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# Transcription of viral genes in chromatin from adenovirus 2 transformed cells by exogenous eukaryotic RNA polymerases

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## ABSTRACT

The transcription of chromatin from adenovirus 2 transformed rat cells by murine plasmacytoma RNA polymerases I, II and III has been studied. Both the total RNA synthesis and transcription of the integrated adenovirus 2 genes by RNA polymerase II represent de novo DNA transcription as assessed by their sensitivity to actinomycin D. It is shown that each RNA polymerase class has characteristic ionic strength activation profiles and metal ion requirements. RNA polymerase II transcribes the integrated adenovirus 2 genes in chromatin at a frequency 25- to 50-fold higher than their sequences are represented in the genome. In contrast, no detectable viral RNA is synthesized when deproteinized DNA is transcribed. In the presence of $Mn^{2+}$, all three RNA polymerases (I,II and III) transcribe the integrated viral genes at approximately the same relative frequency. However, with $Mg^{2+}$ as divalent cation, the proportion of the total RNA which represents viral gene transcripts is increased 3- to 4-fold with RNA polymerase II, while it remains unchanged for RNA polymerases I or III.

## INTRODUCTION

An elucidation of the mechanisms which regulate transcription in eukaryotic cells will undoubtedly require the development of soluble in vitro systems in which macromolecular components can be individually analyzed and regulated. Most previous studies have analyzed either the endogenous RNA polymerase activity in isolated nuclei or the transcription of chromatin by bacterial RNA polymerases (reviewed in 25). While isolated nuclei are useful for identifying the RNA polymerase class which transcribes a given gene as well as determining characteristics of the primary transcript, there is an apparent lack of initiation of RNA chains by RNA polymerase II in these systems (5,14). The regulation of initiation events can be studied, however, using purified RNA polymerases and isolated nucleoprotein templates. It has, in fact, been reported that E. coli RNA polymerase transcribes chromatin, but not deproteinized DNA,

in a manner which reflects the tissue-specific gene expression of the cells from which the chromatin was isolated (3,4,17,33,35,43,44). These findings indicate that chromosomal proteins are involved in the restriction of DNA sequences available for transcription and that E. coli RNA polymerase may recognize and preferentially transcribe such regions of chromatin DNA in vitro. However, no evidence has been presented that the bacterial enzyme correctly initiates and terminates transcription of the given genes. Furthermore, these findings may be complicated by the recent demonstration that E. coli RNA polymerase can utilize endogenous RNA as a template (42,13,21) and possibly as a primer (29).

The existence of three forms of RNA polymerase in eukaryotic cells, and the demonstration that specific genes are transcribed by a given enzyme class (reviewed in 25), indicate that the successful reproduction in vitro of specific transcription events may require the presence of the homologous RNA polymerase. Indeed, it has recently been shown in several systems that only a class III RNA polymerase will selectively and accurately (with correct initiation and termination) transcribe genes which are transcribed by RNA polymerase III in vivo (23,32,41,20). Such studies have not yet, however, been successfully extended to genes which are transcribed by RNA polymerase II in vivo. Thus, the globin (34) and the ovalbumin genes (38) in isolated chromatin were reported to be transcribed somewhat more efficiently by RNA polymerase II than by E. coli RNA polymerase. However, the differences were not striking, in agreement with the results of similar studies of the transcription of avian myeloblastosis DNA sequences in chick myeloblast chromatin (19). Such comparisons of the efficiency of transcription of specific genes by the three eukaryotic RNA polymerases, however, have not yet been reported. These enzymes might be expected to exhibit more discrimination in the transcription of these genes in vitro and hence provide additional information relevant to their regulation in vivo.

As a model system for studying the transcription of nonreiterated chromosomal genes, we have used cloned adenovirus 2 transformed cells which contain several (three to six) integrated copies of only the left 14% of the viral genome (11). By monitoring the endogenous RNA polymerase activity in isolated nuclei, it has been demonstrated that a class II RNA polymerase transcribes the viral genes (5). In view of the apparent necessity of utilizing homologous RNA polymerases in reconstructed systems (above), the present report establishes general characteristics

of chromatin transcription as well as the catalytic properties of murine plasmacytoma RNA polymerases I, II and III with chromatin templates. In addition, the frequency of transcription of the integrated adenovirus genes by each enzyme class has been determined under various reaction conditions.

## MATERIALS AND METHODS

Biochemicals, Cell Culture and Virus Purification. All biochemicals were purchased from sources reported previously (5), except NP-40 which was obtained from BDH Chemicals, Ltd. Adenovirus 2 transformed cells (clone A2/HLREB/10P/B1) were cultured as described (5). Adenovirus serotype 2 (Ad 2)[2] was purified from infected cultures of human KB cells (10) and DNA was purified from virions according to the method of Green and Pina (16).

Preparation of Chromatin and DNA. Nuclei were prepared from subconfluent cultures of B1 cells as described (5) except that 0.5% NP-40 was included in the last wash with hypotonic buffer. Chromatin was prepared by a modification of the procedure of Simpson and Sober (30). Purified nuclei were resuspended (at 1.1 x $10^7$ nuclei/mL) in 50 mM Tris-HCl, pH 7.9 (4°C), 50 mM NaCl, 10 mM EDTA, 0.5 mM DTE and centrifuged at 3,000 g for 20 minutes. The pellet was resuspended in the same volume of 50 mM Tris-HCl, pH 7.9, 1 mM EDTA, 0.5 mM DTE by homogenization in a glass/teflon homogenizer and centrifuged as above. This procedure was next repeated with 25 mM Tris-HCl, pH 7.9, 1 mM EDTA, 0.5 mM DTE and subsequently with 10 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 0.5 mM DTE. The purified chromatin gel was resuspended by gentle homogenization in 10 mM Tris-HCl, pH 7.9 (4°C), 10% glycerol, 0.1 mM EDTA, 0.5 mM DTE and used immediately in transcription reactions. Chromatin purified in this manner had a protein to DNA ratio of 2.1 (DNA was assayed for according to Burton (6) and protein determined by the method of Lowry et al. (22) using bovine serum albumin as a standard).

To prepare DNA, isolated nuclei were made 0.5% $NaDodSO_4$ and extracted with phenol:chloroform (1:1) and then twice with chloroform:isoamyl alcohol (24:1). The DNA was ethanol precipitated, treated with pancreatic RNase A (heat treated to inactivate contaminating DNases) and proteinase K, extracted as above, ethanol precipitated and stored in 0.1 x SSC.

RNA Polymerase Purification. MOPC 315 tumors were propagated as

described (28). RNA polymerase activity was solubilized from whole cell homogenates and assayed according to the procedure of Sklar and Roeder (31). Typically, the F4 fraction from 120 g tissue was diluted to 0.05 M $(NH_4)_2SO_4$ with TGED (50 mM Tris, pH 7.9 (22°C), 25% glycerol, 0.1 mM EDTA, 0.5 mM DTE) and loaded (780 mL/hr) onto a 650 mL column of DEAE-cellulose (Whatman DE52). The column was washed (780 mL/hr) with 0.05 TGAED (TGED which contains 0.05 M $(NH_4)_2SO_4$) until the absorbance at 280 nm of undiluted eluant was 0.05 or less. RNA polymerase activity was eluted with a 3,000 mL linear gradient of 0.05 to 0.50 TGAED at a flow rate of 780 mL/hr. RNA polymerases I and III co-elute at 0.09 M $(NH_4)_2SO_4$ and RNA polymerase II elutes at 0.20 M $(NH_4)_2SO_4$. RNA polymerases I and III were separated by chromatography on a 650 ml column of DEAE-Sephadex (Pharmacia, A-25). Conditions of loading and gradient elution were essentially the same as for DEAE-cellulose chromatography.

The peak fractions of RNA polymerase II activity from DEAE-cellulose chromatography were pooled and diluted with TGED to 0.10 TGAED and loaded (240 mL/hr) onto a 170 mL column of DEAE-Sephadex. The column was washed (200 mL/hr) with 0.10 TGAED as above and RNA polymerase activity eluted with a 600 mL linear gradient of 0.05 to 0.50 TGAED (200 mL/hr). RNA polymerase II activity, which was effectively separated from any contaminating RNA polymerase I and III, was pooled, BSA (Pentex) added to 0.5 mg/mL, and dialyzed against 20-30 volumes of TGED until the ammonium sulate concentration decreased to 0.05 M. The enzyme was then loaded (70 mL/hr) onto a 10 mL column of phosphocellulose (Whatman, P-11) and washed with 40 mL of 0.05 TGAED, 0.5 mg/mL BSA. RNA polymerase activity was eluted with a 30 mL linear gradient of 0.05 to 0.50 TGAED, 0.5 mg/mL BSA (15 mL/hr). Fractions containing RNA polymerase II activity were stored at -70°C. RNA polymerase II purified in this manner was judged to be 90% pure by polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$.

The RNA polymerase I fractions from DEAE-Sephadex chromatography (above) were pooled, made 0.5 mg/mL BSA and dialyzed against TGED until the ammonium sulfate concentration was 0.05 M. The enzyme was loaded (150 mL/hr) onto a 170 mL column of CM-Sephadex (Pharmacia, C-25). After washing with two column volumes of 0.05 TGAED, 0.5 mg/mL BSA, the enzyme activity was eluted with a 600 mL linear gradient of 0.05 to 0.50 TGAED, 0.5 mg/mL BSA (170 mL/hr). Fractions containing RNA polymerase I activity were pooled, diluted with TGED, 0.5 mg/mL BSA to 0.07 M $(NH_4)_2SO_4$

and loaded (100 mL/hr) onto a 12 mL column of phosphocellulose. After washing with 40 mL of 0.05 TGAED, 0.5 mg/mL BSA, the enzyme was eluted with a 30 mL linear gradient of 0.05 to 0.50 TGAED, 0.5 mg/mL BSA and fractions containing RNA polymerase I activity were stored at -70°C. RNA polymerase I prepared in this manner was free of any contaminating II or III activity and judged to be 40% pure by polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$.

The RNA polymerase III fractions from DEAE-Sephadex chromatography (above) were pooled, made 0.5 mg/mL BSA and dialyzed to 0.05 TGAED. The enzyme was further purified by chromatography on CM-Sephadex and phosphocellulose under conditions essentially identical to those used for RNA polymerase I. The RNA polymerase III used in these studies was free of any contaminating I or II activity and judged to be 30% pure by polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$.

One unit of activity is defined as the amount of RNA polymerase which will incorporate one picomole of UMP in 20 minutes at 37°C using calf thymus DNA as template (120 µg/mL). The specific activities of pure RNA polymerases are: 380 units/µg of protein for RNA polymerase I (26), 426 units/µg of protein for RNA polymerase II (27) and 265 units/µg of protein for RNA polymerase III (31).

In vitro Synthesis and Purification of RNA. Standard reactions contained, in a volume of 50 µL, 1.5 µg of chromatin DNA and the appropriate amount of RNA polymerase. Reaction conditions were 65 mM Tris-HCl, pH 7.9 (22°C), 7-9% glycerol, 0.6 mM each of GTP, CTP and ATP, 0.05 mM UTP, 40 µCi/mL [$^3$H]UTP (40-50 Ci/mmole), 0.05 mM EDTA and 0.1 mM DTE. Unless otherwise indicated, reactions contained 2 mM $MnCl_2$ and the optimal ammonium sulfate concentration for each respective enzyme and were incubated for 30 minutes at 30°C. Reactions were terminated by the addition of an equal volume of $NaDodSO_4$ buffer (25 mM Tris-HCl, pH 7.9 (22°C), 200 mM NaCl, 10 mM EDTA, 2% $NaDodSO_4$, an aliquot spotted on a DEAE paper disk and radioactivity in RNA determined as described (24).

When the RNA synthesized in vitro was to be hybridized to adenovirus 2 DNA, reaction volumes were 100 µL (if larger amounts of RNA were required, multiple 100 µL reactions were performed), unlabelled UTP was omitted, [$^3$H]UTP was present at 0.045-0.050 mM, and reactions were incubated for 40 to 60 minutes. The concentration of chromatin DNA was increased 7 to 8-fold with an appropriate increase in the amount of RNA polymerase. Control experiments showed that the incorporation of $^3$H-UMP

was linear through this range of DNA concentrations when the chromatin was transcribed at constant enzyme:DNA ratios (data not shown). Reactions were terminated by the addition of unlabelled UTP to 5 mM and DNase I (RNase free) to 100 µg/mL followed by an additional 5 minute incubation at 30°C. An equal volume of $NaDodSO_4$ buffer was added and the RNA purified by phenol:chloroform extraction, a second DNase I treatment and Sephadex G-50 chromatography as described previously (5). Viral specific RNA was quantitated by filter hybridization under conditions of DNA excess as described (5). Background values were determined as the average of CPM bound to two filters containing no DNA.

## RESULTS

Characteristics of Chromatin Transcription *in vitro*. Figure 1 depicts the kinetics of chromatin transcription in response to exogenous RNA polymerases I and II at saturating enzyme to DNA ratios (see below). The endogenous RNA polymerases in chromatin incorporate 0.6 picomole UMP per µg of DNA. This is about 20% of the activity which is associated with nuclei isolated from these cells (5). When the chromatin is transcribed *in vitro* with RNA polymerase I (Figure 1A), synthesis continues for 40 minutes at 30°C at which point 0.15 µg RNA has been synthesized per µg of DNA. This represents a 200-fold stimulation over the endogenous RNA polymerase activity. The rate of chromatin transcription is slower for RNA polymerase II (Figure 1B). However, synthesis is still approximately linear at 90 minutes of incubation and at this point represents 0.07 µg RNA synthesized per µg of DNA. This reflects a 100-fold increase over the endogenous activity. The *in vitro* synthesis of RNA is completely inhibited by Actinomycin D (see below).

Catalytic Properties of Eukaryotic RNA Polymerases with Chromatin Templates. It has previously been shown (reviewed in 25) that the three forms of eukaryotic RNA polymerase have characteristic salt and metal ion requirements. However, these were determined using deproteinized DNA under conditions of vast template excess. Since we are interested in the functional transcription of specific genes in chromatin, these properties were determined for each enzyme using chromatin as a template.

Figure 2 illustrates the effects of ionic strength on chromatin transcription by exogenous RNA polymerases at saturating enzyme to DNA ratios. RNA polymerase I exhibits an ammonium sulfate optimum of 40 mM (Figure 2A), and the same salt optimum is observed when deproteinized

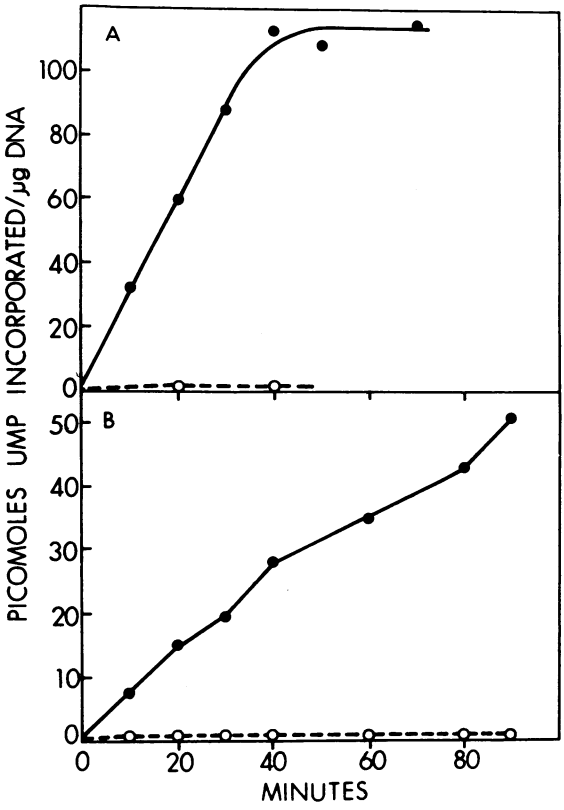


Fig. 1. Kinetics of chromatin transcription by exogenous RNA polymerases. Chromatin was isolated and transcribed with (●–●) or without (0–0) exogenous RNA polymerase as described under Materials and Methods. Reactions with exogenous RNA polymerases contained either 440 units of RNA polymerase I (A) or 500 units of RNA polymerase II (B) per µg of chromatin DNA.

DNA is transcribed. RNA polymerase II has an optimal ammonium sulfate activation of 75 mM for chromatin transcription and optimal activity occurs at 45 mM for DNA transcription (Figure 2B). However, if the enzyme to DNA mass ratio in the reactions is decreased 100-fold, the optimal concentration for deproteinized DNA transcription is 95 mM $(NH_4)_2SO_4$ (data not shown) as reported earlier (28). Such differences in salt optima at various RNA polymerase II to (deproteinized) DNA ratios have been previously reported (15). RNA polymerase III exhibits a biphasic ammonium sulfate activation profile for chromatin transcription

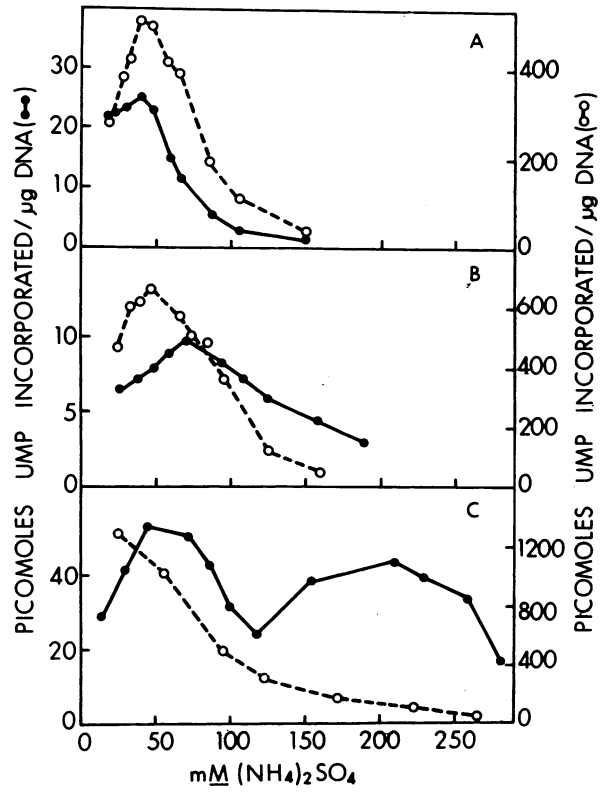


Fig. 2. Effects of ionic strength on chromatin and DNA transcription by exogenous RNA polymerases. Reactions were performed in the presence of various concentrations of ammonium sulfate as indicated. Chromatin was transcribed (●-●) with 380 units of RNA polymerase I (A), 265 units of RNA polymerase II (B) or 275 units of RNA polymerase III (C) per µg of chromatin DNA. The endogenous RNA polymerase activity in isolated chromatin was less than 0.6 picomole UMP per µg DNA. Deproteinized DNA was transcribed (○-○) with 2810 units RNA polymerase I (A), 3450 units RNA polymerase II (B) or 4400 units RNA polymerase III (C) per µg of DNA.

(Figure 2C) with optima occurring at 45 and 210 mM. This profile is similar to that previously reported for RNA polymerase III in the presence of excess calf thymus DNA (28). However, when deproteinized DNA is transcribed at saturating enzyme to DNA ratios (Figure 2C), a single optimum at 25 mM $(NH_4)_2SO_4$ (or less) is observed. Thus, each of the three eukaryotic RNA polymerases have characteristic ionic strength requirements for optimal transcription of chromatin. For RNA polymerases

II and III, these optima are different than those observed for the transcription of deproteinized DNA under similar conditions (saturating enzyme to DNA ratios).

The effects of metal ion concentrations on chromatin transcription by RNA polymerases I, II and III are depicted in Figure 3. All three enzymes exhibit well defined optima at 1-2 mM $MnCl_2$ and broad $MgCl_2$ activation profiles with optima occurring at about 3 to 6 mM. The enzymes each exhibit characteristic optimal $Mn^{2+}$:$Mg^{2+}$ activity ratios. These are 3, 11 and 2.5 for RNA polymerases I, II and III, respectively.

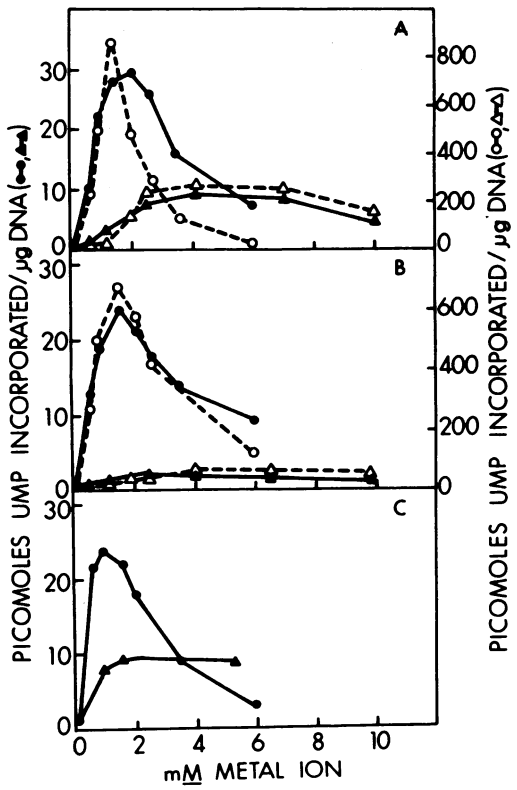


Fig. 3. Effects of metal ion concentrations on chromatin and DNA transcription by exogenous RNA polymerases. Chromatin (solid symbols) or DNA (open symbols) was transcribed in the presence of varying concentrations of $MnCl_2$ (●,○) or $MgCl_2$ (▲,△) as indicated. Chromatin was transcribed with 225 units of RNA polymerase I (A), 204 units of RNA polymerase II (B), or 171 units of RNA polymerase III (C) per µg of chromatin DNA. Deproteinized DNA was transcribed with 7600 units RNA polymerase I (A) or 3560 units RNA polymerase II (B) per µg of DNA.

As shown for RNA polymerases I and II (Figure 3A,B), the metal ion optima are qualitatively similar for chromatin and DNA transcription.

It has previously been shown that E. coli RNA polymerase (8,37) and calf thymus RNA polymerase II (7) have fewer initiation sites in chromatin than in deproteinized DNA. It also appears from these results that chromatin supports fewer initiation events by RNA polymerase II than by E. coli RNA polymerase. As an important aspect of the analysis of chromatin transcription by homologous RNA polymerases (Introduction), we have compared the saturation of sequences available for transcription in chromatin and DNA templates by RNA polymerases I and II. RNA polymerase I (Figure 4A) saturates chromatin at a ratio (µg of enzyme per µg of DNA) of 1.02 while RNA polymerase II saturates at a ratio of 0.61 (Figure

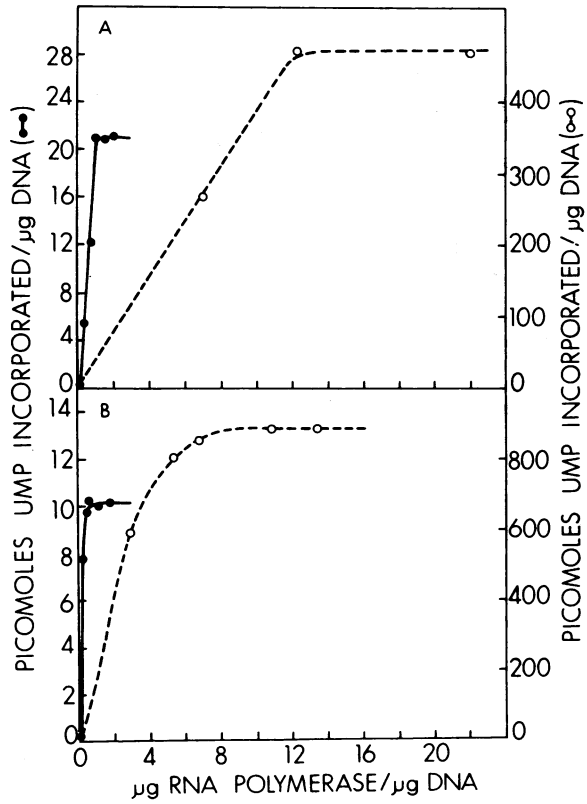


Fig. 4. Saturation of chromatin and DNA templates by exogenous RNA polymerases. Chromatin (●–●) or DNA (○–○) was transcribed by RNA polymerase I (A) or RNA polymerase II (B) as described under Materials and Methods. The ratio (mass:mass) of RNA polymerase to DNA was varied as indicated.

4B). These enzyme to DNA ratios are, respectively, 100- and 130-fold higher than the corresponding ratios of total (free plus bound) RNA polymerase I and II molecules per genome observed *in vivo* (26,27). Thus, these data raise the possibility that isolated chromatin has numerous artificial initiation sites which are not utilized *in vivo*. However, data regarding the transcription of integrated viral genes (below) indicate that this is not so, at least not by the order of magnitude calculated above.

In contrast to the results observed with chromatin, RNA polymerase I saturates DNA at a mass ratio of 11.5 (Figure 4A) while RNA polymerase II (Figure 4B) saturates at 8.2 μg per μg of DNA. Thus, for each enzyme about 12-fold more RNA polymerase is required to saturate a given mass of chromatin DNA after it has been deproteinized. These results suggest, but do not prove, that the presence of chromosomal proteins restrict the sequences of DNA which are transcribed by exogenous RNA polymerases. More direct evidence that this is, in fact, true was obtained by analysis of the transcription of specific chromosomal genes.

Transcription of Viral Genes in Chromatin by Exogenous RNA Polymerase II. Previously, by monitoring the endogenous RNA polymerase activity in isolated nuclei, we demonstrated that a class II RNA polymerase transcribes the integrated viral genes in adenovirus 2 transformed cells (5). Viral specific RNA comprised 0.010 to 0.016% of the total product under conditions where RNA polymerase II accounted for 80% of the total RNA polymerase activity. Thus, 0.012 to 0.020% of the total RNA transcribed from chromatin by exogenous RNA polymerase II is expected to be viral if the exogenous RNA polymerases transcribe the same sequences with the same relative frequency as do the endogenous enzymes in isolated nuclei. Experiment 1 in Table I shows a typical level of viral gene transcription in response to exogenous RNA polymerase II. We previously demonstrated by rehybridization experiments (5) that such analyses of the total *in vitro* product are, in fact, specific for RNA that is complementary to adenovirus 2 DNA. Thus, under these reaction conditions, 0.011% of the total product is viral-specific, a value somewhat lower than expected for accurate transcription by exogenous RNA polymerase II. Viral-specific RNA synthesized by the endogenous RNA polymerases does not account for more than 7% of the observed hybridized RNA (Table I, experiment 1).

It has recently been reported that *E. coli* RNA polymerase utilizes

TABLE I

Hybridization of Exogenous RNA Polymerase II Chromatin Transcripts to Adenovirus 2 DNA

| Experiment | Exogenous RNA Polymerase | Reaction Conditions | Hybridization Input (CPM) | DNA on Filter | CPM Bound | Ad2-Specific CPM | % Input |
|---|---|---|---|---|---|---|---|
| 1 | II | Standard | $2.66 \times 10^6$ | Ad2 | 352 | 281 | 0.011 |
| | | | | None | 71 | | |
| | None | Standard | $0.18 \times 10^6$ | Ad2 | 40 | 17 | 0.009 |
| | | | | None | 23 | | |
| 2[a] | II | Standard | $3.85 \times 10^6$ | Ad2 | 312 | 188 | 0.005 |
| | | | | None | 124 | | |
| | II | Actinomycin D | $0.15 \times 10^6$ | Ad2 | 59 | 18 | 0.011 |
| | | | | None | 41 | | |

[a]Reactions were performed as described under Materials and Methods either in the presence or absence of 166 µg/mL actinomycin D. Recovery of RNA during purification was 80% for the standard reaction and greater than 95% for the reaction containing actinomycin D.

endogenous chromatin RNA as a template (42). In fact, these authors were unable to detect any de novo transcription of globin genes when chromatin was transcribed with E. coli RNA polymerase. We thus examined the DNA dependence of chromatin transcription by eukaryotic RNA polymerases. When chromatin was transcribed in vitro with RNA polymerase II under standard reaction conditions, 98% of the total activity was inhibited by inclusion of 150 µg/mL actinomycin D in the reaction (data not shown). RNA was thus synthesized in the presence or absence of actinomycin D and hybridized to adenovirus DNA (Experiment 2, Table I). In this experiment, total RNA synthesis was inhibited by 98%, while transcription of the viral genes was also more than 90% inhibited. Analogous results were also obtained if chromatin was treated with DNase I (Worthington, RNase free) prior to the transcription reaction (data not shown). Thus, the RNA detected by hybridization analysis represents de novo transcription of the integrated adenovirus genes by exogenous RNA polymerase II.

Figure 5 depicts the level of viral gene transcription observed when chromatin is transcribed by RNA polymerase II at various subsaturating (cf. Figure 4) enzyme to DNA ratios. While the synthesis of viral RNA increases with increasing enzyme to DNA ratios, the total RNA synthesis increases by larger relative increments. Thus, the percentage of the total in vitro product which is viral-specific decreases as the enzyme

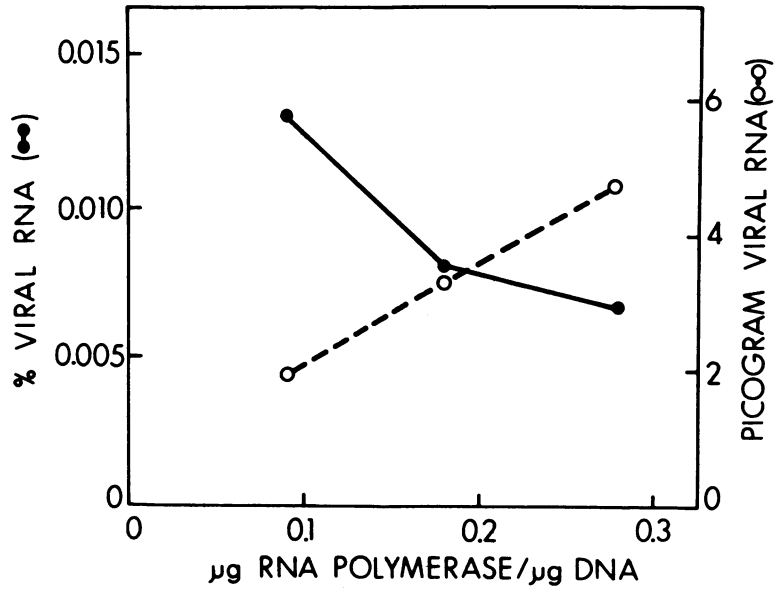


Fig. 5. Effect of varying enzyme to DNA ratios on the frequency of transcription of adenovirus genes in chromatin. Chromatin was transcribed in vitro with RNA polymerase II at various subsaturating enzyme to DNA ratios. Viral-specific RNA was quantitated by hybridization to adenovirus 2 DNA as described under Materials and Methods. The mass of viral RNA synthesized in each reaction was calculated after normalizing to 100% recovery of RNA during purification.

to DNA ratio is increased. Similar results have been reported for transcription of the ovalbumin genes by E. coli RNA polymerase and wheat germ RNA polymerase II (36). However, it is presently not clear whether the synthesis of ovalbumin RNA sequences was entirely DNA-dependent.

Transcription of Viral Genes in Chromatin and DNA Templates by Exogenous Eukaryotic RNA Polymerases. The results presented above demonstrate that exogenous RNA polymerase II transcribes the integrated adenovirus genes in isolated chromatin slightly less frequently (relative to total sequences transcribed) than do the endogenous RNA polymerases. Since it is known that the transcription of these genes in vivo is restricted to RNA polymerase II (5), we next determined whether this level of transcriptional regulation was operative with isolated chromatin and purified eukaryotic RNA polymerases. Experiment 1 in Table II shows the levels of viral gene transcription observed in response to exogenous

TABLE II

Hybridization of Chromatin and DNA Transcripts to Adenovirus 2 DNA

| Experiment | Template | Exogenous RNA Polymerase | Hybridization Input (CPM) | DNA on Filter | CPM Bound | Ad2-Specific CPM | % Input |
|---|---|---|---|---|---|---|---|
| 1 | Chromatin | I | $3.9 \times 10^6$ | Ad2 | 285 | 192 | 0.005 |
| | | | | None | 93 | | |
| | Chromatin | II | $3.2 \times 10^6$ | Ad2 | 341 | 268 | 0.008 |
| | | | | None | 73 | | |
| | Chromatin | III | $8.1 \times 10^6$ | Ad2 | 574 | 418 | 0.005 |
| | | | | None | 156 | | |
| 2 | DNA | I | $5.4 \times 10^6$ | Ad2 | 102 | 10 | 0.0002 |
| | | | | None | 92 | | |
| | DNA | II | $6.9 \times 10^6$ | Ad2 | 129 | 19 | 0.0003 |
| | | | | None | 110 | | |

Chromatin or DNA templates were transcribed in vitro with exogenous RNA polymerases in the presence of 2 mM $MnCl_2$ and the optimal $(NH_4)_2SO_4$ concentration for each respective enzyme. The RNA was purified and hybridized to adenovirus DNA as described under Materials and Methods. Chromatin was transcribed at enzyme to DNA (mass:mass) ratios of 0.42, 0.28 and 0.48 for RNA polymerases I, II and III, respectively. Deproteinized DNA was transcribed by RNA polymerases I and II at enzyme to DNA ratios of 12.2 and 4.9, respectively.

RNA polymerase I, II or III. Under these reaction conditions, all three enzymes transcribe the viral genes with approximately equal efficiency relative to other DNA sequences. Furthermore, the frequency of viral gene transcription is only about half of the apparent in vivo level (see above). In contrast, however, when deproteinized DNA is transcribed by RNA polymerase I or II, no significant synthesis of viral RNA is detected (Table II, Experiment 2). In B1 cells, viral DNA sequences comprise 0.0002% of the genome (11) and viral gene transcription from deproteinized DNA was not observed to occur at a higher frequency. Consistent with the enzyme saturation values with chromatin and DNA templates (above), these results indicate that chromosomal proteins restrict the DNA sequences which are transcribed efficiently by exogenous RNA polymerases. However, the transcription reactions as such do not exhibit the regulatory events manifest in vivo which restrict transcription of the viral genes to RNA polymerase II.

To determine whether the transcriptional specificity of this system could be increased by alterations in reaction conditions, we examined

the effects of different divalent cations on viral gene transcription. Experiment 1 in Table III shows the frequency of viral gene transcription by exogenous RNA polymerase II under reaction conditions which are identical except for the presence of 2 mM $MnCl_2$ or 5 mM $MgCl_2$. In the presence of $Mg^{2+}$, the overall rate of transcription is markedly reduced, in agreement with metal ion effects reported above. However, the frequency of viral gene transcription relative to other DNA sequences is increased more than 3-fold. In fact, the percentage of total RNA which is viral-specific corresponds to the value expected (above) if the exogenous enzymes transcribe specific chromatin DNA sequences with the same relative frequencies as the endogenous RNA polymerase II. Thus, in the presence of $Mg^{2+}$, rather than $Mn^{2+}$, the transcription of the integrated viral genes by exogenous RNA polymerase II appears to resemble more closely the *in vivo* transcriptional pattern.

We next determined whether the increased viral gene transcription frequency observed in the presence of $Mg^{2+}$ was specific for RNA polymerase II, the enzyme which transcribes these genes *in vivo*. The results of such an experiment are depicted in Experiment 2 (Table III). In the presence of $Mg^{2+}$, RNA polymerase II again transcribes the viral genes at a 3 to 4-fold higher frequency (relative to other DNA sequences) than

TABLE III

Effects of Metal Ions on the Transcription of Viral Genes in Chromatin by Exogenous RNA Polymerases

| Experiment | Exogenous RNA Polymerase | Reaction Conditions | Hybridization Input (CPM) | DNA on Filter | CPM Bound | Ad2-Specific CPM | Ad2-Specific % Input |
|---|---|---|---|---|---|---|---|
| 1 | II | $Mn^{2+}$ | $2.0 \times 10^6$ | Ad2 | 193 | 123 | 0.006 |
| | | | | None | 70 | | |
| | II | $Mg^{2+}$ | $0.5 \times 10^6$ | Ad2 | 146 | 100 | 0.020 |
| | | | | None | 46 | | |
| 2 | I | $Mg^{2+}$ | $0.52 \times 10^6$ | Ad2 | 81 | 48 | 0.009 |
| | | | | None | 33 | | |
| | II | $Mg^{2+}$ | $0.41 \times 10^6$ | Ad2 | 185 | 147 | 0.036 |
| | | | | None | 38 | | |
| | III | $Mg^{2+}$ | $0.74 \times 10^6$ | Ad2 | 128 | 52 | 0.007 |
| | | | | None | 76 | | |

Chromatin was transcribed *in vitro* with the indicated exogenous RNA polymerase as described under Materials and Methods, except that the divalent cation was either 2 mM $MnCl_2$ or 5 mM $MgCl_2$ as indicated in the Table. In Experiment 1, chromatin was transcribed by RNA polymerase II at a ratio (µg enzyme:µg DNA) of 0.24. In Experiment 2, the enzyme to DNA mass ratios were 0.43, 0.37 and 0.52 for RNA polymerases I, II and III, respectively.

that observed in the presence of $Mn^{2+}$. Under the same conditions, however, RNA polymerases I and III transcribe the viral genes at frequencies which approximate those observed with $Mn^{2+}$ as divalent cation in the respective transcription reactions (see Table II). The amount of viral-specific RNA synthesized in these reactions is low due both to the low gene copy number (6 copies per diploid DNA content; ref. 11) and to the relatively low total RNA polymerase activity in the presence of $Mg^{2+}$ (cf. Figure 3). However, these experiments have been repeated several times with very similar results. Thus, the increased relative frequency of viral gene transcription in the presence of $Mg^{2+}$ appears to be specific for RNA polymerase II.

## DISCUSSION

One means of studying transcriptional regulation is to attempt to reconstruct specific transcription events *in vitro*. The development of meaningful *in vitro* transcription systems will require that they exhibit the regulatory functions which occur *in vivo*. In eukaryotic cells, one such level of regulation is that of enzyme selection; i.e. regulation of which of the three forms of RNA polymerase transcribes a given gene. In attempting to identify factors responsible for such regulation, we have analyzed the transcription of specific genes, known to be transcribed by RNA polymerase II *in vivo*, by eukaryotic RNA polymerases I, II and III. We have shown, by sensitivity to actinomycin D, that the total RNA synthesis as well as transcription of the integrated adenovirus genes by exogenous RNA polymerase II represents *de novo* DNA transcription. This is in contrast to the RNA templated transcription of sequences complementary to globin DNA reported recently for *E. coli* RNA polymerase (42).

In addition to differences in function, the three eukaryotic RNA polymerases have characteristic catalytic properties when assayed with excess deproteinized DNA (reviewed in 25). Since local salt and metal ion concentrations, as well as enzyme concentrations, may have effects on functional gene expression *in vivo*, we determined the catalytic properties of each enzyme using chromatin as a template. The metal ion optima are qualitatively similar to those observed with the DNA templates. With respect to salt optima RNA polymerases I and II exhibited maximal rates of transcription at 40 and 75 mM ammonium sulfate, respectively,

while RNA polymerase III showed optima at both 45 and 210 mM. In contrast, when deproteinized DNA is transcribed under similar conditions (saturating enzyme to template ratios), all three enzymes exhibit only a single optimum at relatively low ionic strength (less than 50 mM). These results indicate that there may be differences in the mechanism of initiation of chromatin and DNA transcription by eukaryotic RNA polymerases. This has also been suggested from previous studies (39) of the effects of temperature and ionic strength on initiation of chromatin and DNA transcription by E. coli RNA polymerase.

In addition to general catalytic properties we have examined the specificity of transcription of integrated adenovirus genes in chromatin by exogenous RNA polymerases. From an examination of the frequency with which the viral DNA is transcribed, it appears that the RNA polymerases are not transcribing large portions of the genome which are not transcribed in vivo. Thus, under optimal conditions for RNA polymerase II transcription, the integrated adenovirus 2 DNA sequences are transcribed with a frequency (relative to other DNA sequences) which is about 50% of that observed in vivo. The simplest explanation of these results is that the exogenous RNA polymerase transcribes DNA in chromatin which is transcribed in vivo in addition to an equivalent amount of DNA which is not expressed in vivo. Alternatively, it is possible that only those regions of DNA expressed in vivo are transcribed in vitro, but that the relative efficiencies of transcription of different sequences varies. These slight differences between the chromatin and the in vivo transcriptional patterns are in direct contrast, however, to the results observed when deproteinized DNA is transcribed in vitro. No significant synthesis of viral RNA above that expected from a random transcription of the DNA template was detected. Thus, the presence of chromosomal proteins appears to restrict the sequences of DNA which are transcribed by exogenous RNA polymerases such that the frequency of viral gene transcription is at least 25- to 50-fold higher than the frequency with which these sequences exist in the genome. These results are also consistent with the observation that a 10-fold greater level of purified eukaryotic RNA polymerase is required to saturate a deproteinized DNA template. Additionally we have observed that the frequency of viral gene transcription is increased at lower RNA polymerase to chromatin DNA ratios. Thus, the transcription of DNA sequences not expressed in vivo (above) may only occur at high enzyme to chromatin ratios in vitro.

Since the transcription of isolated chromatin by purified RNA polymerase II appears to reflect some of the transcriptional controls operative *in vivo*, we next sought to determine whether discrimination in the transcription of these genes by RNA polymerases I and III occurred *in vitro*. In the presence of $Mn^{2+}$, all three polymerases transcribe the integrated viral genes at approximately the same frequency. Thus, under these reaction conditions, there is apparent selectivity in that the DNA sequences transcribed by exogenous RNA polymerases appear similar to those transcribed *in vivo*. However, there has been a loss of discrimination at the level of RNA polymerase. This could be due to the loss or inactivation of components during chromatin or RNA polymerase purification. Alternatively, incorrect initiation could be caused by the reaction conditions. That this latter explanation is at least partially true was suggested by the fact that the use of $Mg^{2+}$ in place of $Mn^{2+}$ increased the frequency of viral gene transcripton by exogenous RNA polymerase II to a level similar to that which occurs *in vivo*. Furthermore, this increased proportion of viral gene transcripts in the presence of $Mg^{2+}$ was observed only with RNA polymerase II, the enzyme which transcribes these genes *in vivo*. A similar effect of specific metal ions on transcriptive specificity has been reported for the transcription of yeast ribosomal RNA genes in deproteinized DNA (18). Thus yeast RNA polymerase I was reported to synthesize rRNA at a frequency 15- to 30-fold above that expected for a random transcription of the template while RNA polymerase II and *E. coli* RNA polymerase were found to transcribe these genes randomly in the presence of either metal ion. In view of these earlier findings with deproteinized DNA, it seems probable that the preferential transcription of viral genes in chromatin by RNA polymerase II in the presence of $Mg^{2+}$ is due to effects of metal ions on the transcriptional specificity of the RNA polymerases rather than on structural aspects of the chromatin template.

The system described in this paper seems appropriate for analyzing components involved in the transcriptional regulation of nonreiterated chromosomal genes. The synthesis of viral RNA represents *de novo* transcription of the integrated adenovirus 2 genes by exogenous RNA polymerase II. Regulatory components are present in the chromatin such that RNA synthesized *in vitro* appears to be similar to the populations synthesized *in vivo*. Furthermore, in the presence of $Mg^{2+}$, the integrated adenovirus 2 genes are transcribed more efficiently (relative to other DNA sequences)

by RNA polymerase II than by RNA polymerases I or III. Despite these promising observations, however, it is not yet clear whether the purified RNA polymerase II transcribes the integrated genes with fidelity (i.e. that it recognizes proper initiation and termination sites). It also seems improbable that this occurs with the isolated class I and III RNA polymerases which, like pancreatic DNase I (40) and presumably bacterial RNA polymerase (Introduction), may simply serve as crude probes for active gene regions in chromatin. Thus, the viral RNA synthesis observed in vitro in response to RNA polymerases I and III, and possibly II, indicates that some regulatory components have been inactivated or lost during purification. These factors might be recovered, and possibly identified, by using alternate methods of chromatin or RNA polymerase purification or by including various subcellular fractions in transcription reactions and monitoring the synthesis of accurate viral gene transcripts.

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2. Abbreviations used: Ad2, adenovirus serotype 2; BSA, bovine serum albumin; CM, carboxymethyl; 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.
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