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Use of an Inducible Hybrid Viral Gene as a Model for Evaluating Drug Effects on Gene Expression

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SUMMARY

The glucocorticoid-inducible LTL gene [*Cell* 38:29–38 (1984)] was used as a model target to evaluate preferential drug effects on gene expression. Specifically, the potential of bleomycin, neocarzinostatin, and actinomycin D to induce alterations in either transcriptional or posttranscriptional gene expression was assessed. A Northern blot analysis was used to measure transcriptional effects, whereas changes in posttranscriptional expression were determined through an enzymatic assay for the thymidine kinase product of the LTL gene. Comparisons of the results from these assays with results obtained from assays that evaluated drug effects on cellular RNA and protein synthesis showed that none of the drugs were capable of inducing preferential effects on transcription. However, selective drug-induced effects on the expression of thymidine kinase activity were observed.

There are now numerous studies demonstrating an interrelationship between DNA structure and gene activity. In particular, the structure of transcribing chromatin has been shown to be distinct from that of bulk chromatin in that the former appears to be characterized by a more open conformation (see Refs. 1–3 for reviews). Although there is no direct proof demonstrating that a structural alteration is required for inducing a change in transcription, it is interesting that several promoter and enhancer regions have been shown to adopt altered structures (4–6). Indeed, one study has shown that a change in structure within a glucocorticoid-responsive enhancer sequence occurs with a time course that either just precedes or closely parallels the changes in transcriptional activity (7).

Because of the apparent relationship between gene structure and transcription, it seems probable that agents that are capable of inducing changes in DNA structure could also alter gene expression. Indeed, there are studies that indicate that certain antineoplastic drugs have such potential. For example, the strand scission antibiotic BLM has been shown to induce changes in both chromatin structure (8, 9) and gene expression. In particular, this drug has induced differentiation in hematopoietic cells (10, 11) and collagen synthesis in lung fibroblasts (12, 13). Studies with other drugs, which include VM26 (14), ethidium bromide (15), distamycin A (16), and AMD (17), have indicated that the potential to alter gene expression is not confined to a particular type of DNA-interactive drug.

Although in some cases it appears that an interaction between the drug and DNA is responsible for the observed change in gene expression (14–16), there are studies that show that drugs can exert their effects posttranscriptionally (18, 19). Thus, in order to more fully understand the mechanisms by which a drug could alter gene expression, it is necessary to systematically characterize its interaction at the level of transcription and beyond. Consequently, a system that directly assesses drug effects on transcriptional as well as posttranscriptional gene expression is needed.

Accordingly, in the studies described in this report, a mouse fibroblast cell line (L1.2-2) containing several integrated copies of a unique hybrid viral gene was developed for use as a model in which to assess potential drug effects on gene expression. This gene, known as LTL, was originally constructed and characterized by Zaret and Yamamoto (7). It contains the coding sequences for a viral tK gene sandwiched between regulatory elements derived from mouse mammary tumor virus DNA (see Fig. 1). Because the regulatory elements contain both the transcriptional initiation site and a glucocorticoid-responsive enhancer domain, efficient transcription of LTL and expression of its tK product require exogenous glucocorticoids. Having a gene whose efficient expression is dependent upon the presence of exogenous factors (like glucocorticoids) provides a useful model for assessing the ability of a drug to selectively alter gene expression because both the stimulatory and inhibi-

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ABBREVIATIONS: BLM, bleomycin; AMD, actinomycin D; TdR, thymidine; Kb, kilobase; NCS, neocarzinostatin; PBS, phosphate-buffered saline; tK, thymidine kinase; HEPES, *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid.

tory potential of the drug can be evaluated. In particular, because transcription within LTL is almost nonexistent in the absence of glucocorticoids, the ability of a drug to induce transcription can be readily assessed in those cells that do not receive any glucocorticoid treatment. Conversely, when glucocorticoids are added just before drug treatment, the ability of a drug to inhibit the initiation of transcription within the LTL gene can be evaluated. Furthermore, because the tK product of the LTL gene is readily quantifiable through biochemical assays (20), drug effects on posttranscriptional expression can be easily measured.

The drugs chosen for study in the LTL system include the DNA strand scission antibiotics BLM and NCS, as well as the classical RNA synthesis inhibitor AMD. Previous studies with LTL indicated that the transcriptional regulatory region of this gene was a preferential target for BLM- or NCS-induced cleavage (21) and, therefore, it was of interest to evaluate the potential of these antibiotics to selectively alter LTL gene expression. In addition, both AMD and BLM have been shown previously to induce alterations in gene expression (10–13, 17). Therefore, through the use of Northern blots to measure changes in transcription and the use of tK assays, we have systematically evaluated the potential of these drugs to alter transcriptional or posttranscriptional gene expression, and we demonstrate how the LTL gene can be used as a model for such evaluations.

Experimental Procedures

Materials. Blenoxane, the clinical preparation of BLM, was generously supplied by Dr. Bradner from Bristol-Myers. The drug was stored as a solid at −20° and was dissolved in PBS (no. 450-1300; GIBCO, Grand Island, NY) before use. NCS, which was also obtained from Bristol-Myers, was supplied as a 1.0 mg/ml solution in 2.0 ml of 15 mM sodium acetate (pH = 5.0). Stocks were stored frozen at −20° and diluted in PBS immediately before use. The concentrated stocks of NCS used in some experiments were prepared using a ProDiMem (Amicon, Lexington, MA) concentration apparatus with a 10,000 molecular weight exclusion membrane. Dexamethasone (no. D-1756) and AMD (no. A-1410) were purchased from Sigma Chemical Company (St. Louis, MO). Stocks of dexamethasone were prepared in ethanol and stocks of AMD were made in methanol. Both stocks were stored at −20° and diluted in PBS just before use.

[5,6-^{3}H]Uridine (no. MT-799; 1 mCi/ml, 40 Ci/mmol) and [2-^{14}C]TdR (no. MC-104; 0.1 mCi/ml, 40 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). L-[4,5-^{3}H]leucine (no. TRK.510; 1 mCi/ml, 186 Ci/mmol) and [α-^{32}P]TTP (no. PB.10387; 10 mCi/ml, 800 Ci/mmol) were purchased from Amersham (Arlington Heights, IL).

Cell culture. The L1.2-2 cell line, which has been described previously (7, 20), was maintained in Dulbecco's modified Eagle's medium (GIBCO no. 430-2100) containing 10% bovine calf serum (Hyclone no. A2151-L) and 10 mM HEPES (Boehringer-Mannheim, Indianapolis, IN). Stock cultures were grown in 75-cm^2 flasks (Falcon) at 37° in humidified 5% CO_2 incubators and were passaged 1 or 2 times per week.

Effects of drugs on macromolecular synthesis. Drug effects on the synthesis of total cellular RNA were assessed by a [^{3}H]uridine incorporation assay. Cells to be used in these determinations were near-confluent monolayer cultures that had been plated 3 days before drug treatment at a density of 1×10^6 per 25-cm^2 flask in 5.0 ml of medium. On the day of treatment, the spent medium was replaced with 2.5 ml of fresh medium, which contained 1×10^{-7} M dexamethasone and 1.0 μCi/ml [^{3}H]uridine. BLM, NCS, or AMD were diluted in PBS at 4°, and various dilutions of each drug were added in 500-μl aliquots to the appropriate flasks, immediately after the medium change. The cells

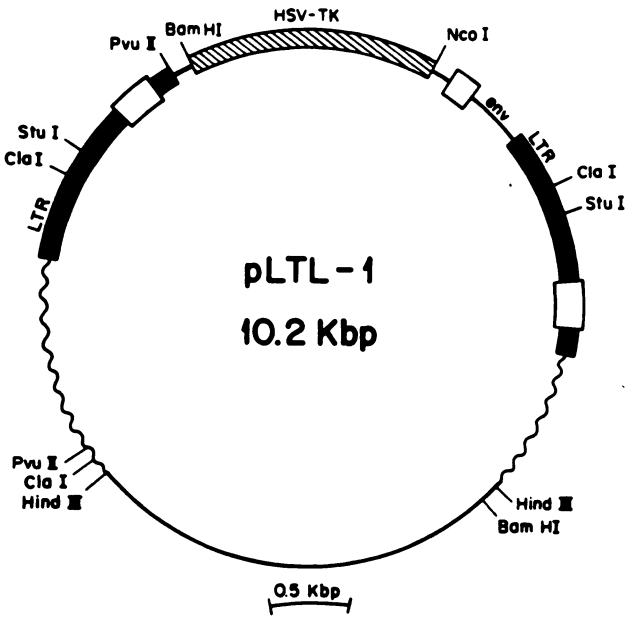

Fig. 1. Structure of plasmid pLTL-1. In this heterologous plasmid, the bulk of the coding sequences for the mouse mammary tumor virus are replaced by a 1.5-Kb *Herpes simplex* virus tK gene (*hatched box*) such that this gene is bounded on each side by the retroviral long terminal repeats (*thick lines*). The glucocorticoid receptor binding regions associated with the mouse mammary provirus are denoted by the *open boxes*. The *wavy lines* denote the flanking rat DNA derived from the original proviral clone. Recognition sites for the restriction endonucleases *Nco*I, *Bam*HI, *Cla*I, *Stu*I, *Pvu*II, and *Hind*III are also shown. Cleavage of pLTL-1 with *Nco*I and *Bam*HI yields, in addition to other fragments, a 1.5-Kb fragment that consists of sequences that hybridize solely with the tK gene. This fragment was used as an LTL-specific probe in the Northern blot analyses. Transcription of LTL is initiated within the left LTR and proceeds rightward. The *curved bar* below the figure indicates 0.5 Kb.

were then incubated at 37° for 30 min. One flask of cells that had both dexamethasone and uridine but not drug was incubated for 30 min on ice. The total amount of radioactivity incorporated into these cells was considered background and was subtracted from the values determined for the other samples.

After incubation, the radioactive medium was removed and the cells were washed once with 5.0 ml of warm PBS. The cells were then detached from the flasks by trypsinization and resuspended in 5.0 ml of medium at 4°. Three separate 1.0-ml aliquots were removed and each was placed into a tube containing 9.0 ml of cold 0.5 M perchloric acid. The remaining 2.0 ml of cells were reserved for the determination of the cell number. Acid-precipitable material was recovered by centrifugation (1500 × *g* for 15 min) at 4°. The resultant pellet was washed three times in 10 ml of 0.4 M cold perchloric acid by alternative resuspension and centrifugation (1500 × *g* for 15 min). The final pellet was resuspended in 0.5 ml of 0.5 M perchloric acid, and the tubes were placed in a water bath at 70° for 1 hr. The entire contents of the tubes were then transferred to a scintillation vial, 10 ml of scintillation fluid (Beckman Instruments, Palo Alto, CA) were added, and the total amount of radioactivity in each sample was determined by scintillation counting.

The three determinations made for each drug treatment were averaged, and the total number of counts per 10^6 cells was determined. Background counts were subtracted, and the values of [^{3}H]uridine incorporation relative to a no-drug control were determined.

In experiments in which the effects of a 4- or 8-hr treatment with NCS or AMD on RNA synthesis were measured, unlabeled uridine (Sigma no. U-3750) was added to a final concentration of 50 μM. Under these conditions, incorporation of the labeled uridine remained linear throughout the duration of the assay.

The relative effects of NCS or AMD on protein synthesis were determined as described for RNA synthesis except that [^{3}H]leucine was used as the labeled precursor rather than [^{3}H]uridine. Control experiments showed that incorporation of [^{3}H]leucine into acid-precipitable material was linear for at least 8 hr.

Isolation of RNA. Monolayer cultures of L1.2-2 cells were plated in 25-cm^2 flask as described above and were allowed to grow to near-confluence. These cultures were subsequently treated with either BLM, NCS, or AMD immediately after the replacement of the spent medium with fresh medium containing either 0.1 μM dexamethasone or ethanol solvent. Total cellular RNA was isolated following various treatment periods at 37° as described below. Flasks were placed on ice, the medium was removed, and the cells were washed once with 5 ml of PBS. The cells were lysed with 2.0 ml of an 8 M guanidine hydrochloride solution (8 M guanidine hydrochloride, 10 mM EDTA, 50 mM Tris·HCl, pH 7.5), and the lysate was scraped off the surface of the flask using a rubber policeman. The lysate was collected, and the DNA was sheared by passing it 20–25 times through a 3-ml syringe fitted with an 18-gauge needle. Enough 1.0 M acetic acid (50 μl) was then added to give a final concentration of 25 mM and a pH of approximately 5. One-half volume of ethanol was added, the contents were vortexed immediately, and the samples were incubated overnight at −20°. The RNA was recovered by centrifugation (10 min in an Eppendorf microfuge), resuspended in a solution containing 6 M guanidine hydrochloride, 10 mM EDTA, 50 mM Tris·HCl, pH 7.5, and subsequently reprecipitated with acetic acid and ethanol. The RNA was again recovered by centrifugation, resuspended in the 6 M guanidine hydrochloride solution, and reprecipitated. Following an overnight incubation at −20°, the RNA samples were centrifuged; the resultant pellets were washed in 70% ethanol/30% H_2O and then dried for 10 min at room temperature in a vacuum dessicator. The RNA was resuspended in 300 μl of 25 mM EDTA (pH = 8.0) and the solution was incubated in a water bath at 60° for 1 min. After incubation, the tubes were vortexed and 300 μl of phenol, previously heated to 60°, was added. The samples were again vortexed and then incubated for an additional 5 min at 60°. The samples were vortexed at 1-min intervals during this period. Subsequently, the samples were removed from the water bath and were allowed to cool to room temperature. The aqueous and organic phases were separated by a 2-min centrifugation, and the aqueous phase was re-extracted once with 300 μl of phenol/chloroform/isoamyl alcohol (25:24:1) followed by an extraction with 300 μl of chloroform/isoamyl alcohol (24:1). The RNA was precipitated using 0.1 volume of 2 M potassium acetate (pH = 5.0) and 2 volumes of ethanol. After an overnight incubation at −20°, the RNA was recovered by centrifugation. The RNA pellet, which was dried at room temperature in a vacuum dessicator, was finally resuspended in 300 μl of H_2O. The concentration of RNA in the solution was determined by diluting a 25-μl aliquot in a total of 1.0 ml of H_2O and measuring the resultant absorbance at 260 nm (1 A_{260} unit = 40 μg/ml). All RNA samples were stored at −70°.

Electrophoresis of RNA and Northern blotting. Electrophoresis of RNA was performed according to the procedure outlined by Maniatis *et al.* (22). Briefly, RNA samples were denatured by heating at 50° for 15 min in a buffer containing 10 mM NaH_2PO_4/Na_2HPO_4 (pH 6.5), 6.5% formaldehyde, and 50% formamide. Subsequently, an appropriate volume of 10-times concentrated loading buffer (50% glycerol, 1 mM EDTA, 100 mM NaH_2PO_4/Na_2HPO_4, pH 6.5 was added, and the samples were loaded onto 1.5% agarose gels (SeaKem; GTG, Rockland, MA). The gels, cast in a buffer containing 10 mM NaH_2PO_4 (pH 6.5) and 6.5% formaldehyde, were run for 15–16 hr at 30 V, during which time the buffer was constantly recirculated.

Following electrophoresis, the gels were stained with ethidium bromide (0.5 μg/ml in 10 mM Tris·HCl, pH 8.0) for 30 min and then were photographed using UV light. Once photographed, the relative amounts of 18S rRNA loaded onto each gel lane was determined by densitometric scanning of the resultant negative. The gel was then soaked for 10–15 min in 10 × SSC (1 × SSC = 0.15 M NaCl and 0.015 mM sodium citrate, pH 7.0), and the RNA within the gel was transferred to nitrocellulose (Schleicher and Schuell no. BA-85) by capillary action. The transfer, done in 10 × SSC, was allowed to proceed for 12–16 hr, after which the nitrocellulose was dried in a vacuum oven for 2 hr at 80°. The resultant Northern blot was hybridized with the 1.5-Kbp *Bam*HI-*Nco*I fragment from pLTL-1 (see Fig. 1) as described previously (20). The origin of this LTL-specific fragment and its labeling with [α-^{32}P]TTP has also been previously described (21).

tK assays. The relative amounts of tK present within cells were determined using a biochemical assay for the enzyme (20). Specifically, the rate of conversion of TdR to its phosphorylated derivatives was assessed in lysates prepared from drug-treated and untreated L1.2-2 cells. The relative amount of tK within drug-treated samples was then determined by expressing the values measured for tK activity in these samples as a percentage of the total activity found within untreated controls.

Cultures of L1.2-2 cells were plated on 25-cm^2 flasks and treated as described above for the RNA isolation experiments. After either a 4- or 8-hr treatment period with drug, the medium was removed and the cells were detached from the flask by trypsinization. Cells were resuspended in 5.0 ml of cold medium and were centrifuged (100 × g for 5 min at 4°). The resultant cell pellet was washed twice with cold PBS by alternate resuspension and centrifugation and the cells were finally resuspended in 200 μl of extraction buffer [10 mM Tris·HCl, pH 7.5, 2.5 mM ATP, 1.5 mM $MgCl_2$, 50 μM TdR, 10 mM KCl, 3 mM dithiothreitol (added fresh)]. Lysates for tK assays were prepared by subjecting cell suspensions to three alternate rounds of quick freezing and thawing followed by a 30-sec sonication period in a water bath sonicator. Cell debris was removed by centrifugation (25 min in a microfuge at 4°), and the supernatants were assayed for tK activity.

Previous experiments had shown that changes in tK activity were linear with respect to both the incubation time and lysate volume. In the studies presented here, each assay consisted of 20 μl of lysate in a total reaction volume of 200 μl. The lysates were combined with 100 μl of tK assay buffer (150 mM Tris·HCl, pH 7.5, 20 mM ATP, 0.02 mM TdR, 3 mM dithiothreitol, 0.75 μCi/ml [^{14}C]TdR, 0.9 mg/ml bovine serum albumin) and were brought to 200 μl by the addition of the appropriate volume of extraction buffer. Triplicate samples were assayed at each drug concentration. All components were combined together on ice and the reaction was initiated by placing the samples in a water bath at 37°. The samples were removed 4 hr later and placed on ice. A 50-μl aliquot was drawn from each sample and was subsequently spotted onto a Whatman DE81 ion exchange filter (2.3 cm). Unphosphorylated [^{14}C]TdR was removed from the filters by three sequential 5-min washes in 1.0 liter of 95% ethanol. The filters were subsequently dried under a heat lamp and were placed into scintillation vials. Scintillation fluid (7.0 ml) was added, and the amount of radioactivity on each filter was determined by scintillation counting. Another 50-μl aliquot was removed from each sample and was spotted onto the ion exchange filters. These filters were dried without prior washing in ethanol, and the amount of radioactivity on each was quantitated as described above. The radioactive counts on these filters were averaged, and this value was used to determine the final specific activity of the TdR within each 50μl spot. TK activity, expressed as pmol of [^{14}C]TdR converted per hr per μg of cell protein, was determined by using this value for the specific activity. The amount of protein in each lysate was determined by the macroassay described by Bradford (23).

Results

Kinetics of LTL gene expression. The dependence of LTL transcription on the presence of exogenous glucocorticoids was initially demonstrated by the studies of Zaret and Yamamoto (7). In this report, we have extended those studies for the purpose of characterizing the LTL system as a potential model for studying drug effects on gene expression. Because our interest was to evaluate potential drug effects on both tran-

scriptional and posttranscriptional events, our initial studies required not only characterization of the kinetics of LTL gene transcription but also an evaluation of the time-dependent relationship between glucocorticoids and the expression of the tK product of the LTL gene. Accordingly, Northern blots were used to evaluate both qualitative and quantitative changes in LTL-specific transcription whereas an enzymatic assay was used to measure changes in the expression of tK.

The inducibility of both transcription and tK activity by the synthetic glucocorticoid dexamethasone is illustrated in Fig. 2. From the Northern blot assay (Fig. 2A), it can be seen that two differentially expressed LTL-specific transcripts are synthesized following dexamethasone addition. The sizes of the major and minor transcripts were found to be 1.6 and 3.5 Kb, respectively, and represent mRNA species that are initiated at a common transcriptional start site but terminate at different

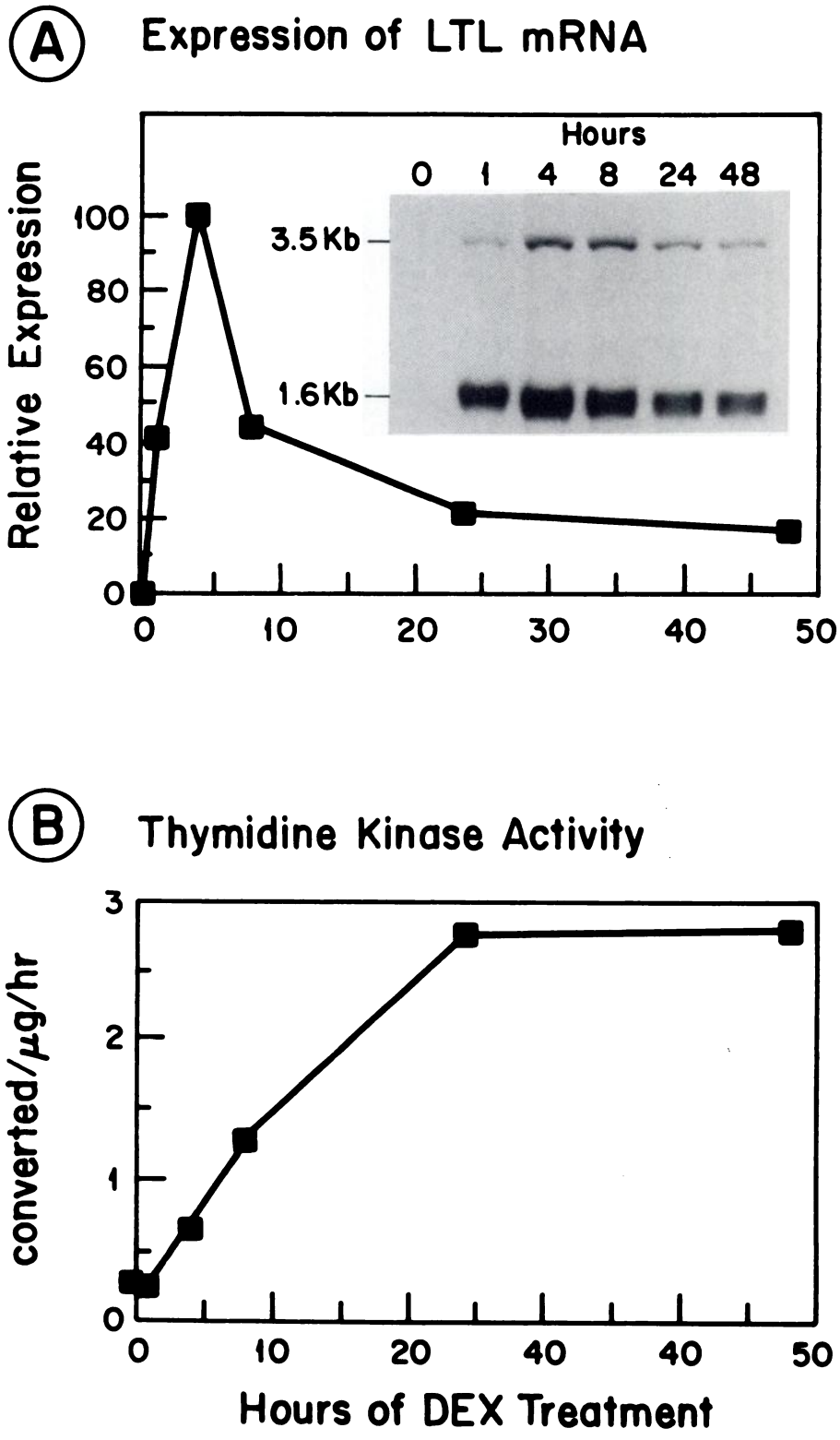

Fig. 2. Dexamethasone-dependent expression of LTL-specific transcripts and tK activity. A, After the treatment of cells with dexamethasone, total cellular RNA was isolated, fractionated on 1.5% agarose gels, stained with ethidium bromide, transferred to nitrocellulose, and subsequently hybridized to a ^{32}P-labeled 1.5-Kb *Bam*HI-*Nco*I fragment from pLTL-1. The expression of the 1.6-Kb and 3.5-Kb transcripts was quantitated by densitometric scanning of the resultant autoradiogram. Densitometric determinations were normalized to the relative amount of 18S RNA present within the appropriate sample. The amount of 18S RNA was determined by densitometric scanning of the negatives prepared from photographs of the stained gel. Normalized values for the expression of both the 1.6-Kb and 3.5-Kb transcripts are expressed relative to the values determined for the 4-hr time point and are graphed as a function of time (hr) following dexamethasone addition. The autoradiogram of the Northern blot is also shown in this figure. The times of dexamethasone treatment (hr) are indicated at the *top* of the autoradiogram. B, After dexamethasone (DEX) treatment, cells were harvested, and lysates were prepared and subsequently analyzed for tK activity as described in Experimental Procedures. Values determined directly from the tK assay are normalized to the amount of total cellular protein and are graphed as a function of time following dexamethasone addition.

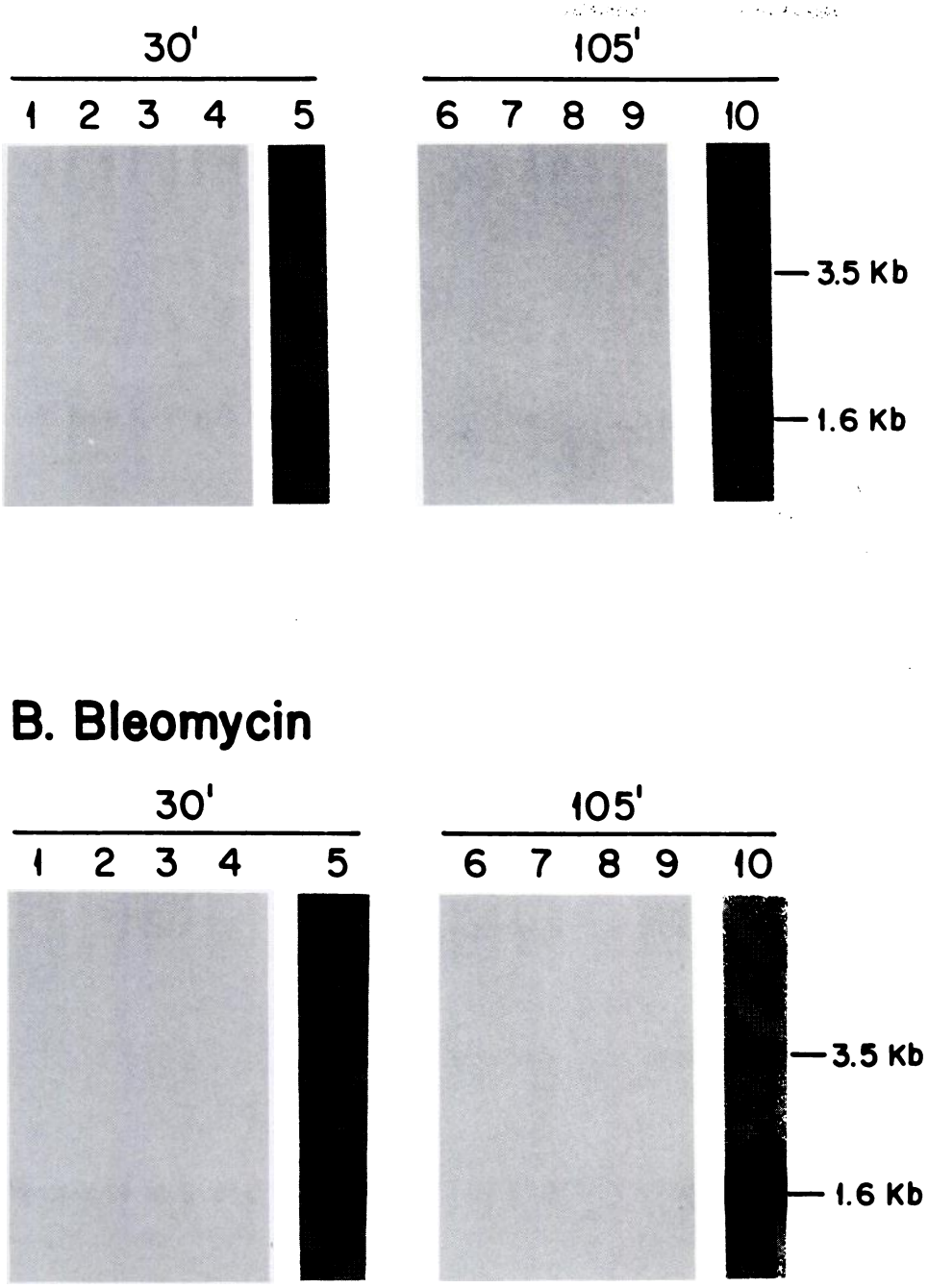

Fig. 3. Northern blot analysis of BLM- or NCS-induced effects on LTL-specific transcription in cells not treated with dexamethasone. L1.2-2 cells, not exposed to dexamethasone, were treated with various concentrations of either NCS (A) or BLM (B) for 30 (*lanes 1–4*) and 105 (*lanes 6–9*) min. Afterwards, total cellular RNA was isolated and processed as described in the legend to Fig. 2. In A, cells were treated with NCS at concentrations of 0 μg/ml (*lanes 1* and *6*), 0.1 μg/ml (*lanes 2* and *7*), 0.5 μg/ml (*lanes 3* and *8*), and 2.5 μg/ml (*lanes 4* and *9*). In B, cells were treated with BLM at concentrations of 0 μg/ml (*lanes 1* and *6*), 1.4 μg/ml (*lanes 2* and *7*), 7 μg/ml (*lanes 3* and *8*), and 35 μg/ml (*lanes 4* and *9*). *Lanes 5* and *10* in both *panels* represent controls in which cells were treated with dexamethasone, but not with drugs, for 30 and 105 minutes, respectively. Molecular sizes (kb) are indicated to the *right* of each *panel*.

loci within the LTL gene (7). The relative amounts of transcripts accumulated as a function of time can be determined by densitometric scanning of the autoradiogram. These data, presented graphically in Fig. 2A, show that a rapid accumulation of both LTL-specific transcripts occurs within the initial 4 hr after dexamethasone addition. The accumulation is followed by a period in which there is a measureable decline in the overall amount of transcripts. However, a steady state is eventually achieved by 24 hr and is maintained for the duration of the assay. Assays conducted for longer time periods have shown that this steady state is maintained for at least 3 days (data not shown).

A similar time-dependence study assessing tK activity is shown in Fig. 2B. The results displayed in the graph represent values determined directly from a tK assay. A linear increase in activity is evident during the initial 24 hr following dexamethasone addition, after which no change in activity is evident.

Based on these initial kinetic studies, it was decided that potential changes in tK activity resulting from drug treatment would be assessed at times between 4 and 8 hr after dexamethasone addition, because it is during this time period that measurable tK activity is first evident and the kinetics of its expression are linear. Likewise, changes in transcriptional ac-

tivity would be studied during the initial 4 hr after glucocorticoid addition.

Evaluation of drug effects on LTL-specific transcription. Previous reports (10, 17, 24) have indicated that agents that are capable of inducing DNA breaks may also possess a potential for inducing the expression of specific genes. To determine whether such activity could be due to a direct induction of transcriptional activity, the potential of DNA cleavage antibiotics to stimulate LTL-specific transcription in cells not treated with dexamethasone was evaluated. Such evaluations for BLM and NCS are presented in Fig. 3. The drug concentrations used in this analysis are roughly equivalent to the D_{10} (concentration that reduces survival to 10%), $1/5 \times D_{10}$, and $5 \times D_{10}$ and represent the spectrum between minimally and extremely cytotoxic concentrations. From these Northern blots, it can be seen that no demonstrable increase in the LTL-specific transcription results following either a 30-min or 105-min treatment with NCS (Fig. 3A, *lanes 1–4* and *6–9*). Likewise, treatment of L1.2-2 cells with BLM in the absence of dexamethasone gives similar results (Fig. 3B, *lanes 1–4* and *6–9*). Consequently, it appears that neither NCS or BLM are effective in directly stimulating transcription of the LTL gene.

Subsequent studies evaluated whether NCS and BLM were specific inhibitors of LTL mRNA synthesis. AMD was also included in these studies as an example of a classical RNA synthesis inhibitor that has been shown to induce changes in gene expression (17). The potential of NCS to inhibit LTL-specific transcription in dexamethasone-treated cells is evaluated in Fig. 4A. Transcription of the LTL gene appears to be qualitatively unchanged by NCS treatment. Both the 1.6-Kb and the 3.5-Kb transcripts are evident and are expressed to the same degree relative to each other in both NCS-treated and untreated cells. In addition, the sites of initiation and termination of both transcripts, as well as processing of the mRNAs, do not appear to be affected by NCS treatment, inasmuch as the relative electrophoretic mobilities of the two transcripts are unaltered by the drug.

Quantitative evaluations of NCS-induced effects on LTL-specific transcription, as determined by densitometric scanning, are presented in Table 1A. It is evident from this analysis that NCS inhibits transcription of the LTL gene. However, the level of this inhibition does not appear to be concentration dependent. Nevertheless, significant levels of inhibition (40–60%) are achieved. Comparing the effects of NCS on both the synthesis of LTL-specific mRNA and total cellular RNA (Table 1A) indicates that the degree of inhibition of the former closely parallels the degree to which the synthesis of all cellular RNA is inhibited. For example, an NCS concentration of 500 μg/ml results in a reduction in the rate of cellular RNA synthesis to 57% while the rate of LTL-specific synthesis is 60%. Consequently, it appears that neither cellular RNA synthesis nor LTL-specific transcription are preferentially affected by NCS.

Treatment of cells with BLM in the presence of dexamethasone gives results that are qualitatively similar to those just described for NCS (Fig. 4B). Both the 1.6-Kb and the 3.5-Kb transcripts are produced in significant quantities in BLM-treated and untreated cells. Furthermore, the expression of these two transcripts relative to one another, as well as their electrophoretic mobilities, do not appear to be changed as a result of BLM treatment. This indicates that the sites of transcription initiation and termination are not significantly altered by the drug.

The quantitative assessment of BLM-induced effects on the transcription of the LTL gene is presented in Table 1B. No demonstrable inhibition of transcription of this gene is evident even at concentrations as high as 500 μg/ml. Comparison of the two data columns in Table 1B shows that the effects of BLM on LTL-specific transcription are similar to those observed for the synthesis of total cellular RNA.

AMD-induced changes in transcription of the LTL gene are evaluated in the Northern blot presented in Fig. 4C. In contrast to BLM and NCS, that a 30-min treatment with AMD leads to significant inhibition of LTL-specific transcription is readily apparent on Northern blots. Densitometric scanning (Table 1C) shows that this inhibition is concentration dependent and is as great as 95% at a concentration of 5 μg/ml. However, as with NCS, the inhibition of LTL-specific mRNA synthesis by AMD is nearly equivalent to that of total cellular RNA (Table 1C).

Evaluation of drug effects on tK Activity. The Northern blot analyses demonstrate how the LTL system can be used to study preferential drug effects at the level of gene transcription. However, phenotypic expression of the LTL gene requires not only efficient synthesis of LTL-specific transcripts but also efficient posttranscriptional processing of the transcripts and their resultant tK products. Therefore, the expression of tK can be used to assess potential drug effects on posttranscriptional expression of LTL. Accordingly, the ability of either NCS, BLM, or AMD to alter tK expression was evaluated through the use of a biochemical assay for the enzyme.

Treatment of cells with BLM for either 4 or 8 hr results in only slight decreases in tK activity (Table 2). In particular, a concentration of 500 μg/ml causes only a 20% decrease in activity during an 8-hr treatment period. The possibility that BLM decreased tK expression by directly inhibiting enzymatic activity was eliminated by a lysate-mixing experiment. Specifically, when lysates from drug-treated (500 μg/ml) and untreated samples are mixed, the observed activities (0.48 and 0.91 pmol/μg/hr for 4 and 8 hr, respectively) are nearly equivalent to those predicted on the basis of additive behavior (i.e., 0.50 and 0.97 pmol/hr/μg for 4 and 8 hr, respectively).

In contrast to BLM, treatment of cells with NCS for either 4 or 8 hr results in significant reductions in tK activity (Table 3A). These reductions are evident at all three NCS concentrations tested and become more significant with increases in treatment time, such that a greater than 70% reduction in activity is achieved following an 8-hr treatment with 100 μg/ml. As with BLM, lysate-mixing experiments in which NCS-treated (100 μg/ml) and untreated samples are combined show activities that are nearly equivalent to those predicted for additive behavior (see legend to Table 3).

To assess whether inhibition of tK synthesis by NCS is preferential, it is necessary to determine the effects of the drug on total cellular protein synthesis. Therefore, by using a [^{3}H] leucine incorporation assay, NCS-induced effects on this process were evaluated. It is apparent that treatment with 100 μg/ml for either 4 or 8 hr results in equivalent levels of inhibition for both protein synthesis and the expression of tK activity (Table 3A). In contrast, treatment of cells with either 2.5 or 10 μg/ml results in an apparent decrease in tK synthesis approximately 1.5–2-fold greater than the decrease observed for cel-

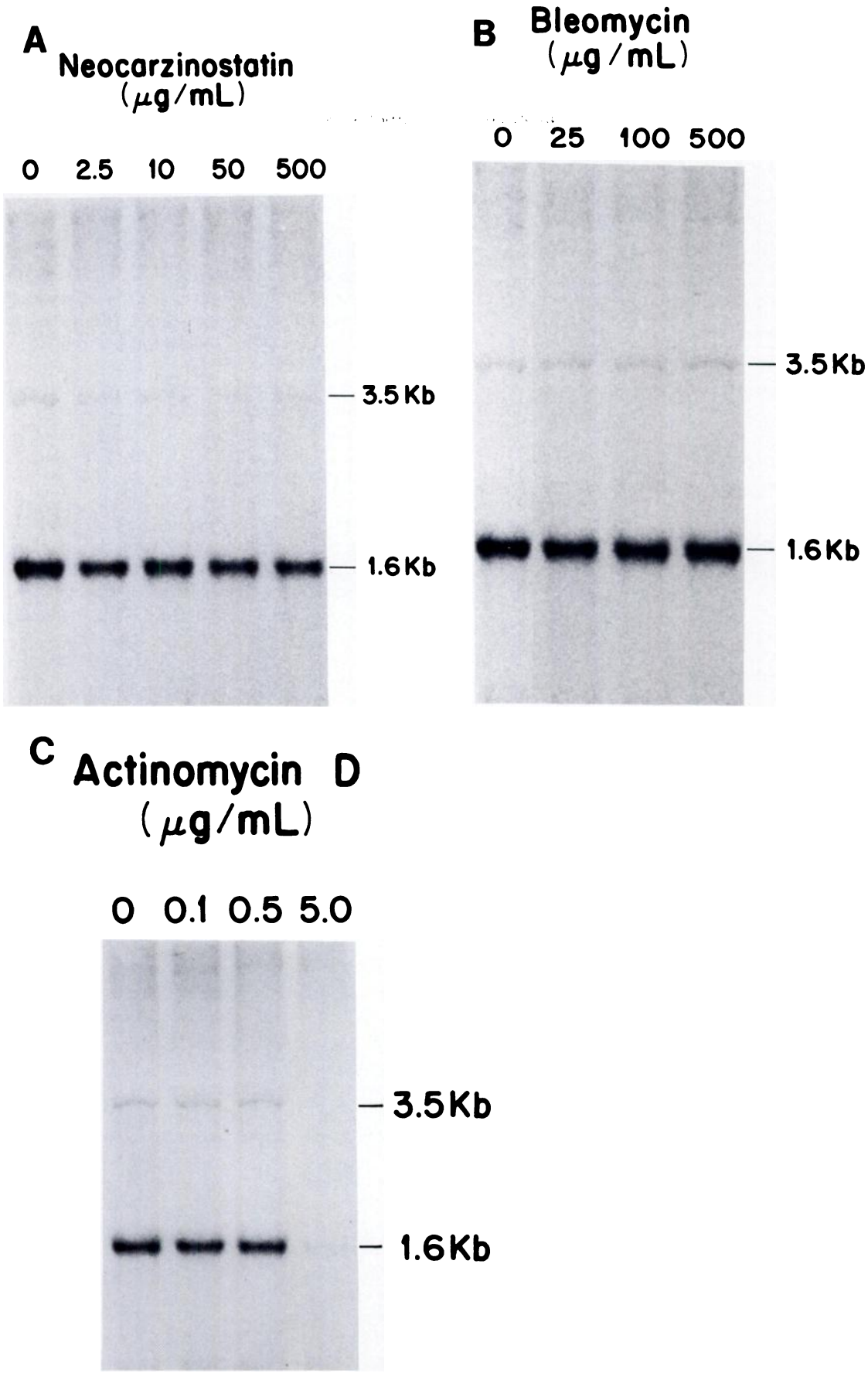

Fig. 4. Northern blot analysis of drug-induced effects on LTL-specific transcription in dexamethasone-treated cells. L1.2-2 cells were treated with 0.1 μM dexamethasone and various concentrations of either NCS (A), BLM (B), or AMD (C) for 30 min. Total cellular RNA was isolated subsequently and processed as described in the legend to Fig. 2. The concentrations (μg/ml) of drugs used in the different analyses are given at the *top* of each *panel*. Molecular sizes (kb), as determined by denatured DNA markers, are indicated by the *numbers* shown to the *right* of each *panel*.

TABLE 1

Effects of short term drug treatment on the synthesis of LTL-specific mRNA and total cellular RNA

Effects of drug treatment on cellular RNA synthesis was determined using a [^{3}H] uridine incorporation assay. In these assays, L1.2-2 cells were incubated with 0.1 μM dexamethasone, 1.0 μCi/ml [^{3}H]uridine, and various concentrations of either NCS (A), BLM (B), or AMD (C) for 30 min. Cells were subsequently harvested, and large molecular weight material was precipitated with perchloric acid. The acid-insoluble fraction was collected, and the amount of radioactivity incorporated into this fraction was quantitated by scintillation counting. Three determinations were made for each drug treatment, and the average number of counts per 10^6 viable cells was determined. Values for incorporation are expressed relative to no-drug controls and represent averages obtained from at least two different experiments. Values for LTL-specific mRNA synthesis are determined by densitometric scanning of the appropriate autoradiograms presented in Fig. 4 using the method described in the legend to Fig. 2. The values presented in the tables are expressed relative to the no-drug (0 μg/ml) controls.

Concentration	Relative synthesis of LTL-specific RNA	Relative synthesis of cellular RNA
μg/ml	% control	
A. NCS		
0	100	100
2.5	64	74
10	72	74
50	52	75
500	60	57
B. BLM		
0	100	100
25	106	99
100	89	99
500	104	93
C. AMD		
0	100	100
0.1	98	95
0.5	79	76
5.0	5.9	14

TABLE 2

Effects of BLM on tK activity

After either a 4- or 8-hr treatment with 0.1 μM dexamethasone and various concentrations of BLM, L1.2-2 cells were harvested, lysed, and subsequently analyzed for tK activity as described in Experimental Procedures. The values for tK activity presented in this table were determined directly from a single tK assay but are representative of results obtained from other experiments. These values are normalized to the amount of total cellular protein and represent the average of triplicate samples.

Concentration	tK Activity			
	4 Hr		8 Hr	
μg/ml	pmol/hr/μg	% control	pmol/hr/μg	% control
0	0.54	100	1.08	100
10	0.54	100	1.11	103
100	0.49	91	0.98	91
500	0.45	83	0.86	80
M*	0.48	—	0.91	—

* In M, equal volumes of lysate from untreated and BLM-treated (500 μg/ml) samples were mixed and assayed as described above. The predicted activities based on additive behavior are 0.50 and 0.97 pmol/hr/μg for 4 and 8 hr, respectively.

lular protein synthesis. Moreover, this preferential decrease in tK expression is observed under conditions where there are not significant changes in total cellular RNA synthesis (Table 3A).

AMD, like NCS, induces significant reductions in tK activity (Table 3B). At all three concentrations of AMD tested, tK activity is inhibited to a greater extent than is cellular protein synthesis. In particular, the differences in inhibition between cellular protein synthesis and tK activity are as great as 3–5-fold for a concentration of 5 μg/ml. This inhibition of tK activity does not appear to be due to a direct AMD-induced inhibition of enzymatic activity because mixing experiments (see legend to Table 3) gave activities that were equivalent to those predicted for additive behavior. Comparisons of the effects of AMD on the expression of tK activity and on cellular RNA synthesis (Table 3B) show that both processes are strongly inhibited by the drug and that the levels of inhibition, especially for the 8-hr time point, are similar.

Discussion

The overall goal of this study was to develop the LTL gene system as a model to 1) assess the potential of drugs to induce selective alterations in gene expression and 2) study whether such selective changes could be correlated with relatively early changes in transcriptional or posttranscriptional events. Northern blot studies (Fig. 3) showed that short term treatment with either BLM or NCS did not result in the induction of LTL-specific transcription. Interestingly, previous studies with BLM demonstrated that this drug had a potential to induce long term changes in gene expression within hematopoietic cells (10, 11) and lung fibroblasts (12, 13). The results of our studies indicate that such effects do not result from direct drug-induced alterations in gene transcription. Thus, it may be possible that BLM exerts its effects through pathways that do not directly involve the gene (or genes) under study. Indeed, there are studies that support this notion. One noteworthy example demonstrates that BLM causes an increase in extracellular matrix components by inhibiting the expression of the enzymes responsible for their degradation (25, 26).

The evaluation of BLM, NCS, and AMD as inhibitors of LTL-specific mRNA synthesis was presented in Fig. 4 and Table 1. Although all three drugs had varying effects on LTL-specific mRNA synthesis, none of them were capable of selectively affecting the synthesis of this particular RNA when compared with the synthesis of total cellular RNA. Thus, we conclude that LTL-specific transcription is not preferentially sensitive to inhibition by any of the drugs used in our study. Other studies from our laboratory indicate that there is no relationship between the induction of DNA damage and the inhibition of mRNA synthesis. At the lowest levels of NCS (2.5 μg/ml), estimates from filter elution measurements revealed that the number of single-stranded breaks induced within genomic DNA was equivalent to the number produced by 0.4 krad of ionizing radiation. This same concentration of drug inhibited LTL-specific transcription by approximately 40%. Incremental increases in drug concentration produced linear increases in DNA damage but did not cause a concomitant increase in inhibition of mRNA synthesis. Furthermore, a comparison between NCS and BLM shows that the latter drug is never as effective as the former in producing inhibitory effects on transcription, even at concentrations that produce more damage to DNA.

Certain studies have indicated a potential for some drugs to alter gene expression through posttranscriptional pathways (18, 19). Therefore, in order to completely assess whether a drug can alter gene expression, it is necessary to evaluate the potential of a drug to alter both transcriptional and posttranscriptional events. The TK assay provided a method to evaluate the latter. Reductions in tK activity were observed in lysates isolated from cells treated with any one of the three drugs. However, whereas significant reductions were noted in both NCS- and AMD-treated samples (Table 3), only minimal reductions (20%) in tK activity were observed in those cells treated with BLM (see Table 2).

TABLE 3

Effects of drug treatment on tK activity and on macromolecular synthesis

The effects of either NCS (A) or AMD (B) on RNA synthesis were quantitated as described in the legend to Table 1 except that both the label and the drug were present for either 4 or 8 hr. In addition, unlabeled uridine was added to a final concentration of 50 μM. Drug effects on protein synthesis were measured as described above except that [^{3}H]leucine was used as the labeled precursor rather than [^{3}H]uridine. tK activity was assessed as described in the legend to Table 2 and is expressed as a percentage relative to a no-drug control. The differential effects of drugs on protein synthesis and on tK activity can be directly compared by dividing the inhibition values of the former by the values of the latter.

Concentration	Relative RNA Synthesis		Relative Protein Synthesis		Relative tK Activity		Protein Synthesis/tK Activity	
	4 Hr	8 Hr	4 Hr	8 Hr	4 Hr	8 Hr	4 Hr	8 Hr
μg/ml	% of control							
A. NCS								
0	100	100	100	100	100[a]	100[a]	1.00	1.00
2.5	100	113	112	112	64	54	1.75	2.07
10	89	96	93	90	68	44	1.37	2.05
100	59	48	50	28	53	30	0.94	0.93
B. AMD								
0	100	100	100	100	100[b]	100[b]	1.00	1.00
0.1	55	55	98	98	86	81	1.13	1.21
0.5	26	25	89	79	48	32	1.85	2.47
5.0	5.1	3.9	80	70	27	14	2.96	5.00

[a] The results of an experiment in which lysates from NCS-treated (100 μg/ml) and untreated cells were mixed gave tK activities of 0.44 and 0.78 pmol/hr/μg for 4 and 8 hr, respectively. The predicted activities based on additive behavior are 0.45 and 0.82 pmol/hr/μg for 4 and 8 hr, respectively.

[b] The results of an experiment in which lysates from AMD-treated (5 μg/ml) and untreated cells were mixed gave tK activities of 0.40 and 0.71 pmol/hr/μg for 4 and 8 hr, respectively. The predicted activities are 0.40 and 0.70 for 4 and 8 hr, respectively.

Further analyses in NCS-treated cells showed that RNA synthesis, protein synthesis, and tK activity were all differentially affected (Table 3B). Notably, NCS concentrations of 2.5 and 10 μg/ml resulted in significant reductions in tK activity, whereas, under identical conditions, protein and RNA synthesis were only minimally affected. The fact that low concentrations of NCS (2.5 and 10 μg/ml) affected tK expression to a greater extent than protein synthesis suggests that NCS-induced effects on the expression of tK are preferential. Interestingly, such preferential effects disappear at higher concentrations of NCS, inasmuch as at 100 μg/ml, the observed inhibition of protein synthesis, RNA synthesis, and tK activity become nearly equivalent.

Mixing experiments confirmed that the presence of NCS within the lysate was not directly responsible for the observed reductions in tK activity. If NCS directly inhibited enzymatic activity, then mixing of lysates from drug-treated and untreated cells would have resulted in a less than additive response. Conversely, if NCS affected the synthesis of a cofactor required for tK activity, then mixing of the two different lysates would have resulted in a greater than additive response. Because additive behavior was evident in the mixing experiments (see legend to Table 3), it was apparent that NCS did not directly interfere with the tK assay. Although the outcome of our studies demonstrates that NCS has a potential to inhibit selectively the expression of a specific gene product, the precise mechanisms by which this occurs remain to be elucidated.

Like NCS, AMD also preferentially inhibited tK activity (Table 3B). However, unlike the inhibition observed for NCS, the preferential inhibition induced by AMD was present at all concentrations tested; the degree of this preferential inhibition became greater with increases in concentration and treatment time (see the two rightmost columns in Table 3B). Mixing experiments also showed that the AMD-induced inhibition on tK expression was not due to direct effects on enzymatic activity (see legend to Table 3).

Interestingly, the relative effects of AMD on RNA synthesis are greater than those observed for cellular protein synthesis. In fact, protein synthesis is only moderately affected at concentrations that severely inhibit RNA synthesis (i.e., 0.5 and 5 μg/ml). Notably, tK activity is also significantly reduced at concentrations of AMD that inhibit RNA synthesis but that do not significantly affect protein synthesis. These results imply that the selective reductions in tK activity are a consequence of an overall decrease in the synthesis of LTL-specific transcripts. However, our studies have already indicated that the effects of AMD on LTL-specific mRNA synthesis and total cellular RNA are equivalent (Table 1C). Therefore, a preferential decrease in the synthesis of tK may reflect differences between the turnover rates of LTL-specific mRNA and other cellular RNAs, rather than differences in the rates of synthesis. If the turnover rate of LTL-specific transcripts is greater than the rate for other RNAs, then the observed preferential inhibition of tK synthesis would be expected.

In summary, this report describes the use of a model system for evaluating the potential of various DNA interactive drugs to induce alterations in gene expression. Specifically, by using the hybrid viral LTL gene as a representative model of a normal cellular gene, drug effects on both transcriptional and posttranscriptional gene expression can be readily assessed. Such molecular analyses may provide novel information about drug action, which may aid in the search for new and more effective chemotherapeutic strategies.

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