Transcription of Viral Genes by RNA Polymerase II in Nuclei Isolated from Adenovirus 2 Transformed Cells†

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ABSTRACT: An in vitro transcription system utilizing nuclei isolated from adenovirus type 2 transformed cells has been developed. Cloned cell lines which contain viral DNA sequences complementary to only the left 14% of the adenovirus genome [Gallimore, P. H., Sharp, P. A., and Sambrook, J. (1974), *J. Mol. Biol. 89,* 49] have been used in the present study. The sensitivity to α-amanitin of RNA synthesis in isolated nuclei from B1 cells demonstrates the presence of the three classes of RNA polymerase which are characteristic of eukaryotic cells. In the presence of Mn^{2+}, the endogenous RNA polymerase activity (predominantly RNA polymerase II) shows a biphasic ammonium sulfate activation profile. At the lower salt optimum (0.03 M ammonium sulfate), synthesis continues for 40 min at 30 °C. Pulse-chase experiments demonstrate that the in vitro product is stable for an extended period of time after synthesis. In addition, studies with heparin indicate that negligible initiation of RNA chains occurs in the isolated nuclei. Thus, the observed synthesis appears to consist entirely of elongation of RNA chains which were initiated in vivo. Under optimal conditions for RNA synthesis, the transcription of the integrated viral genes has been detected by hybridization to adenovirus DNA. In nuclei isolated from B1 cells, viral-specific RNA represents about 0.01% of the total RNA synthesized in vitro. The synthesis of viral RNA in isolated nuclei is completely (>95%) inhibited by concentrations (0.6 μg/mL) of α-amanitin which completely and selectively inhibit RNA polymerase II. These results demonstrate, for the first time, the function of an RNA polymerase II in the transcription of viral genes in cells transformed by DNA tumor viruses. In addition, the transcription of the viral genes has been further analyzed by hybridization of RNA synthesized in isolated nuclei to the purified separated strands of adenovirus 2 DNA. While the stable cytoplasmic viral RNA is complementary to only the *r* strand of the viral genome [Flint, S. J., Gallimore, P. H., and Sharp, P. A. (1975), *J. Mol. Biol. 96,* 47], both strands of the integrated viral genes are transcribed. The relevance of these findings to transcription of cellular genes by RNA polymerase II is discussed.

The control mechanisms which regulate transcription in eukaryotes are at present largely undefined. The existence of three distinct forms of DNA-dependent RNA polymerase in eukaryotes (reviewed in Roeder, 1976) suggests that a coarse level of control may be effected in vivo by restricting the transcription of certain genes or groups of genes to a given enzyme class. It has, in fact, been shown that RNA polymerases I and III transcribe, respectively, the ribosomal RNA genes and the genes coding for transfer and 5S RNA. RNA polymerase II synthesizes heterogeneous nuclear RNA and, presumably, messenger RNA (reviewed in Roeder, 1976). Class II RNA polymerases have also been implicated in the synthesis of the RNA populations which contain precursors to viral mRNAs in cells productively infected with DNA tumor viruses or transformed by RNA tumor viruses (reviewed in Roeder, 1976). To date, however, only one specific cellular messenger RNA has been identified as an RNA polymerase II product (Suzuki and Giza, 1976). Modifications of chromatin structure have been implicated in the transcriptional regulation of genes which encode mRNAs and which are presumably transcribed by RNA polymerase II (Axel et al., 1973; Gilmour and Paul, 1973; Harris et al., 1976; Weintraub and Groudine, 1976; Garel and Axel, 1976). However, a more detailed knowledge of the mechanism of transcription of specific genes by RNA polymerase II may be necessary for understanding many aspects of genetic control.

Cells which have been transformed by DNA tumor viruses provide a useful system for analyzing the transcription of nonreiterated genes. We have begun a study of viral RNA synthesis in rodent cells transformed by adenovirus 2. All cloned cell lines thus far examined contain DNA sequences complementary to the left 14% of the adenovirus genome, and in six lines these are the only viral DNA sequences present (Gallimore et al., 1974). Moreover, the stable cytoplasmic RNA in these latter lines is complementary only to the *r* strand[1] of the adenovirus genome and has a complexity of about 2500 bases (Flint et al., 1975).

Here we report the development of an in vitro system capable of detecting the synthesis of viral-specific RNA in isolated nuclei from adenovirus 2 transformed cells which contain several (3–6) integrated copies of only the left 14% of the adenovirus genome. Optimal conditions for total RNA synthesis in isolated nuclei were first determined, and characteristics of the in vitro system are presented. The α-amanitin sensitivities of the endogenous RNA polymerases demonstrate that a class II enzyme transcribes the viral genes. Furthermore, although the mature transcripts are complementary to only the *r* strand, the endogenous RNA polymerase II transcribes both strands of the integrated viral genes.

Materials and Methods

Biochemicals. Joklik's modified minimal essential medium

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[1] The nomenclature used is that of Sharp et al. (1974). The *r* and *l* strands are transcribed to the right and left, respectively, on the conventional adenovirus 2 genome map.

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and dialyzed fetal calf serum were purchased from Kansas City Biologicals. Vitamins and amino acids were obtained from Grand Island Biological. Nonradioactive ribonucleoside triphosphates and poly(uridylic,guanylic) acid (lot no. 508-103-31) were purchased from P-L Biochemicals. [^{3}H]UTP (40–50 Ci/mmol) was obtained from Amersham Searle. Phosphoenolpyruvate (trisodium salt), pyruvate kinase (type III, 350–500 units/mg), *Escherichia coli* tRNA (type XXI), calf thymus DNA (type I), bovine pancreatic RNase A (type IIIA), heparin (sodium salt, 158.1 USP units/mg), NP-40, and α-amanitin were purchased from Sigma. DNase I (RNase free) was obtained from Worthington.

Cell Lines and Virus Purification. Adenovirus 2 transformed cell lines were cloned by Dr. P. H. Gallimore (Gallimore, 1973). Cell lines B1 and F17 were obtained from Drs. A. R. Dunn and J. K. McDougall, Cold Spring Harbor Laboratory, and line F18 was obtained from Dr. A. Levine, Princeton University. Cell cultures were grown on plastic petri dishes (Falcon) in Joklik's media supplemented with 5% dialyzed fetal calf serum, 1% vitamins, 1% essential amino acids, 1% nonessential amino acids, and 2 mM glutamine. Adenovirus 2 was purified from infected cultures of human KB cells as described (Craig et al., 1975) with omission of the sonication step. DNA was purified from virions according to the method of Green and Pina (1964). MOPC-315 solid tumors were obtained as described previously (Schwartz et al., 1974).

Protein and DNA Determination. Protein was quantitated according to Lowry et al. (1951), and DNA was measured as described by Burton (1956).

RNA Polymerase Purification. MOPC-315 RNA polymerase II was solubilized, separated from RNA polymerases I and III by chromatography on DEAE-cellulose, and assayed as described by Sklar and Roeder (1976). One unit of activity represents the incorporation of 1 pmol of UMP during a 20-min incubation at 37 °C. These procedures yielded an enzyme with a specific activity of 0.75 unit/μg of protein when assayed with calf thymus DNA as template and in the presence of 0.07 M $(NH_4)_2SO_4$.

Preparation of Nuclei. All glassware and buffers, where possible, were autoclaved before use. To prepare nuclei, subconfluent cultures were released from the plates with phosphate-buffered saline which was made 0.04% EDTA.[2] Subsequent operations were performed at 0–4 °C. After washing twice with Earle's balanced salt solution (Earle, 1943), the cells were suspended at 1–2 × 10^7 cells/mL in hypotonic buffer (10 mM Tris-HCl, pH 7.9 at 4 °C, 24 mM KCl, 10 mM $MgCl_2$, 1 mM DTE). The cells were pelleted by centrifugation (400·g/5 min) and resuspended in the same volume of hypotonic buffer. The cells were allowed to swell for 3–5 min, made 0.5% in NP-40, and homogenized (10–15 strokes) in a dounce homogenizer with a type B pestle. This resulted in greater than 95% cell lysis as judged by phase contrast microscopy. The nuclei were collected by centrifugation, washed once more in hypotonic buffer, and resuspended in TGMED (50 mM Tris-HCl, pH 7.9 at 22 °C, 25% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTE). Nuclei isolated in this manner from 10^8 B1 cells contain 1.14 mg of DNA and 3.38 mg of protein. Isolated nuclei were used immediately in RNA synthesis reactions.

In Vitro Synthesis and Purification of RNA. RNA polymerase activity in isolated nuclei was assayed in 50-μL reactions containing 10–25 μg of nuclear DNA, 70 mM Tris-HCl, pH 7.9 (22 °C), 10% glycerol, 0.4 mM DTE, 0.04 mM EDTA, 2 mM $MnCl_2$, 2 mM $MgCl_2$, 0.6 mM each of GTP, CTP, and ATP, 0.05 mM UTP, 6 mM NaF, 4 mM phosphoenolpyruvate, 20 μg/mL pyruvate kinase, and 20 μCi/mL [^{3}H]UTP. Unless otherwise indicated, $(NH_4)_2SO_4$ was present at 0.03 M. After incubation at 30 °C for the indicated time, reactions were terminated by the addition of DNase I to 100 μg/mL. After incubation at 30 °C for 5 min, 50 μL of $NaDodSO_4$ buffer (25 mM Tris-HCl, pH 7.9 (22 °C), 200 mM NaCl, 10 mM EDTA, 2% $NaDodSO_4$) was added. An aliquot of the reaction mixture was spotted on a DEAE paper disk, and radioactivity in RNA was determined as described (Roeder, 1974).

In experiments where the RNA synthesized in vitro was to be hybridized to adenovirus DNA, reaction volumes were 0.5–1.0 mL, unlabeled UTP was omitted, [^{3}H]UTP was present at 0.045–0.050 mM, and reactions were for 30 min. When the RNA was to be hybridized to separated strands of adenovirus DNA, unlabeled UTP was added to 10 mM just prior to the addition of DNase. After the addition of $NaDodSO_4$ buffer, *E. coli* tRNA was added as carrier and the reactions were extracted with 2 volumes of phenol–chloroform (1:1). The aqueous phase was reextracted two more times and the organic phase extracted with 0.5 volume of 10 mM Tris-HCl, pH 7.9 (22 °C), 100 mM NaCl, 1 mM EDTA, 0.5% $NaDodSO_4$. The nucleic acids in the pooled aqueous phases were precipitated with 2 volumes of ethanol, concentrated by centrifugation, resuspended in 50 mM Tris-HCl, pH 7.9 (22 °C), 15 mM $MgCl_2$, 0.5% $NaDodSO_4$, and ethanol precipitated a second time. The nucleic acids were resuspended in 50 mM Tris-HCl, pH 7.9 (22 °C), 5 mM $MgCl_2$, and DNase I was added to a final concentration of 100 μg/mL. After incubation at 30 °C for 5–10 min, an equal volume of $NaDodSO_4$ buffer was added. The sample was extracted with phenol–chloroform as described above, ethanol precipitated, resuspended in 10 mM Tris-HCl, pH 7.9 (22 °C), 100 mM NaCl, 1 mM EDTA, 0.5% $NaDodSO_4$, and passed over a 0.6 × 25 cm Sephadex G-50 column. RNA which eluted in the void volume was pooled and ethanol precipitated before use in hybridization reactions.

Adenovirus DNA Strand Separation. The complementary strands of adenovirus 2 DNA were separated and purified essentially according to the method of Tibbetts et al. (1974). The lot of poly(U,G) used resulted in a 12 mg/cm^3 peak separation of the two strands in the first CsCl equilibrium centrifugation. The peak fractions of each strand were pooled and rebanded in CsCl. The purified strands were then exhaustively self-annealed (48 h at 67 °C in bouyant CsCl). The fidelity of the strand separation was routinely monitored by control hybridizations of radioactive 5.5S RNA (generously provided by Drs. J. A. Jaehning and B. Harris) purified from adenovirus 2 infected KB cells (see text).

RNA–DNA Hybridization. DNA was denatured in NaOH and immobilized on nitrocellulose filters (Schleicher and Schuell, BA 85). Purified separated strands were loaded directly onto filters without a denaturation step. RNA–DNA hybridization was performed in 150–300 μL of 6 × SSC, 0.1% $NaDodSO_4$ for 42–48 h at 67 °C. After hybridization, the filters were washed extensively with 2 × SSC and treated with pancreatic RNase A (20 μg/mL in 2 × SSC) for 45 min at 37 °C. The filters were washed again in 2 × SSC, dried, and counted in a toluene-based scintillation fluor (Roeder, 1974). In hybridization experiments with separated strands of adenovirus DNA, the RNA was first heated at 90 °C for 5 min in 6 × SSC and transferred immediately to reaction vials con-

[2] Abbreviations used: Ad 2, adenovirus serotype 2; DEAE, diethylaminoethyl; DTE, dithioerythritol; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; SSC, 0.15 M NaCl–0.015 M $Na_3C_6H_5O_7$; Tris, tris(hydroxymethyl)aminomethane.

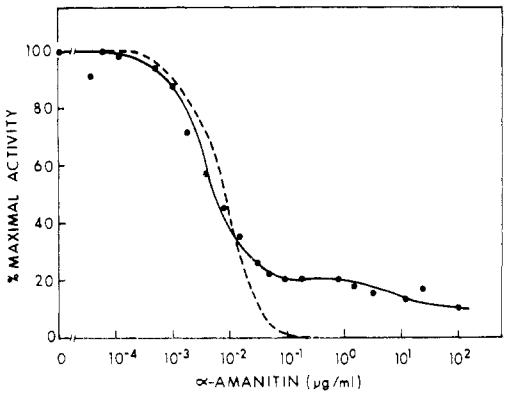

FIGURE 1: α-Amanitin sensitivity of the endogenous RNA polymerase activity in isolated nuclei from B1 cells. Reactions were performed in the presence of varying concentrations of α-amanitin, and the level of synthesis was plotted as a percentage of that synthesized in a control reaction with no α-amanitin: (●-●) sensitivity of RNA synthesis in isolated nuclei; (- - -) sensitivity of MOPC-315 RNA polymerase II.

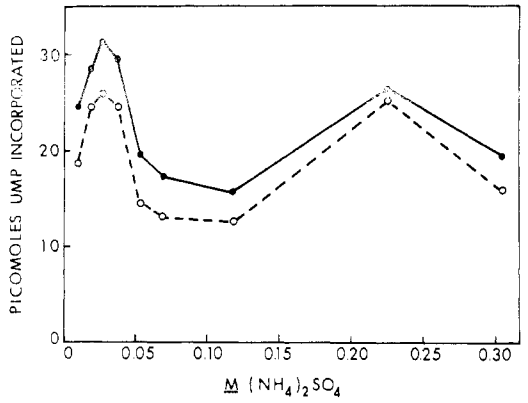

FIGURE 2: Effect of ammonium sulfate on RNA synthesis in isolated nuclei from B1 cells. Nuclei were incubated in vitro for 20 min in the presence of varying concentrations of ammonium sulfate: (●-●) total RNA polymerase activity; (O-O) RNA polymerase activity which is sensitive to 0.6 μg/mL α-amanitin.

taining the DNA–membrane filters. All hybridizations were performed under conditions of DNA excess (data not shown).

Results

Characteristics of the in Vitro System. When isolated nuclei from B1 cells are incubated in vitro as described under Materials and Methods, the endogenous RNA polymerases incorporate [^{3}H]UMP into RNA. The tritiated product is sensitive to alkali (99% hydrolyzed after 60-min incubation at 37 °C in 0.4 N NaOH) and RNase (92% degraded after 30-min incubation at 37 °C with 0.1 μg/mL pancreatic RNase A). The incorporation of isotope is completely dependent on the presence of exogenously added ribonucleoside triphosphates and exhibits an apparent K_m for UTP of 0.049 mM (data not shown). This latter value is similar to that reported for purified RNA polymerases (Chambon, 1974), indicating that endogenous pools of ribonucleoside triphosphates are negligible.

Three classes of nuclear DNA-dependent RNA polymerase have been reported to exist in a wide variety of eukaryotic cell types (reviewed in Roeder, 1976). Previous studies have shown that the three classes of eukaryotic RNA polymerase can be readily distinguished by their distinct sensitivities to the fungal toxin α-amanitin (Weinmann and Roeder, 1974). Furthermore, class II and III enzymes, which are inhibited at low and

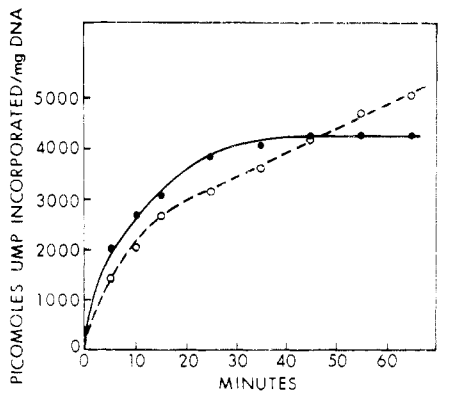

FIGURE 3: Kinetics of RNA synthesis in isolated nuclei from B1 cells. Isolated nuclei were incubated in vitro for the indicated time, and total RNA synthesized was determined as described under Materials and Methods. The level of synthesis per mg of nuclear DNA was calculated using the determined value of 1.14 mg of DNA/10^8 nuclei: (●-●) total RNA synthesis in the presence of 0.03 M $(NH_4)_2SO_4$; (O-O) total RNA synthesis in the presence of 0.20 M $(NH_4)_2SO_4$.

high concentrations of the toxin, respectively, are inhibited equally well whether free in solution or bound to template in transcription complexes (Weinmann and Roeder, 1974). Thus, the three RNA polymerase activities may be distinguished in isolated nuclei by their differential α-amanitin sensitivities.

The α-amanitin sensitivity of the endogenous RNA polymerase activity in isolated nuclei from cell line B1 is shown in Figure 1. Under these reaction conditions, 80% of the total activity is inhibited by α-amanitin in a fashion identical to that of purified RNA polymerase II. About 10% of the activity is inhibited by high concentrations of the toxin and represents synthesis of RNA polymerase III products. The remaining 10% of the activity is insensitive to α-amanitin and represents RNA polymerase I activity. Thus, isolated nuclei from adenovirus 2 transformed rodent cells contain the three classes of eukaryotic RNA polymerase, and their respective α-amanitin sensitivities appear to be identical to those of the corresponding enzymes in other mammalian cells (Roeder, 1976).

Since adenovirus 2 transformed cells contain only a few viral gene copies per cell, the level of viral RNA synthesis is expected to be quite low. Therefore, we determined optimal conditions for total RNA synthesis in isolated nuclei before attempting to detect the synthesis of viral RNA. Among the parameters tested were the effects of ionic strength. The endogenous RNA polymerase activity in isolated nuclei from B1 cells exhibits two salt optima which occur at 0.03 and about 0.2 M ammonium sulfate (Figure 2). At the low salt optimum, RNA polymerase II represents 80% of the total activity, while it accounts for 90% of the activity at the high salt optimum. At the high salt optimum, RNA synthesis proceeds beyond 60 min of incubation (Figure 3). However, continued synthesis at this ionic strength probably reflects disruption of the native chromosome structure (the nuclei appear to be lysed), and we have chosen to work exclusively at the low salt optimum in subsequent studies. At 0.03 M ammonium sulfate, total RNA synthesis reaches a plateau value after 35–40 min of incubation at 30 °C (Figure 3). The ultimate levels of synthesis, about 4000 pmol of UMP incorporated per mg of nuclear DNA, are about the same when reactions are performed at 26 °C but slightly lower if conducted at 37 °C (data not shown). The incorporation of isotope is linear over a concentration range of nuclear DNA from 0.2 to 0.5 mg/mL. The plateau values of UMP incorporation observed after 40 min of incubation at the low salt optimum could reflect either a lack of initiation

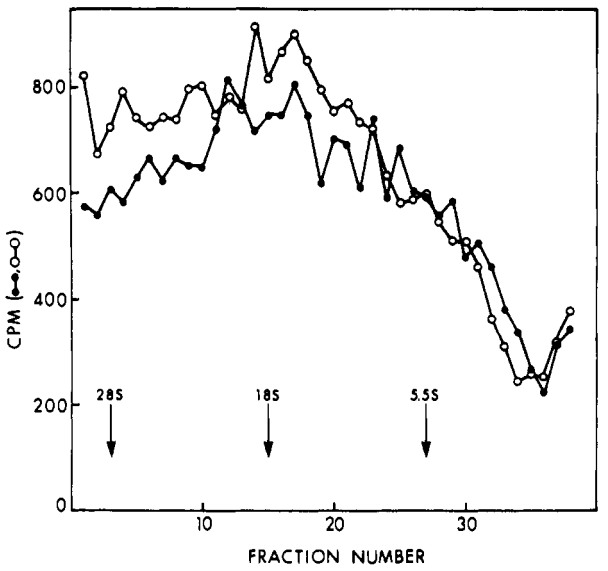

FIGURE 4: Stability of in vitro synthesized RNA. Isolated nuclei from B1 cells were incubated in vitro in a final reaction volume of 0.5 mL. After 20 min of synthesis, unlabeled UTP was added to a final concentration of 10 mM in both reactions: (●-●) DNase I was added to 100 μg/mL simultaneously with the UTP; after incubation for another 5 min, 0.5 mL of $NaDodSO_4$ buffer was added and the RNA extracted; (O-O) incubation was continued for another 30 min after the addition of UTP before DNase treatment. RNA was purified from both reactions as described under Materials and Methods with omission of the gel-filtration step. RNA samples were layered over 4-mL gradients of 10–30% sucrose in 10 mM Tris-HCl, pH 7.9 (22 °C), 100 mM NaCl, 1 mM EDTA, 0.5% $NaDodSO_4$ and centrifuged in an SW56 rotor at 55 000 rpm for 170 min at 18 °C. Gradients were collected from the bottom of the tube directly onto DEAE paper disks, and radioactivity in RNA was determined as described under Materials and Methods. 28S, 18S, and 5.5S RNAs were centrifuged in a separate gradient as markers. Slightly different amounts of total radioactivity in the control RNA and chase RNA gradients were due to unequal recoveries of the two RNA samples during purification.

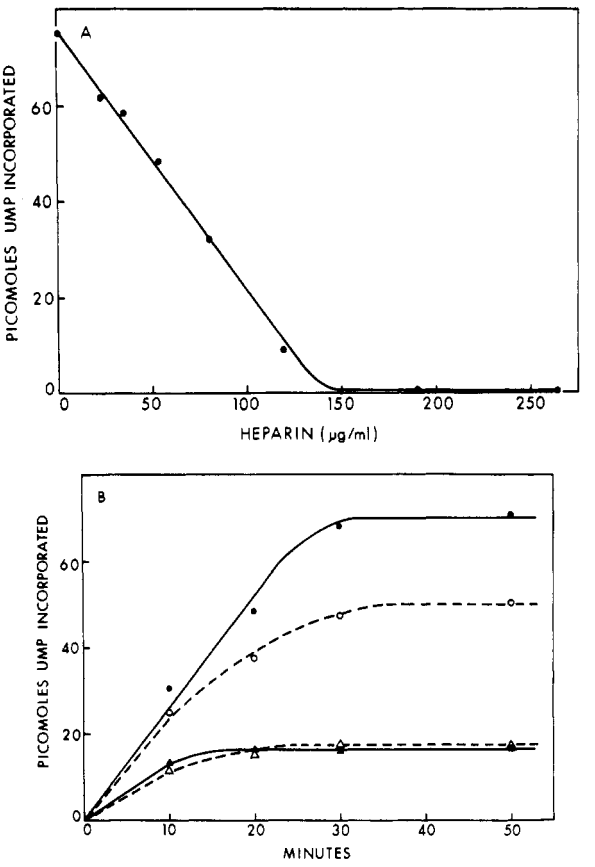

FIGURE 5: Effects of heparin on initiation of transcription by MOPC-315 RNA polymerase II. Assays were performed in the presence of 0.07 M $(NH_4)_2SO_4$ as described under Materials and Methods. (A) The enzyme was preincubated for 8 min at 37 °C with varying concentrations of heparin in the standard reaction mixture without any template. Reactions were started by the addition of calf thymus DNA to 120 μg/mL. (B) The enzyme was preincubated for 10 min at 37 °C in the standard reaction mixture without any UTP. Reactions were started by the addition of a solution containing UTP and the appropriate amount of heparin and allowed to proceed for the indicated length of time: (●-●) control reaction; (O-O) 35 μg/mL heparin; (▲-▲) 150 μg/mL heparin; (△-△) 300 μg/mL heparin.

of RNA chains or a steady-state level of synthesis and degradation. Accordingly, these possibilities were investigated.

When RNA was synthesized in vitro for various time intervals followed by the addition of unlabeled UTP to 10 mM (resulting in a 200-fold dilution of the [^{3}H]UTP), no further incorporation of isotope was observed and the [^{3}H]RNA already synthesized, measured as described under Materials and Methods, was stable for more than 30 min (data not shown). This type of analysis, however, is not expected to be sensitive to a small amount of endoribonuclease activity. Consequently, the RNA synthesized in vitro was subjected to sucrose-$NaDodSO_4$ sedimentation velocity analysis (Figure 4). RNA synthesized under the conditions described under Materials and Methods ranges in size from 4 to greater than 28 S with a modal distribution around 18 S. When RNA is synthesized under identical conditions followed by a 30-min chase with unlabeled UTP, the size distribution of the in vitro synthesized RNA is essentially identical to the RNA synthesized in the control reaction. These results indicate that there is little or no nuclease activity in the in vitro system.

Initiation of RNA chains has been reported to occur in isolated nuclei (Busiello and DiGirolamo, 1975; Marzluff et al., 1974). However, the conclusions of these studies were based on the incorporation of [γ-^{32}P]ribonucleoside triphosphates as an assay for initiation and may be complicated by the presence of phosphatases and kinases in nuclei (Winicov, 1977). In the present studies, which have focused on the major (i.e., RNA polymerase II) nuclear products, we have tested for initiation in isolated nuclei using heparin, a known inhibitor of RNA chain initiation (Zillig et al., 1976). Partially purified RNA polymerase II was first used to determine the minimal amount of heparin necessary to inhibit initiation (Figure 5A). When the RNA polymerase is preincubated with increasing concentrations of heparin and the reaction started by addition of the DNA template, a heparin concentration of 150 μg/mL is necessary to completely inhibit initiation of synthesis by the free enzyme. This result is corroborated by the experiment depicted in Figure 5B. The RNA polymerase was allowed to form preinitiated complexes with DNA by incubating the enzyme and DNA in the presence of only three ribonucleoside triphosphates. The reaction was started by the addition of a solution containing heparin and the fourth nucleoside triphosphate. When the kinetics are examined in the presence of heparin, the plateau level of synthesis is less than in the control reaction. The extent of synthesis is the same in the presence of 150 or 300 μg/mL heparin, indicating that 150 μg/mL is, in fact, a concentration sufficient to completely inhibit initiation. The observed synthesis at these concentrations probably reflects synthesis initiated at "heparin-resistant complexes" (Zillig et al., 1970) formed during the preincubation. At a heparin concentration (40 μg/mL) which is insufficient to completely inhibit initiation by the unbound RNA polymerase (cf. Figure 5A), an intermediate level of synthesis is observed,

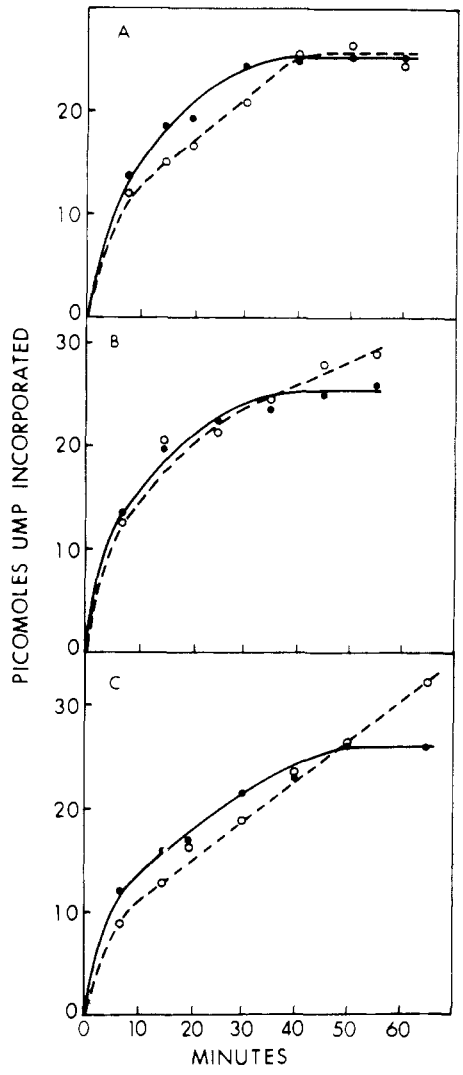

FIGURE 6: Effects of heparin on RNA synthesis in isolated nuclei from B1 cells. Nuclei were isolated and incubated under standard conditions either in the absence or presence of 45 (A), 100 (B), or 150 μg/mL (C) heparin: (●-●) control reactions; (○-○) reactions containing heparin.

presumably due to an incomplete inhibition of reinitiation. These experiments indicate that heparin can inhibit initiation by RNA polymerase free in solution as well as the reinitiation by enzymes which were originally bound to the template.

Heparin is a nonspecific inhibitor of RNA polymerase (Zillig et al., 1976) and is expected to interact electrostatically with a wide variety of molecules. Thus, the effects of heparin on solubilized RNA polymerase II and the endogenous RNA polymerase activity in isolated nuclei are not strictly comparable, since the molecular environment is different in the two reactions. However, the specific activity of the partially purified RNA polymerase II used in these experiments (0.75 unit/μg of protein) is similar to that calculated (Schwartz and Roeder, 1975) for the RNA polymerase II present in the nuclear reactions (0.63 unit/μg of protein). Moreover, the total protein concentration is tenfold higher in the reactions with solubilized RNA polymerase II (Figure 5A,B) than in the experiments (described below) with the endogenous nuclear RNA polymerases. Thus, a concentration of less than 150 μg/mL heparin may in fact be sufficient to inhibit any initiation in isolated nuclei if it does occur. The kinetics of RNA synthesis in isolated nuclei were thus examined in the presence of different concentrations of heparin. At the lowest concentration tested (45 μg/mL), the plateau value of UMP incorporation is the same as the control reaction without heparin (Figure 6A). At higher heparin concentrations (100 and 150 μg/mL; Figure 6B,C), there is again no decrease in the final levels of synthesis but rather a slight stimulation of synthesis. This is correlated with the apparent disruption of nuclei at these heparin concentrations and is similar to the effects observed at high ammonium sulfate concentrations (cf. Figure 3). Thus, the total RNA synthesis (predominantly RNA polymerase II activity, see above) is insensitive to inhibition by heparin. These results suggest, but do not prove, the absence of any significant level of RNA chain initiation in the in vitro system.

Transcription of Integrated Viral Genes. The viral DNA sequences present in cell lines which contain only the left 14% of the adenovirus genome must code for viral functions which are required to maintain the transformed state. It was of interest, therefore, to determine which of the three forms of RNA polymerase transcribes these genes. Experiment 1 in Table I shows the level of synthesis of viral-specific RNA in isolated nuclei from B1 cells as analyzed by filter hybridization under conditions of DNA excess. The amount of radioactive RNA hybridized to adenovirus 2 DNA is significantly more than that bound to a heterologous DNA filter (*E. coli*) or a filter containing no bound DNA, indicating the synthesis of viral RNA. The validity of the hybridization method was verified as follows. RNA was synthesized in isolated nuclei and viral-specific RNA was purified by hybridization of the in vitro product to total adenovirus 2 DNA as above. The radioactive RNA was then melted from the adenovirus DNA filter, ethanol precipitated, and rehybridized to a different set of DNA filters (Table I, experiment 3). Such experiments have resulted in 20–40% hybridization efficiencies, representing a 2000- to 4000-fold increase in efficiency over that of the first hybridization. This demonstrates that the hybridization analyses of the total in vitro product are, in fact, specific for RNA which is complementary to adenovirus DNA.

In various experiments with isolated nuclei from B1 cells, viral-specific RNA has represented from 0.010 to 0.015% of the total RNA synthesized in vitro. When isolated nuclei were incubated either in the presence or absence of 0.6 μg/mL α-amanitin, a concentration which completely and selectively inhibits RNA polymerase II (Figure 1), total RNA synthesis was inhibited by 80% (Table I, experiment 2). Synthesis of viral-specific RNA, however, was 95% inhibited. Analogous experiments with cell line F18 have also demonstrated that viral RNA synthesis is completely inhibited by this concentration of α-amanitin (data not shown). These results conclusively identify RNA polymerase II as the enzyme which transcribes the integrated viral genes in adenovirus 2 transformed cells.

It is generally assumed that transcription is asymmetric; that is, only one DNA strand of a gene is used as a template for RNA synthesis. This principle has been formulated predominantly from studies in prokaryotic systems. While the reiterated ribosomal genes of *Xenopus laevis* appear to be transcribed asymmetrically (Miller and Beatty, 1969; Reeder and Roeder, 1972) by RNA polymerase I, it has not been firmly established that transcription in eukaryotes is in fact always asymmetric. Since we were able to detect the transcription of unique gene sequences, it was of immediate interest to determine the strand selectivity of the endogenous RNA polymerase II.

The complementary strands of adenovirus 2 DNA were preparatively separated as described under Materials and Methods and immobilized on nitrocellulose filters. RNA was synthesized in vitro in isolated nuclei from B1 cells under

TABLE I: Hybridization of RNA Synthesized in B1-Isolated Nuclei to Adenovirus 2 DNA.

Expt	Source of RNA	Hybridization input (cpm)	DNA on filter[a]	cpm bound	cpm minus background	% input
1	Total in vitro product	5.5×10^6	Ad2	730	640	0.012
			E. coli	90		
			None	96		
2	Total in vitro product (control)	4.1×10^6	Ad2	509	414	0.010
			E. coli	95		
			None	64		
	Total in vitro product (0.6 μg/mL α-amanitin)	1.0×10^6	Ad2	76	24	0.002
			E. coli	52		
			None	48		
3	In vitro product purified by hybridization to Ad2 DNA[b]	459	Ad2	208	178	39
			E. coli	30		
			None	27		

[a] Filters contained either 6 μg of adenovirus 2 DNA or 2 μg of *E. coli* DNA. [b] RNA was synthesized in vitro, purified, and hybridized to Ad2 DNA as described under Materials and Methods. After determining the amount of radioactivity bound to each filter, the filter containing adenovirus DNA was rinsed several times with chloroform and dried. The filter was placed in a sterile tube and 100 μL of 10 mM Tris-HCl, pH 7.9, 2 mM EDTA was added. After heating at 90 °C for 5 min, the solution was collected and *E. coli* tRNA was added as carrier. The sample was made 0.2 M NaCl, 1% $NaDodSO_4$, and the RNA was ethanol precipitated before rehybridization.

TABLE II: Hybridization of RNA Synthesized in Transformed Cell Isolated Nuclei to Separated Strands of Adenovirus 2 DNA.

Expt	Cell line	Source of RNA	Hybridization input (cpm)	DNA on filter[a]	cpm bound	cpm minus background	% input	*r/l*
1	B1	Total in vitro product	3.1×10^6	Ad2 *r* strand	406	313	0.010	2.8
				Ad2 *l* strand	206	113	0.004	
				E. coli	93			
				None	84			
2	B1	Total in vitro product	3.5×10^6	Ad2 *r* strand	414	353	0.010	2.8
				Ad2 *l* strand	186	125	0.004	
				Ad2 Sal A fragment	61			
				None	50			
3	B1	In vitro product purified by hybridization to total Ad2 DNA	974	Ad2 *r* strand	179	147	15	3.6
				Ad2 *l* strand	73	41	4	
				E. coli	32			
				None	33			
4	F18	Total in vitro product	3.4×10^6	Ad2 *r* strand	163	117	0.003	3.2
				Ad2 *l* strand	83	37	0.001	
				Ad2 Sal A fragment	46			
				None	42			
Control		Ad2-infected cell 5.5S RNA	5.8×10^4	Ad2 *r* strand	206	193		24.1
				Ad2 *l* strand	21	8		
				None	13			

[a] Reactions contained filters with 1.5 μg of each strand of Ad2 DNA and either 2 μg of *E. coli* DNA or 0.35 μg of *Streptomyces albus* restriction fragment A of Ad2 DNA. The control reaction contained 0.8 μg of each strand immobilized on filters.

standard reaction conditions and then hybridized to the separated strands. Table II shows two representative experiments (experiments 1 and 2). A nonspecific background value was determined using *E. coli* DNA in the first experiment and *Streptomyces albus* restriction fragment A of the adenovirus 2 genome in the second experiment. This fragment encompasses the right 54% of the viral genome[3] and is thus not present in B1 cells (Gallimore et al., 1974). In both experiments, it is clear that the endogenous RNA polymerase II transcribes both the *r* and *l* strands of the viral DNA in a ratio of about three to one. As a control, viral 5.5S RNA, which is known to be complementary to the *r* strand (Pettersson and Philipson, 1975), was isolated from productively infected human KB cells and hybridized to the separated strands (Table II). This RNA hybridizes specifically to the *r* strand with an asymmetry ratio of 24, demonstrating that the hybridization of B1 nuclear transcripts to the *l* strand cannot be due to cross-contamination of the strands.

To unequivocally demonstrate that the radioactivity bound to the adenovirus *l* strand filter represented *l* strand transcripts, a rehybridization experiment was performed. RNA was syn-

[3] J. R. Arrand, P. A. Myers, and R. J. Roberts, unpublished observations.

thesized in vitro and viral-specific RNA was purified by hybridization to total adenovirus DNA as described in the footnote to Table I. This RNA was then rehybridized to the separated strands of viral DNA (Table II, experiment 3). Once again, the RNA purified by hybridization rehybridized with a much higher (2000-fold) efficiency, indicating that the hybridized counts are, in fact, viral gene transcripts. Furthermore, this purified viral RNA hybridized to both strands of the viral DNA. Thus, although the major viral transcripts in B1 cells are derived from the *r* strand, significant *l* strand transcription also occurs.

Cell line B1 contains six copies of only the left 14% of the adenovirus 2 genome per cell (Gallimore et al., 1974). Cell lines F18 and F17, which contain 2.9 and 3.5 copies, respectively, of the same viral DNA sequences (Gallimore et al., 1974), have been shown to contain cytoplasmic viral RNA complementary only to the *r* strand of the viral genome (Flint et al., 1975). Since nuclei from line B1 have been shown (above) to synthesize RNA from both strands of the viral DNA, the transcriptional pattern in these latter lines was also examined. Nuclei were isolated from cloned line F18 and incubated under the same conditions used for B1 isolated nuclei. The RNA synthesized in vitro was then hybridized to the separated strands of adenovirus 2 DNA (Table II, experiment 4). While the percentage of total RNA synthesis which is viral specific is only about 50% of that in B1 cells (perhaps reflecting a gene dosage effect), it appears that both strands of the integrated viral genes are also transcribed. Analogous results were also obtained with nuclei isolated from line F17 (data not shown). Thus, in these adenovirus 2 transformed cell lines which contain stable cytoplasmic viral RNA complementary only to the *r* strand, both strands of the integrated viral DNA are transcribed by RNA polymerase II within the nucleus.

Discussion

This study describes an in vitro system in which the transcription of specific genes, present in only a few copies per cell, has been analyzed. Since the total RNA synthesized in isolated nuclei is stable for an extended time after synthesis, the observed plateau values of UMP incorporation cannot be due to a steady-state level of synthesis and degradation but reflect a true cessation of synthesis. In addition, the total RNA synthesis is not inhibited by concentrations of heparin which completely inhibit initiation by solubilized RNA polymerase II. The heparin sensitivities of the solubilized and the endogenous RNA polymerase II activities may not be identical. However, all in vitro transcription initiation events thus far studied, including those in prokaryotic systems, have been shown to be sensitive to heparin. It is not unreasonable to expect the same to be true for the nuclear transcription events, especially at the high concentrations of heparin used. Hence, the results indicate an apparent lack of initiation. The mild conditions used for nuclei isolation and the low ionic strength reaction conditions make artifactual elongation of the nascent RNA chains due to chromosome disruption an unlikely possibility. In the case of the integrated viral genes, aberrant transcription also seems improbable, since the viral-specific RNA synthesized in vitro accounts for less than 0.5 complete transcript per gene. Thus, the RNA synthesis observed appears to reflect the continuation of transcription events initiated in the living cell and may be used as a probe to determine the transcriptional pattern of specific genes.

One major conclusion of this study is that the integrated viral genes in adenovirus 2 transformed cells are transcribed by RNA polymerase II, a result predicted from previous studies of the host RNA polymerases (introductory statement) but not heretofore demonstrated for viral genes in cells transformed by DNA tumor viruses. A second important observation is that both strands of the integrated viral genes are transcribed, whereas the stable cytoplasmic viral RNAs have been shown to be exclusively synthesized from one (the *r*) strand. Since it is not presently known whether the nuclear *r* and *l* strand viral transcripts are complementary (symmetrical transcription) or whether they are synthesized from nonoverlapping sequences of DNA, we refer to this type of transcription as bistranded transcription. In either case, this observation implies the existence of processing and/or transport mechanisms which selectively stabilize viral *r* strand transcripts in the cytoplasm. Moreover, these findings emphasize a new aspect of RNA processing in eukaryotic cells other than the intramolecular processing which has been well documented for the ribosomal RNAs as well as some messenger RNAs (reviewed in Perry, 1976). Thus, they indicate that RNA polymerase II may synthesize molecules (in this case, viral *l* strand transcripts), no sequences of which apparently reach the cytoplasm. Such transcripts could account for some of the nucleus-specific viral RNA sequences reported in adenovirus 12 transformed cells (Green et al., 1976) as well as a fraction of cellular heterogeneous nuclear RNA, most of which appears to never leave the nucleus (Brandhorst and McConkey, 1974).

The general applicability of these findings may be limited by the fact that the genes examined are viral or that they have been integrated into the host chromosome. Thus, there may be RNA polymerase II promoters on both strands of the integrated viral DNA sequences reflecting a characteristic of adenovirus gene expression but having little relevance to cellular mRNA biogenesis. Alternatively, the viral genes could be integrated within transcriptionally active host sequences in different orientations, such that one or both strands could be transcribed as a result of initiation events on adjacent (upstream) host promoters. This possibility is compatible with the fact that the three cell lines examined in this study all contain multiple copies of the adenovirus DNA sequences. However, regardless of the mechanism responsible for bistranded transcription events, the transformed cell has a processing mechanism which allows the accumulation of only *r* strand transcripts in the cytoplasm. As yet, however, there is relatively little comparable information on the transcription of specific cellular genes which encode messenger RNAs. Wilson et al. (1975) reported that transcription of the globin genes in rabbit marrow cells was asymmetric, since anti-sense strand transcripts were not detected in cellular, nuclear, or chromatin-associated steady-state RNA populations. However, their probes would not have detected transcripts from DNA regions contiguous to sequences found in mRNA nor would transcripts with a very short half-life in vivo been detected with the methodology employed (cf. Curtis and Weissmann, 1976). Such difficulties have been avoided in the present system. The adenovirus DNA used as hybridization probe clearly contains all the viral gene sequences present in the transformed cells. In addition, nascent nuclear transcripts, apparently stable in the nuclear transcription system, have been monitored under DNA-excess hybridization conditions.

As described in this paper, in vitro RNA synthetic systems utilizing isolated nuclei can be useful for defining the in vivo mode of transcription. RNA chains initiated in vivo can be labeled via the endogenous RNA polymerases to a high specific activity in vitro, and RNA processing, which may alter the products of the primary transcriptive event, appears to be minimal. The further refinement of these systems to include correct initiation as well as elongation events will allow further

investigations of the mechanism and regulation of gene transcription. Studies of this sort may necessitate utilizing purified homologous RNA polymerases and isolated nucleoprotein templates (cf. Parker and Roeder, 1977; Sklar and Roeder, 1977; Jaehning and Roeder, 1977). Such analyses of the integrated viral genes in adenovirus 2 transformed cells are now in progress.

Note Added in Proof

After this manuscript was submitted for publication, Yanagi et al. (1977) reported the presence of nuclear transcripts complementary to both strands of integrated viral DNA sequences in an SV-40 transformed cell line which accumulates completely asymmetric cytoplasmic viral RNA.

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