JOURNAL OF VIROLOGY, Jan. 1977, p. 139-146

Vol. 21, No. 1
Printed in U.S.A.

Steroid Induction of Mouse Mammary Tumor Virus: Effect upon Synthesis and Degradation of Viral RNA

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Received for publication 20 July 1976

Steroid hormones have been demonstrated to induce in tissue culture the production of mouse mammary tumor viral (MMTV) RNA, proteins, and particles 10-fold compared with constitutive levels. However, previous data of increased viral RNA levels did not distinguish between an increased rate of viral-specific RNA synthesis and a slower rate of viral RNA degradation. According to the recently developed assay of Coffin et al. (1974) for measuring rates of viral RNA synthesis, short-term labeling experiments of a mouse mammary tumor cell line indicate that the glucocorticoid hormone dexamethasone stimulates a 3-fold increase in the synthesis of MMTV-specific RNA within 10 min after the addition of hormone and that stimulation of RNA synthesis reaches 5- to 10-fold within 30 to 60 min, while the synthesis of Moloney leukemia virus-specific RNA in the same cell is unaffected by steroids. The decay rates of pulse-labeled and accumulated MMTV RNA in the presence or absence of dexamethasone show this RNA to have a half-life of greater than 8 h. In addition, hormone-stimulated MMTV RNA appears to have an increased rate of decay compared to basal MMTV RNA, thus ruling out an increased stability of MMTV RNA in the presence of steroid hormones as the basis for increased RNA levels. Thus, the magnitude, rapidity, and specificity of hormone action on MMTV RNA synthesis indicate a primary effect upon transcription.

Hormones and type B viruses are known to be important cofactors in naturally occurring murine mammary tumorigenesis. Thus, it is especially interesting to examine the molecular interaction between steroid hormones and murine mammary tumor virus (MMTV) expression. In previous studies with established mammary tumor cell lines (9, 18), it was observed that glucocorticoids stimulated MMTV expression to 10-fold higher levels than those observed in untreated cultures (5, 6, 11-13). Subsequent work (14, 16) has shown that this hormone effect occurs within 30 min of addition of the hormone and is first manifested by increased levels of MMTV-specific RNA (14, 16).

The most likely mechanism of MMTV induction is a transcriptional event because the MMTV viral genome is integrated as DNA proviral sequences into high-molecular-weight DNA of murine cells, and because in mammary cell cultures steroid hormones bind to and "activate" cytoplasmic receptors that are subsequently transported to the nucleus (14, 17, 19). Actinomycin D, but not inhibitors of DNA or protein synthesis, blocks MMTV RNA induction, indicating that induction is associated with RNA synthesis (14, 16). Despite the indirect evidence that supports the view of a primary effect of the hormone-receptor complex on the rate of MMTV RNA synthesis, direct evidence for such a hypothesis has not been presented.

In this report, previous work (14, 16) was confirmed by using a different approach to demonstrate that MMTV RNA synthesis and stability are significantly altered in glucocorticoid-treated cells. Using the polyinosinic acid [poly(I)]-Sephadex assay of Coffin et al. (4) to directly measure newly synthesized RNA after hormone treatment we demonstrate that MMTV RNA increased within 10 min of steroid addition. Because the time course and the magnitude of the hormone effect are immediate and rapid, we concluded that the major stimulation by glucocorticoids of MMTV expression occurs at the levels of transcription and specifically affects MMTV RNA synthesis.

MATERIALS AND METHODS

Materials. Actinomycin D was purchased from Calbiochem (La Jolla, Calif.). [^{3}H]dCTP (23 Ci/mmol), [^{3}H]uridine (26 Ci/mmol), and ^{32}P were obtained from New England Nuclear Corp. (Boston, Mass.). Dexamethasone was obtained from Sigma Chemical Co. (St. Louis, Mo.).

Cells. The cells utilized in these experiments (34I

cl 101) are clonally derived from the mammary tumor cell line originally isolated by Owens and Hackett (9). This cell line has been described previously (10, 11, 16). For certain experiments, these cells were superinfected with Moloney leukemia virus (MoMuLV) in the following manner. Cells were plated at 10^5 cells per 60-mm plastic petri dish in the presence of 2 μg of polybrene per ml. After 24 h they were infected with 5 to 10 XC PFU of MoMuLV for 60 min at 37°C. The medium was removed, fresh medium was added, and the cells were grown for 1 week as described below. Supernatant reverse transcriptase was monitored as described previously (11), and single-cell clones were isolated from polymerase positive plates by the technique of Goldsby and Zipsser (7). Bat cells (CCL88; American Type Tissue Culture Collection, Rockville, Md.) and all other cells were maintained in the Dulbecco modification of Eagle minimal essential medium containing 10% fetal calf serum (Colorado Serum Co., Denver, Colo.). For induction experiments, cells were seeded on 100-mm plastic petri dishes (Falcon, Oxnard, Calif.) at 37°C at 1×10^6 to 2×10^6 cells per plate. Four days after seeding, when there were approximately 10^7 cells per plate, the medium was removed and replaced with fresh medium containing 200 μCi of [^{3}H]uridine (26 Ci/mmol) per ml. After the plates were incubated for 15 min at 37°C, dexamethasone (10 mg/ml in absolute ethanol) was added to a final concentration of 5 μg/ml, and the cells were harvested at 0 (zero time), 10, 20, and 30 min after dexamethasone addition. Parallel plates received equivalent amounts of alcohol and were incubated for 30 min at 37°C. RNA was then extracted as described below. For the pulse-chase experiments, cells were labeled with 200 μCi of [^{3}H]uridine (26 Ci/mmol) per ml at 37°C for 15 min in the presence of 1 μg of dexamethasone per ml after being pretreated with 1 μg of dexamethasone per ml for 1 h at 37°C. The [^{3}H]uridine-containing medium was removed and replaced with fresh medium containing 1 μg of dexamethasone and 5 μg of actinomycin D per ml. The plates were then incubated for an additional 4 or 8 h at 37°C. For studies involving accumulated RNA the same procedure was followed except that the 15-min [^{3}H]uridine labeling was omitted. Parallel cultures did not receive any dexamethasone. As indicated in a previous report (16), actinomycin D at 5 μg/ml inhibited RNA synthesis >99%.

RNA extraction. Cells were prepared for RNA extraction as follows. The surfaces of the plates were rinsed twice with phosphate-buffered saline, and the cells were then lysed directly on the plates by the addition of 2 ml of lysing buffer containing 0.01 M Tris (pH 7.4), 0.1 M NaCl, 0.001 EDTA, and 1% sodium dodecyl sulfate (SDS). Self-digested Pronase (Calbiochem) was added to a final concentration of 100 μg/ml, and the lysate was incubated for 20 min at 37°C. Equal portions of phenol and chloroform-isoamyl alcohol (24:1) were then added and the protein was extracted. After dialysis for 16 to 18 h, the aqueous phase was adjusted to 0.01 M Tris (pH 7.4), 0.005 M magnesium acetate, and 0.1 M NaCl. DNase I (Sigma) was added to a final concentration of 30 μg/ml, and the mixture was incubated at 37°C for 1 h. SDS was added to a final concentration of 0.5%, and the protein was reextracted by the phenol-chloroform method. The aqueous phase was then dialyzed, lyophilized, suspended in water, and passed over a Sephadex G-75 column (1.5 by 25 cm). The void volumn fractions were combined, concentrated, and used for hybridization.

^{32}P-labeled or unlabeled 70S viral RNA preparation. Viral RNA labeled with ^{32}P was prepared by incubating cell cultures with carrier-free ^{32}P (500 μCi/ml; New England Nuclear Corp.) in phosphate-free Dulbecco medium with 10% calf serum and 1 μg of dexamethasone per ml. Cultures were first incubated with the ^{32}P-containing medium for 16 h, and the supernatant fluid was discarded. Fresh ^{32}P medium was added, and three collections of virus-containing fluid were harvested at 3-h intervals. Each 3-h collection was clarified at 3,000 rpm just after harvest of the fluid and stored at 4°C. The maximum time of storage at 4°C was 6 h. The ^{32}P-labeled viral particles were then concentrated by centrifugation through a 30% sucrose solution containing 0.01 M Tris-hydrochloride (pH 7.2) and 10^{-3} M EDTA onto a cushion of 60% sucrose; centrifugation was performed in an SW27 rotor at 25,000 rpm at 4°C for approximately 14 to 18 h. The virus was aspirated from the 60% cushion of sucrose, diluted approximately fourfold with a solution containing 0.01 M Tris-hydrochloride (pH 7.2) and 10^{-3} M EDTA, and concentrated again by centrifugation at $100,000 \times g$ for 1.5 h at 4°C. The pelleted virus was disrupted at room temperature with 1.0 ml of a solution containing 1% SDS and 0.2% (vol/vol) diethylpyrocarbonate in 0.01 M Tris-hydrochloride (pH 7.2). The disrupted virus was applied to a 15 to 30% sucrose gradient containing 0.01 M Tris-hydrochloride (pH 7.2) and 10^{-3} M EDTA and centrifuged at 39,000 rpm in an SW41 rotor for 2 h at 15°C. The 70S RNA was collected by puncturing the tubes from below and collecting approximately 25 equal fractions. The 70S RNA migrated consistently in fractions 8 through 12 in the gradient. MMTV type B or Moloney type C unlabeled viral RNA was isolated by velocity sedimentation in linear, 15 to 30% sucrose gradients containing 0.01 M Tris (pH 7.4), 0.1 M NaCl, and 0.001 M EDTA (1).

Preparation of MMTV cDNA. MMTV cDNA was prepared by using avian myeloblastosis virus reverse transcriptase, viral 70S RNA, and chick DNA (Calbiochem) fragments as described by Taylor et al. (J. Taylor, R. Illmensee, and J. Summers, Biochim. Biophys. Acta, in press). The final reaction cocktail (20 ml) included the following: Tris (pH 8.3), 0.05 M; KCl, 0.06 M; magnesium acetate, 0.005 M; dithiothreitol, 0.002 M; actinomycin D, 20 μg/ml; Triton X-100, 0.01%; chick DNA fragments, 200 μg/ml; dCTP, dATP, dGTP, and TTP, each at 0.5 mM; and partially purified avian myeloblastosis virus reverse transcriptase (G-200 purified), 1,200 U (850 μg). One unit of enzyme represents the incorporation of 1 pmol of [^{3}H]TTP into trichloroacetic acid-precipitable material as defined for viral reverse transcriptase (11). Incubation was at 37°C for 1 h. SDS was then added to a final concentration of 0.5%, and the cDNA was processed as described previously (1). In a typical

experiment, 150 μg of 70S RNA in a 20-ml reaction mixture yielded 78 μg of cDNA. The unlabeled cDNA was then elongated with a stretch of polydeoxycytidylic acid [poly(dC)] by calf thymus terminal transferase (P-L Biochemicals, Milwaukee, Wis.) as described by Coffin et al. (4). The yield of elongation is virtually quantitative, because 162 μg of elongated product was obtained from 74 μg of cDNA. For the preparation of ^{3}H-labeled cDNA, [^{3}H]dCTP (23 Ci/mmol) was added to a final concentration of 100 μCi/ml (4.3×10^{-2} mM) in place of the non-radioactive dCTP. The use of AMV reverse transcriptase in this procedure results in the synthesis of up to 50% S1 nuclease-sensitive material that does not hybridize to either viral 70S RNA or total cellular RNA (unpublished data). Thus, at saturating levels of RNA, only 45 to 50% of the input cDNA becomes S1 resistant. At 1:1 molar ratios of cDNA and viral 70S RNA, the MMTV cDNA hybridizes to 33% of the viral genome, and at 5:1 molar ratios the cDNA hybridizes to over 95% of the viral genome.

Hybridization. Hybridization analysis using ^{3}H-labeled cDNA and cellular RNA was carried out with S1 nuclease (8); C_rt values were determined as described previously (2). Hybridization with non-radioactive poly(dC)-cDNA was performed by the method of Coffin et al. (4) as modified in this laboratory. The hybridization reaction mixture contained 0.01 M Tris (pH 7.5), 0.75 M NaCl, 0.5 mM EDTA, 0.1% SDS, 15 nmol of oligo(dC), 0.8 to 1.0 μg of MMTV poly(dC)-cDNA, 100,000 or 200,000 cpm of [^{3}H]uridine-labeled RNA, and 600 to 800 cpm of ^{32}P-labeled 70S RNA in a final volume of 50 μl. The reaction was incubated for 18 h at 66°C, and poly(I)-Sephadex chromatography was performed in the presence of polyuridylic acid as described previously (4), except that the RNase was added for 1 h in 0.3 M NaCl instead of 0.5 M NaCl and the column was washed immediately before the SDS wash with Tris buffer containing 1.5 M NaCl. Bound radioactivity was eluted from the column by the addition of 0.2 N NaOH. After the 0.2 N NaOH wash was neutralized with 1 N HCl, the resulting column wash was dissolved in 10 ml of Beckman Ready-Solv VI (Fullerton, Calif.), and the radioactivity was determined in a Beckman L350 scintillation counter with a 50% efficiency for tritium.

RESULTS

Previous experiments in this laboratory have demonstrated that a twofold increase in accumulated MMTV-specific RNA can be detected as early as 30 min after hormone addition (16). The reverse type of experiment, using a non-radiolabeled cDNA and a pulse-labeled RNA, is necessary to determine the initial rate of MMTV RNA synthesis upon hormone stimulation. The use of poly(I)-Sephadex chromatography as reported by Coffin et al. (4) allowed us to measure initial rates of MMTV RNA synthesis. Basically, this technique involves the hybridization of a cDNA elongated with poly(dC) to pulse-labeled RNA. This poly(dC)-cDNA-RNA hybrid is then passed over a poly(I)-Sephadex column that binds the poly(dC) regions of the hybrid. Single-stranded RNA is then digested with RNase A, leaving only the poly(dC)-cDNA-RNA hybrid bound to the column.

When the poly(I)-Sephadex assay was applied to the 34I cl 101 cell line, in the absence of poly(dC)-cDNA, very low amounts of ^{3}H-labeled RNA (0.0025%) bound to the column (Table 1). When MMTV poly(dC)-cDNA was used as the probe, 0.22 to 0.32% of pulse-labeled RNA bound to the column. In cells exposed to hormones for 30 to 60 min, the levels increased approximately 5- to 10-fold (1.5 to 3%) in several experiments. A significant amount of background hybridization was observed both in heterologous bat cells (CCL88) (0.05%) and NIH-3T3 cells not producing any MMTV (0.12%). These background levels of hybridization may be due either to the interaction of the poly(dC) end of the cDNA with guanosine-rich regions of the pulse-labeled RNA as discussed by Coffin et al. (4) or to the presence of some cellular sequences in the cDNA. These background levels of hybridization could not be completed by increasing levels of viral 70S RNA. In any case, the levels of MMTV-specific RNA are above this background value and increase dramatically in hormone-treated cultures.

Specificity of the hybridization. The specificity of hybrid formation was tested by hybridizing the ^{3}H-labeled RNA and ^{32}P-labeled 70S RNA to MMTV poly(dC)-cDNA in the presence of increasing amounts of MMTV or MoMuLV 70S RNA. Thus MMTV, but not MoMuLV, RNA should compete with the hybridized ^{3}H-labeled cellular RNA. The results (Fig. 1) demonstrate hybridization of the ^{3}H-labeled RNA, and the ^{32}P-labeled MMTV 70S RNA to the MMTV poly(dC)-cDNA was almost completely eliminated in the presence of MMTV 70S RNA but not in the presence of MoMuLV 70S RNA. The percent inhibition was the same for both the ^{32}P-labeled 70S RNA and the ^{3}H-labeled RNA, but 10 to 15% of the [^{3}H]RNA hybridization was not inhibited by MMTV 70S RNA. This background level of hybridization is probably due to the nonspecific hybridization of MMTV poly(dC)-cDNA to RNA as discussed in the previous section. Controls for the integrity of the MoMuLV 70S RNA indicated that the MoMuLV 70S RNA was an effective competitor in its homologous reaction (data not shown).

Kinetics of dexamethasone induction. As shown previously (5, 6, 11-14, 16), dexamethasone stimulates an increase in intracellular MMTV RNA, proteins, and extracellular virus but does not stimulate an increase in RNA spe-

TABLE 1. *Hybridization of in vivo RNA to poly(dC)-cDNA*[a]

Cell type	Poly(dC)-cDNA	Internal standard: ^{32}P-labeled 70S RNA hybridized (%)	Input cpm (× 10^5)	Hybridized cpm	Input hybridized (%)	Viral RNA content (%)[b]
Expt 1	MMTV					
34I cl 101	–	0	2	5	0.0025	
34I cl 101	+	46	2	203	0.10	0.22
34I cl 101 + dexamethasone	+	42	2	2,531	1.26	3.0
NIH-3T3/MoMuLV	+	39	2	94	0.047	0.12
Bat (CCL88)	+	42	3	60	0.02	0.05
Expt 2	MMTV					
34I cl 101/MoMuLV	+	37	2	245	0.12	0.32
34I cl 101/MoMuLV + dexamethansone	+	37	2	818	0.40	1.1
	MoMuLV					
34I cl 101/MoMuLV	+	36	2	298	0.15	0.42
34I cl 101/MoMuLV + dexamethasone	+	38	2	358	0.18	0.47

[a] Cells were treated for 60 min with 1 μg of dexamethasone per ml and labeled for 30 min with [^{3}H]uridine (200 μCi/ml). The RNA had a specific activity of 30,000 cpm/μg (experiment 1) or 75,000 cpm/μg (experiment 2). Hybridization was performed for 18 h as described in the text. ^{32}P-labeled 70S RNA (ca. 600 to 700 cpm, 10^6 cpm/μg) was added to each assay to monitor its hybridization efficiency (4, 15). A background of 30 cpm was subtracted from all values.

[b] Viral RNA content was calculated from percent hybridization and ^{32}P-labeled RNA hybridized as follows: [(percent input hybridization)/(percent ^{32}P-labeled 70S RNA hybridized)] × 100.

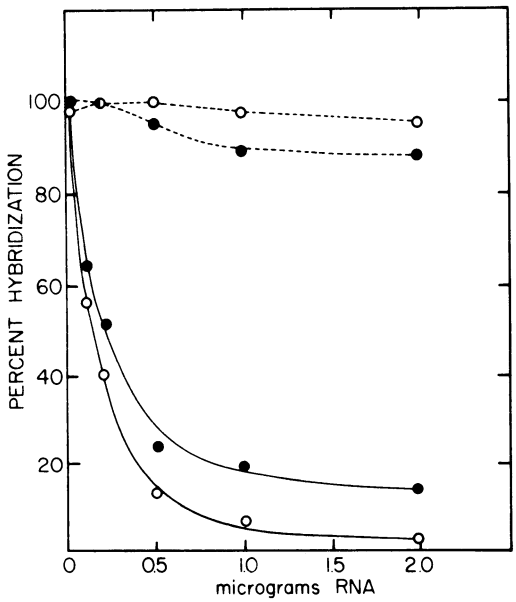

FIG. 1. *Specificity of MMTV poly(dC)-cDNA hybridization. RNA (75,000 cpm/μg, 200,000 cpm input) from 34I cl 101/MoMuLV pulse-labeled for 30 min in the presence of 5 μg of dexamethasone per ml was hybridized to MMTV poly(dC)-cDNA as described in the text. One hundred percent hybridization (850 cpm of ^{3}H-labeled RNA and 265 cpm of ^{32}P-labeled RNA) represents the RNA bound to poly(I)-Sephadex in the absence of any competing viral 70S RNA. A scintillation counter background (30 cpm) has been subtracted from all values. Micrograms of RNA is expressed as micrograms of 70S RNA added per 50 μl of reaction mixture. Symbols: (●——●) Percent ^{3}H counts per minute with MMTV 70S RNA as competing RNA; (○——○) percent ^{32}P counts per minute with MMTV 70S RNA as competing RNA; (●- -●) percent ^{3}H counts per minute with MoMuLV 70S RNA as competing RNA; (○- -○) percent ^{32}P counts per minute with MoMuLV 70S RNA as competing RNA.*

cific for the endogenous type C viruses. However, it has not yet been possible to detect increased levels of accumulated MMTV RNA much sooner than 30 min after the addition of hormone. By using poly(I)-Sephadex chromatography and short-term pulsing with [^{3}H]uridine we have been able to study the kinetics of MMTV RNA synthesis in vivo immediately after the addition of steroid hormone. A 3-fold increase in the levels of MMTV-specific RNA can be detected as early as 10 min after the addition of dexamethasone, and a 5- to 10-fold increase is observed after 30 to 60 min (Fig. 2). No further increase is observed after approximately 30 to 60 min of hormonal stimulation. The magnitude of the observed increase in MMTV RNA levels may fully account for the 10-fold increase in viral proteins and extracel-

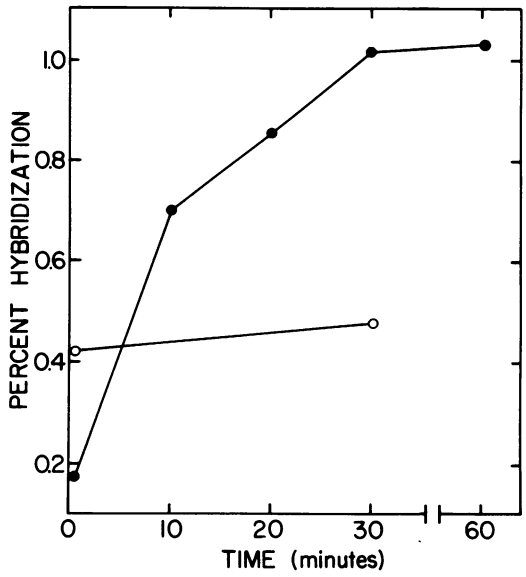

FIG. 2. *Kinetics of dexamethasone stimulation of MMTV RNA synthesis. 34I cl 101/MoMuLV was labeled with 200 μCi of [³H]uridine per ml (23 Ci/mmol) for 15 min prior to addition and was continuously labeled during exposure to dexamethasone. Hybridization was performed for 18 h as described in the text with either MMTV poly(dC)-cDNA (●) or MoMuLV poly(dC)-cDNA (○). RNA (25,000 to 75,000 cpm/μg; 200,000-cpm input) hybridized to the respective poly(dC)-cDNA was determined by poly(I)-Sephadex chromatography, and viral RNA content was corrected using ³²P-labeled 70S viral RNA as an internal standard ("hybridization efficiency") as described in footnote b of Table 1 and previously (4, 15). The percent hybridization of MMTV poly(dC)-cDNA to NIH-3T3/MoMuLV RNA (0.12%) has been subtracted from the MMTV hybridization values plotted.*

lular virus in cells treated for 24 to 48 h with dexamethasone. No significant increase was noted in the syntheis of MoMuLV RNA, indicating that the steroid effect is not only rapid, but that it is also highly specific. Although these results show that the MoMuLV is not affected by hormone treatment, they are in agreement with previous data showing that no increase is observed in RNA homologous to the endogenous type C viruses (11), suggesting that neither class of type C expression is affected by dexamethasone at the level of transcription. Type C RNA also serves as an internal biochemical control for any effects on the intracellular pool of [³H]UTP. The constant level of type C RNA after dexamethasone treatment indicates that this treatment results in no appreciable difference in [³H]UTP specific activity and that the increased incorporation of radioactivity into MMTV RNA represents a true increase in RNA synthesis.

Half-life of MMTV RNA. Although previous work has suggested that the effect of steroid hormones is primarily upon transcription, an effect of steroid hormones on RNA stability had not been excluded. We extended previous observations on the effect of hormones on MMTV RNA stability (14, 16) by investigating the half-life of both pulse-labeled and accumulated MMTV RNA in the presence and absence of hormone. Previous data from this laboratory indicated that, in the presence of dexamethasone and actinomycin D, MMTV RNA had a half-life of at least 6 h (16). In the present experiments we utilized cultures treated with dexamethasone for 60 min since maximal rates of MMTV RNA synthesis were obtained within this time period. A sixfold increase in accumulated MMTV RNA was observed after 60 min of dexamethasone treatment. This increase was somewhat more rapid than that seen in previous data (16) indicating that maximal levels of MMTV RNA were obtained by 4 h of hormone treatment. This discrepancy cannot be fully explained at this time.

In the first set of experiments, the fate of pulse-labeled RNA was monitored by incubating the cells with dexamethasone for 60 min, pulse-labeling in the presence of dexamethasone, and treating with actinomycin D for 4 and 8 h in the continued presence of the hormone. A decrease of approximately 20% was observed in ³H-labeled RNA that was capable of hybridizing to MMTV poly(dC)-cDNA in the initial 4 h of actinomycin D treatment (Table 2). An additional decrease of 10% was observed within the next 4 h. Experiments of this type were also attempted in non-hormone-treated cells, but rates of synthesis of the MMTV-specific RNA were too low to provide any accurate determination of half-life after actinomycin D addition (data not shown). Additional experiments were thus designed to investigate the fate of accumulated viral RNA.

In these experiments, cells were incubated with or without steroid for 60 min, and actinomycin D was added for 4 and 8 h in the presence or absence of hormone. The RNA was extracted from each set of cells and hybridized with MMTV ³H-labeled cDNA. The results of this experiment, expressed as a function of RNA concentration and time (C_rt) (2), can be seen in Fig. 3. Dexamethasone-treated cells show a 10 to 20% decrease in MMTV RNA after 4 h of actinomycin D treatment and a further 4 to 8% decrease after an additional 4 h. Thus, a total decrease of accumulated MMTV RNA of 15 to

TABLE 2. *Decay of dexamethasone-stimulated RNA*[a]

RNA from:	^{32}P-labeled 70S RNA hybridized (%)		Input cpm ($\times 10^5$)		Hybridized cpm		Input hybridized (%)		Viral RNA content (%)	
	I	II	I	II	I	II	I	II	I	II
34I cl 101	50	46	2	4	159	292	0.08	0.07	0.16	0.16
34I cl 101/MoMuLV + dexamethasone (zero time)	45	37	2	4	1,377	2,103	0.68	0.53	1.5	1.4
34I cl 101/MoMuLV + dexamethasone (4 h, actinomycin D)	46	35	2	4	1,138	1,519	0.57	0.38	1.24	1.09
34I cl 101/MoMuLV + dexamethasone (8 h, actinomycin D)	46	35	2	4	954	1,324	0.48	0.33	1.04	0.94

[a] Cells were treated for 60 min with 1 μg of dexamethasone per ml before pulse-labeling for 15 min with 200 μCi of [^{3}H]uridine (23 Ci/mmol) per ml. Actinomycin D (5 μg/ml, final concentration) was added, and the plates were incubated for 4 and 8 h at 37°C. 34I cl 101 RNA (2,500 to 8,000 cpm/μg) was hybridized to MMTV poly(dC)-cDNA (1.6 μg/assay) for 18 h as described in the text. Percent viral RNA content was determined as described in footnote *b* of Table 1.

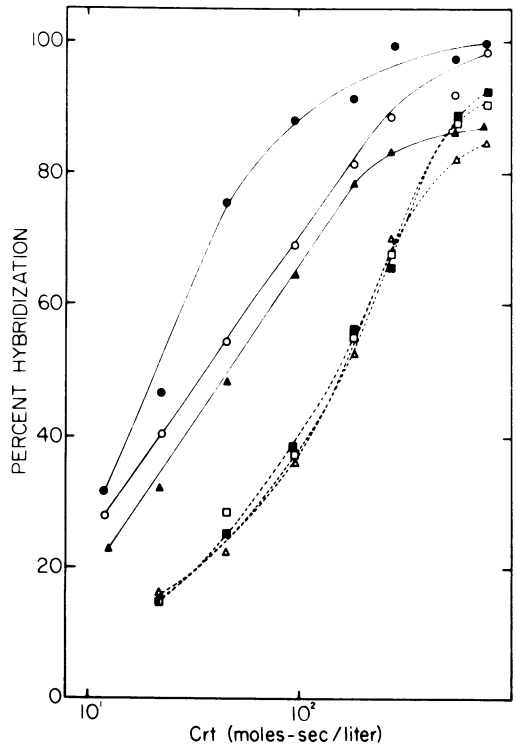

FIG. 3. *C_rt analysis of MMTV RNA in actinomycin D-treated cultures. Cells were first treated with 1 μg of dexamethasone per ml for 1 h and then treated with 5 μg of actinomycin D per ml for 0, 4, and 8 h prior to RNA extraction. Total RNA was then extracted as described in the text. Hybridization was analyzed by the use of S1 nuclease as described previously (8). Approximately 2,400 cpm of MMTV cDNA (1×10^7 to 2×10^7 cpm/μg) was tested per point. The final percent hybridization (46% of input) is normalized to 100% value. Background levels of S1-resistant ^{3}H counts per minute (85 cpm) were subtracted before calculation of percent hybridization. C_rt (2) is expressed as moles · seconds per liter (2) and was calculated by the formula of Kohne {[(absorbancy at 260 nm per milliliter)/2] × hours} and corrected to 0.18 M NaCl (3). An RNA solution of 1 mg/ml was taken to have an absorbancy at 260 nm of 20. Symbols: (●——●) Dexamethasone-treated cultures, no actinomycin D; (○——○) dexamethasone-treated cultures, actinomycin D, 4 h; (▲——▲) dexamethasone-treated cultures, actinomycin D, 8 h; (■----■) non-dexamethasone-treated cultures, no actinomycin D; (□----□) non-dexamethasone-treated cultures, actinomycin D, 4 h; (△----△) non-dexamethasone-treated cultures, actinomycin D, 8 h.*

30% is observed after 8 h of actinomycin D treatment, which is in close agreement with the 30% decrease observed in the pulse-labeled RNA. A different result was obtained in the non-hormone-treated cells. No decrease in the basal levels of MMTV RNA was observed after 8 h of actinomycin D treatment, suggesting that the hormone-stimulated RNA has a decreased half-life in the presence of actinomycin D when compared to the basal MMTV RNA (Fig. 3). However, the results indicate a protracted half-life of MMTV RNA in the cells both in the presence and absence of hormone and suggest that the immediate effect of steroids must be upon transcription. When the fate of MoMuLV RNA was investigated under the same conditions, the results obtained from both hormone-treated and non-treated cultures were similar, closely resembling the results obtained with hormone-stimulated MMTV RNA (unpublished data).

DISCUSSION

Until recently, a reliable nucleic acid hybridization assay for measuring newly synthesized RNA in intact cells infected with type C viruses was not available. Coffin et al. (4) described a novel assay that allows the detection of radiolabeled newly synthesized viral RNA and is free of several technical problems that affected earlier studies of transcription. Labeled RNA is first hybridized to a cDNA probe previously elongated with poly(dC); radioactive hybrids are then recovered by poly(I)-Sephadex chromatography, treated with RNAse, eluted with formamide, and assayed. The assay system of Coffin et al. (4) provides a direct measurement of the rate of viral RNA transcription in whole cells, consequently providing a means to resolve the process primarily involved in glucocorticoid induction. Glucocorticoid addition to the cell nutrient culture medium results in an increase in MMTV-specific RNA within 5 to 10 min; within 30 to 60 min a 5- to 10-fold increase in the amount of newly made MMTV RNA is noted. Although these results strongly suggest that increased transcription of MMTV DNA sequences accounts for most, if not all, of the observed increase in MMTV RNA, we have not identified the specific transcriptional step or steps primarily affected by the hormone-receptor complex. There may be an increased rate of initiation or elongation of RNA synthesized; alternatively, the hormone-receptor complex may cause nontranscribed proviral sequences to be transcribed without a direct effect on the rates of transcriptional events. Although the latter possibility of increased sites for transcription exists (since there are multiple copies of MMTV sequences integrated in the DNA of the murine mammary tumor cells [16]), the requirement for preexisting basal levels of MMTV RNA before hormone stimulation occurs leads us to believe that some transcriptional event at sites already active in the process of transcription is the primary effect of hormonal stimulation.

Since glucocorticoid hormone treatments result in a constant 10-fold increased rate of synthesis of MMTV RNA, either the chemical degradation or excretion of the MMTV RNA must increase by some mechanism related to the rates of synthesis to achieve a steady-state level of accumulated RNA higher than that found in the uninduced cell. While experiments using actinomycin D to determine RNA half-life must be interpreted with caution, it is interesting that the chemical decay of MMTV RNA was greater in induced cultures than in uninduced cultures and that the degradation of MMTV RNA in induced cultures occurred faster in the initial 4 h than in the subsequent 4 h. The data suggest that, as the RNA levels decrease, the rate of degradation also decreases, and that the rate of degradation of MMTV RNA may be directly related to the rate of synthesis of this RNA. However, the role of viral proteins in stabilizing and packaging the viral RNA is unknown at this time; these may be important factors in regulating the fate of newly synthesized viral RNA in the cell. Thus, it is difficult to establish any meaningful half-life measurements from the data presented here. In addition, we cannot determine whether the hormone plays any role in the rate of degradation of the MMTV RNA, although in these studies the degradation of MoMuLV RNA was unaffected by hormone treatment. The results do indicate, however, that glucocorticoid hormone affects primarily the rate of MMTV RNA transcription but does not affect the stabilization of newly synthesized MMTV RNA.

Our results and those from previous studies indicate the following characteristics of dexamethasone-stimulated MMTV RNA accumulation: (i) the effect occurs within minutes of hormone addition; (ii) the effect is primarily on the rate of RNA synthesis; and (iii) the increased rate of synthesis does not require new cellular DNA or protein synthesis (14, 16). Thus, the hormone-mediated event has all the prerequisites for further study in in vitro transcription systems.

ACKNOWLEDGMENTS

We thank Thomas Parsons, University of Virginia, for helpful discussions about the poly(I)-Sephadex assay; John Taylor, Fox Chase Cancer Research Center, Philadelphia, for advice on the use of fragment priming of reverse transcription; and David Williams for excellent technical assistance.

This work was supported in part by a contract from the Virus Cancer Program. H. A. Young is supported by American Cancer Society fellowship PF-1011.

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