40 DNA alone.

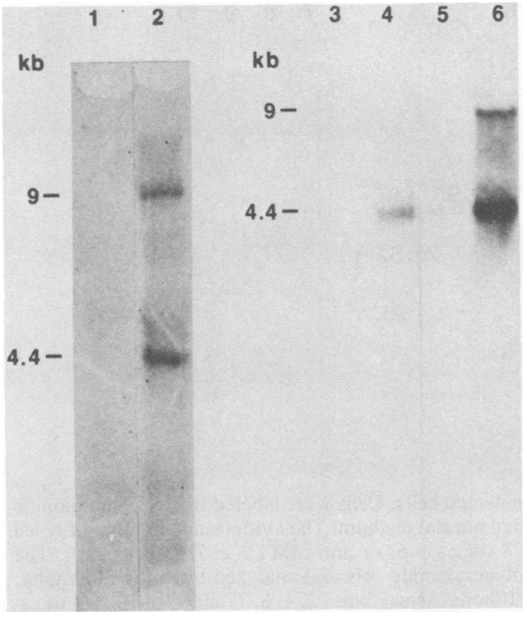

FIG. 3. Size determination of MMTV-specific RNA in transfected cells. Total poly(A)-containing RNA (5 μg) from cells grown in the presence (lanes 2, 4, and 6) or absence (lanes 1, 3, and 5) of 10^{-6} M dexamethasone was transferred to nitrocellulose filters after electrophoresis on denaturing agarose gels and hybridized with ^{32}P-labeled MMTV cDNA. Lanes: 1–4, two cell clones transfected with exogenous MMTV DNA clone H; 5 and 6, cells transfected with endogenous clone GR-40 DNA.

Lanes 11 and 12 of Fig. 2 show the same *Eco*RI-digested DNA as in lanes 1 and 3 hybridized with a nick-translated SV40 DNA probe. DNA from cells transfected with SV40 alone is shown in lanes 7 through 10. No hybridization was seen with a MMTV-specific probe (Fig. 2, lanes 7 and 8), whereas the nick-translated SV40 probe hybridized with many bands (lanes 9 and 10). The heterogeneous patterns of integration of SV40 DNA in the transfected cells are consistent with reports that SV40 DNA integrates randomly with respect to the viral genome (5).

MMTV RNA in transfected cells. Total cellular RNA was prepared from cells by hot phenol extraction (32) 10 to 20 weeks after transfection. Poly(A)-containing RNA was isolated and transferred to nitrocellulose filters (36) after electrophoresis in agarose gels under denaturing conditions (21). Figure 3 shows MMTV-specific RNA detected by hybridization of filters with MMTV cDNA. Lanes 1 and 3 show poly(A)-containing RNA from two cell clones transfected with the exogenous DNA clone H, grown without added hormone in the culture medium. RNA from the same cells grown for 20 h in the presence of 10^{-6} M dexamethasone is shown in lanes 2 and 4. Levels of both the 9-kb mRNA, representing the entire viral genome, and the spliced 4.4-kb mRNA, which codes for the viral envelope proteins, were increased in the presence of hormone. The increase of about fivefold in viral RNA transcripts is comparable to the hormone effect seen in GR mammary tumor cells (6, 16) and in mink cells infected with MMTV (19).

Cells infected with the endogenous MMTV DNA also showed a fivefold dexamethasone-stimulated increase in MMTV-specific RNA levels (Fig. 3, lanes 5 and 6).

As a control for the specificity of the hormone stimulation, the same RNA as in lanes 3 through 6 of Fig. 3 was hybridized with a nick-translated SV40 DNA probe. No hormone-dependent increase in the expression of SV40-specific RNA (Fig. 4) was observed.

Synthesis of MMTV-specific proteins. Cultures grown in either the presence or the absence of 10^{-6} M dexamethasone were labeled with [^{35}S]methionine for 1 h and then incubated for 2 h with added normal medium. Cells were then washed, lysed, and immunoprecipitated with an antiserum directed either against the major viral core protein (anti-MMTV p28) or against the major envelope glycoprotein (anti-MMTV gp52) (10, 35). Immunoprecipitates were electrophoresed in SDS-polyacrylamide gels and analyzed by autoradiography (Fig. 5). When cells were grown in the presence of dexamethasone, small amounts of the precursor protein Pr77^{gag}, from which p28 is processed (8, 29, 33), were found in cells transfected with either the exogenous or the endogenous MMTV DNA clones (Fig. 5, lanes 3 and 5). In the absence of dexamethasone this protein was not detected (lanes 4 and 6). As compared with levels of Pr77^{gag} found in mouse

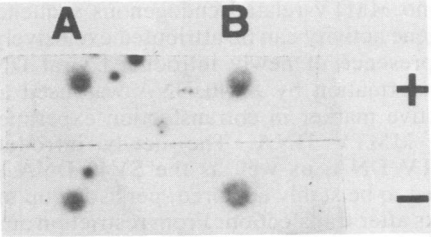

FIG. 4. SV40-specific RNA in cells cotransfected with SV40 DNA and MMTV clone H DNA (A) or clone GR-40 DNA (B). Total poly(A)-containing RNA (2 μg) from cells grown in the presence (+) or absence (—) of 10^{-6} M dexamethasone was spotted onto a nitrocellulose filter and hybridized to ^{32}P-labeled SV40 DNA.

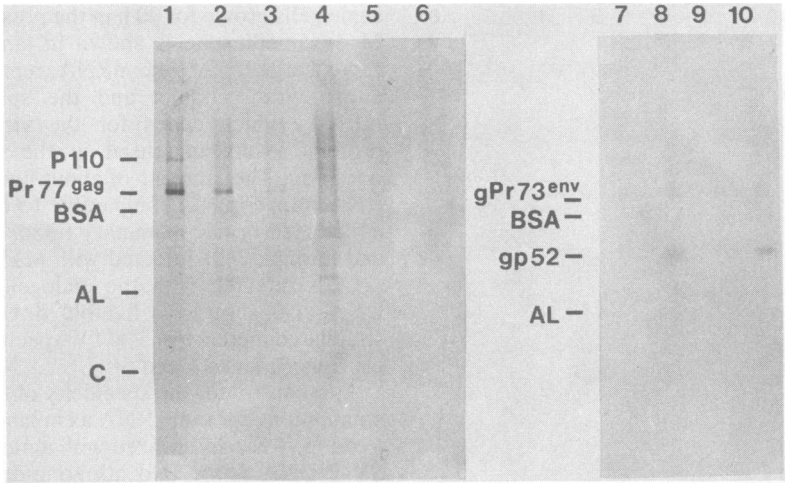

FIG. 5. Immunoprecipitation of MMTV proteins in transfected cells. Cells were labeled with [^{35}S]methionine in Earle saline for 1.5 h and then incubated for 2 h with added normal medium. The cytoplasmic fraction of lysed cells was immunoprecipitated with rabbit anti-MMTV p28 (lanes 1–6) or anti-MMTV gp52 (lanes 7–10). The immunoprecipitates were electrophoresed in 15% SDS-polyacrylamide gels and analyzed by autoradiography. The cells were grown in the presence (lanes 1, 3, 5, 8, and 10) or absence (lanes 2, 4, 6, 7, and 9) of 10^{-6} M dexamethasone. Lanes: 1 and 2, mouse L-cells transfected with MMTV clone H; 3, 4, 7, and 8, CCL64 cells transfected with clone H; 5, 6, 9, and 10, CCL64 cells transfected with clone GR-40. Trichloroacetic acid-precipitable radioactivity measured for each lysate (expressed as ^{35}S counts per minute $\times 10^5$ per 2-μl aliquot): 2.2 (lane 1), 2.7 (lane 2), 1.8 (lane 3), 8.2 (lane 4), 1.8 (lane 5), 1.5 (lane 6), 1.4 (lane 7), 1.5 (lane 8), 1.9 (lane 9), 2.1 (lane 10). The following size markers were run in parallel slots: bovine serum albumin (BSA) (molecular weight, 68,000); aldolase (AL) (40,000); chymotrypsin (C) (35,000).

L-cells transfected with clone H (lanes 1 and 2), the amounts found in transfected mink cells were low.

Immunoprecipitation with anti-MMTV gp52 is shown in Fig. 5, lanes 7 through 10. Synthesis of the viral envelope glycoprotein gp52 and its precursor gPr73 (9) was also dexamethasone dependent.

DISCUSSION

In this report we have demonstrated expression of viral genes in mink cells transfected with cloned MMTV DNA. Because these cells contain no MMTV-related endogenous sequences, the gene activity can be attributed exclusively to the presence of newly introduced viral DNA. Transformation by SV40 DNA was used as a selective marker in cotransfection experiments with MMTV DNA. The newly introduced MMTV DNA, as well as the SV40 DNA, appeared to be stably acquired, persisting up to 20 weeks after transfection. From restriction analysis alone, however, it was not possible to determine whether the intact MMTV DNA copies present in these cells were integrated into cellular DNA or into large extrachromosomal structures. Mink cells transfected with either exogenous or endogenous cloned MMTV DNA were able to synthesize both of the major viral mRNAs found in an MMTV-producing GR tumor cell line (14). In all of the clones examined, the level of the 4.4-kb mRNA coding for the viral envelope proteins was higher than that of the 9.0-kb species which codes for the core proteins and also constitutes the viral genome. The reason for this difference, which is also observed in transfected mouse L-cells, is unclear, especially as it is not seen in MMTV mRNA populations from the virus-producing GR cell line (14). Amounts of MMTV RNA and viral proteins found in the transfected mink cells were low as compared with those in the GR mouse mammary tumor cell line or in MMTV-transfected L-cells (6, 16, 17; Diggelmann et al., in press). Previous studies of mink lung cells infected with MMTV have shown that virus production in these cells is lower than in cultured mammary tumor cells (19). The low levels of viral proteins might also be due to the transformed phenotype of these cells induced by SV40, which could impair normal transcription or translation of viral mRNA. For this reason it would be desirable to do future transfection experiments in these cells by using selective markers which do not significantly alter cellular metabolism (25).

A hormonally stimulated increase in MMTV RNA levels was clearly evident in transfected mink cells. This supports the hypothesis that

both the endogenous and the exogenous MMTV DNA contain the sequences necessary for hormonal control of viral transcription, as previously proposed (6, 16, 37; Diggelmann et al., in press). These experiments demonstrate that hormone-dependent expression of transfected MMTV DNA is not due to interaction with endogenous cellular MMTV sequences. The possibility that other cellular sequences near the site of integration are involved in hormonal regulation cannot be excluded. However, hormone receptor binding studies using cloned MMTV fragments suggest that this is unlikely (11a, 28). In addition, several recent reports point to sequences in the long terminal repeat as being responsible for hormonal regulation (11, 15, 20). Further transfection experiments with MMTV DNA containing mutations or deletions in this region should make it possible to localize the site of hormone receptor recognition.

ACKNOWLEDGMENTS

We thank N. E. Hynes and B. Groner for kindly providing the recombinant λ phage DNA clone GR-40 and E. Buetti for the recombinant λ phage clone H. We are grateful to P. M. Osterrieth for a generous gift of anti-gp52 antiserum and to G. Fey for anti-T-antigen antiserum. We thank P. Beard for SV40 DNA, helpful discussions, and critical reading of the manuscript. E. Buetti contributed much useful criticism and advice. The assistance of L. Bucher with the immunofluorescent microscopy was very much appreciated. The expert technical assistance of A. L. Vessaz and D. Petrelli-Sem was invaluable.

This work was supported by grants from the Swiss National Science Foundation and the Swiss Cancer League.

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