JOURNAL OF VIROLOGY, Nov. 1984, p. 420–430
0022-538X/84/110420-11$02.00/0

Vol. 52, No. 2

Use of the Mouse Mammary Tumor Virus Long Terminal Repeat to Promote Steroid-Inducible Expression of v-*mos*

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Received 11 June 1984/Accepted 9 August 1984

We used the mouse mammary tumor virus long terminal repeat to promote dexamethasone-regulated expression of the Moloney murine sarcoma virus (M-MSV) transforming gene, v-*mos*. A recombinant DNA vector containing the mouse mammary tumor virus long terminal repeat fused to the M-MSV 124 v-*mos* gene was cotransfected with a plasmid containing the herpes simplex virus thymidine kinase gene (*tk*) into 3T3TK$^-$ cells. Individual clones of cells which grew in hypoxanthine-aminopterin-thymidine medium were tested for dexamethasone-regulated expression of p37mos as well as several transformation-specific phenotypic parameters. In the absence of dexamethasone, the v-*mos* transfectants appeared morphologically similar to the control cells despite low basal levels of p37mos expression. Upon hormone treatment, the levels of p37mos increased 5- to 10-fold, coincident with morphological changes typical of M-MSV transformation of 3T3 cells. The ability to form foci in monolayers also correlated with p37mos induction. The extent of morphological changes varied in individual clones of cells with similar levels of induced p37mos. Although the induced levels of p37mos were comparable to those seen in stable M-MSV 124 virus-transformed NIH 3T3 cells, the transfectants were unable to grow in soft agar under conditions which support growth of the virus-transformed cells. Acute infection of the transfectants with M-MSV 124 virus, a situation which resulted in elevated levels of p37mos, allowed these cells to grow in soft agar. The results described in this paper suggest that different threshold levels of p37mos may be necessary for the expression of various parameters of the transformed phenotype and also that continued expression of p37mos is necessary for maintenance of the transformed state. However, it also appears that the sensitivity to given levels of p37mos varies among clonal cell lines.

The transforming gene, v-*mos*, of Moloney murine sarcoma virus (M-MSV) encodes a 37,000-dalton phosphoprotein, p37mos (6, 16, 21–23, 25). p37mos is a soluble cytoplasmic protein (24) with limited homology, based on nucleic acid sequence data, to several retroviral tyrosine protein kinases as well as the catalytic subunit of the cAMP-dependent protein kinase (1, 8, 26). Despite these homologies, p37mos has no detectable tyrosine protein kinase activity and appears to be phosphorylated only at serine (25). The normal cellular homolog of v-*mos*, c-*mos*, differs from all other protooncogenes in that it is not known to be expressed in any cell or tissue type examined to date (7, 18). In addition, p37mos is a scarce protein in M-MSV 124 virus-transformed cell lines, constituting only ca. 0.0005% of total cellular protein (22, 25). NIH 3T3 cells acutely infected with M-MSV 124 virus, however, transiently contain 30- to 100-fold more p37mos (22, 25), which appears to correlate with extensive cell death. Survivors of the acute infection contain the characteristic low levels of p37mos detected in stable M-MSV 124 virus-transformed cell lines. These results imply that a maximum intracellular concentration of p37mos can be tolerated and that stable M-MSV 124 virus-transformed cell lines represent survivors which, for reasons unknown, express low levels of p37mos. On the other hand, it is not known whether a threshold level of p37mos is necessary to produce the various phenotypic changes characteristic of M-MSV 124 transformation. In the absence of c-*mos* expression, it is possible in principle that, by virtue of its being expressed in an abnormal context, even a small quantity of p37mos would produce all of the characteristic phenotypic effects. Similarly, it appears that v-*fos*-induced transformation may be a consequence of its expression in an atypical cell type (4, 27). However, whether a threshold level of v-*fos* protein is required for transformation has not yet been determined. To examine the effect of p37mos dosage on various phenotypic aspects of v-*mos*-induced transformation and to examine directly the role of p37mos in both initiation and maintenance of the transformed state, a recombinant DNA vector was constructed in which a dexamethasone-inducible promoter, the mouse mammary tumor virus long terminal repeat (MMTV LTR), was fused to the entire coding region for p37mos. Previous reports show that the MMTV LTR contains sequences that confer hormone-regulated expression upon heterologous genes fused downstream (11, 15). Upon transfection into appropriate cells, the intracellular levels of p37mos should be modulated by treatment with hormone. In this report, we exploited the ability of the MMTV LTR to promote steroid-inducible expression of a downstream gene to show that a threshold level of p37mos is required for both induction and maintenance of various aspects of v-*mos*-induced transformation.

MATERIALS AND METHODS

Construction and purification of recombinant DNA. Restriction enzymes and DNA-modifying enzymes were obtained commercially and used as prescribed in the instructions of the supplier. Standard procedures for manipulation and gel purification of cloned DNAs were described previously by Maniatis (17). The pSV2TK and pMTVdhfr recombinant DNAs were constructed as described previously (15; M. Fromm, Ph.D. thesis, Stanford University, Stanford, Calif., 1983). The recombinant DNA clone containing the

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entire M-MSV 124 provirus, pMSV1L, was constructed as previously described (26) and provided by I. M. Verma (Salk Institute). The MMTV–v-*mos* hybrid recombinant DNA was prepared as follows. pMTVdhfr was digested with *Pvu*II-*Bgl*II to liberate the entire *dhfr* coding region together with 135 nucleotides at the 3′ end of the fragment carrying the MMTV LTR. The remaining vector, which contained most of the MMTV LTR (ca. 1,200 nucleotides) as well as bacterial and eucaryotic regulatory sequences, was agarose gel purified and linked with T4 ligase to the v-*mos* DNA fragment which was prepared as follows. pMSV1L was cleaved with *Xba*I, and the resulting 5′ overhang was filled in with Klenow DNA polymerase. The entire v-*mos* coding region, along with 346 nucleotides of PBR322 sequences at the 3′ end of v-*mos*, was purified by subsequently digesting with *Bam*HI. The *Xba*I to *Bam*HI v-*mos* DNA fragment was agarose gel purified and linked to the MMTV-containing DNA as described above. Recombinant DNA plasmids were grown in *Escherichia coli* strains C600 or HB101. The plasmids were purified by cesium chloride density gradient centrifugation. The structure of the pMTVmos64 clone was confirmed on the basis of filter hybridization with a v-*mos* probe as well as restriction endonuclease digestion.

Cells. All cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. The M-MSV 124 virus-transformed producer NIH 3T3 cell line has been described previously (14) and was provided along with uninfected NIH 3T3 cells by T. Hunter (Salk Institute). The thymidine kinase-deficient 3T3 cell line (3T3TK$^-$) was derived as previously described (28) and provided by I. M. Verma. Acute infections with virus-containing medium from the M-MSV 124 virus-transformed 3T3 cell line were performed as described previously (25).

Transfections and dexamethasone inductions. To generate stable cell lines containing the MMTV–v-*mos* hybrid plasmid, approximately 10^6 3T3TK$^-$ cells grown in 9-cm tissue culture dishes were cotransfected with 5 μg of pMTVmos64 DNA plus 150 ng of pSV2TK DNA by the calcium phosphate co-precipitation procedure, essentially as described by Wigler et al. (29), except that no carrier DNA was used. Two days after transfection the, cells were passaged into hypoxanthine-aminopterin-thymidine (HAT) medium. Individual colonies were picked 10 days later and recloned in microtiter dishes. The resulting cell lines were grown continuously in HAT medium. These cell lines are referred to as 64-1, 64-4, 64-9, and 64-10.

For analysis of hormonal control of v-*mos* expression, cells were plated in 5-cm dishes and treated, at 50 to 60% confluence, with HAT medium containing 10^{-6} M dexamethasone. For the reversion experiment (see Fig. 4) 64-4 transfectants were treated with dexamethasone as described above for 2 days, washed several times with HAT medium, and grown in HAT medium for the remainder of the experiment. The medium was changed every 2 to 3 days. Dishes of 64-4 cells were plated and grown in parallel with HAT alone or HAT plus dexamethasone.

Soft agar assays. Agar assays were done in 24-well microtiter dishes. ca. 10^4 cells in a final volume of 0.6 ml were plated per well. Cells in agar were carefully aspirated through a 21-g needle with a 1-ml syringe to eliminate clumps. The agar-containing growth medium consisted of DMEM supplemented with 10% fetal calf serum (Irvine), 10 mM HEPES (*N*-2-hydroxyethyl piperazine-*N*′-2-ethanesulfonic acid) buffer (pH 7.5), and 0.35% agarose (Calbiochem-Behring). The agar bases, poured the day before, consisted of 0.4 ml of the same agar-containing medium. One day after plating, the wells were fed with 200 μl of DMEM plus 10% fetal calf serum. In some cases, the agar-containing medium was supplemented with 10^{-6} M dexamethasone. After 10 to 14 days, the wells were examined for colonies and photographed. Negative wells contained ≤1 colony, whereas positive wells contained 10 to 100 colonies; the negative wells had mostly single cells and occasionally double- or triple-cell microcolonies which were not scored as positives. Each cell line was plated in parallel into soft agar, and experiments were repeated three times.

Focus assay. For each 9-cm dish, ca. 10^2 pMTVmos64 transfectants or control cells were mixed with ca. 10^5 NIH 3T3 cells and plated in DMEM plus 10% fetal calf serum. Where indicated, the medium was supplemented with 10^{-6} M dexamethasone. The medium was changed every 3 to 4 days, and dishes were photographed after 2 weeks.

Photography of cells. Cells were examined with an inverted Leitz microscope with phase-contrast optics. Photographs were taken with a Nikon camera and Polaroid Land pack film, type 665. The final magnification of all photographs is ×65.

Immunoprecipitation and gel electrophoresis. The antiserum anti-C3, which is directed against a peptide consisting of the 12 COOH-terminal amino acids of the v-*mos* gene product, has been described previously (23). Cells grown in 5-cm dishes were labeled for 4 h with 300 μCi of [^{35}S]methionine in 0.5 ml of methionine-deficient DMEM. Appropriate dishes were supplemented with 10^{-6} M dexamethasone. Cell lysates and immunoprecipitations were carried out as outlined previously (25). An equal number of [^{35}S]methionine counts per minute of each sample were subjected to immunoprecipitation. The cell lysate from each 5-cm dish was immunoprecipitated with either 2 μl of anti-C3 serum or 2 μl of anti-C3 serum preabsorbed with 2 μg of C3 peptide. One-half of each immunoprecipitate was analyzed on a 12.5% sodium dodecyl sulfate-polyacrylamide gel as previously described (25).

RESULTS

Construction of plasmid pMTVmos64 and transfection of 3T3 cells. To obtain steroid-inducible expression of v-*mos*, a plasmid was constructed which contains, in addition to other elements necessary for gene expression, the MMTV LTR fused to the entire v-*mos* gene. We used a recombinant DNA clone containing the *dhfr* gene fused to the MMTV LTR (pMTVdhfr) and a recombinant DNA clone containing the entire M-MSV provirus (pMSV1L) as starting materials. pMTVdhfr was used to provide the MMTV LTR and other regulatory elements, and the entire v-*mos* coding region was prepared from pMSV1L (Fig. 1; see above). The gel-purified MMTV and v-*mos*-containing fragments were ligated to yield pMTVmos64. In this construction, transcription of the v-*mos* gene is expected to initiate at the steroid-inducible promoter within the MMTV LTR, and the RNA should be polyadenylated within the simian virus 40 sequence elements downstream from the v-*mos* coding region. The first v-*mos* AUG codon is 146 nucleotides downstream from the MMTV LTR cap site and 11 nucleotides downstream from the junction between MMTV and M-MSV sequences. The terminator codon used for synthesis of the v-*mos* protein is within the M-MSV sequences.

3T3TK$^-$ cells were transfected by the calcium phosphate co-precipitation procedure with a mixture of the plasmids pMTVmos64 and pSV2TK, a plasmid which expresses herpes simplex virus thymidine kinase. Two days after transfection, the cells were passaged into HAT medium, and 10 days

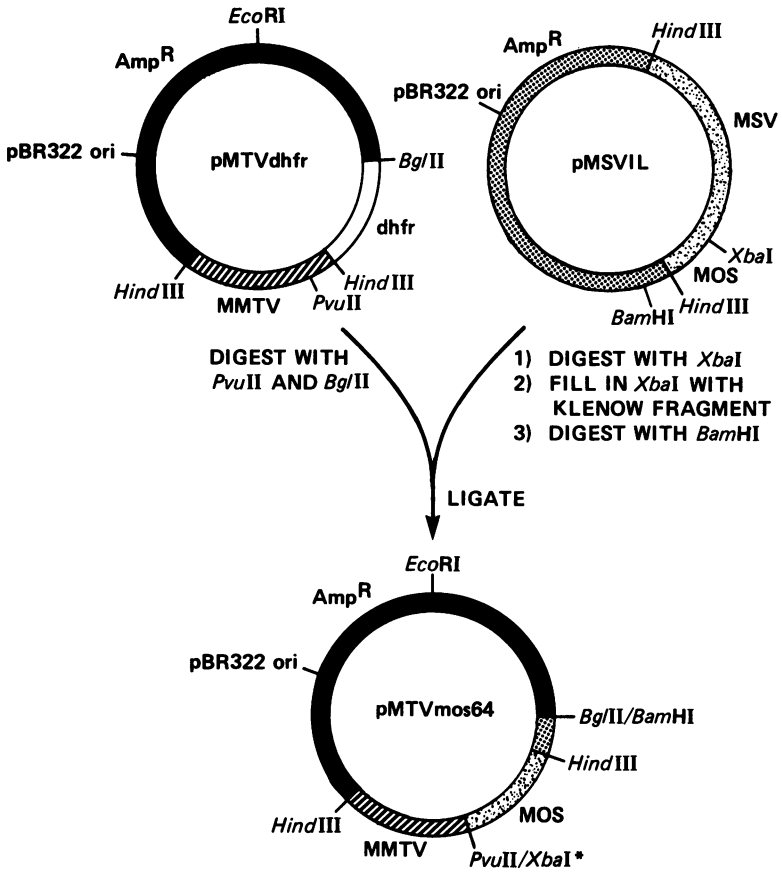


FIG. 1. Construction of the pMTVmos64 vector. The construction of the pMTVmos64 vector is outlined in this figure and described in detail in the text. The two plasmids used as starting materials, pMTVdhfr and pMSV1L, have been published elsewhere (15, 26) and are shown at the top of the figure. The solid black segment of pMTVdhfr denotes a segment of PBR322 (*Hin*dIII to *Eco*RI in pMTVdhfr) which contains the lactamase gene (ampicillin resistance) and the origin of replication as well as simian virus 40 sequences (*Eco*RI to *Bgl*II in pMTVdhfr) which contain the intron for small t antigen and site for polyadenylation for early mRNA. The hatched region denotes the MMTV LTR. The heavy stippled region of pMSV1L contains PBR322 sequences, including the lactamase gene and the origin of replication. The coding regions for dhfr and the M-MSV 124 genome are denoted by open and lightly stippled regions in pMTVdhfr and pMSV1L, respectively. MOS, *Xba*I to *Hin*dIII v-*mos* portion of the M-MSV 124 genome.

later, colonies were picked. The colonies which grew in HAT medium were screened for p37mos expression by immunoprecipitation. To generate control cell lines, the 3T3TK$^-$ cells were transfected with pSV2TK alone.

3T3 Cells transfected with pMTVmos64 express dexamethasone-inducible p37mos. Individual transfected cell lines were plated at 50 to 60% confluence and treated for 4 days with either control HAT medium or HAT medium containing 10^{-6} M dexamethasone. These cells were subsequently labeled for 4 h with [^{35}S]methionine and immunoprecipitated with an antiserum (anti-C3) directed against a synthetic COOH-terminal v-*mos* peptide, C3 (23). To identify p37mos conclusively, each cell lysate was immunoprecipitated with both anti-C3 serum and anti-C3 serum preabsorbed with an excess of C3 peptide. Under these conditions, p37mos can be identified as the only protein which can be reproducibly blocked from the anti-C3 immunoprecipitate by C3 peptide (23). The immunoprecipitation experiment in Fig. 2 revealed that four different cell lines transfected with pMTVmos64 (64-1, 64-4, 64-9, 64-10) express both basal and steroid-inducible p37mos (see Fig. 2, panels 1, 4, 9, and 10). The level of induction, as determined by scanning densitometry of the autoradiograms, varied from 5- to 10-fold among these clones. p37mos migrated as a characteristic doublet which represents both the phosphorylated and unphosphorylated species of p37mos (25). As expected, a control cell line transfected with pSV2TK alone (panel C) did not express the p37mos doublet with or without dexamethasone treatment. In this experiment, equal numbers of cells from an M-MSV 124 virus-transformed 3T3 cell line were labeled and immunoprecipitated in parallel with the transfectants (panel S). The amounts of immunoprecipitable p37mos in all of the steroid-induced transfectants were quite low and comparable to or slightly greater than the amounts found in the virus-transformed cell line. The levels of p37mos were not dexamethasone inducible in the M-MSV 124 virus-transformed 3T3 cell line (data not shown).

Induction of p37mos correlates with changes in cell morphology. The same cells used for the immunoprecipitation experiment shown in Fig. 2 were photographed after 4 days of treatment with either control HAT medium or HAT medium containing 10^{-6} M dexamethasone. These photographs (Fig. 3) demonstrate that dexamethasone-induced morphological changes in pMTVmos64 transfectants correlate with the induction of p37mos. The morphological changes produced by dexamethasone induction, although

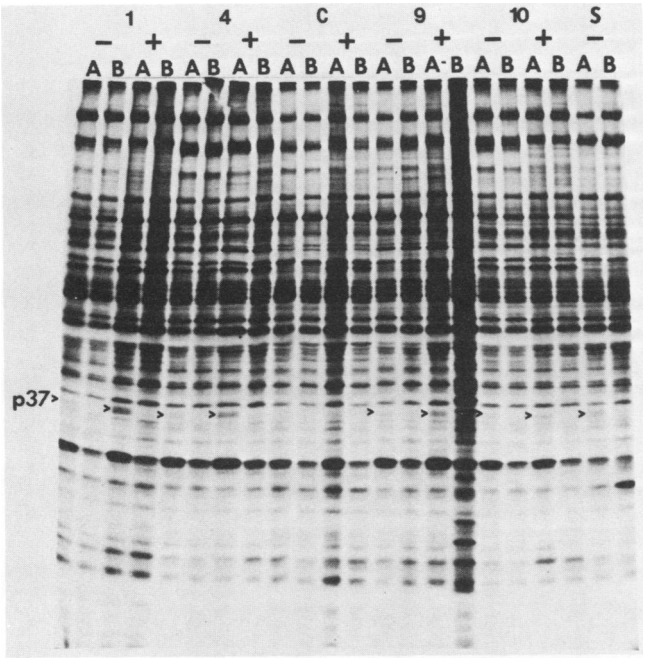


FIG. 2. Immunoprecipitation of extracts from pMTVmos64 transfectants. After being photographed, the same cells shown in Fig. 3 were labeled with [^{35}S]methionine for 4 h and immunoprecipitated with anti-C3 serum as described in the text. Two equivalent samples of a lysate from each cell type were immunoprecipitated with either 2 μl of anti-C3 serum (lanes A) or 2 μl of anti-C3 serum preabsorbed with 2 μg of C3 peptide (lanes B). One-half of each immunoprecipitate was analyzed on a 12.5% polyacrylamide gel. The gel was fluorographed and exposed to film for 3 days. Panels 1, 4, 9, and 10, pMTVmos64 transfectants; panel C, PSV2TK transfectant; +, cells treated with dexamethasone; −, untreated cells; panel S, immunoprecipitates of the stable M-MSV 124 virus-transformed cell line. The p37mos doublet is marked in each case with an arrow.

somewhat varied among the individual transformants, were characteristic of M-MSV 124 virus-transformed 3T3 cells. There appeared to be some correlation between the extent of morphological change and the level of p37mos produced since the clones containing higher levels of p37mos in the presence of dexamethasone (e.g., clone 1) exhibited a more dramatic alteration in morphology. The presence of low basal levels of p37mos in the pMTVmos64 transfectants did not appear to be sufficient to produce morphological changes. The control pSV2TK transfectants which did not express p37mos also did not respond morphologically to dexamethasone treatment.

The photographs shown in Fig. 3 give the impression that p37mos induction inhibited cell growth. This is not generally the case as determined by quantitation of cell numbers (data not shown). The apparent discrepancy can be accounted for by the observation that dexamethasone caused ca. a 1.5-fold decrease in cell number, irrespective of v-*mos* expression; also, cells with transformed morphologies were more rounded and therefore appeared to be less confluent. Despite these differences in cell number, Fig. 3 clearly demonstrates that dexamethasone induction of p37mos correlated with striking changes in cell morphology.

Induced changes in cell morphology are reversible. Figure 4 demonstrates that the morphological changes produced upon dexamethasone induction of p37mos could be reversed by the removal of dexamethasone. In this experiment, the 64-4 transfectants were treated with hormone for 2 days to induce the characteristic morphological changes. After 2 days of treatment, the cells were photographed, rinsed several times, and fed with standard HAT medium. Within 2 days after dexamethasone removal, the cells began to revert, and by 5 days, the cells appeared to be morphologically similar to a parallel culture of 64-4 cells grown for the entire 7 days in the absence of dexamethasone. Thus, continued expression of p37mos at the hormonally induced level was necessary for maintenance of the transformed phenotype.

Induction of p37mos correlates with ability to form foci in monolayers. Since induction of p37mos in the pMTVmos64 transfectants correlated with morphological changes, it was of interest to examine whether other phenotypic aspects of transformation could be modulated in a similar fashion. In the experiment shown in Fig. 5, the 64-1 and 64-4 pMTVmos64 transfectants were tested for their ability to form foci in monolayers in the presence and absence of dexamethasone. In this experiment, a small number of 64-1 or 64-4 cells (ca. 10^2) were mixed with an excess of uninfected NIH 3T3 cells. Each mixture was plated in a 9-cm dish with either standard medium or medium containing 10^{-6} M dexamethasone. At 14 days, the dishes were scored for foci. The dishes grown in the absence of dexamethasone were devoid of foci, but those grown with dexamethasone contained numerous foci, presumably due to the induction of p37mos in the 64-1 and 64-4 transfectants (Fig. 5). The uninfected NIH 3T3 cells or the 3T3TK$^-$ cells did not form foci in response to dexamethasone treatment (data not shown).

Induction of p37mos in pMTVmos64 transfectants is not sufficient to allow growth in soft agar. The 64-1 and 64-4 pMTVmos 64 transfectants, the pSV2TK transfectants, and the stable M-MSV 124 virus-transformed 3T3 cell line were seeded into 0.35% agarose with and without 10^{-6} M dexamethasone as outlined above. Within 7 to 10 days, both the dexamethasone-treated and untreated M-MSV 124 virus-transformed 3T3 cells had formed numerous colonies (see Fig. 6). The dexamethasone did not appear to have any significant effect on the number, size, or morphology of the colonies. In contrast, none of the transfected cells were able to grow in soft agar either with or without dexamethasone (see Fig. 6), even after 1 month. To test whether the 3T3TK$^-$ cells used for the original pMTVmos64 transfections and the resulting transfected cell lines were capable of growing in soft agar under conditions of M-MSV 124 virus transformation, these cells were newly infected with M-MSV 124 virus and challenged for growth in agar. In this experiment, the original 3T3TK$^-$ cells and the 64-4 transfectants were infected with M-MSV 124 virus, and 4 days postinfection, they were seeded in soft agar in parallel with the stable M-MSV 124 virus-transformed 3T3 cell line and the uninfected 64-4 transfectants. Within 10 days, both of the newly infected cell populations and the stable M-MSV 124 virus-transformed cells produced colonies in agar (Fig. 6), with and without dexamethasone. As before, the 64-4 transfectants were completely negative for growth in agar and the stable M-MSV 124 virus-transformed 3T3 cell line formed numerous colonies (Fig. 6). Immunoprecipitation of extracts from the cells used for the agar assay (4 days postinfection) showed that, as expected from previous experiments (5, 6), the newly infected cells had elevated levels of p37mos when compared with either the 64-4 transfectants (Fig. 7) or the stable M-MSV 124 virus-transformed cell line (data not shown). These data suggest that a higher level of p37mos

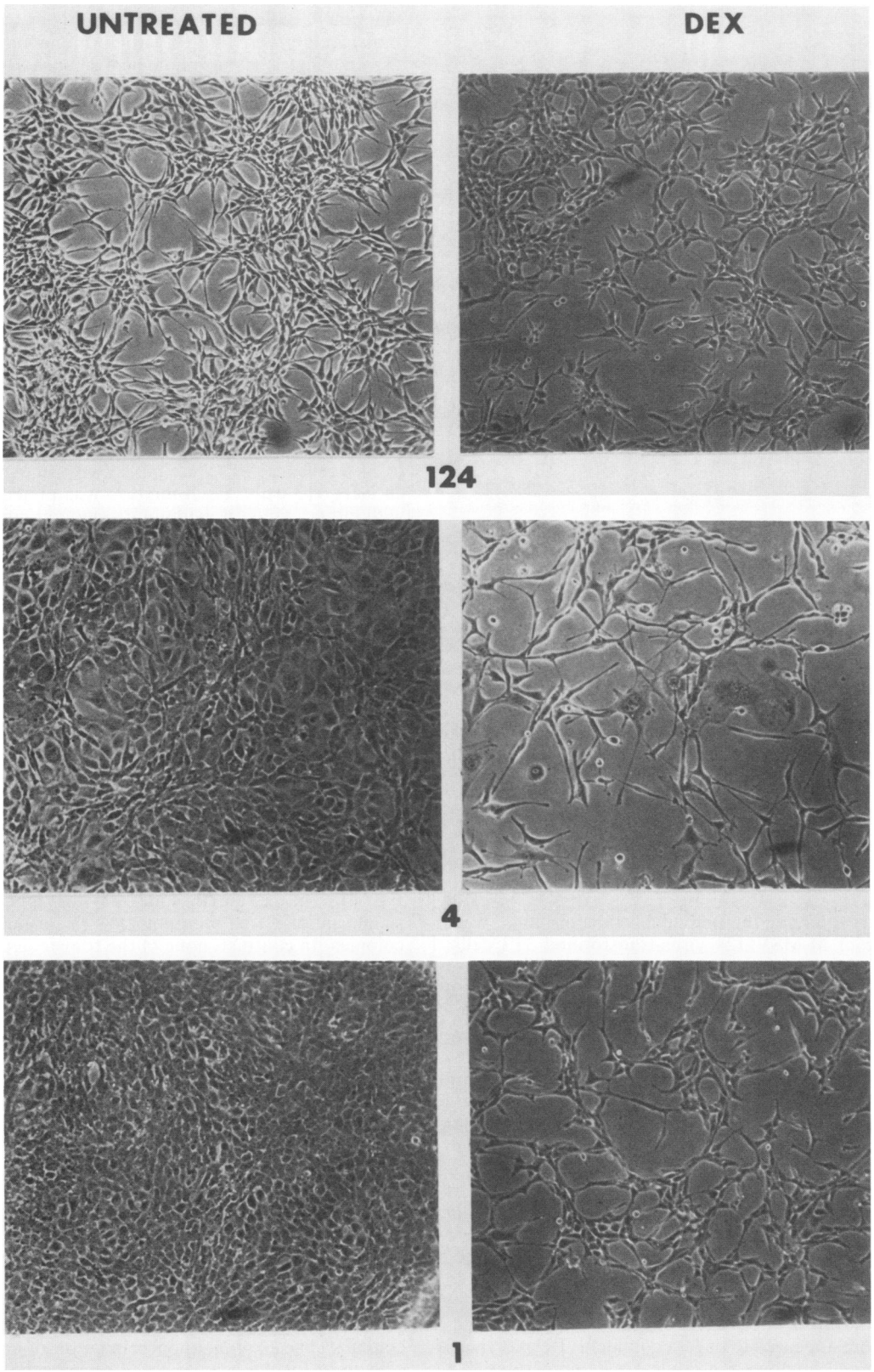
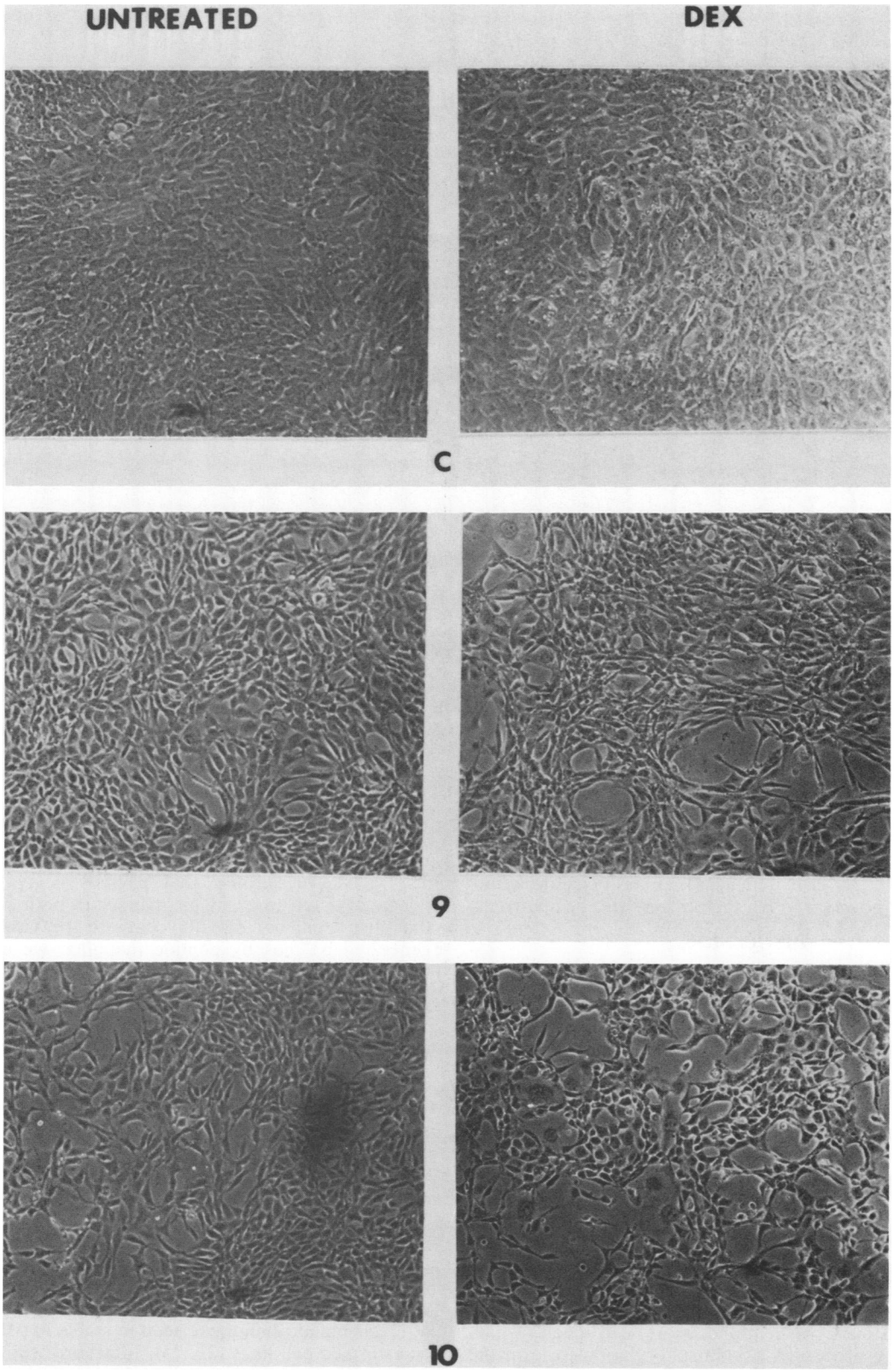


FIG. 3. Photographs of cells grown with and without dexamethasone. The pMTVmos64 transfectants as well as a PSV2TK transfectant were grown in 5-cm dishes in either HAT medium or HAT medium plus 10^{-6} M dexamethasone. The M-MSV 124 virus-transformed NIH 3T3 cell line was grown in either standard medium or standard medium plus 10^{-6} M dexamethasone. After 4 days, the cells were photographed (magnification, ×65). Panels 1, 4, 9, and 10, pMTVmos64 transfectants; panel C, PSV2TK control transfectant; and panel 124, M-MSV 124 virus-transformed NIH 3T3 cell line.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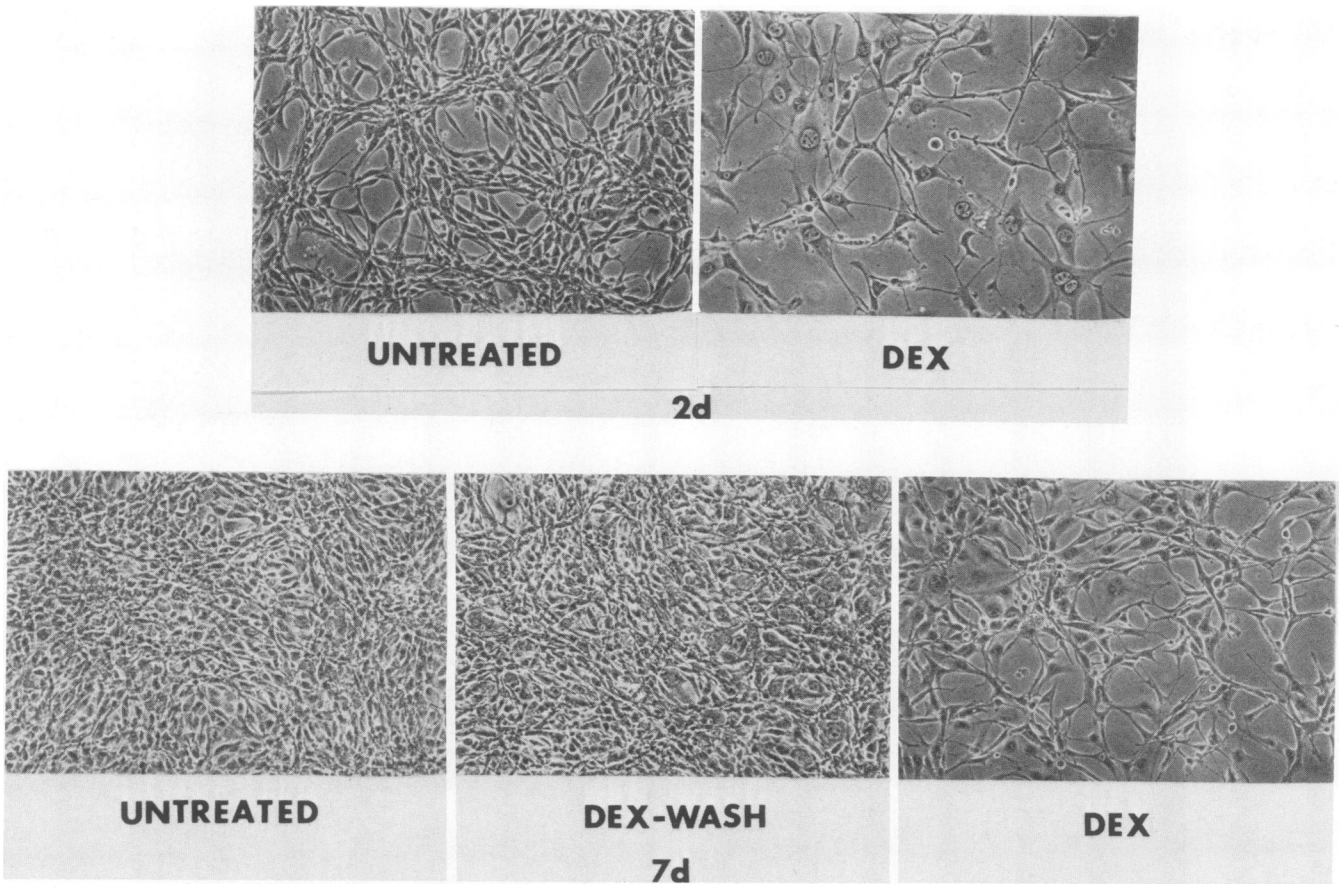

FIG. 4. Reversion of induced changes in cell morphology. 64-4 transfectants were plated in 5-cm dishes and treated for 2 days with either HAT medium or HAT medium plus 10^{-6} M dexamethasone as described in the text. After 2 days, the cells were photographed (top two panels), and one dish of the dexamethasone-treated cells was washed several times and refed with HAT medium lacking dexamethasone. At 7 days, the cells were photographed (center bottom panel [DEX-WASH]). Also photographed were cells treated with dexamethasone for the entire 7 days (right bottom panel) and cells treated with HAT medium alone for 7 days (bottom left panel).

might be required for growth in agar than for focus formation in monolayers; however, other factors may also be involved.

DISCUSSION

We have constructed a recombinant DNA vector, pMTVmos64, in which the MMTV LTR is fused to the coding region for the oncogene, v-*mos*. Upon transfection into 3T3 cells, the intracellular levels of the viral oncogene product, p37mos, can be modulated by dexamethasone. This system permits various transformation-specific phenotypic parameters to be examined as a function of intracellular levels of p37mos.

Immunoprecipitation of extracts from [^{35}S]methionine-labeled cells revealed that several individual pMTVmos64 transfectants expressed steroid-inducible p37mos. The p37mos immunoprecipitated from both uninduced and dexamethasone-induced cells appeared as a doublet and migrated with the same apparent molecular weight as p37mos immunoprecipitated from M-MSV 124 virus-transformed NIH 3T3 cells did. Previous experiments showed that the lower band of the p37mos doublet is an unphosphorylated species which is subsequently processed by phosphorylation to form the upper band of the doublet (25). The half-life for this processing is ca. 3 h. Since the ratio of the two species of the p37mos doublet appears to be the same in the pMTVmos transfectants after 4 h as it does in M-MSV 124 virus-transformed 3T3 cells, we surmise that p37mos is synthesized and phosphorylated in a similar manner in both situations. Although we did not directly compare the viral and vector-generated p37mos by peptide mapping, we can infer that these proteins must have the same sequence, since they have the same apparent molecular weight and are both recognized by an antiserum directed against the 12 COOH-terminal amino acids predicted from the v-*mos* DNA sequence.

The induced levels of p37mos detected in the transfectants are comparable to the levels of p37mos seen in the stable virus-transformed NIH 3T3 cell line. Since p37mos constitutes ca. 0.0005% of total cellular protein in the virus-transformed cells (25), it must also be expressed at a similarly low level in the transfectants. This low level of expression in the transfectants is not unexpected since the MMTV LTR is known to be a weak promoter in transfection experiments (14; Ringold, unpublished data). Furthermore, it is possible that transfectants could not be generated which express higher levels of p37mos, since previous acute infection experiments suggested that p37mos is lethal to 3T3 cells at levels higher than those seen in stable M-MSV 124 virus-transformed cell lines (22, 25). In accord with this idea, we attempted to coamplify the pSV2TK and pMTVmos DNAs in the HAT-selected pMTVmos64 transfectants by growing the cells in limiting concentrations of thymidine. However, all of the cells surviving this selection were devoid of

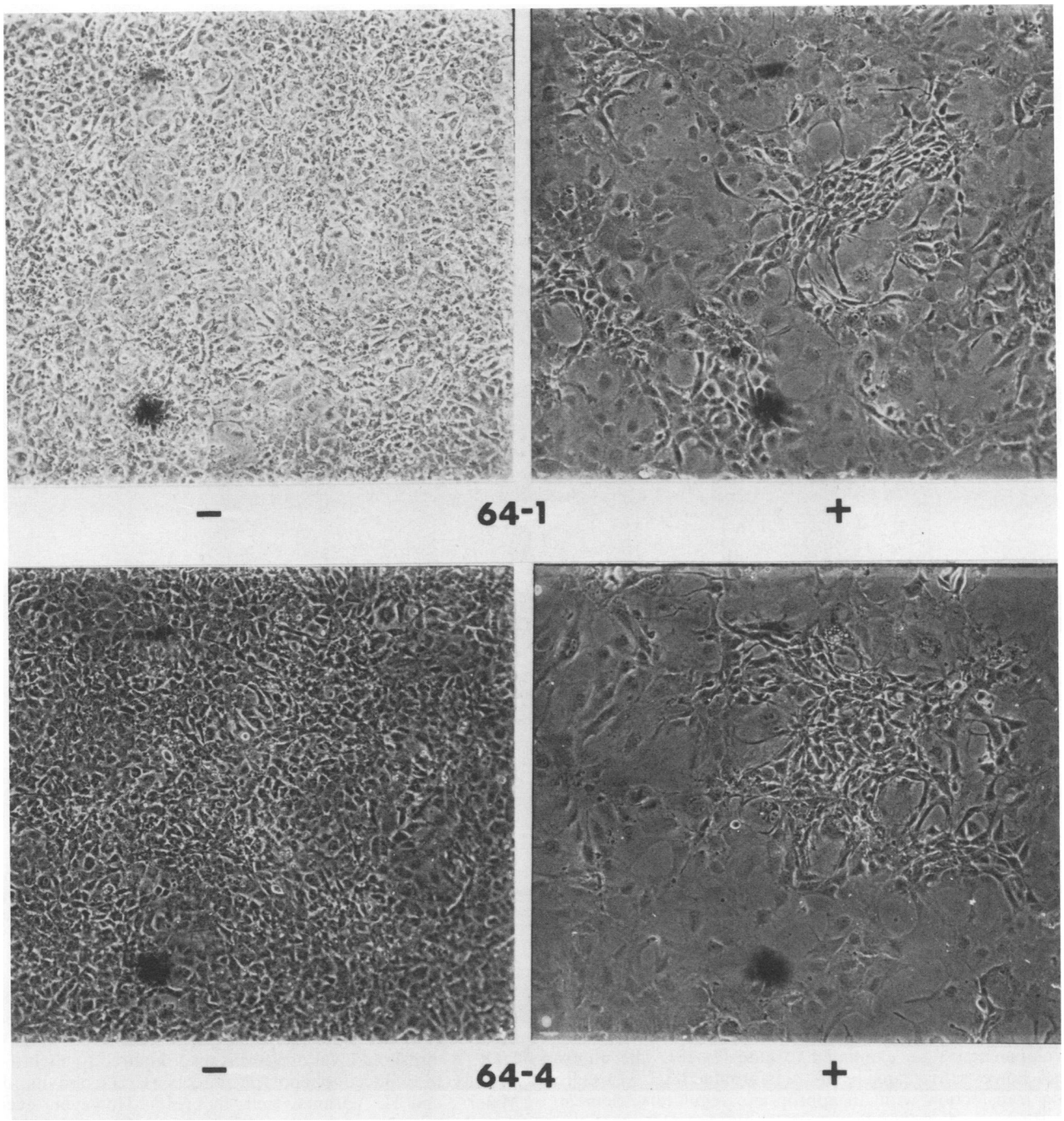

FIG. 5. Focus formation in monolayers. As outlined in the text, the 64-1 and 64-4 transfectants were challenged for their ability to form foci in monolayers. Ca. 10^2 64-1 or 64-4 cells were mixed with ca. 10^5 uninfected NIH 3T3 cells and plated in 9-cm dishes containing medium with or without dexamethasone. The photographs were taken at 2 weeks (magnification, ×65). −, Results obtained in the absence of dexamethasone; +, results obtained when the assay was carried out in the presence of 10^{-6} M dexamethasone.

immunoprecipitable p37mos (data not shown). This result could be explained if the expression of p37mos was inactivated to allow the survival of cells containing amplified pSV2TK plasmids.

Although high levels of p37mos may not be tolerated in the 3T3 transfectants, it appears that a certain basal level is necessary to produce morphological changes characteristic of M-MSV transformation. All of the pMTVmos64 transfectants express trace levels of p37mos in the absence of dexamethasone induction. This basal level is ca. 5- to 10-fold less than that seen in the same dexamethasone-induced cells or the virus-transformed 3T3 cell line. It is significant to note that despite low basal synthesis of p37mos, the transfectants do not respond with transformation-specific morphological

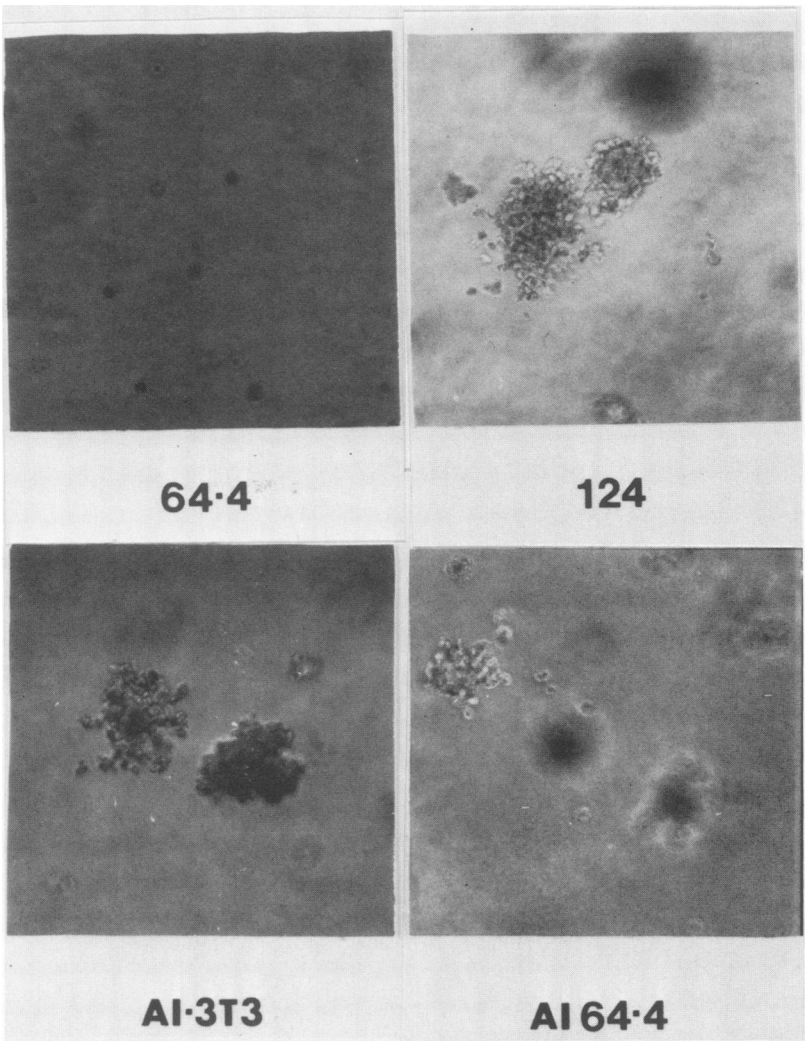

FIG. 6. Soft agar assays. The soft agar assays and acute infections were carried out as described in the text. Representative colonies in soft agar were photographed at 10 days. Since the colonies formed with and without dexamethasone were equivalent, only the colonies formed in the presence of dexamethasone are shown. Panels 64-4 and 124, Uninfected pMTVmos64 transfectants and the M-MSV 124 virus-transformed 3T3 cell line, respectively; panels AI3T3 and AI64-4, newly infected 3T3 and pMTVmos64 cell lines, respectively (magnification, ×65).

changes and do not form foci in monolayers or in soft agar. Unlike other protooncogenes, the cellular homolog of v-*mos* is not known to be expressed in any tissue, cell type, or developmental state examined to date (7, 18). The c-*mos* gene, however, is capable of transforming NIH 3T3 cells upon transfection with an appropriate regulatory element (3). Based on these findings, one could speculate that the normally silent *mos* gene is oncogenic whenever it is expressed out of context. However, the dosage effect described in this report implies that oncogenic transformation by v-*mos* is not simply the result of expression of a normal cellular product in an inappropriate context.

The mechanism of transformation by v-*mos* is further addressed by the experiment which shows that the v-*mos*-induced morphological changes can be reversed upon removal of the dexamethasone. This observation suggests that p37mos is required for both the maintenance and initiation of transformation. Previous experiments with *ts*110 MSV, an M-MSV variant which encodes a *gag-mos* fusion protein ($p8S^{gag\text{-}mos}$), suggested that $p85^{gag\text{-}mos}$ was necessary for the maintenance of the transformed phenotype (2, 9, 10, 20).

The results discussed above are comparable in some respects with the results of similar experiments performed by others with the v-*ras* and v-*src* genes fused to the MMTV LTR. A threshold level of v-*src* is also required to elicit the various *src*-induced phenotypic effects (E. Jacobovits, J. Majors, and H. Varmus, Cell, in press). However, even though Rous sarcoma virus-transformed cells contain relatively high levels of v-*src*, the same transformation-specific phenotypes could be obtained with much lower levels of v-*src* expression. This is unlike the situation observed for v-*mos* which is never seen at high levels in transfectants or virus-transformed cell lines (22, 25; Papkoff, unpublished observations). One can infer from the v-*ras* experiments that a threshold level of v-*ras* is also needed to produce morphological changes (11).

Since the morphologies of the pMTVmos transfectants could be modulated by changes in intracellular levels of p37mos, it was of interest to see whether other transformation-specific parameters responded in a dosage-dependent fashion. We found a correlation between the morphological changes induced by p37mos and the ability of the transfec-

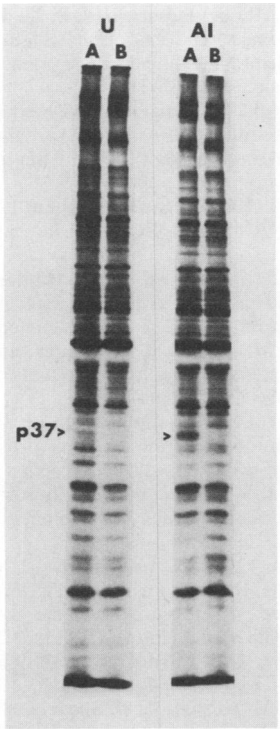

FIG. 7. Immunoprecipitation of extracts from pMTVmos64 transfectants after acute infection. The pMTVmos64 transfectants and acutely infected pMTVmos64 transfectants used for the soft agar assay (Fig. 6) were plated in 5-cm dishes (on day 4 postinfection) and treated for 2 days with dexamethasone. These cells were labeled with [^{35}S]methionine for 4 h and immunoprecipitated with anti-C3 serum as described in the text. Two equivalent samples of a lysate from each cell type were immunoprecipitated with either 2 μl of anti-C3 serum (lanes A) or 2 μl of anti-C3 serum preabsorbed with 2 μg of C3 peptide (lanes B). One-half of each immunoprecipitate was analyzed on a 12.5% polyacrylamide gel. The gel was fluorographed and exposed to film for 1.5 days. Panel U, Uninfected pMTVmos64 transfectant; panel AI, acutely infected pMTVmos64 transfectant. The position of p37mos is marked in each panel with an arrow.

tants to form foci in monolayers. The 64-1 and 64-4 cells cannot form foci unless p37mos is induced with dexamethasone (Fig. 5). On the other hand, these transfectants will not grow in soft agar, with or without dexamethasone, under conditions which support growth of the M-MSV 124 virus-transformed 3T3 cell line. We did not assess the ability of the transfected cells to form tumors in glucocorticoid-treated nude mice. Considering the negative results in agar, we would not predict tumor formation; however, these experiments would be interesting to pursue in future studies. There are several possible explanations for the apparent paradox that the dexamethasone-induced pMTVmos transfectants do not grow in soft agar although the MMSV 124 virus-infected cell line, which expresses comparable levels of p37mos, is capable of growing under the same conditions in soft agar. Since transforming growth factors (TGFs) are known to play a crucial role in the induction of growth in agar (5, 12, 13, 19), it is possible that the inability of the pMTVmos64 transfectants to grow in agar could be a consequence of insufficient TGF production or an insensitivity of this particular line of 3T3 cells to the effects of TGFs. The latter possibility seems unlikely since these cells respond with changes in morphology and increased 2-deoxyglucose uptake after treatment with conditioned medium containing TGFs from the M-MSV 124 virus-transformed 3T3 cell line (data not shown). Since the 3T3TK$^-$ cell line and its transfected descendants can be induced to grow in agar by infection with M-MSV 124 virus, it is possible that in addition to a crucial intracellular level of p37mos, other factors associated with virus infection are necessary for full conversion to the malignant phenotype. The nature of these other factors associated with virus infection is unclear; however, elevated levels of v-*mos* itself, alterations in sensitivity to the biochemical consequences of v-*mos* expression, or changes in growth factor production or sensitivity may be involved.

Despite sequence homology between v-*mos* and other retroviral oncogenes which encode tyrosine protein kinases (1, 8, 26), the biochemical function of p37mos is still unknown. However, the experiments presented in this paper do provide some insight into the mechanism of p37mos-induced transformation. These experiments suggest that a critical level of p37mos is required for both the initiation and maintenance of the transformed phenotype. This threshold level varies somewhat, depending on the phenotypic characteristic examined, and is likely to vary, depending on the cell type analyzed. The pMTVmos64 transfectants described here should be useful for the study of the biochemical activities and effects of p37mos once a function for this protein has been found.

ACKNOWLEDGMENTS

We thank Tony Hunter and Inder Verma for providing cells and recombinant DNAs. We thank Harold Varmus for advice and discussions, Paul Kaplan for advice on the soft agar assays, and Mark Danielsen for his comments on the manuscript.

This research was supported by Public Health Service grant GM25821 from the National Institutes of Health. J.P. was a fellow of the American Cancer Society and the National Institutes of Health during the course of this work. G.M.R. is an Established Investigator of the American Heart Association.

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