Synthesis of Mouse Mammary Tumor Virus Ribonucleic Acid in Isolated Nuclei from Cultured Mammary Tumor Cells†

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ABSTRACT: Glucocorticoid hormone treatment of GR cells, a cultured line derived from mouse mammary tumor tissue, selectively stimulates the rate of transcription of integrated proviral genes specifying mammary tumor virus (MTV). We have incubated isolated nuclei from these cells under conditions in which all three endogenous RNA polymerases appear to be active. RNA synthesized in vitro is distinguished from preexisting nuclear RNA by labeling the in vitro products with [^{3}H]CTP, and the level of MTV RNA synthesis is measured by molecular hybridization with unlabeled viral DNA. Synthesis requires the addition of nucleoside triphosphates and is inhibited by actinomycin D. Pretreatment of GR cells with dexamethasone, a synthetic glucocorticoid, has no significant effect on the amount of total RNA synthesis in isolated nuclei. In contrast, synthesis of MTV RNA is stimulated 10–20-fold in nuclei from dexamethasone-treated cells relative to untreated control nuclei; the sensitivity of in vitro viral RNA synthesis to inhibition by α-amanitin suggests that it is carried out exclusively by RNA polymerase II. The fraction of total RNA synthesis which is viral specific (about 0.2–0.4% in nuclei from dexamethasone-treated cells and 0.01–0.03% in controls) is similar to that detected in pulse labeled RNA from whole GR cells in culture. Our procedures for labeling and hybridization of RNA appear to avoid artifacts recently noted in other in vitro transcription systems.

Steroid hormones appear to act by a common molecular mechanism (King & Mainwaring, 1974; Jensen et al., 1971) initiated by a specific high affinity interaction of the hormone with soluble receptor proteins. Binding of the steroid changes the properties of the receptor, increasing its affinity for binding sites in the cell nucleus, and thereby triggering the biological effect of the hormone. The nature of the nuclear binding sites is controversial, since biochemical experiments implicating virtually every component in the nucleus have been reported (Jackson & Chalkley, 1974; O'Malley et al., 1972; Puca et al., 1974; Baxter et al., 1972; King & Gordon, 1972; Yamamoto & Alberts, 1972); genetic evidence suggests that the nuclear sites are at least partially composed of DNA (Gehring & Tomkins, 1974; Yamamoto et al., 1974, 1976). The actual reactions involved in the alteration of gene expression are not understood, although some potential mechanisms have been proposed (Schwartz et al., 1975; Palmiter et al., 1976; Yamamoto & Alberts, 1976).

In several cases it has been demonstrated that steroid hormones elicit an increase in the intracellular concentration of specific messenger RNA species (Chan et al., 1973; Schutz et al., 1975; Ringold et al., 1975b; McKnight et al., 1975; Harris et al., 1975; Palmiter et al., 1976; Tata, 1976). Thus, it is tempting to assume that the receptor–nucleus interaction selectively alters the rate of gene transcription in a manner functionally analogous to the activities of prokaryotic gene regulatory proteins (e.g., Wilcox et al., 1974; Dickson et al., 1975). However, the apparent complexity of mRNA metabolism, especially in eukaryotes (Lewin, 1974; Berget et al., 1977; Broker et al., 1978), suggests many other points at which receptors might affect the equilibrium concentration of a transcript.

Genes specifying mammary tumor virus (MTV)[1] exist in an integrated state (Morris et al., 1977) in GR cells, a cultured cell line derived from a murine mammary carcinoma. When GR cells and other mouse mammary tumor cell lines are treated with dexamethasone, the accumulation of MTV RNA is selectively stimulated (Parks et al., 1974, 1975; Ringold et al., 1975a), in a reaction which appears to be mediated by the glucocorticoid receptor protein (Ringold et al., 1975b). Using this system, it has been directly demonstrated for the first time that a steroid hormone can act in whole cells to rapidly increase the rate at which a specific RNA is produced (Young et al., 1977; Ringold et al., 1977). The fact that the response occurs in the presence of inhibitors of protein synthesis (Ringold et al., 1975b), and that the stimulated rate of synthesis becomes maximal with no detectable lag (Ringold et al., 1977), suggests that the receptor protein may act directly on the genetic locus coding for MTV RNA. Taken together, these observations imply that a detailed biochemical examination of the role of dexamethasone in MTV gene transcription might reveal important information concerning the initial steps in steroid action.

As a first step toward this long term goal, we have begun to characterize the cell free synthesis of RNA in nuclei isolated from GR cells. The experimental procedures reported here avoid certain in vitro transcription artifacts brought about by aberrant polymerase reactions (Zasloff & Felsenfeld, 1977a,b; Shih et al., 1977; G. Shutz, manuscript in preparation). Under our conditions, we find that GR cell nuclei prepared from control and hormone-treated cells synthesize MTV RNA at relative rates similar to those measured in analogously treated whole cells.

Experimental Procedures

Materials. NCS tissue solubilizer and [5-^{3}H]CTP (21 Ci/mmol) were purchased from Amersham. Nucleoside tri-

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[1] Abbreviations used: MTV, mammary tumor virus; BSA, bovine serum albumin; $NaDodSO_4$, sodium dodecyl sulfate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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phosphates (sodium salts) and actinomycin D were obtained from Calbiochem. Pronase (Calbiochem, Grade B) was self-digested for 60 min at 37 °C at 10 mg/mL in 20 mM Tris-Cl, pH 7.4, before use in RNA preparations. Deoxyribonuclease (DNase) I (DPFF, RNase-free) was from Worthington Biochemical Corp. Dexamethasone (9-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione) and 17β-estradiol were from Sigma Chemical Co. Pentex bovine serum albumin (BSA) was from Miles Laboratories. NP-40 detergent was a gift of the Shell Oil Co. Sodium dodecyl sulfate ($NaDodSO_4$) was from BDH Chemicals. Phenol (Mallinckrodt) was redistilled and saturated with 20 mM Tris-Cl, pH 7.4. α-Amanitin was from Boehringer-Mannheim.

Growth of Cells. GR cells are derived from a spontaneous mammary carcinoma in the GR strain of mouse (Muhlbock, 1965); they were grown in monolayer culture in Dulbecco's modified Eagle's medium supplemented with 10% horse serum. For preparation of nuclei, cells were harvested within 3 days after reaching confluency.

Preparation of Nuclei. Nuclei were prepared by a method similar to those described by Marzluff et al. (1973) and Ernest et al. (1976). Confluent cultures of GR cells in three 150-cm^2 culture flasks ($1-2 \times 10^8$ cells) were rinsed with phosphate-buffered saline, chilled to 4 °C, and removed from the flasks with a rubber scraper. All subsequent operations were carried out at 0–4 °C. After centrifugation for 5 min at 300g, the cells were resuspended in 2.5 mL of hypotonic buffer (20 mM Tris-Cl, pH 8.0, 4 mM $MgCl_2$, 6 mM $CaCl_2$, 0.5 mM dithiothreitol) and allowed to swell on ice for 5 min; 2.5 mL of 0.6 M sucrose, 0.2% NP40, 0.5 mM dithiothreitol was added, and the cells were immediately broken by 12 strokes with the tight-fitting pestle of a Dounce homogenizer. The nuclei were pelleted by centrifugation for 10 min at 1500g and resuspended in 2.5 mL of 0.25 M sucrose, 20 mM Tris-Cl, pH 8.0, 10 mM $MgCl_2$, 0.5 mM dithiothreitol. After adding 2.5 mL of 2 M sucrose, 10 mM Tris-Cl, pH 8.0, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, the crude nuclear suspension was layered over 2.5 mL of the 2 M sucrose buffer in two 5-mL tubes and centrifuged for 45 min at 48 000g. The nuclear pellets were drained and resuspended in 0.4 mL of 25% glycerol, 50 mM Na-Hepes, pH 8.0, 5 mM magnesium acetate, 0.5 mM dithiothreitol, 1% BSA. Light microscopic examination revealed that the final preparation was free from contamination by cytoplasmic debris and whole cells. DNA concentration was measured by the diphenylamine reaction (Burton, 1956).

Cell-Free RNA Synthesis. Conditions were essentially those of Ernest et al. (1976). Nuclei were suspended at $\sim 10^8$/mL ($\sim$1 mg/mL of nuclear DNA in 50 mM Na-Hepes, pH 8.0, 150 mM NH_4Cl, 5 mM magnesium acetate, 0.5 mM $MnCl_2$, 2 mM dithiothreitol, 1 mM ATP, 1 mM GTP, 1 mM UTP, 60 μM [^{3}H]CTP, 10% glycerol, and 1% BSA. The reaction mix was incubated for 60 min at 25 °C in a shaking water bath. Light microscopy confirmed that the nuclei were still intact following incubation.

To measure total RNA synthesis, 0.05-mL reactions were carried out containing [^{3}H]CTP at 1 Ci/mmol. After incubation, the reactions were terminated by rapid chilling and addition of 0.7 mL of 0.1% $NaDodSO_4$; 0.2 mL of 50% trichloroacetic acid was then added with vortexing. Precipitates were collected on Whatman GF/C filters, washed thoroughly with cold 5% Cl_3CCOOH, rinsed with 95% ethanol, and dried under a heat lamp. Filters were counted in toluene based scintillation fluid containing 4 g/L of Omnifluor (New England Nuclear), 4 mL/L of H_2O, 25 mL/L of NCS tissue solubilizer; the data shown have been corrected for an unincubated blank value of 0.03–0.09 pmol of CMP/μg of DNA.

To measure MTV-specific RNA synthesis, 0.5-mL reactions were carried out with [^{3}H]CTP at 10–21 Ci/mmol. The RNA synthesized was prepared for analysis as described below.

Isolation of RNA from the Reaction Mixture. DNase I was added to 50 μg/mL, and incubation was continued for 25 min at 25 °C. A 5-μL aliquot was removed for determination of total RNA synthesis as described above; the remainder was added to 4.5 mL of 20 mM Tris-Cl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.5% $NaDodSO_4$, 0.5 mg/mL Pronase and incubated for 60 min at 37 °C. RNA was then extracted at room temperature with a mixture of 1 volume of phenol and 0.5 volume of chloroform. After centrifugation and phase separation, the aqueous phase was brought to 0.25 M NaCl, and 2 volumes of 95% ethanol was added. RNA was precipitated at −20 °C, collected by centrifugation for 20 min at 16 000g, rinsed with 95% ethanol, drained, allowed to dry, and dissolved in 1 mL of 20 mM Tris-Cl, pH 8.0, 10 mM $MgCl_2$, 2 mM $CaCl_2$. A second DNase I digestion (5 μg/mL) was performed for 30 min at 37 °C and the Pronase digestion, phenol–chloroform extraction, and ethanol precipitation steps were repeated. The RNA pellet was dissolved in 0.5 mL of 20 mM Tris-Cl, pH 7.4, 0.3 M NaCl, 0.5% $NaDodSO_4$ and passed over a 1.5 × 5.5 cm column of Sephadex G-25 to remove free [^{3}H]CTP. RNA was ethanol precipitated from the excluded volume fraction, dissolved in 40 μL of 10 mM Tris-Cl, pH 8.0, 10 mM EDTA, and stored at −20 °C. Final recoveries of the initial acid-precipitable counts in this procedure were 40–60%.

Measurement of MTV RNA Synthesis. Molecular hybridization probes were prepared by detergent activation of the virus-associated RNA-dependent DNA polymerase, using virions secreted from dexamethasone-treated GR cells; these reagents have been extensively characterized (Varmus et al., 1973; Ringold et al., 1975a, 1976); they are essentially fully specific for MTV sequences under our conditions. To determine the amount of MTV RNA synthesized in the cell-free reaction, hybridization with unlabeled MTV "tailed duplex" DNA was performed using essentially the method devised by E. Stavnezer and J. M. Bishop (manuscript in preparation); Ringold et al. (1977) have described in detail the exact procedure we used. Briefly, the [^{3}H]RNA products of the cell free reaction were hybridized with MTV "tailed duplex" DNA in reactions containing 10 μL of the [^{3}H]RNA sample to be analyzed ($0.3–1.3 \times 10^6$ cpm) in a total volume of 20 μL; a large excess of "nonspecific" competitor RNA ($\sim$1 mg/mL total rat RNA) was present in every reaction. MTV 70S [^{32}P]RNA ($\sim 10^3$ cpm; 0.1–0.2 ng) was added as an internal standard for measurement of the efficiency of hybridization. After hybridization, the mixture was treated with pancreatic ribonuclease and then chromatographed on a column of hydroxylapatite under conditions in which only double-stranded DNA is directly bound to the column; MTV RNA specifically hybridizes to the single stranded tails of the MTV "tailed duplex" DNA and is thus indirectly bound. The bound radioactivity represents the fraction of MTV RNA among the products of the cell free system.

Results and Discussion

General Characteristics of Cell-Free RNA Synthesis by Endogenous RNA Polymerases in GR Cell Nuclei. Incorporation of radioactivity into Cl_3CCOOH-precipitable material continues for about 60 min in the cell-free RNA synthesis reaction (Figure 1). When measured as a function of nuclear DNA concentration in the reaction, about two- to fourfold more RNA is synthesized per μg of DNA at 1 mg/mL than at 0.1 mg/mL (data not shown); at 1 mg/mL, 0.5–2.0 pmol

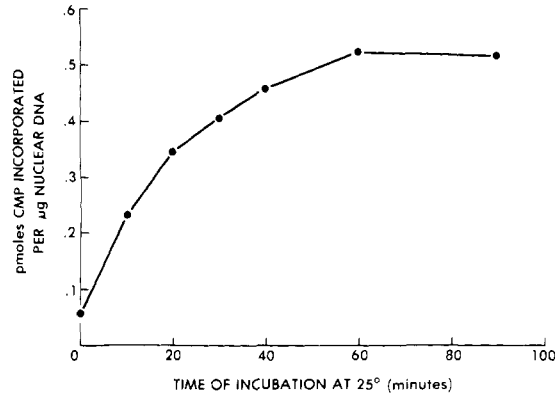

FIGURE 1: Time course of [^{3}H]CMP incorporation in isolated GR cell nuclei. Nuclear preparations and transcription reactions were performed as described in Experimental Procedures; the nuclear DNA concentration in the reactions was 0.6 mg/mL. Unincubated control values have not been subtracted from the data shown.

CMP is incorporated per μg of nuclear DNA. The radioactive product of the cell free reaction is completely degraded by ribonucleases A and T_1, and its synthesis is almost completely dependent upon the presence of added ribonucleoside triphosphates (Table I). The product is insensitive to deoxyribonuclease I and to Pronase.

Factors Affecting Cell-Free Synthesis of MTV RNA. Nuclei were prepared from cultures of GR cells which had been exposed to 10^{-6} M dexamethasone prior to harvest and from cells which were not hormone treated. Total RNA synthesis by the different nuclear preparations varies somewhat, but is not affected in a consistent or predictable way by the hormone treatment. However, nuclei from cells exposed to dexamethasone for either 30 min or 16 h prior to harvest synthesize 10–20-fold more MTV-specific RNA than nuclei from control cells (Table II). The RNA products of nuclei from control and dexamethasone-treated cells contain approximately the same relative amounts of newly synthesized MTV sequences as pulse labeled RNA from whole GR cells which had received the corresponding hormone treatment (Ringold et al., 1977). In contrast, pretreatment of the cells with high concentrations (5×10^{-5} M) of another steroid, 17β-estradiol, has no effect on the synthesis of MTV RNA in the isolated nuclei; moreover, estradiol does not inhibit the activity of dexamethasone (10^{-6} M) added to the cultures subsequently (Table II, part II). Estradiol has no glucocorticoid activity and does not interact with the glucocorticoid receptor (Samuels & Tomkins, 1970); thus, the effect observed appears to be hormone specific. By these criteria, MTV RNA synthesis in the cell-free system reflects some of the characteristics of the process in whole cells.

It appears that, in whole cells, maintenance of hormone-induced levels of MTV RNA requires the continued presence of dexamethasone (Ringold et al., 1978); this implies that synthesis of MTV RNA at the hormone-induced rate requires receptor–steroid complexes bound to loci in the cell nucleus. However, Table II, part III, reveals that in nuclei isolated from dexamethasone-treated cells, the continued presence of dexamethasone (10^{-6} M) during preparation and incubation of nuclei has no further effect on either total or MTV-specific cell-free RNA synthesis. One interpretation is that the rate at which dexamethasone dissociates from the receptor is slow under these conditions, such that added hormone is not required to maintain the interaction between receptors and their nuclear sites. An alternative possibility is that the receptor affects the rate of initiation of MTV gene transcription in vivo, whereas nascent chains are elongated but new transcripts are not initiated in the cell-free system. Since initiation events have not yet been measured (see below), we cannot presently distinguish between these possibilities.

TABLE I: Cell-Free Synthesis; Dependence on Ribonucleoside Triphosphates and Sensitivity of the Product to Ribonuclease.[a]

	Total RNA synthesis (pmol of CMP incorp per μg of nuclear DNA)	% act.
I. RNase		
Control	1.54	100
+ RNase	0.03	2
II. Ribonucleoside triphosphates		
Complete	0.780	100
−ATP	0.078	10
−GTP	0.093	12
−ATP, GTP, UTP	0.017	2

[a] Assays were performed as described in Experimental Procedures. The concentration of nuclear DNA in the assays was 1.3 mg/mL. In Part I, RNase A and RNase T_1 (200 μg/mL and 20 U/mL, respectively) were added after completion of RNA synthesis, and incubation was carried out for 60 min at 37 °C before measurement of acid-insoluble radioactivity. In part II the concentration of each ribonucleoside triphosphate was 0.1 mM instead of 1 mM as usual.

The effects of divalent cations on cell free synthesis were also examined. The activities of RNA polymerases are strongly influenced by Mg^{2+} and Mn^{2+}. With bacterial RNA polymerases, Mn^{2+} stimulates a number of presumably non-physiological reactions which do not occur in the presence of Mg^{2+} alone, e.g., RNA dependent (Steck et al., 1968) and template-independent RNA synthesis (Smith et al., 1967). The specific activity of eukaryotic RNA polymerase II on chromatin or naked DNA templates is increased as much as tenfold when Mn^{2+} is included in the reaction, whether in the presence or the absence of Mg^{2+} (Schwartz et al., 1974; Monroy et al., 1975). The qualitative nature of the stimulation of the eukaryotic enzymes by Mn^{2+} has not been as thoroughly characterized as the effect on prokaryotic polymerases. In view of these observations, it seemed important to determine if the 0.5 mM Mn^{2+} included in our standard reaction mix has detectable effects under our conditions for RNA synthesis. Table II, part III, shows that Mn^{2+} can be omitted from the reaction with no effect on either total or MTV RNA synthesis. Thus, none of the RNA synthesis observed appears to be of a type which is stimulated exclusively by Mn^{2+}.

α-Amanitin and Actinomycin D Sensitivity of Total and MTV RNA Synthesis. Schwartz et al. (1974) have described in detail the differential sensitivities of eukaryotic RNA polymerases I, II, and III to α-amanitin. In GR cell nuclei, 45–50% of the total cell-free RNA synthesis is sensitive to a level of α-amanitin (0.3 μg/mL) that inhibits only RNA polymerase II, while an additional 45–50% of the incorporation is insensitive to 1 mg/mL α-amanitin, a characteristic of RNA polymerase I activity (Figure 2). A small amount of activity, about 5–10% of the total, may be inhibited at intermediate levels of α-amanitin (100 μg/mL); RNA polymerase III is generally affected at this concentration of toxin. Thus, polymerases I and II and perhaps polymerase III appear to be active under our conditions for cell-free RNA synthesis.

It is well established that the synthesis of the bulk of cellular messenger RNAs is mediated by RNA polymerase II (Lindell et al., 1970; Somers et al., 1975). Furthermore, it has been

TABLE II: Synthesis of MTV RNA in Nuclei Prepared from GR Cells Grown in the Presence or Absence of Dexamethasone.[a]

Hormone treatment	Additional conditions	Total RNA synthesis (pmol of CMP/ μg of DNA)	% hybridization A MTV[^{32}P]-RNA Comp.[b] (−)	B MTV[^{32}P]-RNA Comp.[b] (+)	C Δ% [^{32}P]-RNA ann.[b]	D [^{3}H]RNA Comp.[b] (−)	E [^{3}H]RNA Comp.[b] (+)	F Δ% [^{3}H]-RNA ann.[b]	G Corr.[b] % [^{3}H]-RNA ann.[b]	Rel MTV RNA synthesis
I. Duration of whole cell dexamethasone treatment										
Untreated control		0.38	34.4	0.8	33.6	0.040	0.031	0.009	0.025	1
Dex 16 h		0.63	17.7	2.6	15.1	0.071	0.036	0.035	0.231	9
Dex 30 min		0.60	16.9	1.2	15.7	0.115	0.042	0.073	0.465	18
II. Effect of whole cell estradiol treatment										
Untreated control		0.90	42.7	6.8	35.9	0.018	0.012	0.006	0.016	1
Dex 30 min		0.81	19.1	5.9	13.2	0.067	0.027	0.040	0.300	19
17β estradiol 30 min		0.79	44.6	6.8	37.8	0.019	0.007	0.012	0.031	2
17β estradiol +dex 30 min		0.69	20.2	5.7	14.5	0.056	0.022	0.034	0.232	15
III. Effects of added Mn^{2+} and dexamethasone in the cell free reaction										
Untreated control		0.66	21.7	0.8	20.9	0.015	0.013	0.002	0.011	1
Dex 30 min		1.07	12.1	0.6	11.5	0.042	0.015	0.027	0.239	22
Dex 30 min	$-Mn^{2+}$	0.94	10.6	0.8	9.8	0.042	0.015	0.027	0.278	25
Dex 30 min	+Dex in assay and nuclear prep.	0.71	9.9	0.7	9.2	0.037	0.013	0.024	0.260	24

[a] Cells were exposed to hormones for the indicated length of time; nuclei were prepared and RNA synthesis was carried out as described in Experimental Procedures. The nuclear DNA concentration in the assays was 0.8–1.3 mg/mL. The hybridization reactions to measure MTV RNA in the labeled product were performed as described by Ringold et al. (1977). Column A gives the percentage of purified 70S MTV [^{32}P]RNA, added in tracer levels to each reaction, which annealed to tailed duplex MTV DNA; this serves as an internal determination of the efficiency of hybridization. Note, for example, that the hybridization efficiency in nuclei from control cells is ca. twofold higher than that from the dexamethasone-treated counterparts. This reflects the difference in the equilibrium concentration of endogenous MTV RNA present in the nuclei. Column D gives the percentage of the [^{3}H]RNA which annealed. In columns B and E 200–500 ng of unlabeled 70S MTV RNA (prepared from virus particles produced by GR cells) was present in the hybridization reaction as competitor against viral [^{32}P]- and [^{3}H]RNA. Columns C and F represent percentages of annealed [^{32}P]- and [^{3}H]RNA which are competed by unlabeled 70S MTV RNA; C = A − B and F = D − E. Column G is the net percentage of MTV sequences in the [^{3}H]RNA, corrected for efficiency of hybridization (C); G = (F/C) × 100. Dexamethasone was used at a concentration of 10^{-6} M in parts I and III and at 5×10^{-7} M in part II. In part II, 17β-estradiol was used at 5×10^{-5} M and was added 30 min before the dexamethasone. In the last assay in part III, 10^{-6} M dexamethasone was present in the nuclear preparation buffers and in the reaction mixture. [b] Comp., competitor; ann., annealed; Corr., corrected.

shown that transcription of the integrated genes specifying avian C-type viruses is also carried out by this enzyme (Rymo et al., 1974; Monroy et al., 1975). Table III, part I, shows that MTV RNA synthesis is 97% inhibited by 0.3 μg/mL of α-amanitin, supporting the idea that RNA polymerase II is responsible for the cell-free synthesis of MTV RNA in GR nuclei.

Actinomycin D, a drug which intercalates into double-stranded DNA adjacent to GC base pairs (Sobell & Jain, 1972), inhibits DNA-directed RNA synthesis, but does not affect RNA directed or template independent RNA synthesis. When actinomycin D was added to the cell-free reaction at a concentration of 10 μg/mL, total RNA synthesis and MTV-specific RNA synthesis were inhibited by about 80% and 90%, respectively (Table III, part II). This concentration of actinomycin D is sufficient to inhibit 98% of RNA synthesis in GR cell cultures (Ringold et al., 1975b); however, the suspended nuclei are ~100-fold more concentrated than the cell cultures, and the molar ratio of actinomycin D to GC base pairs is only ~1:40 in the cell-free reaction. This may account for our failure to achieve more extensive inhibition of RNA synthesis at 10 μg/mL of actinomycin D. In any case, these results suggest that MTV RNA synthesis in the cell-free system is at least 90% DNA directed.

Methods for Detection of Newly Synthesized RNA. Preparations of chromatin or intact nuclei contain various classes of cellular RNA: nascent transcripts, nuclear RNA, and contaminating cytoplasmic RNA. When the chromatin or nuclei are utilized for cell-free transcription, the presence of these RNA species imposes two technical constraints which complicate interpretation of experimental data. First, those sequences transcribed under cell-free conditions must be unequivocally distinguished from the preexisting RNAs; transcription in most cell-free systems is transient and relatively inefficient, producing much less newly synthesized product than the amount of endogenous RNA present. Second, the endogenous RNA sequences might artifactually affect the type and quantity of the newly synthesized RNAs.

Dale & Ward (1975) have described a novel technique in which mercurated pyrimidine nucleoside triphosphates are employed as substrates by RNA polymerases and are incorporated into RNA synthesized under cell-free conditions. When the total RNA is then chromatographed on sulfhydryl Sepharose, the mercurated sequences are retained while the bulk of the endogenous RNA passes through. Subsequent analysis of the retained fraction as a reliable measure of newly

TABLE III: Sensitivity of Cell-Free RNA Synthesis to α-Amanitin and Actinomycin D.[a]

A	Total RNA synthesis			MTV RNA synthesis	
	B	C		D	E
Concentration of inhibitor (μg/mL)	pmol of CMP/μg of DNA	% of uninhibited act.	Active RNA polymerases	% of total RNA synthesized	% of uninhibited act.
I. α-Amanitin					
0	2.25	100	I, II, (III)	0.319	100
0.3	0.88	39	I, (III)	0.021	2.6
300	0.69	31	I	0.021	2.0
II. Actinomycin D					
0	1.13	100		0.309	100
10	0.22	19		0.138	8.5

[a] The assays were performed as described in Experimental Procedures. Assays contained 1.4 and 0.4 mg of nuclear DNA per mL in parts I and II, respectively. MTV-specific RNA synthesis (column D) was calculated as in Table II, column G. Column E gives the amount of MTV RNA synthesis relative to the uninhibited case; E = (C × D)/0.319 for part I and E = (C × D)/0.309 for part II.

synthesized RNA requires quantitative removal of nonmercurated RNA from this material.

Recently, several investigators using the mercurated RNA technique have shown that aberrant polymerase reactions occur in chromatin and nuclear transcription systems under certain conditions. Zasloff & Felsenfeld (1977a) and G. Schutz (manuscript in preparation) have detected RNA-dependent RNA synthesis; that is, endogenous RNA can be used as a template for production of "minus strand" RNA in cell-free systems. In addition, Shih et al. (1977) have suggested that terminal addition onto endogenous sequences by exogenously added polymerase might also take place. Thus, unless specific precautions are taken (Zasloff & Felsenfeld, 1977b), both of these reactions result in retention of endogenous RNA sequences on sulfhydryl-Sepharose; these nonmercurated RNAs would be incorrectly scored as newly synthesized sequences in molecular hybridization assays.

Although mercurated nucleotides were used in the detection of these artifacts, there is no reason to believe that such aberrant polymerase reactions occur only in the presence of these derivatized substrates. Analogous reactions have been extensively described in simpler transcription systems employing bacterial polymerases (Steck et al., 1968; Smith et al., 1967). In any case, the work of Zasloff & Felsenfeld, Schutz and Shih et al. shows that retention of RNA on a sulfhydryl-Sepharose column followed by hybridization of the RNA with a labeled cDNA is not a sufficient criterion from which to conclude that the RNA in hybrid was indeed synthesized in the cell-free state. At the minimum, if mercury is the only marker of new synthesis, then it must be demonstrated that mercury is contained in the sequences that actually enter the hybrid state with the cDNA.

In the cell-free RNA synthesis experiments described here, radioactively labeled nucleoside triphosphates, rather than mercurated nucleotides, have been used as RNA precursors; newly synthesized mammary tumor virus RNA, transcribed from integrated proviral DNA sequences, can be directly detected by molecular hybridization with an unlabeled "tailed duplex" viral DNA. This hybridization reagent is the product of the endogenous reverse transcriptase of MTV virions and is composed of partially double-stranded molecules with random segments of minus strands available for hybridization with viral RNA (Ringold et al., 1976). The tailed duplex DNA is used at concentrations that approximate the total concentration (endogenous plus newly synthesized) of MTV RNA in the reaction mix, and hybridization efficiency is monitored internally in each reaction (see Table II and Ringold et al., 1977).

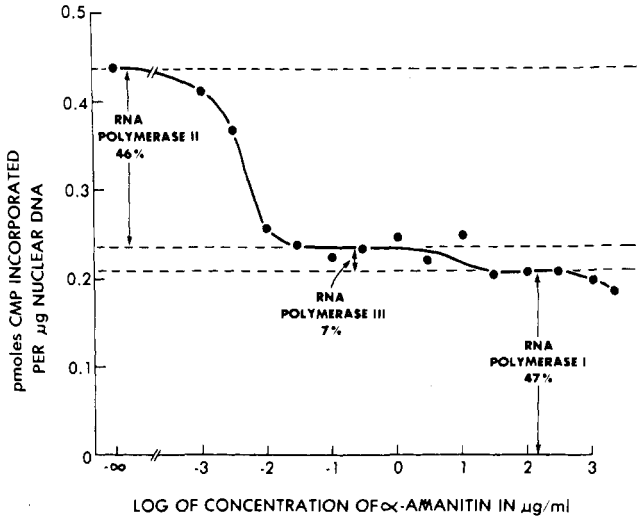

FIGURE 2: Inhibition of cell free RNA synthesis by α-amanitin. Preparation of nuclei and transcription reactions were performed as described in Experimental Procedures, except that varying concentrations of α-amanitin were included in the reaction mix. The nuclear DNA concentration in the reactions was 1.2 mg/mL.

Fortunately, the synthesis of MTV RNA, both before and after stimulation of transcription by dexamethasone, can be examined under conditions in which a relatively small amount of endogenous viral RNA is present.

Our procedure for assaying newly synthesized MTV RNA specifically avoids the difficulties noted above. As shown in Figure 3A, DNA directed synthesis is detected, whereas artifactual products (Figures 3B and 3C) are eliminated by a two-step process: (1) RNase treatment to degrade unhybridized RNA, followed by (2) chromatography on hydroxylapatite under conditions in which DNA:DNA duplexes bind, but single-stranded nucleic acids, RNA:RNA duplexes, and RNA:DNA duplexes do not bind (Smith et al., 1974; Stavnezer & Bishop, manuscript in preparation; see also Ringold et al., 1977). Endogenous MTV RNA is not detected in the assay since it is not radioactively labeled.

Conclusions

Nuclei have been prepared and incubated under conditions in which certain aspects of the biological activity of the transcription machinery appear to be conserved; we have shown here that the relative rate of MTV RNA synthesis in vitro is

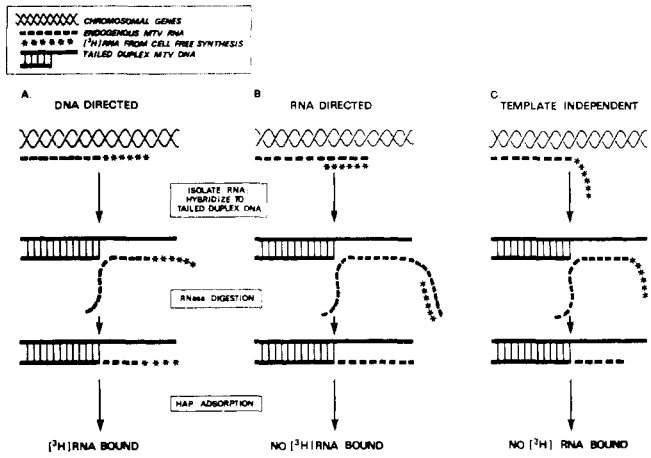

FIGURE 3: Possible products of cell free RNA synthesis reactions. (A) DNA-directed synthesis. Reaction shown is elongation of a nascent chain; initiation events might also occur. (B) RNA-directed synthesis. Endogenous MTV RNA (plus or genome strand) serves as a template for synthesis of complementary (minus strand) RNA; the template and product may remain together in a partial duplex structure after synthesis. (C) Template-independent synthesis. RNA containing a random (or other non-MTV) sequence is terminally added to a chain of endogenous MTV RNA.

similar to that detected in whole cells, both before and after dexamethasone treatment.

It is less encouraging that the duration of the cell-free reaction is only about 1 h; moreover, it would not be surprising if proper initiation and/or termination, two processes which probably require extraordinarily high specificity and selectivity, were defective or altogether absent. Optimal conditions for promoting correct initiation by RNA polymerase II have not been reported; we have not yet directly assayed for RNA chain initiation in our system. A major problem in this regard is the general lack of understanding of RNA polymerase II initiation in vivo; thus, good biological correlates are not yet available. New techniques, such as those recently devised in the laboratories of Huang (Reeve et al., 1977) and Reeder (Reeder et al., 1977) should be extremely useful in these analyses.

A long-term goal of this work is to establish an experimental system in which transcription of a specific genetic locus can be hormonally stimulated under cell-free conditions. It seems likely that initiation of new RNAs at correct loci is a minimum requirement for achieving this goal; whether systems such as this one can be made to satisfy that criterion is an open question. The present results reflect the observation of a simple "partial reaction" in an undoubtedly complex process. Nevertheless, our experimental conditions appear at least to maintain the capacity of isolated nuclei to continue faithfully the synthesis of RNA molecules initiated in whole growing cells.

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Length Dependence in Reassociation Kinetics of Radioactive Tracer DNA†

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ABSTRACT: The reassociation kinetics have been measured for radioactive *Escherichia coli* DNAs (tracers) of various average single-strand lengths reassociated alone and in the presence of excess unlabeled DNA (driver) of two different average lengths. Hydroxylapatite binding was used to follow the reaction time course. The length dependence of the rate constant determined in the tracer self-reassociation reactions is in agreement with the square-root dependence previously determined (Wetmur, J. G., & Davidson, N. (1968) *J. Mol. Biol. 31,* 349-370) using optical methods to follow the time course. However, for the driver-tracer reactions, where the radioactive DNA reassociates largely with DNA of a different average length, the dependence of the rate constant upon average tracer length is increased and approaches an $\bar{L}$ to the first power dependence. In 0.18 M Na^+, the variation of the rate constant for tracer reassociation with the lengths of the reassociating strands has been shown to fit the simple equation $k = (0.0077)\cdot(\bar{L}_T)(1/\bar{L}_T^{0.55} + 1/\bar{L}_D^{0.55})$, where k is the observed rate constant in L mol^{-1} s^{-1} and $\bar{L}_T$ and $\bar{L}_D$ are the weight average tracer and driver lengths, respectively, in nucleotides. This dependence suggests that the rate of nucleation of two free strands is proportional to the sum of the reciprocals of the hydrodynamic radii of the two strands.

The measurement of the reassociation kinetics of a set of DNA sequences is often carried out using trace amounts of radioactive DNA (tracer) and an excess of unlabeled DNA (driver) which contains the complementary sequences. In many experiments, some by design, the tracer sequences are not carried on fragments of the same average length as the driver DNA sequences with which they reassociate. In such cases, the tracer sequences would not be expected to reassociate at the same rate as the same sequences found in the driver DNA (Wetmur & Davidson, 1968). The effect of the lengths of the reassociating strands on the rate of reassociation must be known to permit an accurate comparison of the driver and tracer rate constants, or the rate constants of two different tracers having different average lengths.

A functional relationship between the rate of reassociation of free strands and their lengths based on the assumption of excluded volume effects in reassociation was proposed by Wetmur (1971) and found to be in qualitative agreement with the observed rate of renaturation of T2 DNA when the complementary strands were of different average lengths. The kinetics were measured by following the decrease in absorbance at 260 nm. Using hydroxylapatite binding, we have made measurements at 0.18 M Na^+ on the rate of total *E. coli* DNA tracer self-reassociation, and reassociations with drivers of two different average lengths, over a range of tracer fragment lengths. We have found that the dependence of the rate constant on the average lengths of the driver and tracer fragments appears to be simple in form over the range of lengths studied, and to deviate from that predicted by Wetmur's treatment of excluded volume effects. Our result for tracer self-reassociation reactions is close to that obtained previously at 1.0 M Na^+

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