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Genetics

Glucocorticoid and cAMP induction mechanisms are differentially affected by the p85$^{gag\text{-}mos}$ oncoprotein

(oncogenic transformation/signal transduction/metallothionein 1)

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ABSTRACT **The inability to perceive and coordinate both internal and external signals that function to regulate cellular growth and proliferation is a hallmark of oncogenic transformation. To examine the effects of the v-*mos* oncogene on distinct signal transduction pathways, the 6m2 cell line was used, in which expression of the p85$^{gag\text{-}mos}$ oncogene, and consequently transformation, are temperature sensitive. Through the analysis of endogenous metallothionein 1 (*Mt-1*) gene expression in 6m2 cells, p85$^{gag\text{-}mos}$ effects on glucocorticoid, cAMP, and heavy-metal induction were examined. While heavy-metal induction of Mt-1 mRNA was found to be unaffected by p85$^{gag\text{-}mos}$, differential effects were exerted upon glucocorticoid and cAMP induction of *Mt-1*. Glucocorticoid induction of Mt-1 mRNA in p85$^{gag\text{-}mos}$-transformed 6m2 cells was initiated normally but not maintained to the same extent as in nontransformed 6m2 cells. In contrast, cAMP did not induce Mt-1 mRNA in p85$^{gag\text{-}mos}$-transformed 6m2 cells, although a significant induction was noted in nontransformed 6m2 cells. Thus, an oncoprotein interferes with different steps in each particular signal transduction pathway, ultimately causing abnormalities of inducible gene expression.**

Many alterations in cellular metabolism associated with oncogenic transformation reflect changes in gene expression (1). Initially, Weintraub and Groudine (2) noted changes in the profile of total cellular proteins resulting from v-*src* transformation of chicken embryonic cells. Subsequent molecular analyses have revealed both increased and decreased expression of specific genes resulting from transformation (3, 4). In fact, specific DNA sequences have been identified that are required for transcriptional activation mediated by nuclear oncoproteins, c-*myc* (5) and v-*jun* (6).

In addition to direct interactions with DNA (7, 8), oncoproteins influence expression via interactions with specific transcription factors (9, 10). However, despite detailed molecular analyses, there are no obvious correlations between the biochemical activities of particular oncoproteins and associated alterations in gene expression. For example, tropomyosin synthesis is suppressed in NIH 3T3 cells transformed by a number of functionally unrelated oncogenes such as v-*ras*, v-*mos*, v-*src*, v-*fes*, and v-*fms* (3). Likewise, type I collagen synthesis is inhibited in cells transformed by v-*mos*, v-*src*, Ki-*ras*, and N-*ras* (11).

Specific signal transduction pathways are also affected by transformation. For example, transformation with Ha-*ras* and other oncogenes results in alterations of inositol phospholipid metabolism (12). Likewise, hormonal stimulation of adenylate cyclase activity is reduced by EJ-*ras* transformation of NIH 3T3 cells (13). Steroid hormone regulation of gene expression may also be influenced by transformation as evidenced by v-*mos* inhibition of glucocorticoid-induced expression of mouse mammary tumor virus (MMTV) sequences (14). The v-*mos* oncogene is derived from the Moloney murine sarcoma virus (MoMSV) genome and encodes a 37-kDa cytoplasmic protein-serine/threonine kinase (p37^{mos}; ref. 15). Since MoMSV infection inhibits corticosteroid-induced differentiation of bone marrow preadipocytes (16), v-*mos* effects may be exerted on various glucocorticoid responses.

We were interested in extending the analysis of v-*mos* effects on signal transduction. To facilitate our experiments, we have used a MoMSV ts110-infected NRK cell line (6m2 cells; ref. 17). At 33°C (permissive temperature), 6m2 cells are transformed and express an 85-kDa *gag–mos* gene fusion protein (p85$^{gag\text{-}mos}$) with an associated protein kinase activity (18). The production of p85$^{gag\text{-}mos}$ in 6m2 cells is strictly dependent on a temperature-sensitive splicing event that generates a 3.5-kilobase (kb) mRNA (19). An in-frame translational stop codon is contained within the v-*mos* coding region of the 4.0-kb unspliced viral mRNA that accumulates at the nonpermissive temperature (39°C). The resultant translation product is a 58-kDa *gag* gene-encoded protein (p58^{gag}) that possesses no transforming or associated protein kinase activity (18, 19).

To examine responses to various inducing agents, we have focused our analysis on expression of the endogenous metallothionein 1 (*Mt-1*) gene in 6m2 cells. Transcription of metallothionein genes is regulated by various inducers such as glucocorticoids, heavy metals, cyclic nucleotides, and tumor promoters, which act via independent regulatory pathways (20). The analysis of glucocorticoid, cAMP, and heavy-metal induction of *Mt-1* expression in 6m2 cells demonstrated different effects of p85$^{gag\text{-}mos}$ on these distinct signal transduction pathways, indicating diverse molecular mechanisms through which v-*mos* influences gene expression.

MATERIALS AND METHODS

Cell Lines. 6m2 (17) and 54-5A4 (21) rat cell lines (provided by R. B. Arlinghaus, Jr., Anderson Hospital, Houston, TX) were maintained at 33°C in McCoy's 5A medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO). For inductions performed at the nonpermissive temperature, cells were shifted to 39°C and maintained for at least 16 hr prior to any treatment. Dexamethasone and $CdCl_2$ (Sigma) were added to approximately 60–80% confluent cell cultures in normal growth media to attain a final concentration of 1 μM. Cells were harvested at the indicated times following the initiation of induction and were stored at −70°C prior to RNA isolation. Before induction by 8-(4-chlorophenylthio)adeno-

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Abbreviations: MMTV, mouse mammary tumor virus; MoMSV, Moloney murine sarcoma virus; GRE, glucocorticoid response element; CPT-cAMP, 8-(4-chlorophenylthio)adenosine-3′:5′-monophosphate, cyclic.

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sine-3':5'-monophosphate, cyclic (CPT–cAMP; Boehringer Mannheim), cells were shifted to serum-free medium for 16 hr. Subsequently, CPT–cAMP was added directly to these media to attain a final concentration of 5×10^{-4} M.

RNA Isolation and Analysis. Total RNA was isolated from frozen cells and subjected to agarose gel electrophoresis and Northern blot analysis as described (22). The plasmid pMT-1 (provided by R. D. Palmiter, University of Washington, Seattle) containing mouse Mt-1 cDNA sequences was used to prepare ^{32}P-labeled probes for hybridization to Northern blots (22). Rat Mt-1 mRNA levels were quantified from densitometric scans of autoradiograms, using several exposures to assure linearity of the film response. To normalize for different amounts of RNA loaded per lane, all Northern blots were rehybridized with a chicken β-actin DNA probe, as illustrated in Fig. 1. The following data are representative in each case of at least three independent experiments.

RESULTS

Mt-1 mRNA Levels Are Transiently Induced by Dexamethasone in p85$^{gag\text{-}mos}$-Transformed 6m2 Cells. 6m2 cells grown at 33°C accumulate p85$^{gag\text{-}mos}$ and are transformed phenotypically (17, 18). The effects of p37^{mos} on glucocorticoid-induced expression of transfected MMTV sequences reported previously (14, 23) raised the question that p85$^{gag\text{-}mos}$ might alter glucocorticoid induction of an endogenous gene, *Mt-1*. Northern blots were used to quantify Mt-1 mRNA levels in 6m2 cells treated with the synthetic glucocorticoid dexamethasone. As shown in Fig. 1 *Upper*, Mt-1 mRNA was induced only transiently by dexamethasone in 6m2 cells grown at 33°C and was observed first 2 hr after the addition of dexamethasone (data not shown). Maximum induction (3.5-fold) of Mt-1 mRNA levels followed a 12-hr dexamethasone treatment (Fig. 1 *Upper*). However, the induced levels of Mt-1 mRNA were not maintained in spite of the continued presence of dexamethasone. Within 12 hr after maximal induction (Fig. 1 *Upper*), Mt-1 mRNA dropped to almost basal levels. The poly(A) tail at the 3' end of Mt-1 mRNA has been shown to be shortened following dexamethasone and copper chloride treatments in both rat liver and kidney (24). This most likely accounts for the decreased size of Mt-1 mRNA that is observed after dexamethasone (Fig. 1 *Upper*) and $CdCl_2$ (see Fig. 4) induction. As shown in Fig. 1 *Lower*, β-actin mRNA levels were not affected by dexamethasone treatment of p85$^{gag\text{-}mos}$-transformed 6m2 cells.

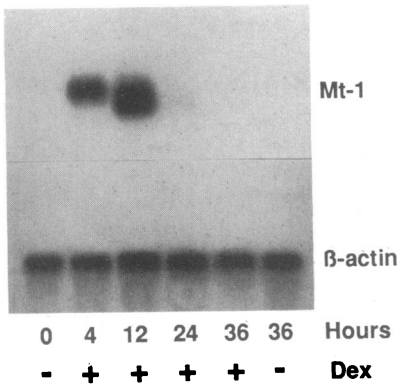

FIG. 1. Time course of Mt-1 and β-actin mRNA response to dexamethasone (Dex) in 6m2 cells grown at 33°C. 6m2 cells grown at 33°C were treated with 10^{-6} M dexamethasone for the amounts of time indicated. Total RNA (5 μg per lane) was electrophoresed, blotted, and hybridized to ^{32}P-labeled mouse Mt-1 (*Upper*) or chicken β-actin (*Lower*) cDNA probes. Autoradiographs were exposed for 10 days (*Upper*) or 12 hr (*Lower*).

Dexamethasone-Induced Mt-1 mRNA Levels Are Maintained in Nontransformed 6m2 Cells. At the nonpermissive temperature (39°C), 6m2 cells do not express p85$^{gag\text{-}mos}$ and are not transformed (17, 18). To establish whether the transient induction of Mt-1 mRNA by dexamethasone in 6m2 cells was a consequence of p85$^{gag\text{-}mos}$ activity, a time course of Mt-1 mRNA induction by dexamethasone was performed at 39°C, the temperature that is nonpermissive for p85$^{gag\text{-}mos}$ expression. As shown in Fig. 2, Mt-1 mRNA levels were rapidly elevated in response to dexamethasone treatment and a 3-fold induction was attained within 1 hr of hormone treatment. In contrast to the transient induction of Mt-1 mRNA observed in 6m2 cells grown at 33°C, dexamethasone-induced Mt-1 mRNA levels were maintained to a significant extent (3-fold induction) for 36 hr at 39°C (Fig. 2). Longer autoradiographic exposures did not reveal any significant difference between the basal levels of Mt-1 mRNA at 33°C and at 39°C (data not shown). Thus, in the presence of p85$^{gag\text{-}mos}$, dexamethasone induction of Mt-1 mRNA is initiated but not maintained to the extent observed in nontransformed 6m2 cells.

Transient Dexamethasone Induction of Mt-1 mRNA at Both 33°C and 39°C in Revertant 54-5A4 Cells. Spontaneous revertants of the 6m2 cell line have been isolated that are transformed at both 33°C and 39°C (21). In one revertant cell line (54-5A4 cells), a 5-base-pair (bp) deletion within the MoMSV ts110 provirus eliminates both a splicing site and a premature translational termination codon (21). As a result, the genomic 4.0-kb viral mRNA in 54-5A4 cells is not spliced but is translated at both 33°C and 39°C into a 100-kDa *gag–mos* gene-encoded fusion protein (p100$^{gag\text{-}mos}$) that is responsible for transformation (21). Although both p85$^{gag\text{-}mos}$ and p100$^{gag\text{-}mos}$ possess an associated protein-serine/threonine kinase activity, they differ in at least one property—i.e., the thermal stability of the kinase activity (18). We therefore analyzed dexamethasone induction of Mt-1 mRNA in 54-5A4 cells to establish whether p100$^{gag\text{-}mos}$ altered glucocorticoid induction in analogy to p37^{mos} and p85$^{gag\text{-}mos}$. Since 54-5A4 cells are transformed at 33°C and 39°C, we could also determine whether the kinetic profile of glucocorticoid induction was influenced by the temperature at which the cells were grown.

As shown in Fig. 3*A*, Mt-1 mRNA was rapidly induced in 54-5A4 cells grown at 39°C, with a 6-fold induction apparent within 1 hr. Maximum induction (>20-fold) occurred within 4 hr, followed by a precipitous drop in Mt-1 mRNA to almost basal levels 12 hr after the initiation of hormone treatment (Fig. 3*A*). This same pattern of transient induction was observed in 54-5A4 cells treated with dexamethasone at 33°C (Fig. 3*B*). Thus, transient glucocorticoid induction of Mt-1 mRNA is characteristic of both p85$^{gag\text{-}mos}$- and p100$^{gag\text{-}mos}$-transformed cells. The kinetics of transient dexamethasone induction of Mt-1 mRNA was similar at 33°C in 6m2 (Fig. 1 *Upper*) and 54-5A4 cells (Fig. 3*B*). At 39°C, nontransformed 6m2 cells (Fig. 2) and p100$^{gag\text{-}mos}$-transformed 54-5A4 cells (Fig. 3*A*) required only 4 hr of dexamethasone treatment to

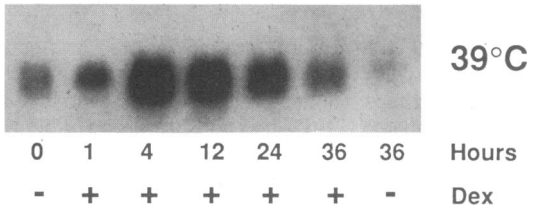

FIG. 2. Time course of Mt-1 mRNA response to dexamethasone (Dex) in 6m2 cells grown at 39°C. 6m2 cells grown at 39°C were treated with 10^{-6} M dexamethasone for the amounts of time indicated. Total RNA was prepared and processed as described in the legend to Fig. 1.

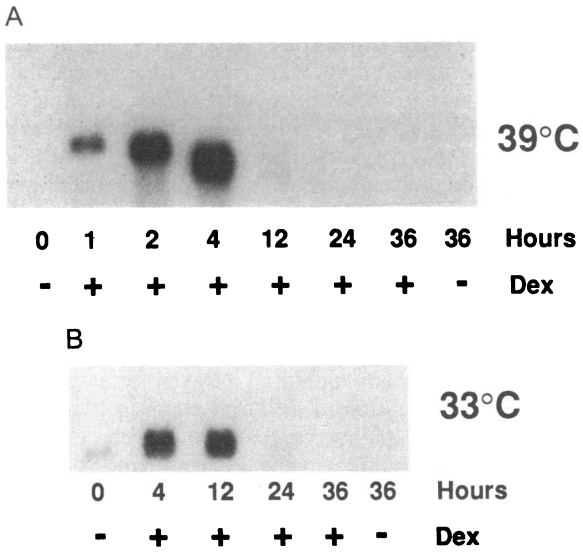

FIG. 3. (*A*) Time course of Mt-1 mRNA response to dexamethasone (Dex) in revertant 54-5A4 cells grown at 39°C. 54-5A4 cells grown at 39°C were treated with 10^{-6} M dexamethasone for the amounts of time indicated. Total RNA was prepared and processed as described in the legend to Fig. 1. (*B*) Time course of Mt-1 RNA response to dexamethasone in revertant 54-5A4 cells grown at 33°C. 54-5A4 cells grown at 33°C were treated with 10^{-6} M dexamethasone for the amounts of time indicated. Total RNA was prepared and processed as described in the legend to Fig. 1.

attain maximally induced Mt-1 mRNA levels. Thus, the initial kinetics of glucocorticoid induction of Mt-1 mRNA is temperature dependent in both 6m2 and 54-5A4 cells and is not influenced by v-*mos* transformation.

$CdCl_2$ Induction of Mt-1 mRNA Levels Is Not Affected by $p85^{gag\text{-}mos}$ in 6m2 Cells. Glucocorticoid and heavy-metal inductions of human metallothionein 2_A (*$MTII_A$*) gene transcription are mediated by distinct sequences termed glucocorticoid (GRE) and metal (MRE) response elements, respectively (25). Although functional GREs have not been mapped, MREs mediating heavy-metal induction of rat *Mt-1* gene expression are reported to be located within 500 bp of 5′ flanking sequences (26). The specificity of $p85^{gag\text{-}mos}$ effects was analyzed by the analysis of heavy-metal induction of Mt-1 mRNA in 6m2 cells. As shown in the Northern blot in Fig. 4 *Upper*, Mt-1 mRNA was maximally induced (5-fold) in 6m2 cells 12 hr after $CdCl_2$ treatment at 33°C. In spite of a decrease following this peak induction, Mt-1 mRNA levels

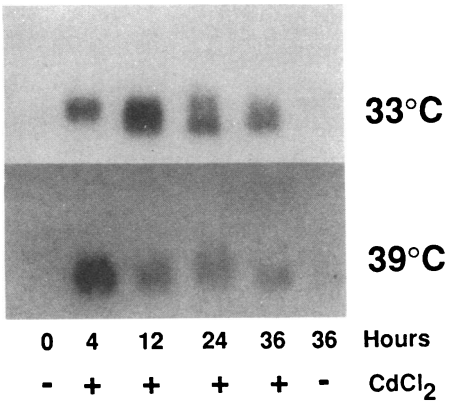

FIG. 4. Time course of Mt-1 mRNA response to $CdCl_2$ in 6m2 cells grown at 33°C and 39°C. 6m2 cells grown at 33°C (*Upper*) and 39°C (*Lower*) were treated with 10^{-6} M $CdCl_2$ for the amounts of time indicated. Total RNA was prepared and processed as described in the legend to Fig. 1.

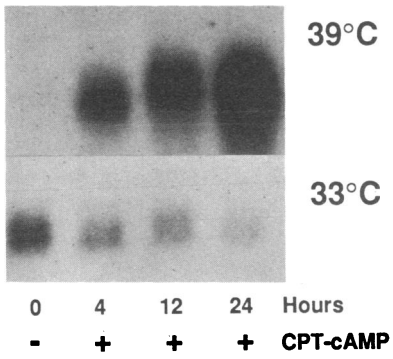

FIG. 5. Time course of Mt-1 mRNA response to CPT-cAMP in 6m2 cells grown at 33°C and 39°C. 6m2 cells grown at 39°C (*Upper*) and 33°C (*Lower*) were treated with 5×10^{-4} M CPT-cAMP for the amounts of time indicated. Total RNA was prepared and processed as described in the legend to Fig. 1.

remained elevated (3-fold) after 36 hr of $CdCl_2$ treatment (Fig. 4 *Upper*). $CdCl_2$ induction of Mt-1 mRNA was also maintained for 36 hr at 39°C to the same extent (Fig. 4 *Lower*). As observed with dexamethasone treatments, the initial kinetics of $CdCl_2$ induction and the time required to attain maximally induced Mt-1 mRNA levels are temperature dependent in both 6m2 (Fig. 4) and 54-5A4 cells (data not shown).

Complete Inhibition of cAMP Induction in $p85^{gag\text{-}mos}$-Transformed 6m2 Cells. Both polypeptide and steroid hormones have been shown to regulate Mt-1 transcription (20). Intracellular cAMP serves as the second messenger for many hormones and is involved in the induction of Mt-1 and $MTII_A$ transcription in cultured rat and human cells, respectively (27, 28). Since glucocorticoids and cAMP regulate transcription via independent pathways (29), cAMP induction of Mt-1 mRNA was examined in transformed and nontransformed 6m2 cells.

As shown in Fig. 5 *Upper*, treatment of nontransformed 6m2 cells (39°C) with the cAMP analogue CPT-cAMP led to a rapid and substantial induction (>40-fold within 24 hr) of Mt-1 mRNA. In contrast, Mt-1 mRNA was not induced by CPT-cAMP in $p85^{gag\text{-}mos}$-transformed 6m2 cells (33°C) but was even slightly reduced after a 24-hr hormone treatment (Fig. 5 *Lower*). The apparent difference in the basal levels of Mt-1 mRNA at 33°C and 39°C (Fig. 5) was not consistently observed. Importantly, the effect of the $p85^{gag\text{-}mos}$ on cAMP induction was quite different from that on glucocorticoid induction since the cAMP response was never initiated. CPT-cAMP also did not induce Mt-1 mRNA in $p100^{gag\text{-}mos}$-transformed 54-5A4 cells grown at both 33°C and 39°C (data not shown), demonstrating that the lack of cAMP induction was caused by v-*mos* transformation and not by temperature.

DISCUSSION

Oncoproteins that affect transcription are likely to interact with different components of the transcriptional machinery. Using the temperature-sensitive MoMSV ts110-transformed 6m2 cell line, we were able to carefully examine v-*mos* effects on induction of rat *Mt-1* gene expression by various agents. In this report, we have shown that cytoplasmic v-*mos*-encoded oncoproteins (both $p85^{gag\text{-}mos}$ and $p100^{gag\text{-}mos}$) impair, in different ways, the response of *Mt-1* to glucocorticoids and cAMP but do not affect heavy-metal induction. Since *Mt-1* expression in 6m2 cells is below the level of detection for nuclear run-on assays, direct transcriptional effects of v-*mos* have not been demonstrated, and differential effects on Mt-1 mRNA stability cannot be excluded.

Since glucocorticoid and cAMP induction pathways are distinct (29), the targets of $p85^{gag\text{-}mos}$ and $p100^{gag\text{-}mos}$ action

that are ultimately responsible for the observed effects are most likely not identical. This is suggested by the different steps in the respective signal transduction pathways that were affected by v-*mos*. cAMP induction of *Mt-1* was not even initiated, while glucocorticoid induction was initiated but not maintained. Since $CdCl_2$-induced and constitutive expression of *Mt-1* were identical in 6m2 cells at both permissive and nonpermissive temperatures, apparently not all regulators of *Mt-1* expression are affected by v-*mos*. Interestingly, analogous effects on *Mt-1* expression have been observed in the liver of newborn mice homozygous for lethal deletions in chromosome 7 (22). While heavy-metal induction of Mt-1 mRNA is unaffected in these mice, no induction of Mt-1 mRNA by glucocorticoids is obtained in the liver of newborn deletion homozygotes, in contrast to normal littermates (22, 30). In 6m2 cells, the v-*mos*-encoded oncoprotein is most likely responsible for causing defective hormonal induction. This lack of hormonal inducibility of gene expression is similar to that reported previously in the deletion homozygotes for several liver-specific genes normally inducible by glucocorticoids and cAMP (30).

Transient glucocorticoid induction of gene expression in v-*mos*-transformed cells had been observed previously from transfected MMTV DNA (14, 23). In $p85^{gag\text{-}mos}$-transformed 6m2 cells, Mt-1 mRNA cannot be reinduced by an additional exposure to dexamethasone 24 hr after the initial treatment (unpublished data). Thus, the effects of v-*mos* on glucocorticoid induction are reminiscent of "desensitization" observed after prolonged treatment with various hormones and neurotransmitters (31). In this context, desensitization refers to the inability of a response to be propagated or maintained at maximum levels despite the continued presence of the initial stimulus. Desensitization can result from covalent modification or degradation of receptor proteins or induction of specific transcriptional repressors (31). In fact, Darnell and co-workers have identified an interferon-induced nuclear factor that binds to interferon-stimulated response element (ISRE) sequences and mediates desensitization of *ISG54*, an interferon-inducible gene (32).

In analogy to desensitization of the interferon response, dexamethasone might induce or activate a transcriptional repressor protein in transformed 6m2 cells that specifically inhibits *Mt-1* expression. In this case, where desensitization is dependent on the transformed phenotype, $p85^{gag\text{-}mos}$ and $p100^{gag\text{-}mos}$ would be expected to activate, perhaps through phosphorylation, either this putative repressor or an intermediate required for induction of repressor synthesis by dexamethasone. Such a mechanism would require the repressor to bind to GRE sequences *in vivo*. Support for this speculation is provided by the actual detection from many different cell types of nuclear factors distinct from glucocorticoid receptors that bind to GRE sequence *in vitro* (33).

Another explanation for transient glucocorticoid induction of *Mt-1* expression could be the existence of v-*mos* effects on the recycling of the glucocorticoid receptor. A number of independent investigations have provided evidence for such recycling of glucocorticoid receptors into and out of the nucleus during the normal induction process (for example, see refs. 34 and 35). As of yet, no physiological role for this process has been established, although phosphorylation of cytoplasmic glucocorticoid receptors at serine residues has been suggested as an important component of the recycling pathway (34). Glucocorticoid receptors are known to be phosphorylated on serine residues, but it is not known whether these modifications affect any glucocorticoid receptor function (36).

The lack of cAMP induction of Mt-1 mRNA in transformed 6m2 cells could be manifested at various levels. 6m2 cells grown at the permissive temperature undergo a rapid but reversible change in morphology in response to CPT-cAMP treatment (unpublished data). This reaction to cAMP makes it unlikely that transformed 6m2 cells have completely lost their responsiveness to cAMP. There are precedents for oncogene effects on cAMP responses. For example, EJ-*ras*-transformed NIH 3T3 cells have reduced hormone-stimulated adenylate cyclase and intracellular cAMP levels (13). In addition, both Ha-*ras* and cAMP have been shown to affect the activity of the enhancer binding-protein PEA1/AP1 (37, 38).

Abnormal expression of genes mediating growth control is no doubt critically involved in the mechanism of cellular transformation. Various signal transduction pathways are concerned with the normal regulation of cellular growth and therefore provide many potential targets for oncoprotein action. The identification of different steps in two distinct signal transduction pathways that are targets of v-*mos*-encoded oncoprotein effects appears significant. It provides the potential for characterizing further specific components of a particular signal transduction pathway. Eventually this may lead to a better understanding of the mechanisms of cellular transformation as well as those normally regulating inducible gene expression.

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