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# Differential Transformation of C3H10T1/2 Cells by v-*mos*: Sequential Expression of Transformation Parameters

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**Extremely small quantities of the product of the transforming gene v-*mos* of Moloney murine sarcoma virus are able to efficiently transform cells. Recent data indicate the existence of a threshold level for v-*mos* transformation of NIH3T3 cells. Using mouse mammary tumor virus long terminal repeat sequences or hybrid promoters consisting of mouse mammary tumor virus and Moloney murine sarcoma virus long terminal repeat elements to express v-*mos* in C3H10T1/2 cells, we established cell lines representing different stages of morphological transformation in vitro. The threshold level for v-*mos* transformation was considerably lower than that for NIH3T3 cells, because no treatment with dexamethasone or primary selection other than transformation was necessary during standard transfection procedures. Using the cell lines mentioned we established an association of the level of v-*mos* expression with the transformation parameters examined, but not with p53 levels. Furthermore, the characterization of the different promoters showed (i) that the distal binding site confers hormone responsiveness to Moloney murine sarcoma virus promoter elements and (ii) that artifactual transcription initiation sites can be detected in mouse mammary tumor virus-Moloney murine sarcoma virus hybrid promoters which are, however, not regulated by the hormone.**

Transformation of cells in vivo leading to the appearance of tumors is considered to be a multistep process. By analysis of a large number of tumors and tumor-derived cell lines the involvement of oncogenes in one or another of the stages of the process of carcinogenesis has been suggested (26, 41). Among these genes several members of the *ras* proto-oncogene family (8) might play a role in the final stage of the multistep process, because the biological activity of mutated *ras*-type oncogenes could efficiently be detected in in vitro transformation assays using NIH3T3 mouse fibroblasts as recipients (3). Other oncogenes like c-*myc* (6) do not morphologically transform NIH3T3 cells, although v-*myc* can transform these cells (46). However, the activity of the c-*myc* gene has been shown to be necessary for stable transformation of primary fibroblasts by the mutated *ras* oncogene (26) and was able to confer tumorigenicity to NIH3T3 cells (25), and elevated levels of *myc* RNA were detected in several tumors (7, 17). Also, several stages of morphological transformation in vivo of cells by oncogenes might not be represented by the NIH3T3 transfection assays employed. From results of recent studies it can be concluded that NIH3T3 cells, often used in transfection experiments, exhibit a particular threshold level below which no transformation is apparent (37). We examined the possibility to use C3H10T1/2 mouse fibroblasts as recipient cells in transfection experiments designed to obtain transformed cells of differential phenotype. C3H10T1/2 cells have been used widely for transformation studies by chemicals and x rays (18). Such cell lines might allow the examination of several of the parameters associated with different stages of in vitro and in vivo transformation (14, 28, 42). For this purpose we used the v-*mos* gene (43), because it was shown that extremely small amounts of $p37^{mos}$ are sufficient to transform NIH3T3 cells (36). To control its expression the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) (11) or parts of it were used. The promoter in this LTR is regulated by upstream sequences which confer hormone responsiveness (4, 5, 29). In the absence of hormone only low base levels of transcription were found. The level of transcription can be induced manyfold by the addition of glucocorticoid hormones. To obtain promoters exhibiting transcriptional activity over a wider range, parts of the MMTV LTR were deleted and replaced by parts of the Moloney murine sarcoma virus (M-MSV) LTR. This allowed construction of two hybrid promoters, one of which appeared to be transcriptionally weaker than the MMTV promoter in the absence of hormone. C3H10T1/2 cells could be transformed by all constructs, and the DNA and RNA specific for v-*mos* were analyzed in all transformants. Depending on the amount of v-*mos* product, different stages of morphological transformation could be observed, and several transformation parameters and their association to v-*mos* expression could be determined.

## MATERIALS AND METHODS

**Cells.** C3H10T1/2 cells (38) were grown in minimal essential medium supplemented with 10% heat-inactivated fetal calf serum (GIBCO Laboratories) and antibiotics. Transfections were performed by the calcium phosphate coprecipitation method (15) as described previously (44). Foci of transformed cells were picked after various times (2 to 4 weeks) with cloning cylinders and single-cell cloned in microtiter wells. Single-cell clones were grown and used for analyses. When indicated, cells were treated for 72 h with $10^{-6}$ M dexamethasone. Cells were photographed with a Leitz phase-contrast microscope equipped with a Leitz camera on Ilford FP-4 film at ×100 magnification. The soft agar assay was performed as described previously (37). For determination of p53, lysates were prepared from cells labeled with [$^{35}$S]methionine, and p53 was immunoprecipitated with the monoclonal antibody RA3-2C2 (39) and protein A-Sepharose. Analysis of precipitates on sodium dodecyl sulfate-polyacrylamide gels was as described previously (45). The amount of $p37^{mos}$ in cell lines was determined as described previously for p53 with anti-C3 antiserum (36).

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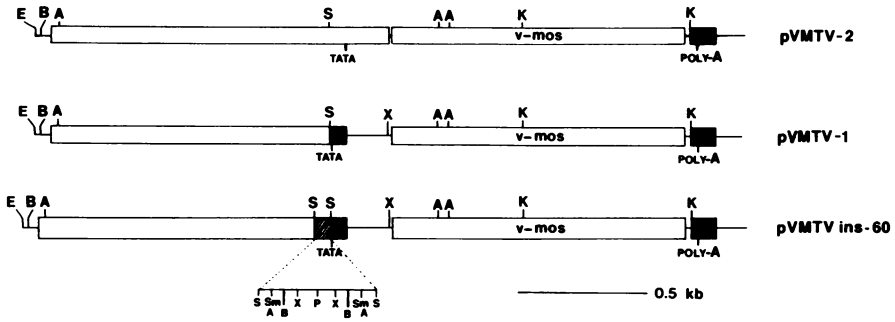


FIG. 1. All plasmid constructs used contained as vector the pBR322 *Eco*RI-*Pst*I fragment, which conferred tetracycline resistance. The constructs, described in detail in the text, contained different promoter elements, the v-*mos* oncogene, and a viral polyadenylation signal isolated from the M-MSV LTR as indicated by the black box. The promoter elements were taken from the MMTV LTR (unlabeled white box), the M-MSV LTR (black box), and a bacteriophage M13mp11 fragment (hatched box). TATA and poly-A denote, respectively, the positions of the TATA box in the promoters and the polyadenylation signal that was used. Abbreviations: A, *Ava*I; B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; *P*, *Pst*I; *S*, *Sac*I; Sm, *Sma*I and X, *Xba*I.

**v-*mos* Constructs. (i) pVMTV-2.** Plasmid pVMTV-2, called p-2, contained as an insert in the pBR322 *Eco*RI-*Pst*I sites the following different elements: a complete MMTV LTR present on a 1.6-kilobase (kb) *Bgl*II-*Pvu*II fragment (11), the v-*mos* coding sequences located on a 1.16-kb *Xba*I-*Hin*dIII fragment isolated from M-MSV DNA (43), and the viral polyadenylation site present on a 0.5-kb *Sma*I-*Pst*I fragment (see Fig. 1) also isolated from M-MSV DNA.

**(ii) pVMTV-1.** Plasmid pVMTV-1, called p-1, was identical to p-2 with one exception. The 0.24-kb *Sac*I-*Pvu*II fragment of the MMTV LTR was replaced by a similarly sized *Sac*I-*Xba*I fragment, which contained a 60-base-pair (bp) *Sac*I-*Sma*I fragment harboring the M-MSV TATA box and viral cap site (43) (see Fig. 1) and a 170-bp *Bgl*II-*Xba*I fragment harboring the sequences which flank v-*mos* in M-MSV.

**(iii) pVMTV-ins60.** Plasmid pVMTV-ins60, called p-ins60, was derived from plasmid p-1. A *Pst*I-*Sac*I fragment was isolated from the synthetic linker present in bacteriophage M13mp11 DNA (33) and self-ligated generating a 60-bp *Sac*I-*Pst*I-*Sac*I fragment. The latter fragment was inserted in the unique *Sac*I site of plasmid p-1 (see Fig. 1).

**DNA and RNA analysis.** High-molecular-weight DNA was isolated and analyzed by Southern blotting as described previously (44). For hybridization of Southern blots we used two different probes nick-translated as described previously: a 1.45-kb *Pst*I fragment harboring the MMTV LTR sequences (11) (see Fig. 3) and a 1.16-kb *Xba*I-*Hin*dIII fragment containing *mos*-specific sequences (see Fig. 3). Cytoplasmic RNA was isolated from cell cultures by the method of Maniatis et al. (30). S1 nuclease mapping of RNA was performed with 5'-end-labeled probes as described previously (23). The protected fragments were separated on 5% urea-polyacrylamide gels and visualized by autoradiography on Kodak XAR films.

## RESULTS

**Features of the contructs used in transformation assays.** The elements in the MMTV LTR which confer hormone responsiveness to the promoter have recently been characterized by deletion studies (4, 29) and by hormone receptor DNA binding experiments (40). From the deletion experiments it was concluded that sequences to −204 (relative to the cap site) can be deleted without impairment of hormonal response. The protein DNA binding studies established the presence of a distal and a proximal receptor binding site located between positions −192 and −163, and −124 and −71. In construct p-2 (Fig. 1), a *Bgl*II-*Pvu*II fragment containing the complete MMTR LTR (11) was employed. The v-*mos* oncogene was present on a 1.16-kb *Xba*I-*Hin*dIII DNA fragment, and the polyadenylation site was provided by a 0.51-kb *Kpn*I-*Pst*I fragment isolated from M-MSV DNA which contained $U_5$ sequences of the M-MSV LTR (43). For construction of p-1 (Fig. 1), the MMTV LTR sequences from the *Sac*I site (position −108) to the *Pvu*II site were replaced by a DNA fragment of identical size containing M-MSV LTR sequences from the *Sac*I site (position −36) to the *Sma*I site (position +28) linked to an 170-bp DNA fragment which normally preceeds v-*mos* in M-MSV. Therefore, p-1 and p-2 differed in several respects: (i) p-2 contained the complete proximal receptor binding site located between −124 and −71 in the MMTV LTR while p-1 contained a truncated version of it; (ii) in p-1 the remaining hormone regulatory sequences were located 68 bp closer to the TATA box (5 bp upstream) than in p-2 (Fig. 1); and (iii) MMTV sequences between −108 and +134 which might be important for efficient transcription are present in p-2 but deleted in p-1. The difference in distance between p-2 and p-1 mentioned under (ii) might cause a spacing problem between the hormone receptor complex and RNA polymerase II, leading to a loss of hormone response (29). Therefore, p-ins60 (Fig. 1) was constructed. p-ins60 was derived from p-1 by the insertion of a 60-bp *Sac*I-*Pst*I-*Sac*I palindromic DNA fragment prepared from an isolated bacteriophage M13mp11 *Sac*I-*Pst*I fragment (33). As a result of this insertion the distance between the hormone regulatory sequences and the TATA box in p-ins60 is only eight nucleotides shorter than in the natural situation. Both p-1 and p-ins60 contained the polyadenylation site, also used in p-2 (Fig. 1).

**Morphological transformation of cells with plasmid DNAs.** C3H10T1/2 fibroblasts were transfected with the constructs shown in Fig. 1, using the calcium phosphate coprecipitation method, in the absence of dexamethasone. The transfection efficiencies for p-1, p-ins60, and p-2 were approximately 25, 100, and 110 foci per μg of plasmid DNA, respectively. It is important that (i) no selection other than transformation was used in contrast to cotransfection type experiments that have been described by several other groups (19, 35, 37) and (ii) transfected C3H10T1/2 cells could be maintained as confluent cultures up to 6 weeks after transfection, which allowed the isolation of slowly growing foci consisting of cells with minimally transformed morphologies. In all cases

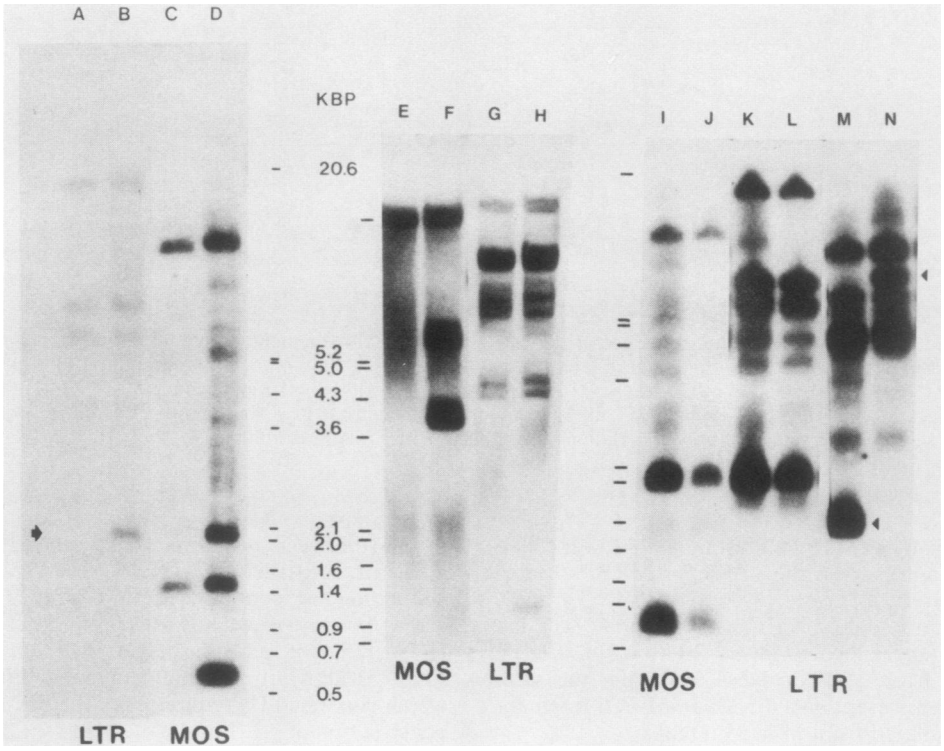


FIG. 2. Plasmid DNA in transformed cells. DNA, isolated from the normal or the transformed C3H10T1/2 cells, was examined by restriction analysis and hybridization to specific DNA probes. The probes used were a fragment specific for the MMTV LTR (LTR) and a fragment specific for the *mos* sequences (MOS). The restriction enzymes used in the analysis were *Kpn*I-*Eco*RI (lanes A, B, C, D, I, J, K, and L), *Bam*HI (lanes E, F, G, and H), and *Eco*RI-*Xba*I (lanes M and N). Sources of the DNA were C3H10T1/2 cells (lanes A, C, E, and G), p-2-transformed cells (lanes J, L, and N), p-1-transformed cells (lanes B, D, I, K, and M), and p-ins60-transformed cells (lanes F and H).

transformed foci were isolated in the absence of dexamethasone, single-cell cloned in microtiter wells, and grown for analyses of DNA and RNA content and morphology. We observed that a particular construct always led to the appearance of transformed cells with the same morphology, which was stable upon single-cell cloning. Thus, foci of p-1 transformants appeared late, and single p-1-transformed cells had normal morphology and they might represent a minimal transformed stage. In contrast, p-ins60 transformants were spindle-shaped, and p-2 transformants had morphologies intermediate between normal and spindle shaped. The effect of hormone treatment of these cell cultures was examined. Within 72 h after the addition of dexamethasone to p-2 transformants, all cells became round and spindle shaped and were highly refractile, the effect of which was reversible. The washing of hormone-treated cells led to a reversion of the transformed to the intermediately transformed morphology within 72 h. In contrast, p-1 and p-ins60-transformed cells did not show any change of cell morphology on the addition of dexamethasone even after prolonged incubation with the hormone.

**Plasmid DNA in transformed cells.** For DNA analysis of the plasmids in the transformants we chose restriction enzymes which would generate fragments of sizes which (i) can be detected with labeled MMTV LTR and *mos*-specific DNA probes and (ii) can be distinguished from endogenous hybridizing DNA fragments. For each plasmid transfection several independent transformants were analyzed and gave identical results. The results for one of each such transformants are shown in Fig. 2.

C3H10T1/2 cells contain three endogenous MMTV proviruses (16) which on cleavage with *Bam*HI (Fig. 2, lane G) or with *Kpn*I-*Eco*RI (Fig. 2, lane A) give rise to six fragments, as recognized with a probe specific for the MMTV LTR. Similarly, the unique endogenous c-*mos* gene in C3H10T1/2 cells, recognized with a *mos*-specific DNA probe, generates one fragment of 21 kb on cleavage with *Bam*HI (Fig. 2, lane E) and two fragments of 11.5 and 1.5 kb on cleavage with *Kpn*I-*Eco*RI (Fig. 2, land C) (23). For analysis of p-1 and p-2 transformants the enzyme combination *Kpn*I-*Eco*RI was used, and the fragment of 1.95 kb, expected to be recognized both by the MMTV LTR and the *mos*-specific probes, was detected (Fig. 2, lanes B and K, and lane L, respectively). In addition the *mos* probe identified the expected 0.65-kb fragment (Fig. 1) in p-1 (Fig. 2, lanes D and I) and p-2 transformants (Fig. 2, lane J). p-1 and p-2 cells were distinguished with the enzymes *Xba*I-*Eco*RI, because the *Xba*I site is only present in p-1 DNA (Fig. 1). The MMTV LTR-detectable fragments were expected to be larger than 2.5 kb for p-2 DNA and 1.6 kb for p-1 DNA and were indeed detected (Fig. 2, lanes N and M, respectively). p-ins60-specific DNA was identified with *Bam*HI, which was expected to yield a MMTV LTR-specific 1.1-kb fragment (Fig. 1), which was detected (Fig. 2, lane H), and *mos*-specific fragments larger than 3 kb. Two *mos*-specific fragments of 6.5 and 4.0 kb (Fig. 2, lane F) were found, indicating the presence of two p-ins60 copies per cell. Densitometer scanning of these films showed the presence of two, six, and two plasmid copies per cell in p-2, p-1, and p-ins60 transformants, respectively (see Table 1).

**Amount of *mos* RNA and p37$^{mos}$ correlate with transformation stage.** Based on the described morphology of the cell

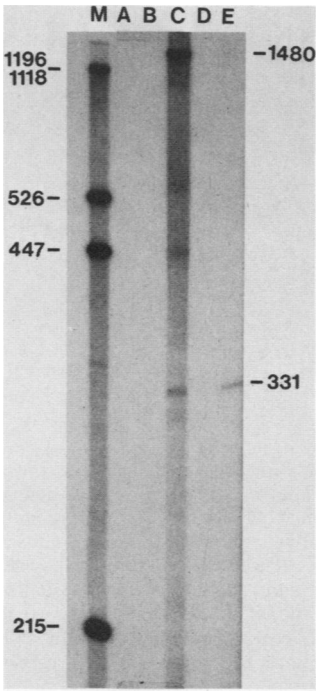


FIG. 3. S1 nuclease analysis of p-2 transcripts. For determination of the RNA initiation site in p-2 and the relative amounts of *mos*-specific RNA in the presence or absence of dexamethasone in p-2 transformants, a 1.48-kb, 5'-end-labeled *Ava*I fragment (Fig. 1) was hybridized to p-2-directed RNA transcripts and digested with S1 nuclease, and the protected fragments were separated electrophoretically on urea-polyacrylamide gels. Sources of the RNA were (i) two independent p-2-transformed cell lines in the absence (lanes B and D) or the presence (lanes C and E) of $10^{-6}$ M dexamethasone and (ii) normal C3H10T1/2 cells (lane A). 3'-end-labeled *Hin*dIII digests of simian virus 40 DNA served as markers (M).

lines and their response to dexamethasone we expected to find hormone-independent amounts of *mos*-specific RNA in p-1- or p-ins60-transformed cells, but increased amounts in dexamethasone-treated, p-2-transformed cells. For determination of the amounts of *mos*-specific RNA, S1 nuclease mapping experiments were performed which allowed the quantitation of the transcripts and establishment of the initiation sites. Two different S1 probes were isolated for this purpose. (i) The 1.48-kb *Ava*I fragment which contained the MMTV LTR and downstream sequences (Fig. 1) was isolated from p-2 DNA, 5'-end-labeled with polynucleotide kinase, and used for characterization of RNA from p-2 transformed cells. (ii) p-1 DNA was digested with *Xba*I, 5'-end-labeled to the same specific activity with polynucleotide kinase, and used for the analysis of RNA from p-1- and p-ins60-transformed cells. Several independent transformants, also used for DNA analysis, were analyzed and gave similar results.

Figure 3 shows the result of RNA analysis of two independent p-2-transformed cell lines. RNA isolated from hormone-treated cell cultures protected a 331-base fragment which had the size expected for proper initiation of transcription at the normal MMTV cap site (11) in plasmid p-2 (Fig. 3, lanes C and E). On overexposure of the same gel the 331-base fragment could also be seen in the nontreated cultures (Fig. 3, lanes B and D). Results of densitometer scanning of such films indicated that the hormone induction was approximately 10-fold in p-2-transformed cells (Table 1). Therefore, the increase in *mos*-specific RNA on the addition of hormone to these cells correlates with the dramatic change in morphology toward a fully transformed one.

RNA from p-1-transformed cells gave rise neither to protection of discrete fragments in the S1 nuclease mapping assay nor to appearance of a discrete band(s) in an RNA size analysis (data not shown). We could however detect extremely low hormone-independent amounts of *mos*-specific RNA in these cells by RNA dot blot analysis (data not shown). Therefore, we conclude that in p-1-transformed C3H10T1/2 cells very low quantities of *mos*-specific RNA, initiated from unidentified sites, are sufficient to cause the minimally transformed morphology of these cells, the phenotype of which is hormone independent, which is in agreement with observations by Huang et al. (19). Figure 4 shows the results of an S1 nuclease mapping analysis of p-ins60 cell RNA. Unexpectedly, three fragments of 224, 200, and 179 bases were protected by the *mos*-specific RNA in these cells (Fig. 4, lanes B and C) but not by RNA of normal C3H10T1/2 cells (Fig. 4, lane A). The 200-base fragment had a size expected for hormone-inducible initiation of transcription at the M-MSV cap site (Fig. 4, lanes B and C). The protection of the 224- and 179-base fragments indicated the use of new cap sites (see Fig. 6 and below).

Based on densitometer scanning of these films we estimated that in p-ins60-transformed cells the RNA species protecting the 224-base fragment was 15 times more abundant than the one protecting the 179-base fragment (Table 1). For comparison of the different strengths of the promoters in the p-ins60 construct to that of a complete M-MSV LTR and for further characterization of the new RNA species in p-ins60-transformed cells, we used the same S1 probe in the analysis of RNA from cells transformed with an M-MSV LTR-v-*mos* construct (pMLVCH-1). The intensity of the protected 200-base fragment (Fig. 4, lane D) suggests that RNA promoted by the M-MSV LTR is approximately 30 times more abundant than the RNA species protecting the 179-base fragment (Table 1). The morphology of the pMLVCH-1 transformants is in agreement with these elevated RNA levels. Cells were round and highly refractile,

TABLE 1. *mos*-RNA expression in transformed cells

| Plasmid | Plasmid copy no. per cell[a] | RNA species[b] | Relative amount of RNA[c] | |
|---|---|---|---|---|
| | | | +dexamethasone[d] | −dexamethasone |
| p-2 | 2 | + 1 | 1.0 | 0.1 |
| p-ins60 | 2 | −23 | 15 | 15 |
| | | + 1 | 1.0 | 0.2 |
| | | +22 | 1.0 | 1.0 |
| p-1 | 6 | ? | 0.05 | 0.05 |
| pMLVCH-1 | 1 | + 1 | ND[e] | 30 |

[a] Plasmid copy number was estimated by comparison of plasmid-specific DNA fragments to endogenous MMTV and c-*mos*-specific DNA fragments.

[b] Indicated are the transcription initiation sites for the RNA species as determined by S1 nuclease mapping. Faithfully transcribed RNA species initiated at position +1. The question mark indicates that no discrete initiation sites were detected.

[c] Relative amounts were determined by comparison of S1-protected labeled fragments, taking the amount of hormone-induced p-2-directed RNA as 1.0. Probes used in S1 experiments were 5' end-labeled to identical specific activities. Comparison of RNA quantities in p-1 and p-2 cells was done by RNA dot blot analysis.

[d] Cells were treated for 72 h with $10^{-6}$ M dexamethasone before RNA isolation.

[e] ND, Not determined.

growing loosely attached to the surface of petri dishes (data not shown). These cells did not express the two new RNA species (Fig. 4, lane D), although pMLVCH-1 and p-ins60 shared all sequences downstream of the *Sac*I site (Fig. 1). To correlate directly the stage of transformation and the amount of p37$^{mos}$ immunoprecipitations were performed on the cell lines. The results (Fig. 5) are in agreement with the RNA data obtained. The amount of p37$^{mos}$ in p-2 transformants is hormone-inducible (Fig. 5, lanes c and e); the level of this protein in p-ins60 cells is comparable to the level in hormone-treated p-2 cells; and very low, but detectable levels are found in p-1 transformants in which RNA was initiated from unidentified sites (Fig. 5, lane b).

Figure 6 shows a summary of the results obtained with the p-ins60 plasmid. The three transcription initiation sites are indicated with respect to the original M-MSV LTR cap site (+1), the TATA box, and the origin of the DNA sequences used in this construct. The quantity of a particular RNA species in the absence or presence of hormone is indicated. Also indicated are GC-rich inverted repeats and the palindromic mp11 fragment.

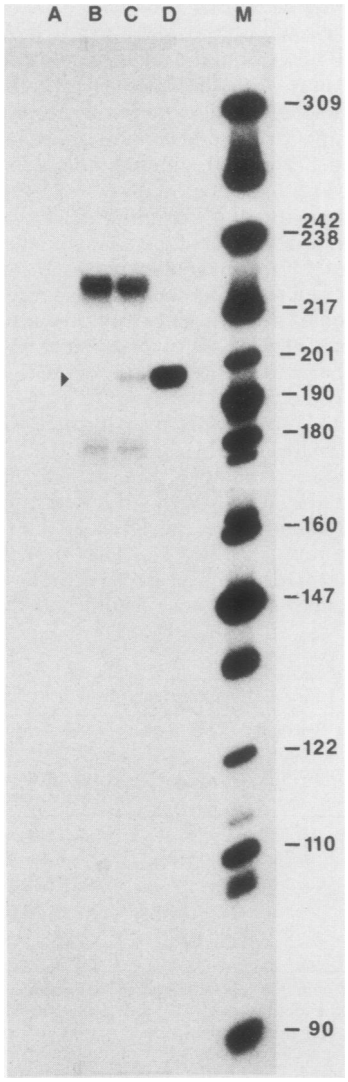


FIG. 4. S1 nuclease analysis of p-ins60 transcripts. The experimental approach for analysis of RNA of p-ins60-transformed cells was as described in the legend to Fig. 3, except that the probe used was a 5′-end-labeled, *Xba*I digest of p-1 DNA (Fig. 1). Sources of the RNA were normal C3H10T1/2 cells (lane A), p-ins60-transformed cells (lanes B and C), and pMLVCH-1 transformed cells (lane D). 3′-end-labeled *Msp*I digests of pBR322 DNA served as markers (M).

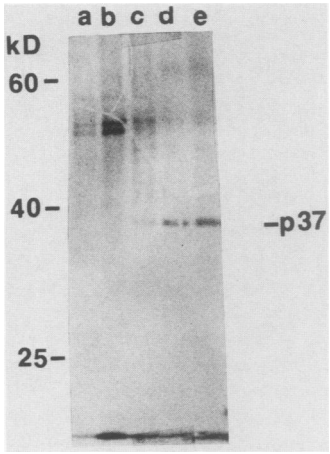


FIG. 5. Correlation between p37$^{mos}$ expression and stage of transformation. The amount of p37$^{mos}$ was determined in the differentially transformed cell lines by immunoprecipitation of the protein from metabolically labeled cells. Precipitates were analyzed by electrophoresis through 10% sodium dodecyl sulfate-polyacrylamide gels, and radioactivity was visualized by exposure of En$^3$Hance- (New England Nuclear Corp.) treated gels to Kodak XAR-5 film for 2 weeks. The sources of the cells were C3H10T1/2 (lane a), p-1 (lane b), p-2 (lane c), p-ins60 (lane d), and dexamethasone-treated p-2 transformants (lane e).

**Differential expression of transformation parameters.** For all cell lines shown we determined several biological characteristics which are considered to be transformation parameters (12, 42), namely, loss of contact inhibition, doubling time in 0.5% serum and colony-forming efficiency in soft agar, loss of actin cables and the relative amount of p53, a transformation-associated nuclear phosphoprotein shown to be involved in immortalization of primary cells (22), and induction of serum-dependent DNA synthesis (32) (Table 2). As expected, all transformed cell lines had lost the strong contact inhibition observed for C3H10T1/2 cells. Furthermore, only the pMLVCH-1 transformants exhibiting a fully transformed morphology were able to grow in 0.5% serum, but several cell lines showing intermediately transformed morphologies were able to grow in soft agar.

Interestingly, dexamethasone treatment of p-2 transformants induced their capacity to grow in soft agar, a result which stands in contrast to the result of similar experiments with NIH3T3 cells (37; see below). From Table 2 we conclude that the gradual increase in v-*mos* expression in C3H10T1/2 cells leading to the appearance of cells with increasingly transformed morphologies is associated with the expression of an increasing number of transformation parameters. Their order of appearance seems to be fixed. The loss of contact inhibition is followed by the ability to grow in soft agar and finally by the ability to grow in 0.5% serum. Interestingly, we did not observe any change in the steady-state level of p53 in the cell lines which showed the differential expression of transformation parameters, thus ruling out that the v-*mos* gene product has an influence on the amount of p53.

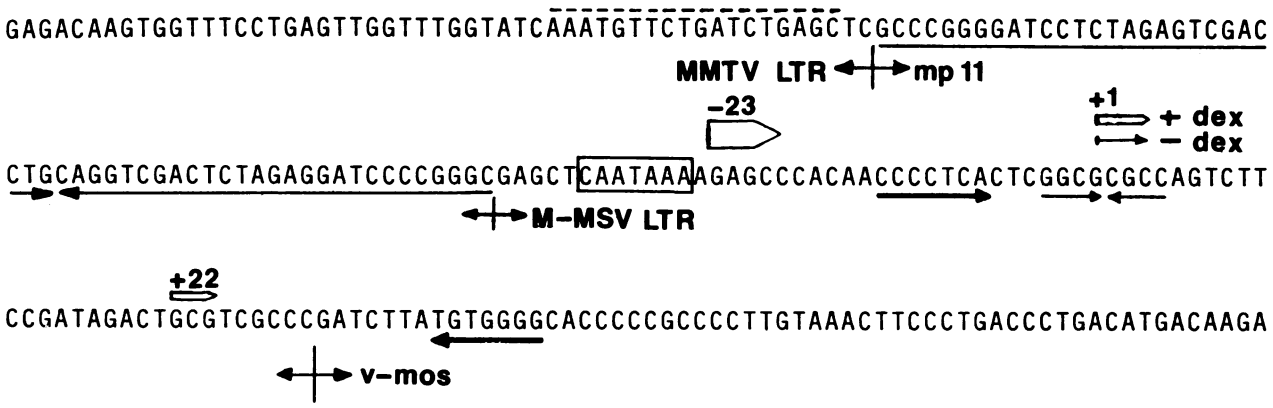


FIG. 6. Transcription initiation sites detected in p-ins60 DNA. The three protected p-ins60-specific fragments shown in Fig. 5 indicate the presence of two new transcription initiation sites denoted by arrows at positions −23 and +22 in addition to the original viral cap site (indicated as +1). The relative amount of the transcripts is indicated by the thickness of the arrows. The sources of the DNA sequences shown are indicated by MMTV LTR, mp11, M-MSV LTR, and v-*mos* under the sequence. The M-MSV LTR TATA box is indicated and was taken from Van Beveren et al. (43). The arrows under the sequence indicate the mp11 palindrome and inverted repeats. The broken line denotes the remaining part of the proximal hormone receptor binding site.

## DISCUSSION

**Transformation of C3H10T1/2 cells.** Several v-*mos* constructs which differ in the promoter and 5′ untranslated region, but not in the coding part or the 3′ untranslated sequences, were used to transform C3H10T1/2 cells. Cell lines with distinguishable stable morphologies representing different stages of transformation in vitro could be reproducibly obtained, depending on both the DNA used for transformation and the treatment with dexamethasone. We show that there is a direct correlation between the amounts of cytoplasmic *mos*-specific RNA and p37$^{mos}$ protein in these cells and their stage of transformation as defined by several transformation parameters. Examination of the cell lines which stably expressed the different phenotypes indicated that the transformation parameters (loss of contact inhibition, integrity of stress fibers, growth in soft agar and in 0.5% serum) are related in a sequential fashion. It remains to be determined whether there exists a causal relationship between them and whether the sequential appearance is related to similar phenomena in vivo (14, 28). These stably transformed cell lines might help to elucidate the mechanism of action of the v-*mos* gene product p37$^{mos}$. Thus, we have shown that the v-*mos* gene product does not have an effect on the steady-state level of p53 over a wide range of p37$^{mos}$ concentrations, although it induced tumor growth factors necessary for anchorage-independent growth (9, 24). It will be of interest to examine the effect of p37$^{mos}$ on cells lacking expression of p53, the protein of which was shown to play a crucial role in the induction of DNA synthesis by serum growth factors (27, 32), possibly using microinjection of p53 antibodies in the cell lines described here (32). Furthermore, the expression of tumor growth factors, which are known to be induced by M-MSV (9), might be regulated in the p-2 transformants which only grow in soft agar in the presence of dexamethasone in a hormone-inducible fashion, because the p37$^{mos}$ expression in these cells is inducible with hormone (Fig. 5). In agreement with this hypothesis we have recently demonstrated that tumor growth factors produced by hormone-treated p-2 cells, but not by untreated p-2 cells, induce the anchorage-independent growth of C3H10T1/2 cells (Van der Hoorn, unpublished data). Thus, the p-2 transformants

TABLE 2. Differential expression of transformation parameters

| Cell line | Cell morphology[a] | Loss of contact inhibition[b] | Growth in soft agar[c] | Doubling time (h) in 0.5 % serum | p53 expression[d] | Loss of actin cables[e] |
|---|---|---|---|---|---|---|
| C3H10T½ | − | − | <1 | >60 | 1.0 | − |
| p-1 (C3H) | − | + | <1 | >60 | 0.9 | − |
| p-2 (C3H), no dexamethasone | + | + | <1 | >60 | 0.9 | + |
| p-ins60 (C3H) | ++ | + | 20 | >60 | 1.0 | + |
| p-2 (C3H), dexamethasone[f] | +++ | + | 35 | >60 | 0.9 | + |
| pMLVCH-1 (C3H) | ++++ | + | 95 | 18 | 1.1 | + |

[a] Based on microscopic observations, the range of cell morphologies was arbitrarily set from normal (−) to supertransformed (++++).
[b] One hundred cells of the cell lines indicated were mixed with $10^4$ normal C3H10T½ cells and plated. Foci were analyzed after 2 weeks.
[c] $10^4$ cells were suspended in 0.3% agarose in complete medium and layered on top of an 0.5% agarose-Dulbecco modified Eagle medium bottom layer. Colonies were counted after two weeks and results expressed as colony forming efficiency (%).
[d] Relative p53 amounts were determined by densitometer scanning of fluorographs of gels used to separate immunoprecipitated labeled p53, taking the amount of p53 in normal C3H10T½ cells as 1.0.
[e] The presence of actin cables was determined by indirect immunofluorescence with a monoclonal anti-actin antibody (Amersham Corp.) and a rabbit-anti-mouse immunoglobulin antibody coupled to fluorescein isothiocyanate.
[f] Cells were grown and assays were performed in the presence of $10^{-6}$ M dexamethasone.

might allow investigation of whether p37$^{mos}$ directly induces the rate of transcription of tumor growth factor genes or whether it's effect is post-transcriptional.

Earlier reports have shown that cells could be transformed in vitro to different morphological stages by infection with *src* mutants of Rous sarcoma virus (1, 20) or *gag-fps* deletion mutants of Fujinami sarcoma virus (13). In each of these cases the oncogenes contained 5′ deletions or point mutations and were found to be expressed at the same levels as the wild type. The authors observed intermediate stages of cell transformation caused by the mutant viruses, in contrast to the full cell transformation caused by the wild-type viruses but, however, could not exclude that the mutated oncogene products and the wild-type products interacted with different cellular targets. Because no such mutants exist for M-MSV, we followed a different approach in this study by introducing the same v-*mos* gene in cells but varying the amount of stable *mos*-specific RNA. The C3H10T1/2 cells appeared to be extremely sensitive to the action of p37$^{mos}$ because (i) we observed transformation of C3H10T1/2 cells, but not NIH3T3 cells (data not shown), in the absence of dexamethasone by p-2 in low copy number (Table 1) which expressed the gene under the control of the MMTV promoter, and (ii) we obtained transformation with the p-1 plasmid, which expressed RNA and protein in quantities lower than those detected for untreated p-2 transformants. Interestingly, p-2-transformed C3H10T1/2 cells, but not NIH3T3 cells expressing a similar construct (37), could be induced to grow in soft agar on hormone treatment.

Other groups have used NIH3T3 cells as recipients for constructs harboring the MMTV LTR and the v-*mos* (37) or v-*ras* (19) genes and obtained different results indicating that NIH3T3 cells exhibit a higher threshold level for transformation. Papkoff and Ringold (37) introduced an MMTV LTR-v-*mos* construct together with the HSV thymidine kinase gene into 3T3TK$^-$ cells; although the cells expressed basal levels of p37$^{mos}$, they had normal morphologies. Addition of dexamethasone led to morphological transformation but did not induce the ability of such cells to grow in soft agar. Experiments reported by Jakobovitz et al. (21) for an MMTV LTR-v-*src* construct showed results similar to the ones described here, using rat-2 cells.

**Activity of different promoter elements.** The correlation between the sequences present upstream of the coding region of the three constructs and of the RNA levels in cells transformed by these constructs suggests the importance of several promoter elements for efficient transcription of the oncogene. (i) The p-2 plasmid (Fig. 1) acted similarly to constructs used by others to promote heterologous genes in a hormone-dependent fashion (19, 35, 37). (ii) The p-1 plasmid, however, lacks the CAT box normally present upstream of the M-MSV TATA box (43), the 3′ half of the proximal hormone receptor binding site (40) in the MMTV LTR, as well as sequences present in the MMTV LTR between the TATA box and the proximal binding site (11). The failure to detect discrete *mos*-specific RNA species in p-1 transformants indicates that the M-MSV TATA box is not functional to detectable levels. Therefore, sequences upstream of the TATA box which are absent from p-1 are important for efficient transcription directed by the TATA box, in agreement with earlier reports, and the remaining MMTV LTR sequences can not functionally substitute for them (2, 10). (iii) In p-ins60 transformants, however, faithful transcription directed by the M-MSV TATA-box was hormone inducible. Therefore, we conclude that the mp11 palindromic DNA fragment present in p-ins60 DNA can functionally substitute for promoter elements necessary for TATA box-directed transcription. Interestingly, the mp11 fragment contained GC-rich stretches similar to those of upstream sequences described for the HSV thymidine kinase gene (31) and the simian virus 40 promoter (2). They might have induced the use of the two new transcription initiation sites on the p-ins60 DNA (Fig. 6). It is not likely that these new sites represent 3′ splice sites, because comparison of the 3′ splice site consensus sequence (34) and the sequences around positions −23 and +22 did not reveal any similarities. From the results shown in Fig. 6 it can be seen that the largest abundant RNA species contained two different GC-rich inverted repeats (Fig. 6) in its 5′-terminal region which might form stable secondary stem-loop structures. Conceivably, these secondary structures could inhibit translation of this RNA species. This would be in agreement with the actual amount of p37$^{mos}$ in p-ins60 transformants. Furthermore, the absence of a visible hormone effect on p-ins60 cells suggests that the two smaller RNA species are both biologically active, because in this case the total level of active transcripts would be induced by a factor of only two by dexamethasone. This induction is probably too low to be reflected in the additional expression of transformation parameters.

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