Volume 11 Number 14 1983 Nucleic Acids Research

Transcription initiation of transfected mouse mammary tumor virus LTR DNA is regulated by glucocorticoid hormones

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Received 20 May 1983; Accepted 24 June 1983

ABSTRACT

A chimeric gene, recombined in vitro, containing a long terminal repeat (LTR) sequence from the proviral DNA of mouse mammary tumor virus and the thymidine kinase (tk) gene of Herpes Simplex Virus was introduced into L tk$^-$ cells. No transcription of LTR RNA was observed in transfected cells when glucocorticoid hormones were absent from the growth medium. Accumulation of LTR initiated RNA was measured upon hormone addition by the single strand specific nuclease RNA mapping procedure. The accumulation was rapid (detectable after 7.5 minutes), independent of simultaneous protein synthesis and mediated by a functional glucocorticoid receptor complex. Glucocorticoid hormones affect LTR transcription at the level of initiation. The rate of initiation (1.8×10^{-2} molecules/cell/sec) and a half life of about 30 minutes could be calculated for LTR RNA. The half life of LTR RNA is independent of the presence of hormone.

INTRODUCTION

Glucocorticoid hormones regulate many important processes during embryonic and fetal development as well as in adult tissues (1,2). The mechanism of glucocorticoid hormone action has therefore received widespread attention and several model systems have been developed to study the molecular details of glucocorticoid controlled gene expression (1). The expression of mouse mammary tumor virus (MMTV) in cultured cells has been recognized to be uniquely suited for this purpose (3,4). Glucocorticoids cause a rapid increase in the rate of synthesis of MMTV RNA in a variety of cell types infected with virus particles (5,6). Proviral gene cloning and DNA mediated gene transfer experiments have allowed the localisation of a DNA sequence responsible for the hormonal induction of MMTV. This sequence is physically linked to the proviral gene (7-9).

Results from experiments using chimeric gene constructions have shown that the hormonal control sequence is located within the long terminal repeat (LTR) sequence of the MMTV proviral gene. The hormonal regulation can be conferred to genes recombined with the LTR sequences in vitro following their transfer into cultured cells (10-12). In vitro mutagenesis of a chimeric MMTV LTR-tk gene, resulting in a series of deletion mutants which lack sequences from the U3 region of the LTR, and the test of these deletions mutants in transfected cells has allowed the delimitation of the hormone response sequence to 202 nucleotides 5' of the LTR RNA initiation site (13). This region also preferentially binds glucocorticoid receptor in vitro (14,15). To investigate the mechanism by which glucocorticoid hormones induce the RNA transcribed from the LTR-tk chimeric gene the kinetics of response of specific RNA synthesis were measured. The response was shown to be rapid. Seven and one half minutes after hormone addition to the growth medium specific LTR RNA synthesis can be detected in the transfected cells. Since this response is independent of simultaneous protein synthesis and requires a functional glucocorticoid receptor complex, we conclude that the initiation of transcription of the transfected LTR DNA is directly regulated by the hormone receptor complex. LTR initiated RNA molecules are synthesized upon hormone addition at an increasing rate. Maximal initiation is reached after 12.5 minutes when 1.8×10^{-2} molecules/cell/sec are made. The half life of the LTR RNA in the presence of dexamethasone is about 30 minutes and can be calculated from the kinetics of LTR RNA induction and from the decay of LTR RNA following inhibition of transcription.

MATERIALS AND METHODS

Cells and DNA mediated gene transfer

The construction of the chimeric LTR-tk plasmid shown in Fig.1A has been described previously (12,13). This plasmid was transfected into L tk^- $aprt^-$ cells by the calcium phosphate precipitation technique (16). L tk^+ transformants were selected and grown into mass culture. The L tk^+ cell clone used in the

experiments described here has previously been characterised (12).

Induction of LTR RNA synthesis

For the experiment described in Fig. 2a, 24 plates of semiconfluent cells were grown in DMEM and 10% fetal calf serum (FCS). At 0 time 10^{-6} M dexamethasone was added to the growth medium. After 0, 7.5, 15, 22, 30, 45, 60 and 120 minutes the cells in 3 plates were lysed and total cellular RNA was prepared as described previously (7). For the experiment described Fig. 2b, 20 µg/ml cycloheximide was added five minutes before the addition of dexamethasone and cellular RNA was prepared after the time intervals indicated.
The cells used in the experiment described in Fig. 3A were prepared as follows: 3 plates of semiconfluent cells were grown in DMEM and 10% FCS (lane 1), in growth medium and 10^{-6}M dexamethasone for 15 hours (lane 2), RU 486 for 3 hours and subsequent addition of 10^{-6}M dexamethasone for 15 hours (lane 4, 10^{-6} M RU 486; lane 5 10^{-5} RU 486). The cells used in the experiment described in Fig. 3B were grown in DMEM and 10% FCS (lane 1) or in growth medium and 10^{-6}M dexamethasone for 2 hours (lane 2). The cells used in lanes 3 to 5 were induced with dexamethasone for 2 hours. Subsequently the cells were washed three times with growth medium and then grown in medium containing 10^{-5}M RU 486 for 1 hour (lane 3), 3 hours (lane 4) and 5 hours (lane 5). Total cellular RNA was prepared and used in the nuclease S1 mapping experiments.

Single strand specific nuclease mapping of LTR initiated transcripts and quantitative evaluation of the signals

The 5' ends of the RNA transcripts were analyzed using the nuclease S1 mapping technique (17,18). The ClaI-Hpa II fragment which was labelled with ^{32}P at the 5' end of the Hpa II recognition site is described in Fig. 1. 50 µg of total cellular RNA were hybridized to the labelled DNA in 0.4 M NaCl, 40 mM Pipes pH 6.4 and 5 mM EDTA and subsequently treated with S1 nuclease (13). The nuclease S1 resistant DNA was isolated, electrophoresed through a 6.5% polyacrylamide sequencing gel and visualised by

autoradiography (13). The radioactive signals corresponding to the 106 nucleotides region of the gel were quantitated. Densitometric scans of the region of the autoradiograms comprising the 106 nucleotides signal were made and compared to a standard signal containing a predetermined number of cpm. The standard signal was electrophoresed and exposed together with the S1 nuclease resistant DNA. The number of cpm present in the 106 nucleotide signals are shown in Fig. 2C. They served to calculate the rate of RNA synthesis and the half life of LTR RNA (Fig. 3B).

Assuming a model in which the steady state concentration of LTR RNA is determined by an equal rate of synthesis and degradation the induction kinetics can be described as $\frac{dR}{dt} = k_o - k_1 R$ (R is the concentration of RNA, expressed as molecules/cell; t is time in seconds; k_o is the synthesis constant (molecules/cell/second) and k_1 is the degradation constant ($seconds^{-1}$). The cellular RNA concentration can be calculated by solving the differential equation for t > 12.5 minutes (see Fig. 2C), $R(t) = k_o/k_1 (1-e^{-k_1 t})$. The maximal concentration, reached at $t - \infty$ is $R_{max} = K_o/k_1$. The following assumptions and measurements were used to calculate the rate of RNA synthesis and the mRNA half life: 1) 350 cpm were found in the 106 nucleotide signal at maximal induction (Fig. 2A, lane 8 and Fig. 2B, lanes 7 and 8). 2) The specific activity of the DNA probe used in the S1 nuclease protection experiment was $1.2x10^6$cpm per pmole. 3) 10^{-2}pmoles (=$1.2x10^4$ cpm) were introduced in the hybridisation reaction together with 50 µg of total cellular RNA. 4) We assume quantitative hybridisation of LTR RNA with the large excess of homologous DNA introduced into the reaction. 5) 350 cpm present in the S1 nuclease signal correspond to $\frac{350x10^2x10^{-12}}{1.2x10^4} = 2.91x10^{-16}$ moles of RNA or $1.75x10^8$ molecules. 6) The RNA content was found to be 11.25 pg/cell. 7) The LTR RNA molecules per cell at maximum induction (R_{max}) therefore correspond to $\frac{1.75x10^8x11.25x10^{-12}}{50x10^{-6}} = 40$. 8) The rate of synthesis increases until 12.5 minutes. If the synthesis is maximal at 12.5 minutes and degradation neglected at this time, the slope in Fig. 2C at 12.5 minutes allows the determination of $k_o = 1.8x10^{-2}$ molecules/cell/second). 9) Since $k_1 = k_o/R_{max}$ a value of $1.8x10^{-2}/40$

(sec^{-1}) = $4.5x10^{-4}$ sec^{-1} can be calculated. 10) The half life of the mRNA is calculated as $t_{1/2} = ln2/k_1$ = 1544 sec = 25.7 minutes.

RESULTS

1) MTV LTR DNA is not transcribed from the transfected LTR tk gene in the absence of dexamethasone

Hormonal effects and developmental control of gene expression have been traced to the level of transcription of several regulated genes. Changes in the rate of transcription could be measured by pulse labeling nuclei or whole cells and determination of the fraction of the newly synthesized radioactive RNA corresponding to a particular gene (19-26). Filter bound,cloned DNA served to select by hybridisation the specific RNA sequences. Radioactively labelled RNA served to distinguish preexisting, non radioactive RNA from the transcripts made during the pulse label period. The ratio of total RNA synthesized during the labeling period to the RNA which hybridized specifically to immobilised cloned DNA allowed the calculation of relative rates of RNA synthesis.

We have used a different principle to measure the rate of synthesis of RNA initiated in the LTR of MMTV. This experimental protocol is based on the observation that in the absence of glucocorticoid hormones no transcripts initiating correctly in the LTR can be detected in cells transfected with a chimeric LTR-tk gene (Fig. 1a). This is in contrast to the transcription of intact proviral genes, aquired by the cellular genome either after transfection of cloned proviral DNA or after infection of cells with viral particles and reverse transcription. In the absence of hormone intact proviral genes show a constitutive level of LTR initiated RNA (Ponta et al., manuscript in preparation). The absence of LTR RNA in the LTR-tk transfected cells grown without hormone obliviates the necessity to radioactively pulse label RNA. Instead, a radioactive DNA probe can be used to quantitative RNA transcripts by the single strand specific nuclease protection procedure (17,18). We used a ClaI-Hpa II fragment which was ^{32}P labelled at the Hpa II site on its 5' end

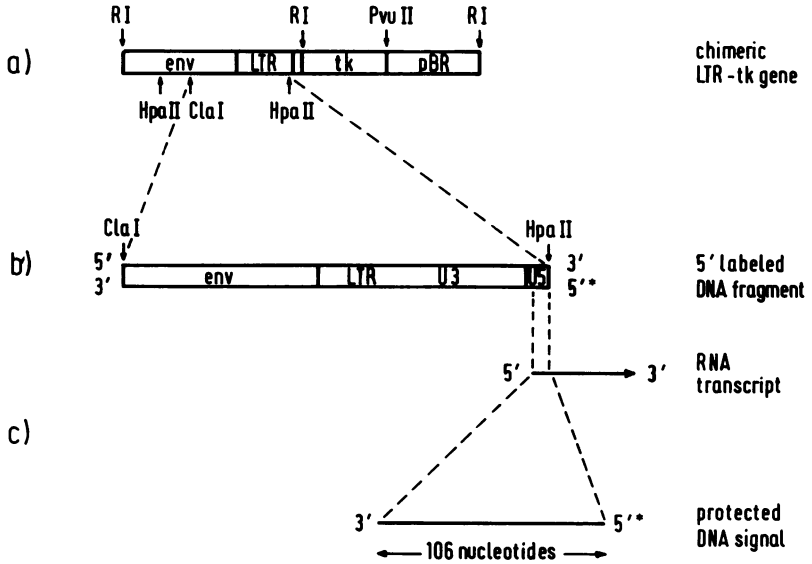
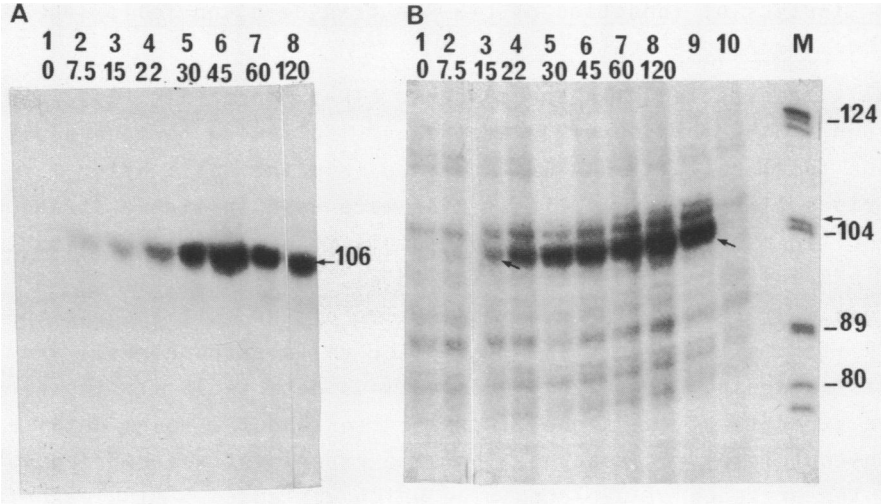


Figure 1. Schematic diagram of A) the chimeric MMTV-LTR thymidine kinase gene consisting of the proviral envelope gene (env), the 3' LTR, a short sequence of 222 nucleotides of mouse DNA derived from the proviral integration site, the HSV tk gene from its Eco RI at position -77 with regard to the tk mRNA initiation site to the Pvu II and a fragment of pBR 322 containing the origin of replication and the ampicillin resistance gene. This plasmid has been described before (12,13). B) Restriction fragment ClaI-Hpa II used for single strand specific nuclease protection assay. The chimeric plasmid LTR-tk was cleaved with Hpa II and the 3 Kb fragment containing part of the env region and the 3' LTR were radioactively labeled at their 5' ends with ^{32}P-ATP (17,18). The fragment was the digested with ClaI and the ClaI-Hpa II fragment shown was isolated. C) The ClaI-Hpa II fragment was hybridized as described (13) to total cellular RNA and then digested with nuclease S1. The nuclease resistant hybrids were isolated and visualised as shown in Fig. 2 and 3. The correctly initiated RNA molecules from the LTR can be monitored as a signal of 106 nucleotides (13).

(Fig. 1b). This DNA is able to protect a RNA fragment of 106 nucleotides (Fig. 1c) which is initiated at the cap site within the MMTV LTR (13). Since only the 5' 106 nucleotides of the RNA molecule is taken into consideration the nuclease protection assay provides a measure of the number of RNA molecules correctly initiated within the LTR.

2) Kinetics of induction of LTR RNA transcription following dexamethasone administration

Cells transfected with the chimeric LTR-tk gene (Fig. 1a) were grown in the absence of dexamethasone. Following hormone addition total cellular RNA was prepared from the cells after various time intervals. These RNAs were used in single strand specific nuclease protection protocols to determine the amount of LTR initiated molecules (Fig. 1b,c). The resulting DNA signals of 106 nucleotides, indicative of RNA initiated at the LTR start site, were visualised after gel electrophoresis and autoradiography (Fig. 2A). No signal is detectable at time zero. The strength of the signal increased for about 2 hours until a constant level was reached. The same experiment was carried out in the presence of cycloheximide which was added 5 minutes before the addition of hexamethasone at a concentration of 20 µg/ml. This resulted in a 94% inhibition of protein synthesis as measured by ^{35}S methionine uptake into total cellular protein (data not shown). The appearance of LTR initiated RNA,however, was unaffected by protein synthesis inhibition (Fig. 2b). Both observations i.e. the rapidity of the response and the independence from ongoing protein synthesis, indicate that the hormonal regulation of LTR transcription is direct and probably not mediated by other synthetic events (27,28).
The 106 nucleotide signals shown in Fig. 2b were quantitated (Fig. 2c). The shape of the curve obtained allows several conclusions to be made concerning the induction process, the rate of RNA initiation and the stability of the chimeric LTR-tk RNA produced. Induction of RNA synthesis starts with an increasing rate which reaches a maximum after about 12.5 minutes. This increase in rate parallels the increase in nuclear glucocorticoid receptor. Nuclear receptor accumulation in cultured mouse cells has been shown by Mayo and Palmiter (29) to reach a maximum after about 15 minutes after dexamethasone addition. Between 15 and 30 minutes after hormone addition LTR RNA accumulates linearly and the rate of synthesis can be calculated to be 1.8×10^{-2} molecules per cell per second. After 30 minutes the rate of accumulation declines and a constant RNA concentration is reached after about 2 hours. Since the steady state concentra-



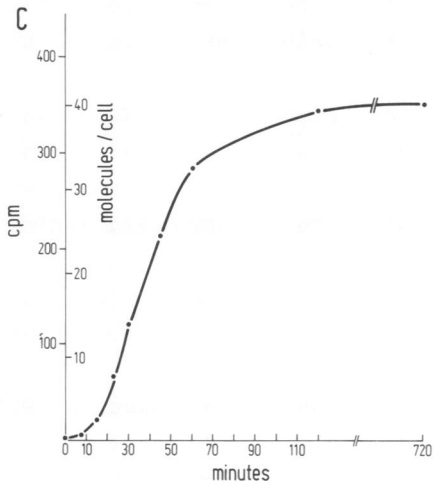


Figure 2. Single strand specific nuclease analysis of LTR initiated transcripts from cells transfected with the LTR-tk gene after hormonal induction. A) Transfected L cells were induced with dexamethasone as described in Material and Methods for 0 (lane 1), 7.5 (lane 2), 15 (lane 3), 22 (lane 4), 30 (lane 5), 45 (lane 6), 60 (lane 7) and 120 minutes (lane 8). B) Transfected L cells were treated with 20 µg/ml cycloheximide and 5 minutes later they were induced with dexamethasone. Cells were harvested 0 (lane 1), 7.5 (lane 2), 15 (lane 3), 22 (lane 4), 30 (lane 5), 45 (lane 6', 60 (lane 7) and 120 minutes (lane 8) after dexamethasone addition. The two control lanes contained RNA from cells induced for 12 hours with dexamethasone in the absence of cycloheximide (lane 9) and the

second control lane RNA from untransfected L tk$^-$ cells (lane 10). Molecular weight markers are indicated in lane M. C) Quantitative evaluation of the 106 nucleotide signal obtained in the kinetic analysis of B). The 106 nucleotide signals (marked by arrows in A and B) were quantitated and then used to calculate the number of RNA molecules/cell present at various times after induction. The assumptions used in this calculation are described in Materials and Methods.

tion results from the equilibrium between rate of synthesis and rate of degradation an average half life ($t_{1/2}$) of 25 minutes can be calculated for the LTR RNA (the details of the calculations are described in the Materials and Methods section).

3) Inhibition of LTR RNA transcription by the antiglucocorticoid RU 486

The effect of the antiglucocorticosteroid RU 486 (30) on the induction process was tested. RU 486 is a strong antagonist of dexamethasone in the receptor dependent cytolytic response of the mouse W7 lymphoid cell line (31). RU 486 binds to the glucocorticoid receptor with a higher affinity than dexamethasone and decreases the receptor affinity to DNA when tested in an vitro assay with a cloned fragment comprising the promoter region of the MMTV LTR (31). This drug is therefore well suited to investigate the involvement of the glucocorticoid receptor in the induction process.

Cells transfected with the chimeric LTR-tk gene (Fig. 1a) were grown in the presence of dexamethasone (Fig. 3A, lane 2) or in the presence of RU 486 (Fig. 3A, lane 3). No transcriptional initiation of LTR RNA was observed when RU 486 was present in the growth medium. Simultaneous addition of equimolar amounts of dexamethasone and RU 486 (10^{-6} M) resulted in a strongly decreased LTR RNA induction (Fig. 3A, lane 4). A 10 fold excess of RU 486 over dexamethasone abolished LTR transcription (Fig. 3A, lane 5). Binding of RU 486 prevents the formation of a functional receptor complex with dexamethasone (31). This experiment suggests that the active glucocorticoid receptor complex is directly mediating the hormonal initiation of transcription. When RU 486 was added to cells fully induced by dexamethasone the inhibition of LTR RNA synthesis and the decay of this RNA could be visualized (Fig. 3B). The 106 nucleotide

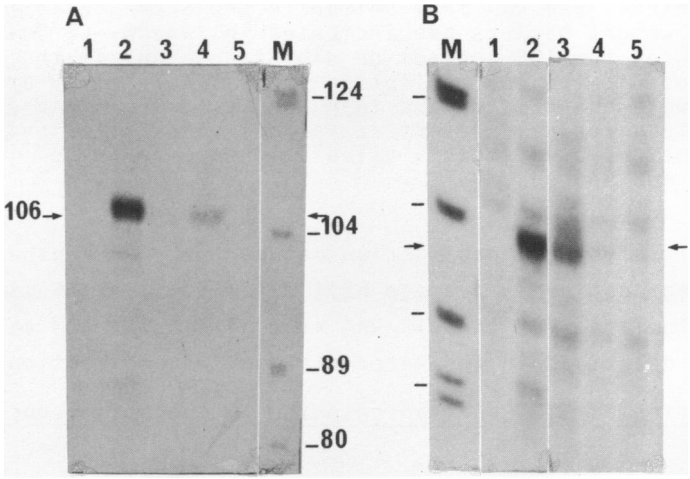


Figure 3. Single strand specific nuclease analysis of LTR initiated transcripts from LTR-tk transfected cells after dexamethasone or RU 486 treatment. A) RNA from cells not treated with dexamethasone (lane 1), treated with 10^{-6} M dexamethasone (lane 2), with 10^{-6} M RU 486 (lane 3), with 10^{-6} M dexamethasone and 10^{-6} M RU 486 (lane 4) and 10^{-6} M dexamethasone and 10^{-5} M RU 486 (lane 5) was introduced into the hybridisation reactions. Treatment was for 12 hours in all cases. The molecular weight markers are shown in M and the 106 nucleotide signal is indicated by an arrow. B) Cells were treated without dexamethasone (lane 1) with dexamethasone for two hours (lane 2). They were washed and medium containing 10^{-5}M RU 486 was added for 1 hour (lane 3), 3 hours (lane 4) and 5 hours (lane 5). RNA was extracted and used for the hybridisation reactions. M indicates the marker lane with the molecular weight standards and the arrow shows the position of the 106 nucleotide signal. The quantitation of the 106 nucleotide signals yielded 152 cpm in lane 2, 60 cpm in lane 3, 4 cpm in lane 4 and 0 cpm in lane 5.

signal, indication of LTR initiated RNA, was quantitated at 1 hr (lane 3), 3 hours (lane 4) and 5 hours (lane 5) after replacement of dexamethasone by RU 486. Evaluation of the signals obtained on a semilogarithmic scale (not shown) revealed linear decay kinetics and an half life of the RNA of 30 minutes. This value is in close agreement with the half life calculated from the accumulation kinetics (Fig. 2) and indicates that the presence of dexamethasone has no effect on the stability of the LTR RNA.

DISCUSSION

MMTV expression in virus infected cells has been shown to be regulated by glucocorticoids at the level of RNA transcription (5,6). The same mode of regulation has been established for the mouse metallothionein-I (MT-1) gene in liver tissue (24) and in cultured mouse cell lines (29). Glucocorticoid inducibility, however, was lost in cells containing amplified MT-1 genes and in human and mouse cells which acquired cloned copies of MT-1 genes after transfection (32). Inducibility of the MMTV proviral gene (7-9) and MMTV-LTR chimeric genes (10-12) was retained upon transfection. Here we demonstrate that the induction effect on transfected DNA can be explained by a change in the rate of RNA initiation. The rapidity of the response, the independence from simultaneous protein synthesis, the kinetics of change of the rate of RNA initiation, the accumulation kinetics of nuclear glucocorticoid receptor in cultured mouse cells (29) and the requirement for a functional hormone receptor complex fit well into a model which postulates the interaction of the receptor complex with a regulatory region of the controlled gene. This interaction results in an increase in the rate of RNA polymerase II initiation events.

Evaluation of the kinetics of LTR RNA induction allowed the calculation of a maximal rate of synthesis. The value obtained of 1.8×10^{-2} molecules/cell/second seems rather low. Blotting analysis of DNA introduced into these cells (see ref. 12, Fig. 4B, lanes 2 and 3) indicates that about 10 LTR-tk genes are present. How many of the genes transfected into the L cells are transcriptionally active can not be decided. An observation made with rat cells cotransfected with an intact MMTV proviral gene and a tk gene (33) might contribute to the explanation of the low transcriptional efficiency. Immunofluorescent staining of cells with an anti gp 52 serum revealed expression of the viral surface protein in only about 10% of a mass culture of the cloned cells. The clonally derived cells are therefore not uniformly expressing the transfected DNA (S. Kozma and B. Salmons, unpublished results). This heterogeneity in turn may be related to cell cycle regulated expression. Since the half life of LTR RNA is only about 30 minutes, the low transcriptional efficiency

observed could be explained by cell cycle related expression. The half life of the LTR RNA also seems low. Messenger RNA turnover in mouse L cells has been investigated and mean life-times for poly(A) + RNA of 15 hours have been measured (34). Differently from egg white protein mRNA stability in the chicken oviduct (35) LTR RNA stability in the L cells is unaffected by the absence or presence of hormone. Sequence analysis of the LTR-tk chimeric gene (Fig. 1A, N.Kennedy, unpublished results) revealed several stop codons in the 222 nucleotides linking the LTR to the tk gene. This makes it unlikely that the LTR-tk chimeric RNA (12) is a functional mRNA and translated into protein. This could be a reason for its instability when compared to the population of L cell mRNA.

ACKNOWLEDGEMENTS

We thank E.E. Baulieu for a gift of RU 486, H. Dertinger for helpful discussions, P. Butkeraitis for excellent technical assistance and C. Heinold for the preparation of the manuscript.

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