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Amplification and Hormone-Regulated Expression of a Mouse Mammary Tumor Virus-*Eco gpt* Fusion Plasmid in Mouse 3T6 Cells

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Mouse 3T6 cells were transformed with a chimeric DNA plasmid, pSVM*gpt*, in which the mouse mammary tumor virus (MMTV) promoter was fused to the *Escherichia coli* gene encoding xanthine-guanine phosphoribosyl transferase (*Eco gpt*). The transformants exhibited glucocorticoid-inducible expression of *Eco gpt*. With limiting xanthine concentrations, conditions were established in which cell growth became hormone dependent. Cells selected for their ability to grow in limiting concentrations of both xanthine and glucocorticoids contained amplified levels of *Eco gpt* DNA, and expression of *Eco gpt* remained glucocorticoid inducible in these amplified cells. Thus, amplification of the MMTV promoter region in itself did not abolish hormonal responsiveness of a gene. In addition to increased levels of *Eco gpt* DNA, some of the selected cells also exhibited increased levels (two- to threefold) of glucocorticoid receptors. Lastly, we found that excessive expression of *Eco gpt* is toxic to 3T6 cells; by maintaining low hormone levels and, therefore, low levels of expression, we were able to select cells with amplified *Eco gpt*. Thus, the MMTV promoter may be of general utility in expressing genes whose products may be lethal if they are produced in excessive quantities.

Steroid hormone-responsive genes serve as useful model systems for studying the control of eucaryotic transcription. Hormones of this class bind to cytosolic receptor proteins, which then enter the nucleus as hormone-receptor complexes (for a review, see references 8 and 35). Although the functions of such complexes are poorly understood at a molecular level, substantial evidence suggests that they stimulate the transcription of responsive genes by interacting with specific DNA sequences (9, 21, 24).

We have been using mouse mammary tumor virus (MMTV) to analyze the mechanism of action of glucocorticoid hormones. MMTV-infected cells produce increased amounts of viral RNA and protein in response to glucocorticoids (23, 30); this effect on MMTV gene expression is due to a rapid and specific increase in the rate of synthesis of viral RNA (29, 36). Recent experiments demonstrate that the long terminal repeats (LTRs) of MMTV DNA contain the viral promoter, as well as sequences that confer glucocorticoid responsiveness on MMTV transcription (13, 17). For example, we have previously constructed a plasmid vector containing a mouse dihydrofolate reductase (DHFR) cDNA fused to the MMTV LTR. When introduced into CHO cells, this hybrid DHFR gene is expressed under the control of the viral promoter and can be induced by the glucocorticoid, dexamethasone (17).

Eco gpt is the *Escherichia coli* gene encoding xanthine-guanine phosphoribosyl transferase (XGPRT). This enzyme will convert xanthine to XMP, a reaction that cannot be carried out by the analogous mammalian purine salvage enzyme, hypoxanthine-guanine phosphoribosyl transferase (HGPRT). As shown by Mulligan and Berg (19), this gene, when fused to a eucaryotic promoter, can be expressed efficiently in mammalian cells. With an inhibitor of IMP dehydrogenase to deplete cells of XMP, this bacterial gene can be used as a dominant selectable marker in cells supplemented with xanthine (20).

In this paper, we describe the construction of a plasmid (pSVM*gpt*) containing the MMTV LTR fused to the coding sequence for *Eco gpt* and demonstrate its hormonal responsiveness in mouse 3T6 cells. Furthermore, we used this plasmid to establish conditions under which the growth of transfected 3T6 cells becomes glucocorticoid dependent. By limiting the xanthine concentration available to a clone of these transformants, we selected cells containing amplified pSVM*gpt* sequences and showed that in such

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cells, *Eco gpt* RNA remains glucocorticoid inducible. Lastly, we found that excessive production of XGPRT is detrimental to 3T6 cells. In this context, we demonstrate the utility of the MMTV LTR for expression of gene products that may be toxic to cells when they are present at high levels.

MATERIALS AND METHODS

Cells and DNA-mediated transformation. Mouse 3T6 fibroblasts were propagated in Dulbecco modified Eagle medium containing penicillin, streptomycin, 5% newborn calf serum, and 3% fetal calf serum. For the experiments leading to pSVM*gpt* amplification, the selective medium also contained hypoxanthine (15 μg/ml), aminopterin (2 μg/ml), thymidine (5 μg/ml), glycine (5 μg/ml), mycophenolic acid (25 μg/ml; gift from Eli Lilly & Co.), and varying concentrations of xanthine.

The plasmid pSVM*gpt* (Fig. 1) was constructed by inserting a 1.4 kilobase (kb) *Pst* MMTV LTR fragment containing *Hin*dIII linkers into the unique *Hin*dIII site of the previously described plasmid pSV2*gpt* (19). The details of the construction are identical to those described by Lee et al. (17) for the generation of pSVM DHFR.

DNA-mediated transformations were by the method of Graham and Van der Eb (10), as modified by Parker and Stark (22), except that carrier DNA was omitted. Briefly, 100-mm plates were seeded with 10^6 cells and then treated with 10 to 20 μg of calcium phosphate-precipitated pSVM*gpt* DNA. At 4 h, the cells were exposed to 20% (vol/vol) glycerol in phosphate-buffered saline (PBS) for 5 min. After growth for 3 days at 37°C in nonselective medium, the cells were passaged (1:10) into selective medium containing mycophenolic acid (25 μg/ml) and xanthine (250 μg/ml), as described by Mulligan and Berg (20). Clones were picked in cloning cylinders 7 to 10 days later.

RNA preparation for S1 nuclease analysis. Total cytoplasmic RNA was prepared as follows from several of the isolated 3T6 pSVM*gpt* transformant clones grown in the presence and absence of 1 μM dexamethasone. Pelleted cells were lysed with isotonic Nonidet P-40 buffer, and nuclei were removed by centrifugation. The supernatant was diluted with an equal volume of 7 M urea–0.35 M NaCl–0.01 M Tris (pH 7.4)–0.1 M EDTA–1% sodium dodecyl sulfate and extracted twice with 1:1 phenol-chloroform (4). Polyadenylate-containing RNA was isolated by binding to oligodeoxythymidylate-cellulose (2). Mapping of the 5′ ends of *gpt* RNA was by the method of Berk and Sharp (5); the end-labeled probe used for the S1 nuclease analysis was the *Bgl*II-to-*Eco*RI fragment containing the MMTV LTR (Fig. 1) and was prepared as described by Ringold et al. (26).

Xanthine and dexamethasone dose-response curves. To assess the hormonal growth requirements of transformants, 20,000 cells were passaged into 60-mm tissue culture dishes and grown for 1 day in selective medium containing 25 μg of xanthine per ml and varying concentrations of dexamethasone. The cells were then washed with PBS twice and grown for 4 days in selective medium containing the amount of xanthine and dexamethasone indicated on the figures. For one experiment (see Fig. 5), the selective medium contained serum that had previously been dialyzed to remove any xanthine it might contain. These cells were grown for 3 days in 25 μg of xanthine per ml and for 7 days in 2.5 μg of xanthine per ml.

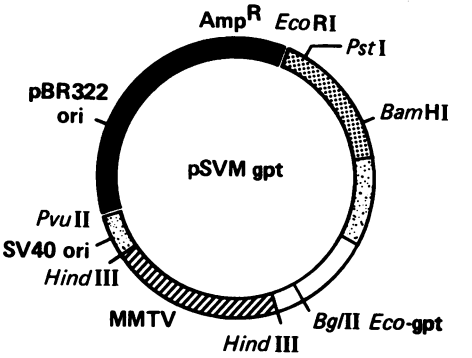

FIG. 1. Structure of the pSVM*gpt* plasmid used to transfect mouse 3T6 fibroblasts. The solid black segment represents a 2.3-kb fragment of pBR322 DNA which allowed replication of the plasmid and selection for ampicillin resistance in *E. coli*. The stippled region represents SV40 DNA which contained the SV40 early promoter, as well as RNA-processing signals. The hatched region represents that part of the MMTV LTR containing the promoter for MMTV RNA synthesis. Immediately downstream from the MMTV promoter is the *E. coli gpt* gene represented by the solid white segment.

Selection of cells containing amplified *Eco gpt*. 3T6.3 cells (10^6) were grown for 1 day in selective medium containing 25 μg of xanthine per ml and either 1, 3, or 10 nM dexamethasone. The cells were then washed with PBS and grown in selective medium with the same concentration of dexamethasone and either 2.5, 0.75, or 0 mg of added xanthine per ml. After 3 days, the cells were passaged and plated at 10^5 cells per 100-mm plate. After 9 days, the largest clones were picked from several populations. As they became confluent, cells were passaged and frozen at −70°C for storage.

DNA and RNA extractions for slot hybridizations. Total cellular DNA was prepared as previously described (28). Briefly, cells were treated with sodium dodecyl sulfate (0.5%) and pronase (500 μg/ml), and protein was removed by extraction with phenol. The DNA was dialyzed extensively against 10 mM Tris (pH 7.4)–0.1 mM EDTA. The DNA concentration was measured by the diaminobenzoic acid fluorometric assay (15). RNA was prepared by the method of Chirgwin et al. (7). Briefly, cells were suspended in 4 M guanidinium thiocyanate. The resulting cell lysate was layered onto a cesium chloride cushion (5.7 M) and centrifuged at 80,000 × *g* for 19 h. The RNA pellet was collected, and its concentration was determined by reading absorbance at 260 nm.

Slot hybridizations were performed by a modification of the method of Kafatos et al. (14). Dilutions of DNA were prepared in 120 μl of 10 mM Tris–0.1 mM EDTA with herring sperm DNA added to bring the

total DNA content per sample to 5 μg. Standards of nonradioactive pSVM*gpt* DNA were also prepared. The DNA samples were denatured in 0.25 M NaOH at room temperature for 10 min and chilled on ice. The samples were then neutralized with 1 M ammonium acetate and applied to nitrocellulose paper with a Plexiglas slot mold. Solutions were drawn through the nitrocellulose by a series of 1 M ammonium acetate saturated filter papers under the nitrocellulose. RNA dilutions were prepared in 120 μl of 20× SSC (3 M NaCl–0.3 M sodium citrate), with yeast RNA to bring the total RNA content up to 5 μg. The samples were then bound to nitrocellulose filters, by the method of Thomas (32), using the Plexiglas slot mold. The filters were air dried and baked in a vacuum oven at 80°C for 2 h. The filters were hybridized with nick-translated pSV2*gpt* DNA (5.4×10^7 cpm/μg). The plasmid pSV2*gpt* is identical to pSVM*gpt* except that it lacks the MMTV fragment.

Glucocorticoid receptor assays. Cells were harvested by using 2.5 mM EDTA in PBS. After washing with PBS, cells were suspended at 4°C in TEGM (20 mM Tris-hydrochloride–2 mM EDTA–10% glycerol–20 mM sodium molybdate [pH 7.5 at 25°C]). The cells were then homogenized on ice and the homogenate was centrifuged at 100,000 × *g* for 25 min at 4°C to yield a cytosol supernatant. The 350-μl reaction mixtures contained cytosol (35 to 66 μg of protein in assay 1; 270 to 440 μg of protein in assay 2) and 26 to 30 nM [^{3}H]dexamethasone (80 Ci/m mol) ± a 200-fold excess of unlabeled dexamethasone. The reaction was incubated for 90 min at 4°C and 250-μl portions were loaded on a Sephadex G-50 column in TEGM to separate bound and free hormone. Fractions were collected in scintillation vials, and radioactivity was determined by using ACS (Amersham) as the scintillant. Specific binding was calculated as the difference between total and nonspecific binding and was at least 90% of the total binding. Protein concentrations were determined by the method of Bradford (6), with gamma globulin as the standard.

RESULTS

Glucocorticoid inducibility of *gpt* RNA in transformants. In an attempt to place *Eco gpt* expression in eucaryotic cells under glucocorticoid hormone control, we constructed a plasmid in which a 1.4 kb LTR fragment of MMTV was inserted into the previously described pSV2*gpt* (19). The resulting plasmid (pSVM*gpt*) contains *Eco gpt* sequences immediately downstream from the MMTV promoter and its associated hormone responsive region (Fig. 1) (17).

Mouse 3T6 fibroblasts were transfected with pSVM*gpt* DNA, and *gpt*$^+$ transformants were selected, using the protocol described by Mulligan and Berg (20). Briefly, treatment of cells with the IMP dehydrogenase inhibitor mycophenolic acid prevents de novo synthesis of XMP, the immediate precursor of GMP. In the absence of exogenous guanine, cells are thus unable to synthesize GMP. However, transformants expressing *Eco gpt* are capable of converting xanthine (supplied in the growth medium) to XMP, thereby overcoming the block to GMP synthesis. The mammalian purine salvage enzyme HGPRT is incapable of utilizing xanthine (20).

Clones expressing *Eco gpt* arose at a frequency of approximately 1 per 10^4 cells exposed to DNA. We selected three independent transformants and prepared RNA from cells grown in the presence and absence of dexamethasone. To quantitate *Eco gpt* RNA and to ascertain whether the RNA was initiating at the MMTV promoter, we performed DNA excess hybridization experiments by using an end-labeled probe, as described by Berk and Sharp (5). The probe, labeled with ^{32}P at the *Bgl*II site within *Eco gpt* (Fig. 1), was hybridized to equivalent amounts of polyadenylate-containing RNA from each of the three clones. In each case a band of approximately 400 nucleotides was protected from digestion by S1 nuclease (Fig. 2). The size of the protected fragment indicates that transcription initiates approximately 270 nucleotides upstream of the *gpt* sequences, consistent with the known start site of MMTV RNA synthesis (33). It is clear that the amount of *gpt* RNA increased in the dexamethasone-treated cells. However, as is apparent from Fig. 2, there is substantial variation in both the basal level of expression of *Eco gpt* sequences and the absolute extent of induction among these clones, 3T6.3 exhibiting the largest induction of about 10- to 15-fold.

In addition to the 400 base pair protected fragment, we found varying amounts of reannealed probe after S1 digestion. The amounts seem to vary with the concentration of viral RNA present in the sample and may represent an artifact of the S1 nuclease digestion conditions. Alternatively, there may be random glucocorticoid-inducible transcripts that span the entire length of the probe. Experiments to determine the nature of this material are under consideration.

Hormone-dependent growth of pSVM*gpt* transformants. In the presence of mycophenolic acid, pSVM*gpt*-transformed cells required both *Eco gpt* expression and xanthine in the medium for survival. In an attempt to define conditions under which levels of *Eco gpt* expression are rate limiting for growth, we determined the xanthine concentration needed for 3T6.3 cell growth in the presence and absence of dexamethasone. Figure 3a shows that 3T6.3 was hormone dependent for growth at xanthine concentrations of ≤2.5 μg/ml. This effect seemed to be due to induction of *Eco gpt*, since in the absence of mycophenolic acid (Fig. 3b), when *Eco gpt* was no longer needed for growth, the effect was not seen. Surprisingly, hormone-treated cells were capable of growing, albeit slowly, even in the absence of exogenously

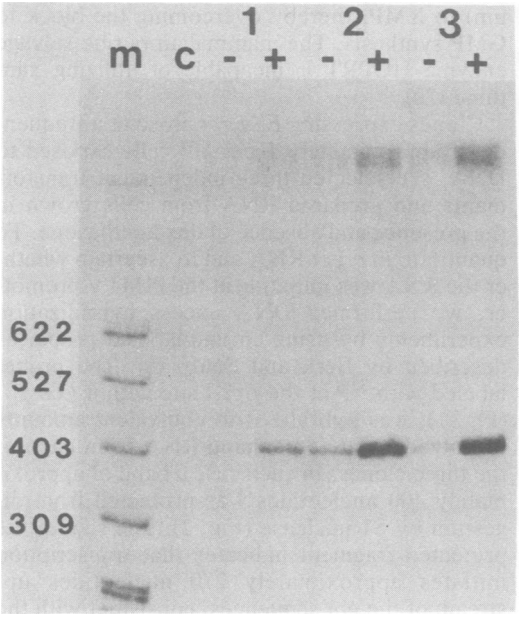

FIG. 2. S1 nuclease mapping and quantitation of pSVM*gpt* RNA in three transformants of mouse 3T6 fibroblasts. Polyadenylate-containing RNA was isolated from three pSVM*gpt*-transformed 3T6 cell clones grown in the presence (+) or absence (−) of 1 μM dexamethasone. The *Bgl*II site within the pSVM*gpt* plasmid was labeled with [^{32}P]ATP, using T4 polynucleotide kinase. The plasmid was then digested with *Eco*RI endonuclease and the labeled fragment containing the MMTV LTR and the 5′ end of *Eco gpt* was isolated after agarose gel electrophoresis. A 5-μg amount of each RNA was hybridized with this probe and subsequently digested with S1 nuclease. The hybrids generated were analyzed on a 6% polyacrylamide gel. The expected size of the resulting hybrid for an RNA initiating at the MMTV promoter is approximately 400 base pairs. Symbols: m, ^{32}P-labeled *Hpa*II digest of pBR322 (sizes are in base pairs); c, RNA from untransformed 3T6 cells; lanes 1 to 3, RNA from clones 1 to 3 of pSVM*gpt*-transformed 3T6 fibroblasts. By densitometer tracings, clone 3 (3T6.3) showed a 15-fold induction of pSVM*gpt* RNA in response to 1 μM dexamethasone. The bands in samples 1 to 3 above the 622-base pair marker represent reannealed probe.

supplied xanthine. We suspect that serum must contain low levels of xanthine or guanine, since this effect was not seen when dialyzed serum was used (data not shown).

The effects of varying dexamethasone concentrations on 3T6.3 cell growth were determined at different xanthine concentrations. The half-maximal response occurs at approximately 30 nM dexamethasone, regardless of the xanthine concentration (Fig. 4). This is a characteristic value for the induction of MMTV RNA by dexamethasone in mouse mammary tumor cells or MMTV-infected rat liver cells (25, 30).

Selection of cells with altered hormone-dependent growth properties. The ability to establish conditions under which pSVM*gpt*-transformed cells are dependent on dexamethasone for growth suggested a possible method for selecting cells that contain increased amounts of glucocorticoid receptor. As described above, cells were grown in low levels of both xanthine (≤2.5 μg/ml) and dexamethasone (10 nM to 1 nM) in the hope that cells expressing increased levels of receptor might respond better to the hormone, thereby providing them with a growth advantage. Indeed, under these conditions, most but not all of the cells died. After 2 weeks, the selected population of cells was growing well, and clones were isolated.

To test the possibility that the selected cells might be more sensitive to glucocorticoids, we analyzed their ability to grow at 2.5 μg of xanthine per ml and various dexamethasone concentrations (Fig. 5). As expected, in low levels of hormone, the selected cells grew better than did the parental 3T6.3 cells. Surprisingly, however, whereas the parental cells exhibited a characteristic increase in growth with increasing hormone concentrations, the selected cells were severely growth inhibited when exposed to high concentrations of dexamethasone. The levels of glucocorticoid receptors in populations and clones of cells selected at various xanthine and dexamethasone concentrations were determined. The results of two such assays are shown in Table 1, and as the data indicate, no significant increase in receptor content was seen in the selected population tested (Pop 1). Thus, for most of the cells selected, the ability to grow in low xanthine and dexamethasone concentrations appeared to be independent of receptor content. Two of the selected clones that were assayed, though, did seem to have a significant increase in receptor levels (two- to threefold); this could have contributed to their survival in the selective media. The unexpected growth inhibition of the selected cells by increasing dexamethasone concentrations did not allow us

TABLE 1. Glucocorticoid receptor assays[a]

Assay no.	Specific binding of [^{3}H]dexamethasone (fmol/mg of protein)			
	3T6.3	Population 1	Clone 1	Clone 3
1	298	241	706	878
2	495	475	978	1285

[a] Assays were performed as described in the text. A description of the conditions used to select Population 1, Clone 1, and Clone 3 is given in the legend to Fig. 6.

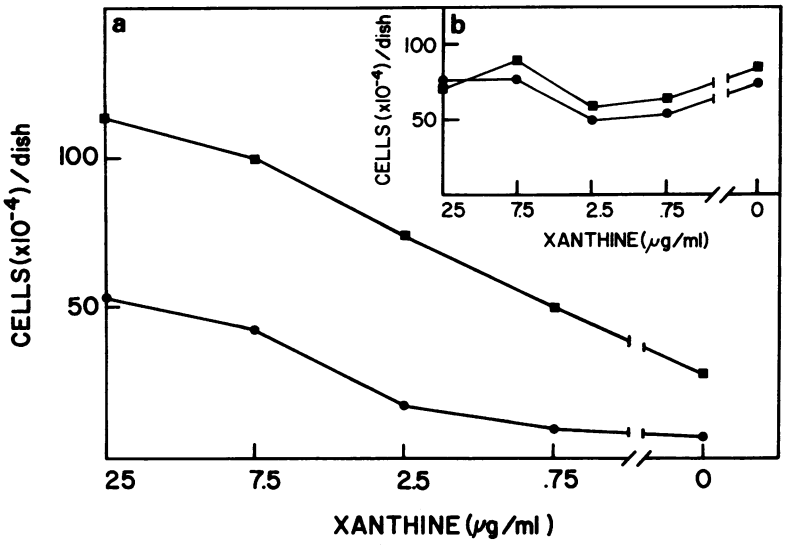

FIG. 3. Xanthine dependence for growth of pSVM*gpt*-transformed mouse 3T6.3 fibroblasts in the presence and absence of dexamethasone. Cells (2×10^4) were passaged into 60-mm dishes containing selective medium, 25 μg of xanthine per ml, and either 1 μM dexamethasone (■) or no dexamethasone (●). After 1 day, the xanthine concentration was changed to that shown in the figure. The cells were grown for 4 more days and then counted. The values represent the average of duplicate determinations. (a) 3T6.3 cells grown in selective media containing mycophenolic acid. (b) 3T6.3 cells grown in identical media lacking mycophenolic acid.

to test for a shift in the log dose versus growth of these cells, and thus we do not know if this two- to threefold increase in receptor levels has a physiological consequence.

Amplification of *Eco gpt* sequences. Cells able to grow in decreased levels of xanthine and dexamethasone may do so as a result of increased production of XGPRT. The levels of *Eco gpt* DNA and mRNA in the selected cells were measured by slot hybridization (see above)

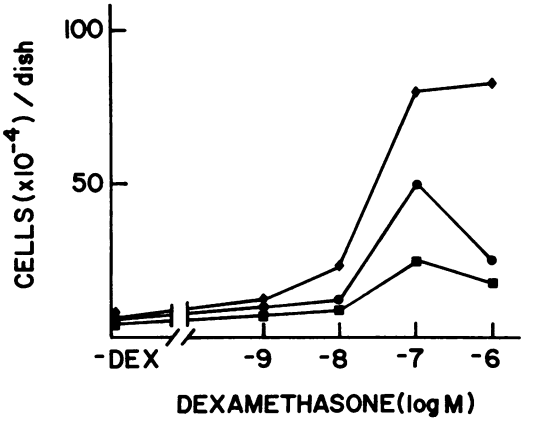

FIG. 4. Effect of dexamethasone concentration on the growth of 3T6.3 cells in limiting concentrations of xanthine. Cells (2×10^4) were passaged into 60-mm dishes containing selective medium, 25 μg of xanthine per ml, and the indicated concentrations of dexamethasone. After 1 day, the xanthine concentration was changed to either 2.5 μg/ml (◆), 0.75 μg/ml (●), or no additional xanthine (■). Four days later the cells were counted. The values represent the average of duplicate determinations.

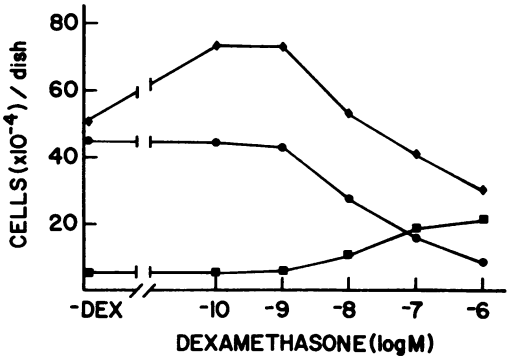

FIG. 5. Effect of dexamethasone concentration on the growth of 3T6.3 cells and selected cells in limiting concentrations of xanthine. Cells (2×10^4) were passaged into 60-mm dishes containing selective medium, 25 μg of xanthine per ml, and the indicated concentrations of dexamethasone. After 3 days, the xanthine concentration was changed to 2.5 μg/ml; 7 days later, the cells were counted. The values represent the average of duplicate determinations. Population 1 (●) was selected from 3T6.3 cells (■) by growth in selective medium containing no additional xanthine and 1 nM dexamethasone. Clone 5 (◆) was picked from Population 1.

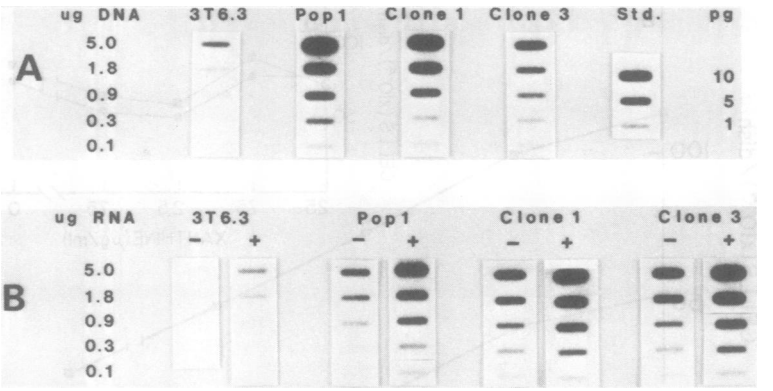

FIG. 6. Quantitation of pSVM*gpt* DNA and *Eco gpt*-specific RNA in 3T6.3 cells and selected cells. Population 1 (Pop 1) was selected in medium containing undialyzed serum and 1 nM dexamethasone without the addition of xanthine. Clone 1 was picked from a population selected in medium containing 3 nM dexamethasone without the addition of xanthine. Clone 3 was picked from a population selected in 0.75 μg of xanthine per ml and 1 nM dexamethasone. The indicated amounts of nucleic acids were blotted onto nitrocellulose filters and processed as described in the text. The filters were hybridized to nick-translated pSV2*gpt* DNA (pSVM*gpt* without the MMTV LTR fragment). (A) Total cellular DNA. Std, Nonradioactive pSV2gpt DNA used as a standard for the amount of specific hybridization. (B) Total cellular RNA isolated from cells grown in the presence (+) or absence (−) of 1 μM dexamethasone.

to directly test the possibility that these cells had amplified the *Eco gpt* DNA. DNA and RNA from the selected cells were bound to nitrocellulose paper and hybridized to nick-translated pSV2*gpt* DNA. Since mouse cells contain endogenous MMTV sequences, pSV2*gpt*, which lacks the MMTV LTR, was used as the probe for *Eco gpt* RNA and DNA. A selected population of cells and two independently isolated clones contained approximately 5- to 10-fold-higher levels of *Eco gpt* DNA than did the unselected 3T6.3 cells (Fig. 6). The selected cells also contained 15 to 20 times as much *Eco gpt* RNA as did the unselected cells. We cannot explain the apparent discrepancy between the relative increases in *Eco gpt* DNA and RNA in the selected cells. Perhaps only expressed copies of pSVM*gpt* underwent amplification to a level consistent with the increase in *gpt* RNA production. Lastly, these *Eco gpt*-amplified cells exhibited a four- to fivefold induction of *Eco gpt* mRNA by 1 μM dexamethasone. This level of induction was comparable to that seen in the starting 3T6.3 cells in this experiment (Fig. 6B), but less than the level of induction seen in the S1 analysis (Fig. 2). The difference in the apparent level of induction in these two experiments could be due either to the methods of the assays themselves or to daily variability in cell induction conditions.

Based on these results, it seems likely that the ability to grow in the selective conditions results from amplification of pSVM*gpt* DNA. The growth inhibition observed at high hormone levels may be due to a detrimental effect of excessive production of XGPRT in the fully induced, amplified cells. Consistent with this interpretation, when cells containing amplified *Eco gpt* DNA were placed into medium containing a high xanthine concentration (in the absence of dexamethasone), their growth was inhibited (data not shown).

DISCUSSION

We showed that the production of *Eco gpt* RNA became glucocorticoid regulated in mouse 3T6 cells transformed with the plasmid pSVM*gpt*. This result confirms previous reports by us and others that the MMTV LTR contains sequences sufficient for glucocorticoid-stimulated accumulation of mRNA (13, 17). Specifically, the 1.4-kb *Pst* fragment of MMTV has now been shown in our laboratory to render both the *Eco gpt* gene and a mouse DHFR cDNA hormone responsive. Similar transfection studies with other genes, such as human growth hormone and α_{2u} globulin, have shown that they also contain sequences sufficient for glucocorticoid responsiveness (16, 31). Studies are in progress in several laboratories to further define the DNA sequences involved in hormonal sensitivity.

The transformed 3T6 clones analyzed here exhibit varying degrees of induction of *Eco gpt* mRNA by dexamethasone, ranging from 6- to 15-fold. This extent of induction is still considerably lower than the 50- to 500-fold induction of MMTV RNA seen in infected HTC rat hepatoma cells but is consistent with the 10- to 15-fold induction of MMTV RNA seen in mouse mammary tumor cells (27). The reasons for these differences are not clear but may have to do with the site(s) of integration of the plasmid or proviral DNAs, the general glucocorticoid responsiveness of the cell, or the removal of the 1.4-kb fragment from the context of the other MMTV sequences. We have recently introduced pSVM*gpt* DNA into MMTV-infected HTC cells to address some of these possibilities.

Our ability to define conditions under which cells become hormone dependent for growth suggested a possible method for selecting cells that contain increased levels of glucocorticoid receptor. If simple mass action applies to the generation of an activated hormone-receptor complex, then cells containing more receptors should be able to respond to lower concentrations of hormone. In the case described here, selection of cells that grow in limiting xanthine and dexamethasone concentrations may yield cells with increased receptor levels. However, since the selection was based on the ability to produce high levels of XGPRT, another likely outcome is amplification of the pSVM*gpt* plasmid itself. Indeed, glucocorticoid receptor assays of the selected population showed very little, if any, change in glucocorticoid receptor level. Rather, the predominant genotype selected was that in which pSVM*gpt* sequences were amplified 10-fold, resulting in overproduction of *Eco gpt* RNA. Two clones of selected cells, however, did show an increase in receptor levels (Clones 1 and 3). These clones also showed a lower level of pSVM*gpt* amplification, suggesting that in these cells an increase in glucocorticoid receptors, as well as an increase in pSVM*gpt* DNA content, is contributing to survival in the selective medium.

Gene amplification in eucaryotic cells seems to be a fairly common response to conditions in which an essential gene product is inactivated by a toxic ligand. Examples include the amplification of DHFR after selection with methotrexate (1); CAD (the multifunctional protein which contains the enzymatic activities carbamyl-P synthetase, aspartase transcarbamylase, and dihydroorotase) amplification after inhibition by *N*-(phosphoacetyl)-L-aspartate (PALA) (34); and metallothionein amplification by inactivation with cadmium (3). In the case described here, gene amplification seems to have occurred in response to limiting the availability of a substrate for an enzyme whose activity is required for growth. It may be possible to utilize this as a general approach to amplifying other genes of interest whose products are normally present at low levels.

We found that pSVM*gpt*-amplified 3T6.3 cells retained hormone-regulated expression of *Eco gpt* mRNA. Our results are in contrast to those of Mayo and Palmiter (18), who found that after a 10-fold amplification of the mouse metallothionein-I gene, the gene loses its responsiveness to glucocorticoids, but not to cadmium. Since the amplified DNA unit usually encompasses large amounts of flanking DNA and since in most cases the glucocorticoid-responsive region is within 1 kb of the promoter (at least for MMTV [17], growth hormone [31], and α_{2u} globulin [16]), it seems unlikely that the metallothionein gene was separated from its regulatory region during amplification. Whatever the explanation, our results demonstrate that loss of glucocorticoid responsiveness upon amplification is not a general phenomenon but rather may be peculiar to the metallothionein gene or a particular cell type. In addition, a 10-fold amplification does not titrate sufficient receptors (by binding to the MMTV DNA) to reduce the available pool to a level that would preclude induction of gene expression. It is possible, however, that greater amplification may have such an effect.

As suggested previously, excessive production of *E. coli* XGPRT may be toxic to cells (20). This can be seen directly in cells containing amplified pSVM*gpt* DNA. As *Eco gpt* RNA and, presumably, XGPRT activity in the amplified cells increases above a certain level after addition of dexamethasone, the cells begin to die. A similar phenomenon may have occurred during amplification of the plasmid pMDSG in transfected CHO cells (26). pMDSG is a plasmid vector containing both a mouse DHFR cDNA and an *Eco gpt* gene. When CHO cells transformed with this plasmid were selected in increasing concentrations of methotrexate, they increased their expression of DHFR 500-fold, whereas *E. coli* XGPRT production was only increased 50-fold. It seems that a maximum tolerable level of XGPRT was reached after 50-fold amplification of pMDSG, and that further increases in DHFR production occurred by other mechanisms. We do not know why excessive *E. coli* XGPRT is harmful to cells, but one possibility is that excessive XMP (or GMP) inhibits the de novo synthesis of adenine, and thus blocks cell replication (12).

From a practical point of view, we point out that fusion of strong promoters with genes whose products are toxic to cells may prevent selection of stable transformants containing the

gene of interest. The utility of a hormone-regulated promoter in such a situation was demonstrated here. Propagation of cells in the absence of glucocorticoids enables maintenance of cells that are producing the toxic substance at low levels. If increased levels of the gene product are desired, it is a simple matter to add the appropriate concentration of hormone. A similar approach could be utilized to study the effects of a protein whose increased activity alters several parameters of cell growth or morphology.

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