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Glucocorticoid Regulation of Thymidine Kinase (*Tk-1*) Expression in L929 Cells*

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Glucocorticoids regulate the proliferation of mouse L cells. Incorporation of [^{3}H]thymidine is inhibited by 70–90% within 24 h after addition of 0.1 μM dexamethasone. This effect on L cells is completely reversible. The expression of the thymidine kinase gene (*Tk-1*) has been examined in L cells that have been treated with 0.1 μM dexamethasone for 24 h. Dexamethasone inhibits thymidine kinase activity 70–90% after 24 h. This is associated with a 90–95% decrease in Tk mRNA abundance. The decrease in Tk mRNA is not caused by a decrease in transcription of *Tk-1*, as shown by nuclear run-on transcription assays. Transient expression of the CAT (chloramphenicol acetyltransferase) gene, driven by the *Tk-1* promoter, was not affected by dexamethasone, and transcription of stably integrated Tk minigenes in LMTk$^-$ cells was not affected by dexamethasone. This effect was observed regardless of whether Tk cDNA was fused to the simian virus 40 promoter or the mouse *Tk-1* promoter region. Conversely, expression of thymidine kinase was inhibited when stable Tk$^+$ transformants of LMTk$^-$ cells were exposed to glucocorticoids; and inhibition of expression was observed irrespective of the promoter that was used to drive transcription of the Tk minigenes. These data indicate that glucocorticoid regulation of *Tk-1* in mouse L cells is, within the limits of detection of the assays used in these studies, entirely due to a posttranscriptional mechanism.

Steroids cause a variety of responses, depending on the cells upon which they act. One of the main target cells for glucocorticoid action is the fibroblast. It has been found that the rate of growth of mouse fibroblasts in culture is depressed by glucocorticoids (Berliner, 1965). A number of investigators have shown that the rate of growth of mouse L929 fibroblastic cells is inhibited by low concentrations (10^{-9} to 10^{-7} M) of glucocorticoids (Berliner, 1965; Ruhmann and Berliner, 1965; Pratt and Aronow, 1966). The activity of steroids in depressing the rate of growth of mouse fibroblasts reflects the clinical efficacy of such compounds as anti-inflammatory agents (Ruhmann and Berliner, 1965, 1967).

Hackney *et al.* (1970) have identified the binding component for glucocorticoids in L929 cells using [^{3}H]triamcinolone acetonide. They also selected for steroid-resistant fibroblasts by growing them for 1.5 years in a high concentration of cortisol. These glucocorticoid-resistant L cells bound much less triamcinolone acetonide than did sensitive cells. These observations suggest involvement of the glucocorticoid receptor in mediating glucocorticoid action. More recently, Housley and Forsthoefel (1989) have selected a variant of glucocorticoid-resistant L cells that contained no specific glucocorticoid binding capacity, no immunoreactive glucocorticoid receptor protein, and no detectable glucocorticoid receptor mRNA.

Early investigations concerning the mechanism of growth inhibition brought about by glucocorticoids have focused on inhibition of the rate of incorporation of radioactive precursors (Pratt and Aronow, 1966 and Seifert and Hilz, 1966) and on inhibition of the rate of nucleic acid synthesis measured in subcellular fractions (Kemper *et al.*, 1969). Pratt and Aronow (1966) observed that 10^{-9} to 10^{-7} M cortisol, which depressed the rate of growth of mouse fibroblasts in culture, also decreased the rate of thymidine and uridine incorporation into macromolecular material. DNA synthesis was depressed earlier and to a greater degree than RNA synthesis. Protein synthesis was not depressed within the first 24 h after steroid addition.

More recent studies on the antiproliferative effects of glucocorticoids on various cell types have focused on the inhibition of cell cycle-specific genes such as c-*myc* (Forsthoefel and Thompson, 1987), ribosomal DNA (Cavanaugh and Thompson, 1983, 1985, 1986; Meyuhas *et al.*, 1987, Cavanaugh *et al.*, 1984, Gokal *et al.*, 1986; Thompson, 1986; Mahajan and Thompson, 1990; Gokal *et al.*, 1990; Mahajan *et al.*, 1990), and thymidine kinase (Barbour *et al.*, 1988). Cytosolic thymidine kinase (Tk; ATP:thymidine 5′-phosphotransferase, EC 2.7.1.21)[1] is of particular interest in studying mechanisms of cell proliferation, because this enzyme is one of several proteins whose expression is regulated during progression through the cell cycle. Cytosolic thymidine kinase activity reaches maximum activity as cells approach and enter the S phase of the cell cycle. This is correlated with accumulation of Tk mRNA, which occurs prior to the onset of S phase (Gudas *et al.*, 1989; Knight *et al.*, 1989). Following the S phase, cytosolic thymidine kinase activity declines. The induction of Tk mRNA at the S phase of the cell cycle involves transcriptional (Coppock and Pardee, 1987; Lieberman *et al.*, 1988; Lipson and Baserga, 1989; Stewart *et al.*, 1987), posttranscriptional (Coppock and Pardee, 1987; Groudine and Casmir, 1984; Hofbauer *et al.*, 1987; Lewis and Matkovich, 1986;

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[1] As is the convention with mouse genes (see *Standard Nomenclature of Inbred Strains of Mice, 7th Listing* (J. Staat (1980) *Cancer Res.* **40**, 2083–2128), italics are used in reference to the name of the thymidine kinase gene (*e.g. Tk-1* gene, *Tk-1* transcription), whereas the abbreviation Tk is used in reference to derivatives of the gene (*e.g.* Tk mRNA, Tk cDNA). The abbreviations used are: kb, kilobase(s); bp, base pair(s); CAT, chloramphenicol acetyltransferase.

Lieberman *et al.*, 1988; Merrill *et al.*, 1984; Stewart *et al.*, 1987), and translational (Gross and Merrill, 1988, 1989; Sherley and Kelley, 1988) controls. Lieberman *et al.* (1988) have investigated the effects of serum stimulation on the expression of *Tk-1* in mouse L cells. The evidence presented clearly showed posttranscriptional control of *Tk-1*. However, they did not eliminate regulation of the gene at the transcriptional level.

The effect of glucocorticoids on *Tk-1* expression have previously been investigated on P1798 mouse T lymphoma cells (Barbour *et al.*, 1988). Thymidine kinase mRNA decreases by >90% within 24 h after addition of dexamethasone. Thymidine kinase activity and nuclear run-on transcription of *Tk-1* decrease in parallel in extracts from glucocorticoid-treated P1798 cells.

As an approach to understanding how *Tk-1* is regulated by glucocorticoids, we undertook the investigation of the effects of dexamethasone on *Tk-1* expression in the mouse L cell line. Unlike P1798 cells, fibroblastic L929 cells grow as adherent monolayers. This facilitates transfection with chimeric genes and a more detailed study of specific sequences involved in *Tk-1* regulation. Furthermore, Tk^- variants of the L cell line ($LMTk^-$) allow for study of thymidine kinase expression after stable transfection of chimeric *Tk* genes fused to different promoters.

The experiments described below were undertaken to test the hypothesis that glucocorticoids regulate transcription of *Tk-1* in L929 cells. Thymidine kinase activity, mRNA, and transcription were assayed. A series of chimeric genes was constructed, introduced into L929 or $LMTk^-$ cells, and assayed for expression in glucocorticoid-treated cells. The results of these experiments are described below.

MATERIALS AND METHODS

Reagents—Restriction endonucleases and enzymes for modification of DNA were purchased from Bethesda Research Laboratories or New England Biolabs (Beverly, MA) and were used according to the manufacturer's suggested procedures. Proteinase K was purchased from Boehringer Mannheim. All ^{32}P-labeled nucleoside triphosphates and D-threo[*dichloroacetyl*-1,2-^{14}C]chloramphenicol were obtained from Du Pont-New England Nuclear. Fetal bovine sera were obtained from Flow Laboratories (McLean, VA). All other chemicals were purchased from United States Biochemical Corp., Sigma, or Fisher. Nitrocellulose (BA85, 0.45 μM) was manufactured by Schleicher and Schuell.

Cell Culture—L929.06 cell line is a subclone of the mouse L cell (NCTC 929; Sanford *et al.*, 1948) selected for their sensitivity to dexamethasone.[2] $LMTk^-$ cells are also derived from the mouse L cell line but are deficient in the thymidine kinase gene (Kit *et al.*, 1963). Both L929.06 cells and $LMTk^-$ cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and subcultured by treatment with trypsin.

Enzyme Assays—Thymidine kinase (EC 2.7.1.21, ATP:thymidine 5'- phosphotransferase) activity was determined by measuring the initial velocity of formation of [^3H]thymidine 5'-monophosphate (TMP), as described previously (Bresnick and Karjala, 1964). A known number of cells (2–3 × 10^7 cells) was homogenized by sonication in 10 mM Tris-HCl (pH 8.0). The homogenate was centrifuged at 100,000 × *g* for 30 min at 4 °C, and an aliquot of the supernatant fraction was assayed for activity. One unit of thymidine kinase activity is defined as the amount of enzyme required to synthesize 1 μmol of TMP in 1 h.

The activity of chloramphenicol acetyltransferase (CAT) was assayed in cell extracts according to the procedure of Gorman *et al.* (1982). Cells harvested after transfection (see below) were sonicated in 100 μl of 0.25 M Tris-HCl (pH 7.8). The homogenate was centrifuged at 12,000 × *g* for 5 min at 4 °C to remove debris. Fifty-μl aliquots of the supernatant fraction (containing 40–100 μg of protein) were incubated with 100 μl of reaction mixture containing 1 μl of D-threo[*dichloroacetyl*-1,2-^{14}C]chloramphenicol (57.9 mCi/mmol, 0.05 μCi/μl) and 0.8 mM acetyl-CoA and 0.25 M Tris-HCl (pH 7.8) at 37 °C for 1 h. The acetylated chloramphenicol products were then separated from the unacetylated form by thin layer chromatography.

[^3H]Thymidine Incorporation—At the indicated times, control and treated cells were pulsed for 1 h with [*methyl*-^3H]thymidine (1 μCi/ml). Cells were harvested with trypsin, and total incorporation was measured by precipitation with 10% trichloroacetic acid followed by liquid scintillation counting.

Recombinant DNA Probes—Plasmid pMTK4 was obtained from Lin *et al.* (1985). pMTK4 contains full-length mouse thymidine kinase cDNA under the control of the SV40 promoter in the pCD expression vector, as shown in Fig. 1. A 1.1-kilobase (kb) *Xho*I fragment of pMTK4 was gel-purified, nick-translated, and used as a probe for analysis of Tk mRNA. Plasmid p5B, which corresponds to mouse 18 S rRNA, was used as an internal control for Northern analysis of mRNA (Bowman *et al.*, 1981). The Syrian hamster 5 S RNA gene (plasmid pTH1; Hart and Folk, 1982) was used as an internal control for hybridization efficiency in nuclear run-on transcription analysis.

A number of plasmids were assembled or constructed and used for transfection protocols. The structures of the relevant plasmids are illustrated in Fig. 1. pMTK4 (see above) is a Tk expression vector in which full-length mouse Tk cDNA is transcribed from the simian virus 40 (SV40) early promoter/enhancer sequences. pSV2CAT (Gorman *et al.*, 1982) contains the SV40 promoter/enhancer fused to the bacterial chloramphenicol acetyltransferase gene. pSA4 (Lieberman *et al.*, 1988) was obtained from Dr. H. B. Lieberman, Columbia University, New York. This Tk expression vector contains full-length Tk cDNA, derived from pMTK4, fused to the mouse *Tk-1* promoter with 2.5 kb of 5'-flanking DNA. Note that pSA4 and its derivative pSA4Δ5' contain the first intron (*IVSI* in Fig. 1) of genomic *Tk-1*. pTK2.5CAT contains the *Tk-1* promoter (extending from −3 bp upstream of the origin of translation plus 2.5 kb of 5'-flanking sequence) fused to the CAT gene. pTK143CAT is deleted of 5'-flanking sequences upstream of −143 bp (relative to the start of translation). The 3' end of the TK143 promoter extends to −3 bp upstream of the origin of translation. pSA4Δ5' is a derivative of pSA4 that has been deleted of 5'-flanking sequences upstream of −143 bp (relative to the start of translation). pGF is a derivative of pSA4 that has been deleted of the first intron by polymerase chain reaction site-directed mutagenesis according to Vallete *et al.* (1989).

Northern Blot Analysis—Total RNA from control and dexamethasone-treated cells was extracted with proteinase K and sodium dodecyl sulfate as described previously (Wood *et al.*, 1984). Total RNA was denatured with 6% formaldehyde and 50% formamide, fractionated on a 1% agarose gel containing 2.2 M formaldehyde, and blotted onto nitrocellulose membranes according to Thomas (1980). The size of the mRNAs was estimated by comparison with ethidium bromide-stained 28 and 18 S rRNA. Nitrocellulose filters were hybridized overnight at 55 °C in the presence of 10% dextran sulfate,

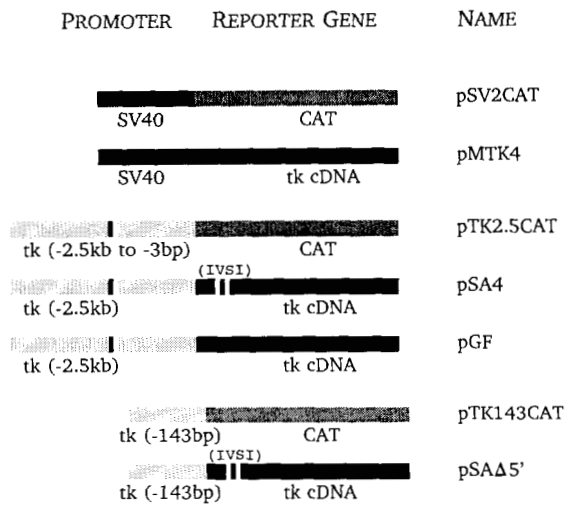

FIG. 1. **Thymidine kinase chimeric reporter and minigenes.** Shown above are schematic diagrams of the 5' sequence and the reporter gene in the hybrid plasmids that were either obtained from other laboratories or constructed in the laboratory, as described under "Materials and Methods." The expression vectors pSA4 and pSA5' contain intron I (designated *IVSI*) of the thymidine kinase gene.

[2] G. H. Frost *et al.*, submitted for publication.

50% formamide, 5 × SSC, 5 × Denhardt's solution, and 100 μg/ml calf thymus DNA. All hybridization filters were washed three times at 65 °C for 1 h (each wash), as described previously (Barbour *et al.*, 1988).

Nuclear Run-on Transcription Assay—Nuclear run-on transcription was carried out as described by Mahajan and Thompson (1987). Briefly, nuclei were isolated from control and dexamethasone-treated cells and transcription was carried out in the presence of [α-^{32}P]UTP. RNA was isolated after digestion with DNAse plus proteinase K in the presence of $CaCl_2$. Labeled RNA was washed by precipitation with trichloroacetic acid. Aliquots were withdrawn after transcription and before hybridization to calculate the recovery of labeled RNA, which was 30–60%. To compare control and dexamethasone-treated cells, equal nuclear equivalents of labeled RNA were hybridized to 3 μg of the appropriate plasmids that were linearized and blotted onto nitrocellulose.

DNA Transfection—L929.06 cells were plated in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum at a density of 1 × 10^6 cells/10-cm tissue culture dish 24 h before transfection. Cells were transfected with pSV2CAT (4 μg) or pTK2.5CAT (10 μg) by the DEAE-dextran method coupled with dimethyl sulfoxide shock treatment as described by Lopata *et al.* (1984). After 24 h, the cells were harvested with trypsin, subcultured into two dishes, and treated with either 70% ethanol (10 μl; final concentration, 0.07%) or 10^{-4} M dexamethasone (10 μl; final concentration, 0.1 μM). After 24 h (or 48 h after transfection), the cells were harvested, and CAT activity in the extracts was determined, as described above.

For stable transfections, LMTk$^-$ cells were plated in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum at a density of 1 × 10^6 cells/10-cm tissue culture dish 24 h before transfection. Cells were co-transfected with either 1) 10 μg of pMTK4 and 10 μg of pSV2CAT, 2) 10 μg of pSA4 and 10 μg of pTK2.5CAT, or 3) 10 μg of pSA4Δ5′ and 10 μg of pTK143CAT. Transfection was carried out by the calcium phosphate co-precipitation technique as described by Kingston (1989) and modified by Graham and van der Eb (1973) and Wigler *et al.* (1978). The cells were exposed to the precipitate for 5 h. The medium was removed and cells exposed to 10% dimethyl sulfoxide in phosphate-buffered saline for 3 min at room temperature. The cells were washed in phosphate-buffered saline and fed fresh medium. After 48 h, the cells were harvested with trypsin and subcultured in complete medium supplemented with 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (HAT; Kaufman *et al.*, 1986). The cells were fed with HAT medium every 4 days until colonies could be seen with the unaided eye. Afterward the cells were subcultured for analysis.

Protein Assay—Protein concentration in cell extracts was measured by the method of Bradford (1976).

RESULTS

Dexamethasone inhibits the proliferation of mouse L929.06 cells. This is shown in Fig. 2 which illustrates thymidine incorporation (*closed circles*) into L929.06 cells measured at intervals after treatment of mid-log phase cells with 0.1 μM dexamethasone. Thymidine incorporation decreased to 40% of control within 12 h. The maximum inhibition of thymidine incorporation by dexamethasone was observed at 24 h, at which time the activity varied between 30 and 10% of the control value in all experiments done. Similar results were obtained when thymidine kinase activity was measured in glucocorticoid-treated L929 cells (Fig. 2, *closed squares*). The coincidence of [^{3}H]thymidine incorporation and thymidine kinase activity reflects the fact that thymidine kinase catalyzes the rate-limiting step in the conversion of exogenous thymidine to DNA. This coincidence enables one to measure [^{3}H]thymidine incorporation as a first order approximation of thymidine kinase expression.

The inhibitory effect of dexamethasone on [^{3}H]thymidine incorporation in L929.06 cells is blocked when L929.06 cells are incubated with dexamethasone in the presence of a 10-fold excess of RU486 (Fig. 2, *open circles*), a competitive antagonist of the glucocorticoid receptor (Bourgeois *et al.*, 1984). Furthermore, dexamethasone did not inhibit thymidine incorporation into E8.2 cells (data not shown), an L929 variant that does not express the glucocorticoid receptor (Housley and Forsthoefel, 1989).

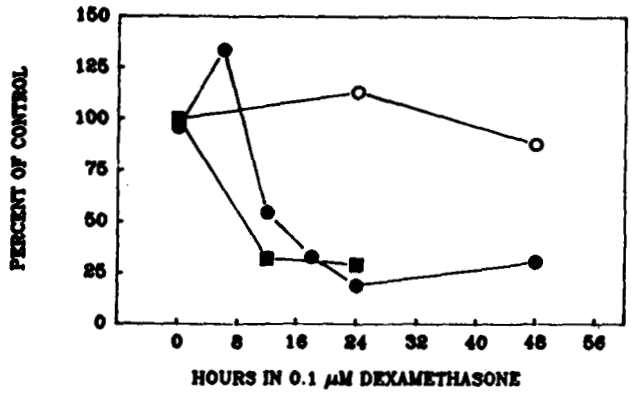

FIG. 2. **Effect of dexamethasone on [^{3}H]thymidine incorporation and thymidine kinase activity in L929.06 cells.** L929.06 cells at mid-log phase were treated with dexamethasone for 0, 6, 12, 18, 24, and 48 h, harvested, and assayed for [^{3}H]thymidine incorporation (*closed circles*). Extracts were also prepared from cultures that had been treated for 0, 12, or 24 h. These were assayed for thymidine kinase activity (*squares*). The data shown in *open circles* represent thymidine incorporation measured in the presence of dexamethasone plus a 10-fold molar excess of RU486 (final concentration of 1 μM). Thymidine incorporation was normalized to cell number (^{3}H counts/min/cell) with 100% of control being that level of incorporation observed in cells that had not been exposed to dexamethasone (0 h). Thymidine kinase activity was normalized to protein concentration in the extracts that were assayed, with 100% of control being the activity that was assayed in extracts from untreated cells (0 h).

Thymidine kinase mRNA was measured in the cells after treatment with glucocorticoids. As shown in Fig. 3, Tk mRNA abundance decreased to <5% of control (*lane C*) upon treatment of cells with 0.1 μM dexamethasone for 24 h (*lane D24*). Cultures that had been treated with dexamethasone for 24 h were washed to remove the steroid and refed with fresh medium without dexamethasone. Such dexamethasone-rescued cells were harvested 12 and 24 h after removal of the steroid and Tk mRNA abundance was assayed (*lanes R12* and *R24*, Fig. 3). The abundance of Tk mRNA increased in rescued cells, demonstrating the reversibility of dexamethasone inhibition. The observation that the abundance of Tk mRNA in rescued cells exceeded that observed in control cells is consistent with the hypothesis that dexamethasone causes L929 cells to arrest at a specific stage of the cell cycle such that rescued cells undergo synchronization of their cell cycle upon addition of fresh medium.

To determine whether or not the decrease in Tk mRNA upon dexamethasone treatment is caused by inhibition of mRNA synthesis, nuclear run-on transcription assays were carried out in nuclei isolated from control and dexamethasone-treated cells. As shown in Fig. 4, dexamethasone did not inhibit nuclear run-on transcription of the *Tk-1* gene (pMTK4) in L929.06 cells. It is known that glucocorticoids do not inhibit the transcription of the 5 S RNA gene (pTH1) in P1798 cells (Cavanaugh *et al.*, 1984). Likewise, in L929.06 cells, transcription of the 5 S RNA gene was not inhibited by dexamethasone.

LMTk$^-$ cells were co-transfected with pairs of expression vectors, including one that conveys expression of thymidine kinase and a second chimeric gene that conveys expression of CAT (see Fig. 1). Stable Tk$^+$ transformants were selected in HAT medium, and expression of thymidine kinase and CAT was measured in control and glucocorticoid-treated cells. One set of cells was transformed to Tk$^+$ by transfection with a pair of genes that encoded thymidine kinase and CAT tran-

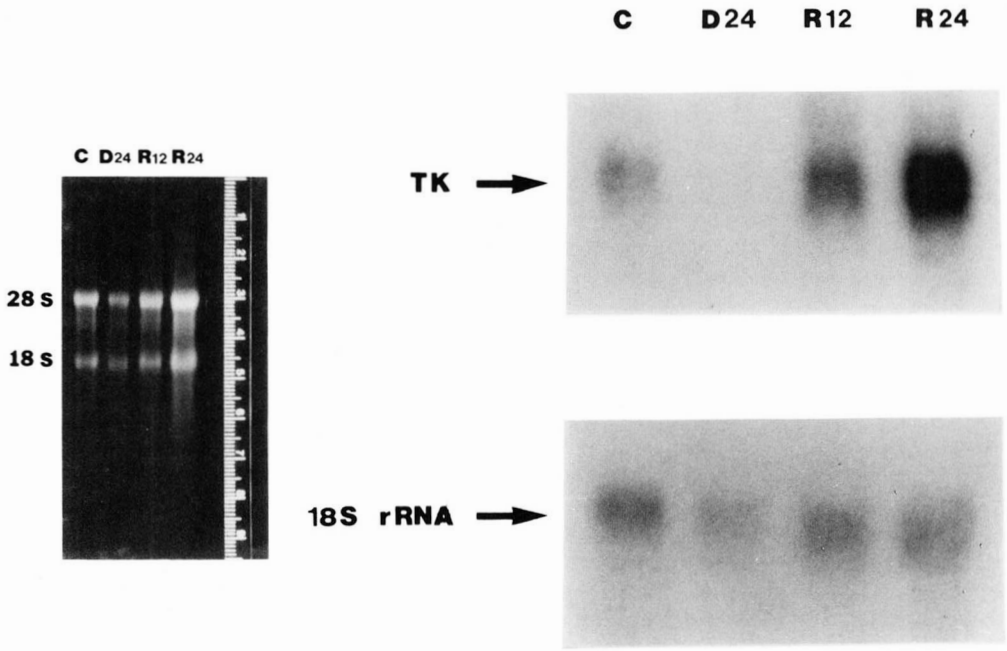

FIG. 3. **Effect of dexamethasone on Tk mRNA levels in L929.06 cells.** L929.06 cells were treated with dexamethasone for 24 h. Some cells were rescued from dexamethasone by removing the steroid-containing medium, washing with warm medium for 30 min, and replacing the medium with fresh warm (37 °C) medium. The control, treated, and rescued cells were harvested at the appropriate times and total cellular RNA was extracted. Total RNA from the cells was denatured and resolved in formaldehyde-denaturing agarose gel (10 μg of RNA/lane). The *panel* on the *left* shows ethidium bromide staining of 28 and 18 S RNA from control cells (*C*), cells treated with dexamethasone for 24 h (*D24*), cells treated with dexamethasone for 24 h and harvested 12 h after rescue (*R12*), and cells treated with dexamethasone for 24 h and harvested 24 h after rescue (*R24*). The *top right panel* shows the nitrocellulose filter that had been hybridized to the Tk cDNA probe, and the *lower right panel* shows the same filter after hybridization with 18 S RNA probe.

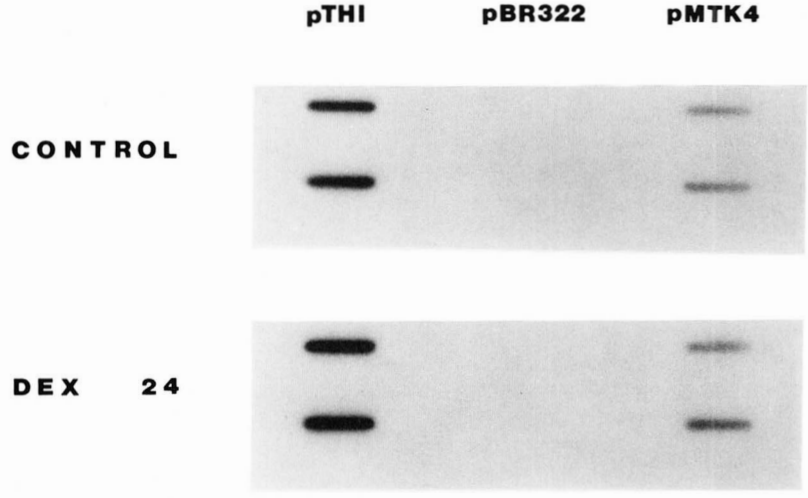

FIG. 4. **Nuclear run-on transcription of Tk-1.** L929.06 cells at mid-log phase were treated with dexamethasone, nuclei were isolated after 24 h, and transcription was carried out in the presence of [α-^{32}P]UTP. ^{32}P-Labeled RNA was isolated and hybridized to filter-immobilized probes (in duplicates), as described under "Materials and Methods." The Tk probe used was full-length mouse Tk cDNA (pMTK4). Transcription of the 5 S RNA gene (pTH1) was measured as an internal standard for recovery and hybridization efficiency. pBR322 was used to detect nonspecific hybridization.

scribed from the SV40 promoter (pSV2CAT and pMTK4). A second population employed the *Tk-1* promoter with 2.5 kb of 5′-flanking sequence (pTK2.5CAT and pSA4). A third set of cells were transformed using genes transcribed from the *Tk-1* promoter with 2.5 kb of 5′-flanking sequence but deleted, in the case of the Tk minigene of intron I (pGF and pTK2.5CAT). The fourth set of cells received chimeric genes containing the *Tk-1* promoter deleted to −143 bp upstream of the start of translation of Tk mRNA (pTK143CAT and pSA4Δ5′). Pooled stable Tk$^+$ transformants were treated with dexamethasone for 24 h; extracts were prepared and CAT assays were carried out. No decrease in CAT activity was observed in dexamethasone-treated cells (data not shown). Similar results were obtained when transient expression of chimeric Tk/CAT genes was assayed in dexamethasone-treated L929 cells (data not shown).

Thymidine kinase activity was measured in stable Tk$^+$ transformants, as shown in Fig. 5. Similar amounts of [^{3}H]thymidine were incorporated by all transformants, suggesting that all express similar amounts of thymidine kinase activity. Thymidine incorporation in all transformants was inhibited following addition of dexamethasone. The abundance of cytoplasmic Tk mRNA was also measured in control and dexamethasone-treated Tk$^+$ transformants of LMTk$^-$cells (data not shown). Irrespective of the promoter used to drive Tk expression, it could be demonstrated that cytoplasmic Tk mRNA abundance correlated with [^{3}H]thymidine incorporation and was reduced by at least 90% in dexamethasone-treated cells. These data indicate that glucocorticoid inhibition of expression of thymidine kinase is not influenced by the promoter from which the Tk cDNA is transcribed. Likewise deletion of intron I (indicated by *−IVSI* in Fig. 5) had

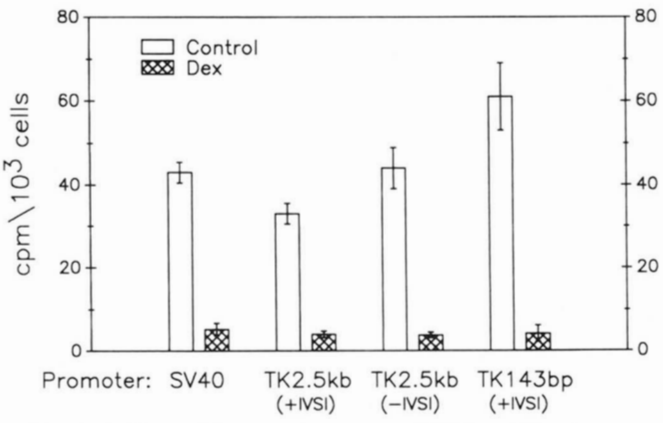

FIG. 5. **Effect of dexamethasone on [³H]thymidine incorporation in LMTk⁻ parent cells and Tk⁺ transformants.** After HAT selection, the stable transformants were plated at 8,000 cells/cm² in T25 flasks. Parallel cultures of the parent LMTk⁻ cells were also set up. At their mid-log phase of growth, the cells were treated with either solvent or dexamethasone (0.1 μM) for 24 h. At this time, the cells were pulsed with [³H]thymidine (1 μCi/ml) for 1 h, and the radioactivity incorporated was measured as described under "Materials and Methods." The number of cells present in aliquots from each flask were counted with a hemocytometer. Each observation is the mean of duplicate determinations ± standard deviation.

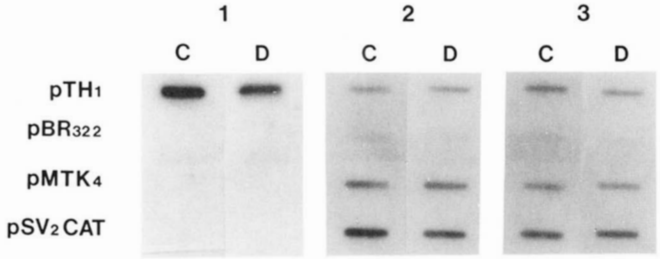

FIG. 6. **Nuclear run-on transcription of Tk and CAT genes in LMTk⁻ cells and Tk⁺ transformants.** LMTk⁻ cells and LMTk⁻ cells that had been stably transfected with pMTK4 plus pSV2CAT or pSA4 plus pTK2.5CAT were prepared and at mid-log phase were treated as described in Fig. 4. Nuclei were isolated after 24 h, and transcription was carried out in the presence of [α-³²P]UTP. ³²P-Labeled RNA was isolated and hybridized to filter-immobilized probes (in triplicates) as described under "Materials and Methods." The Tk probe used was full-length mouse Tk cDNA (pMTK4). The CAT probe used was the bacterial chloramphenicol acetyltransferase coding sequence (pSV2CAT). Transcription of the 5 S RNA gene (pTH1) was measured as an internal standard for recovery and hybridization efficiency. pBR322 was used to detect nonspecific hybridization. Shown are representative slots for control (*C*) and dexamethasone-treated (*D*) cells. *Panel 1*, LMTk⁻ parent cells (untransfected); *panel 2*, LMTk⁻ cells that had been stably transfected with pMTK4 plus pSV2CAT; and *panel 3*, LMTk⁻ cells that had been stably transfected with pSA4 plus pTK2.5CAT.

no detectable effect upon glucocorticoid regulation or basal expression of thymidine kinase.

All data are consistent with the conclusion that dexamethasone has little or no effect upon transcription of *Tk-1* in L929 cells. However, this conclusion is based upon certain assumptions concerning the reliability with which nuclear run-on of *Tk-1* may be measured. For example, the results could be misinterpreted if L cells express a constitutive gene whose products adventitiously cross-hybridize with the Tk probes. To explore this possibility, nuclear run-on of Tk minigenes and CAT chimeric genes was assayed in Tk⁺ transformants of LMTk⁻ cells. The data are shown in Fig. 6. *Panel 1* illustrates the results obtained with untransformed LMTk⁻ cells. These cells actively transcribe 5 S RNA genes (pTH1), and transcription of this gene is similar in nuclei from control (*C*) and dexamethasone-treated (*D*) cells. No signal is observed when labeled RNA is hybridized to probes for Tk (pMTK4) or CAT (pSV2CAT). *Panel 2* contains data from cells that were transformed by co-transfection with a Tk minigene plus a CAT reporter transcribed from the SV40 promoter (pMTK4 and pSV2CAT). Tk⁺ transformants express both thymidine kinase and CAT. No effect of dexamethasone was observed, as would be expected for genes transcribed from the SV40 promoter. The data shown in *panel 3* were obtained with nuclei from cells that had been transformed to Tk⁺ by co-transfection with a Tk minigene and CAT transcribed from the *Tk-1* promoter with 2.5 kb of 5′-flanking sequence (pSA4 and pTK2.5CAT). Dexamethasone did not inhibit transcription of the Tk minigene or the CAT reporter gene driven by the *Tk-1* promoter.

Transcription of the Tk minigenes and CAT reporters was similar in magnitude, irrespective of the promoter from which the genes were expressed. (Compare the signal generated from either CAT or Tk in *panels 2* and *3* of Fig. 6). This observation is consistent with the observation that expression of thymidine kinase is similar in Tk⁺ transformants in which the Tk minigene is driven by either the SV40 or *Tk-1* promoter (Fig. 5).

DISCUSSION

Three conclusions may be drawn from the results of the experiments described in this report. The first, and most obvious, conclusion is that glucocorticoid regulation of expression of thymidine kinase in L929 cells is entirely posttranscriptional. The observation that glucocorticoids down-regulate Tk mRNA, irrespective of the promoter from which the gene was transcribed, would seem to preclude any other interpretation. The turnover rate of Tk mRNA has not been measured, so we cannot safely conclude that glucocorticoids cause a decrease in the half-life of the mRNA. However, preliminary results (discussed below) suggest that this is the mechanism whereby expression of thymidine kinase is inhibited in L929 cells. This is not typical of glucocorticoid regulation. The glucocorticoid receptor is a transcription factor that is known to bind to certain promoters and stimulate transcription (Beato, 1987, 1989; Beato *et al.*, 1989; Pfahl, 1986; Ringold, 1985; Yamamoto, 1985; Hollenberg *et al.*, 1987; Miesfeld *et al.*, 1987). Under other circumstances, binding of the glucocorticoid receptor may be associated with inhibition of transcription (Schena *et al.*, 1989; Drouin *et al.*, 1987, 1989). Finally, it is known that glucocorticoids can regulate the expression of certain transcription factors, thereby affecting secondary regulation of transcription (Mahajan and Thompson, 1990; Mahajan *et al.*, 1990). Most glucocorticoid-regulated genes exhibit some degree of transcriptional regulation, although a few regulatory transitions have been reported to include a posttranscriptional component (Silver *et al.*, 1990; Simmons *et al.*, 1987; Weiner *et al.*, 1987; Peterson *et al.*, 1989). To our knowledge, thymidine kinase is the only gene in which it has been thoroughly demonstrated that there is no transcriptional component to glucocorticoid regulation.

The lack of any transcriptional regulation of thymidine kinase in L929 cells stands in striking contrast to the situation that prevails in P1798 lymphoma cells (Barbour *et al.*, 1988). Thymidine kinase expression is reduced in P1798 cells with kinetics that resemble those observed in glucocorticoid-treated L929 cells (*e.g.* 50% reduction of mRNA abundance within 10–12 h). Both P1798 cells (Forsthoefel and Thompson, 1987) and L929 cells undergo reversible G1 arrest in the presence of glucocorticoids[2] and both cells are of connective

tissue origin. However, P1798 cells exhibit a significant reduction in nuclear run-on transcription activity upon exposure to dexamethasone (Barbour *et al.*, 1988). Recent studies in our laboratory indicate that regulation of thymidine kinase expression in the lymphoid (P1798) cell lines is almost entirely due to inhibition of initiation of transcription.[3] This suggests that fibroblastic and lymphoid cells may respond to glucocorticoids by fundamentally different mechanisms. Observations concerning regulation of expression of c-*myc*[2] and rDNA[4] are consistent with the conclusion that glucocorticoids have very different effects on gene expression in fibroblastic and lymphoid cells. This might not be so surprising were it not for that fact that both cell types exhibit a similar responsive end point, *i.e.* withdrawal from the cell cycle.

There are a number of reports of posttranscriptional regulation by steroid hormones. There is evidence to the effect that glucocorticoids can regulate the stability of certain mRNAs. The effects are usually fairly small and occur within the context of more significant changes in transcriptional activity. Consequently, there is almost nothing known about the mechanism(s) that provide for either stabilization or destabilization of mRNAs in glucocorticoid-treated cells. The glucocorticoid receptor is known to be a DNA-binding protein, and lacking any data to the contrary, one assumes that posttranscriptional regulation is unlikely to reflect a direct interaction between the glucocorticoid receptor and Tk mRNA. Consequently, the effect must almost certainly be secondary. This is a difficult hypothesis to test directly. One normally resorts to inhibitors of protein synthesis to determine if *de novo* protein synthesis is required for down-regulation of mRNA. However, this approach is not practical for thymidine kinase mRNA which is stabilized by inhibitors of protein synthesis (*e.g.* cycloheximide).[5] The phenomenon of "superinduction" is generally believed to be due to the existence of substances of short half-life that either degrade or cause to be degraded specific mRNAs (Almendral *et al.*, 1988). It is possible that glucocorticoids should influence the expression of such degradative principles, although the identity of such principles is unknown and this hypothesis cannot presently be tested. Others have demonstrated that the estradiol-estrogen receptor complex is required for cytoplasmic stabilization of vitellogenin mRNA (reviewed by Nielsen and Shapiro, 1990a, 1990b; Brock and Shapiro, 1983; McKenzie and Knowland, 1990). An estrogen-induced ribonuclease activity is believed to cause cytoplasmic destabilization of mRNAs coding for serum proteins in Xenopus laevis liver (Pastori *et al.*, 1991a, 1991b).

The second point that arises from these studies has to do with the question of whether all agents that cause G1 arrest share similar mechanisms for regulating gene expression. Specifically, the question arises as to whether glucocorticoids and serum starvation affect *Tk-1* expression in different ways. Although the answer to this question is not completely known, it appears certain that thymidine kinase, at least, is not regulated in precisely the same manner by these two agents. The effects of serum on *Tk-1* expression have been studied in some detail by a number of groups. Most of these studies have been carried out in fibroblastic cells (Gudas *et al.*, 1989; Knight *et al.*, 1989; Coppock and Pardee, 1987; Lieberman *et al.*, 1988; Lipson and Baserga, 1989; Stewart *et al.*, 1987; Groudine and Casmir, 1984; Hofbauer *et al.*, 1987; Lewis and Matkovich, 1986; Merrill *et al.*, 1984; Gross and Merrill, 1988; Gross and Merrill, 1989; Sherley and Kelley, 1988), and one series of promoter analysis experiments was carried out in L929 cells (Lieberman *et al.*, 1988). All of these data indicate that there is a major transcriptional component to serum stimulation of fibroblastic cells. Virtually all observations indicate that there is an additional posttranscriptional component to the serum response that may account for a 2–3-fold change in mRNA abundance. The data from serum starvation experiments would indicate that a cell that undergoes G1 arrest will cease to transcribe *Tk-1*. Our data indicate that this is not the case. Of course, it is possible that serum deprivation and glucocorticoids arrest cell proliferation at different restriction points. We have determined that glucocorticoids do not inhibit expression of c-*myc* mRNA in G1 arrested L929 cells.[2] One possible explanation of all of our observations is that glucocorticoids arrest L929 cells at a point very late in G1 at which transcription of *Tk-1* and c-*myc* can be sustained, although Tk mRNA is unstable.

The third and final conclusion is that those sequences that convey glucocorticoid sensitivity lie entirely within the mRNA sequences of *Tk-1*. This is, in fact, the basis for our conclusion that glucocorticoid regulation is entirely posttranscriptional. All intronic sequences can be deleted without loss of regulation. This suggests that changes in mRNA stability are affected by interaction with Tk mRNA rather than by changes in intranuclear processing. There are experimental data that are consistent with this conclusion. During the course of our experiments, we had occasion to measure Tk mRNA derived from the SV40 and *Tk-1* promoter chimera. We determined that when the *Tk-1* promoter was employed, virtually all of the Tk sequences were cytoplasmic, of a discrete size, and responsive to glucocorticoids. On the other hand, a substantial amount of Tk sequences transcribed from the SV40 promoter was disperse in size and never left the nuclei of Tk^+ transformants. Glucocorticoids had no effect upon the abundance of these nuclear Tk sequences. On the other hand, the cytoplasmic Tk sequences disappeared when SV/Tk transformants were treated with glucocorticoids. Based upon these data, we have tentatively concluded that the proximal site of glucocorticoid regulation of *Tk-1* expression in L929 cells is in the cytoplasm. We strongly suspect that nuclear events intervene between the binding of the agonist to the receptor and destabilization of Tk mRNA, but we have no data that preclude alternative explanations.

[3] K. Rhee and E. A. Thompson Jr., manuscript in preparation.

[4] Morrill and E. A. Thompson Jr., unpublished observations.

[5] G. H. Frost, K. Rhee, and E. Aubrey Thompson Jr., unpublished observations.

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