Transcription of Specific Adenovirus Genes in Isolated Nuclei by Exogenous RNA Polymerases*

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Previous studies have shown that nuclei isolated from KB cells infected with adenovirus 2 synthesize discrete low molecular weight RNAs via an endogenous RNA polymerase III activity. The major transcript is the well characterized 5.5 S or VA-RNA which is encoded by the adenovirus genome. Other transcripts include additional low molecular weight viral RNAs and host 5 S RNA and pre-tRNA. In the present studies, partially purified class III RNA polymerases were shown to stimulate nuclear RNA synthesis when incubated with nuclei from adenovirus-infected or uninfected KB cells. These exogenous RNA polymerases stimulate predominantly the synthesis of low molecular weight RNAs. 5.5 S RNA synthesis in nuclei from virus-infected cells is stimulated 3- to 5-fold by KB RNA polymerases III, and III, isolated either from uninfected or from virus-infected KB cells. Similar results were obtained with class III RNA polymerases from mouse plasmacytoma cells and from Xenopus laevis oocytes.

The RNA polymerase III activity in isolated nuclei was found to be reversibly inactivated with low concentrations of N-ethylmaleimide. All class III RNA polymerases which stimulate viral 5.5 S RNA synthesis in untreated control nuclei from virus-infected cells (above) also enhanced the synthesis of 5.5 S RNA in N-ethylmaleimide-treated nuclei. However, the degree of stimulation above the residual endogenous RNA polymerase level was much higher in N-ethylmaleimide-treated nuclei (up to 30-fold). In contrast, neither RNA polymerase II from adenovirus-infected cells nor Escherichia coli RNA polymerase selectively enhanced the transcription of the viral 5.5 S genes in isolated nuclei, although the levels of total RNA synthesis were increased in each case.

Attempts to understand transcriptional control mechanisms

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in eukaryotic cells have focused on the complex nuclear RNA polymerases and chromatin templates and the reconstruction of specific transcription events from subcellular components. It is apparent that one level of regulation is mediated through the existence of the structurally distinct class 1, II, and III RNA polymerases. These enzymes transcribe, respectively, those genes which encode the rRNAs, the HnRNAs and the 5 S and tRNAs (reviewed in Ref. 1). However, structural modifications of chromatin are also of primary importance for selective gene activation. This conclusion has been reached from studies of the accessibility of specific genes in isolated chromatin to exogenous probes, such as bacterial RNA polymerases (2-4) and specific nucleases (5, 6). More recent studies of the transcription of Xenopus laevis 5 S RNA genes (7, 8) and murine 5 S and tRNA genes (9) in reconstructed systems have led to similar conclusions. In these latter studies, however, the 5 S (and tRNA) genes were shown to be very accurately (asymmetrically) and preferentially transcribed, generating discrete RNA products similar to those synthesized in vivo. Significantly, these highly specific transcription events were observed only when the isolated chromatin or nuclear templates were transcribed by the class III RNA polymerases. These studies underscore the importance of using the correct form of RNA polymerase and the advantages of simple reiterated gene systems for detailed studies of eukaryotic gene transcription.

DNA tumor viruses offer similar advantages for the study of transcriptional regulation because of the relative simplicity of the viral genomes, the involvement of host components in viral gene transcription, and the presence of large numbers of viral templates in lytically infected cells. The replication of adenovirus 2 in human cells provides a particularly attractive system, since extensive information is available on the organization and expression of specific viral genes (reviewed in Ref. 10). During the lytic cycle, the viral genome is transcribed by two host RNA polymerases. Viral genes which encode mRNAs are transcribed by RNA polymerase II and viral genes which encode several distinct low molecular weight RNAs are transcribed by RNA polymerase III (11-15). The genes which encode the small viral RNAs are clustered internally on the viral genome (15, 16). The major low molecular weight product is the VA- or 5.5 S RNA (17). The gene for this RNA is transcribed efficiently by endogenous RNA polymerase III in nuclei isolated from cells late in infection (14, 15). Synthesis of 5.5 S RNA appears to be continually
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initiated under these conditions and the product is apparently not subject to further processing (13, 15, 18), therefore facilitating comparisons of in vivo and in vitro transcripts.

In the present study we have focused on the transcription of the viral 5.5 S RNA gene in reconstructed systems. In the studies reported here, isolated nuclei have been used as a template for purified RNA polymerases in order to establish optimal conditions for faithful transcription. Our results show the selective and accurate transcription of the viral 5.5 S RNA gene by exogenous class III RNA polymerases from several sources. This crude reconstituted system thus appears suited for further studies of the various cellular components which regulate transcription.

EXPERIMENTAL PROCEDURES

Cell Culture and Virus Infection - Exponentially growing cultures of KB cells were infected with adenovirus 2 at a multiplicity of 40 plaque-forming units per cell as described by Craig and Raskas (19). After a 1-h adsorption period, the culture was diluted to 3 x 10⁶ cells/ml. The virus used for infection and as a source of DNA was purified by two equilibration centrifugations in cesium chloride (20).

Preparation of in vivo RNAs - Nonradioactive 5.5 S RNA was isolated from KB cells 14 h after infection with adenovirus 2. β-Hexosaminidase (1 mg/ml) was added to the cells at 0°C for 10 min to swell the cells. Cells were then homogenized (10 to 15 strokes) in a tight fitting Dounce homogenizer, and the nuclear suspension was adjusted to 25% glycerol (v/v), 5 mM MgCl₂, and constitutes the cytoplasmic fraction.

Isolation of Nuclei - Cells were collected 14 h after infection with virus by low speed centrifugation and were washed once with Buffer A (10 mM Tris/HCl (pH 7.9, 23°C), 5 mM MgCl₂, 10 mM KCl, 0.05 mM dithioerythritol, and was incubated at 0°C for 10 min to swell the cells. Cells were homogenized (10 to 15 strokes) in a tight fitting Dounce homogenizer. Inspection by phase contrast microscopy revealed complete cellular disruption but maintenance of nuclear integrity. Nuclei were layered over an equal volume of Buffer B (50 mM Tris/HCl (pH 9.2), 25% (v/v) glycerol, 0.1 mM EDTA, 0.5 mM dithioerythritol, and 5 mM MgCl₂) and were collected by low speed centrifugation. The nuclear pellet was resuspended in Buffer B to give a final volume of 25 μl containing the following components: 600 ELM of 3 to 6 Ci/mmole. RNA was isolated as described above with the addition of a second treatment with DNase after two ethanol precipitations. After two further ethanol precipitations, the RNA pellet was lyophilized to dryness and was resuspended in H₂O.

Nitrocellulose filters (1.3 cm) containing approximately 49 μg of total adenovirus DNA were hybridized with radioactive RNA in a total volume of 500 μl under conditions essentially as described previously (27). In some cases, the immobilized DNA was prehybridized with unlabelled 5.5 S RNA (20 μg/ml) under the same conditions. Efficiency of hybridization was calculated for each reaction according to the amount of radioactive RNA which hybridized to adenovirus DNA alone but not to adenovirus DNA prehybridized with unlabelled 5.5 S RNA.

RESULTS

RNA Polymerases in Adenovirus-infected Cells - Previous studies have shown that human KB cells contain RNA polymerases I and II and two chromatin forms of III, and IIIb of RNA polymerase III (27). In addition, the cellular levels and chromatographic properties of the RNA polymerases isolated from KB cells infected with adenovirus type 2 were found to be identical to those of the uninfected cell enzymes. The properties of the KB cell enzymes (21) are, in general, similar to those reported for other mammalian enzymes (28). The class II and III RNA polymerases have been purified by standard procedures, as described under "Experimental Procedures", and used for the studies described here. On the basis of specific activity measurements, the purity of the class II and III RNA polymerases employed here appears to be about 20%.

It is apparent from earlier studies that isolated nuclei contain endogenous RNA polymerase I, II, and III activities (14, 15, 27). The amounts of the various RNA polymerases present in nuclear and cytoplasmic fractions isolated from virus-infected cells have been assessed more directly by solubilization and chromatographic resolution (DEAE-Sephadex) of the RNA polymerases present in these fractions. The results of these experiments are summarized in Table I. The majority of the RNA polymerase I and II activity remains in the nucleus. In contrast, only about one-third of the RNA polymerase III activity is retained in the nuclear fraction. Moreover, only form IIIb is detected in the nuclear extracts whereas approximately equal amounts of forms IIIa and IIIb are found in the cytoplasmic fraction (data not shown). Similar distributions have been observed for the class III RNA polymerases from uninfected KB cells (data not shown) and from mouse plasmacytoma cells (9) using similar fractionation procedures. These observations probably reflect leakage of the RNA polymerases from nuclei during cellular fractionation. As shown previously (15, and in the following experiments), the endogenous RNA polymerase III in isolated nuclei...
is capable of synthesizing discrete viral transcripts. The novel experiments presented here demonstrate the ability of exogenous class III RNA polymerases to transcribe specific viral genes in the nuclear preparations, resulting in the production of discrete RNAs.

Transcription of Specific Host and Viral Genes in Isolated Nuclei in Response to Exogenous Class III RNA Polymerases—Detergent-treated nuclei were employed for the studies reported here. The kinetics and the salt and divalent cation optima of the endogenous RNA polymerase reactions in these nuclei are similar to those described in previous nuclear transcription studies (14, 15). In these experiments, nuclei from uninfected KB cells and from virus-infected cells were incubated in the absence and in the presence of purified RNA polymerases. The incubations were carried out under conditions optimal for the endogenous RNA polymerase III reaction and under conditions where the endogenous RNA polymerase II activity is inhibited (see "Experimental Procedures"). The level of total RNA synthesis in each nuclear preparation was increased from 2- to 3-fold by each of the exogenous class III RNA polymerases examined. The latter included both class III enzymes (IIIa and IIIb) from uninfected cells and from virus-infected cells. Moreover, the actual levels of stimulation of RNA polymerase III activity by the exogenous RNA polymerases were considerably greater since the endogenous RNA polymerase activity represents both RNA polymerase I plus III activities (data not shown, see Ref. 29; cf. Ref. 9).

The RNAs synthesized in isolated nuclei in response to exogenous KB class III RNA polymerases were analyzed by sedimentation in sucrose gradients and shown to consist primarily of low molecular weight transcripts (<10 S) (data not shown, see Ref. 29; cf. Ref. 9). To further characterize the nuclear transcripts, the isolated RNAs were subjected to electrophoresis in 12% polyacrylamide gels. Autoradiograms of the gels in which these 32P-labeled RNAs were analyzed are shown in Fig. 1. As shown in Fig. 1A, the synthesis of 5 S RNA and 4.5 S RNA (tRNA precursors) species is markedly stimulated by RNA polymerases IIIa and IIIb, isolated either from uninfected or from virus-infected cells. These observations are similar to those reported previously for the synthesis of 5 S and 4.5 S RNAs in mouse plasmacytoma cell nuclei (9).

An analysis of the low molecular weight RNAs synthesized in nuclei from virus-infected cells is shown in Fig. 1B. The viral RNAs synthesized by the endogenous RNA polymerase III (lane 1) include the major 5.5 S RNA and a minor 200-nucleotide RNA (designated VΔ0), visible just above the 5.5 S RNA (15). Other low molecular weight viral RNAs synthesized by RNA polymerase III (15) are not apparent in these experiments. The low molecular weight RNAs synthesized in response to exogenous class III RNA polymerases are shown in lanes 2 to 5 in Fig. 1B. The synthesis of the viral 5.5 S and VΔ0 RNAs, as well as the cellular 4.5 S RNAs, is stimulated by RNA polymerases IIIa and IIIb from uninfected and from virus-infected KB cells. The synthesis of some RNA species which are excluded from the 12% acrylamide gel is also stimulated. The nature of these RNAs, apparently greater than 8 S in size, is unknown. Levels of a-amanitin (200 µg/ml) which inhibit RNA polymerase III inhibit the synthesis of all these RNA species in the presence of exogenous RNA polymerase III (data not shown; see also below). Thus, the enhanced synthesis of the various RNAs in the presence of partially purified exogenous RNA polymerase III preparations is clearly due to an RNA polymerase III activity.

To quantify the increased levels of RNA synthesis effected by the exogenous RNA polymerases, polyacrylamide gels similar to those shown in Fig. 1 were sliced and the radioactivity in specific RNAs was determined after solubilization. The upper panel of Fig. 2 shows that exogenous RNA polymerase IIIa stimulates the synthesis of the 5 S RNA and the pre-tRNAs (the broad band between the 4 S and 5 S markers) about 3-fold relative to the level of synthesis by the endogenous activity in uninfected nuclei. The lower panel of Fig. 2 shows the RNAs synthesized in nuclei from virus-infected cells. Exogenous RNA polymerase IIIb stimulates the synthesis of the viral VΔ0 and 5.5 S RNAs about 3-fold. Synthesis of host 5 S RNA and pre-tRNA is also stimulated although the relative levels of synthesis differ from those observed in nuclei from uninfected cells. Possibly this reflects a preferential
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UNINFECTED

INFECTED

FIG. 2. Quantitation of stimulation by exogenous RNA polymerase activity. RNA products were synthesized in 4.4 x 10⁶ uninfected (upper panel) or 5.7 x 10⁶ infected (lower panel) KB cell nuclei in the presence of 0.25 μg of α-amanitin per ml and [α-³²P]UTP (2 Ci/mmol). RNA was analyzed as described in the legend to Fig. 1. The region of the gel slab between the viral V₅₅ and the cellular 4 S RNA was sliced and the radioactivity was determined as described previously (27). O——O, synthesis by the endogenous RNA polymerase activity; ●——●, synthesis in the presence of exogenous RNA polymerase IIIα from uninfected KB cells (206 units in the uninfected nuclear incubation and 309 units in the infected nuclear incubation). Labeled arrows denote positions of ³H-labeled in vivo RNAs included as markers.

stimulation of 5 S RNA synthesis or a preferential inhibition of pre-tRNA synthesis as a result of virus infection. Nevertheless, it is apparent that the exogenous RNA polymerase III promotes a general 3-fold stimulation in the synthesis of low molecular weight RNAs in the KB nuclei, whether from uninfected or from virus-infected cells. Similar levels of stimulation are observed with both forms of the infected and uninfected KB cell class III RNA polymerases.

Other experiments have shown that the viral 5.5 S and the V₂₀₀ RNAs and the cellular 5 S RNA are synthesized in an apparently linear fashion for at least 80 min in the absence and in the presence of exogenous RNA polymerase III (data not shown, see Ref. 29). These observations suggest continued reinitiation by both the endogenous (cf. Ref. 15) and the exogenous RNA polymerases.

Transcription of Specific Viral Genes in Isolated Nuclei in Response to Heterologous RNA Polymerases—To investigate whether the synthesis of the low molecular weight viral RNA species is specifically stimulated by exogenous KB class III RNA polymerases, the effects of heterologous class III RNA polymerases as well as homologous RNA polymerase II were analyzed.

The analyses of the effects of the class III RNA polymerases are shown in Fig. 3, lanes 2 to 6. In these experiments, the endogenous RNA polymerase II activity was inhibited with 0.25 μg of α-amanitin per ml. The endogenous level of 5.5 S RNA synthesis in nuclei from virus-infected cells (lane 2) is significantly stimulated by mouse plasmacytoma RNA polymerase IIIα (lane 4), mouse plasmacytoma RNA polymerase IIIβ (lane 5), and Xenopus laevis oocyte RNA polymerase III (lane 6). These levels of stimulation approximate those attained by the KB RNA polymerase III (lane 3, see also previous section). Quantitation of the radioactivity in the 5.5 S RNA bands by liquid scintillation counting revealed that the exogenous class III enzyme stimulated 5.5 S RNA synthesis 3- to 5-fold.

An analysis of the effects of RNA polymerase II from adenovirus-infected KB cells is also shown in Fig. 3 (lanes 1 and 7). The amount of 5.5 S RNA synthesized in isolated nuclei in the absence of α-amanitin (lane 1) is equivalent to that synthesized in the presence of sufficient α-amanitin to inhibit RNA polymerase II (lane 2) as reported previously (14, 15). The addition of exogenous RNA polymerase II to isolated nuclei in the absence of α-amanitin (lane 7) had no significant effect on the synthesis of 5.5 S RNA. Quantitation of the radioactivity in the 5.5 S RNA bands by liquid scintillation counting showed at most a 10 to 20% increase in 5.5 S RNA synthesis in the presence of RNA polymerase II, even though total RNA synthesis was increased by greater than 50%. The apparent increase shown in Fig. 3 reflects an
increased background radioactivity in the reaction carried out in the presence of exogenous RNA polymerase II.

**Sensitivity of RNA Polymerase III Activities to N-Ethylmaleimide**—Since the endogenous RNA polymerase III activity in isolated KB nuclei is quite high, it seemed desirable to reduce this activity in order to more clearly analyze the effects of exogenous RNA polymerases. N-Ethylmaleimide has been used previously to irreversibly inhibit endogenous RNA polymerase activity in chromatin preparations (30). Fig. 4 shows the effect of N-ethylmaleimide on the activity of a purified KB RNA polymerase III and on the combined endogenous RNA polymerase I plus III activity in nuclei isolated from virus-infected cells. Under the assay conditions used, most (>75%) of the endogenous RNA polymerase activity in these nuclei represents RNA polymerase III activity (27). It is apparent that both RNA polymerase activities are almost completely inhibited by concentrations of N-ethylmaleimide above 1 mM, with 50% inhibition at about 0.3 mM. Other experiments (not shown) have indicated that the inhibition of 5.5 S RNA synthesis (monitored electrophoretically) parallels the inhibition of the activities shown in Fig. 4. These experiments suggested that it might be possible to inhibit most of the endogenous RNA polymerase III activity without severe effects on the template activity of the nuclei.

**Transcription of Specific Viral Genes in N-Ethylmaleimide-treated Nuclei by Exogenous Class III RNA Polymerases**—The objective of these experiments was to investigate the ability of N-ethylmaleimide-treated nuclei to serve as templates for the synthesis of the viral 5.5 S RNA by exogenous class III enzymes. In the experiment shown in Fig. 5, nuclei from virus-infected KB cells were preincubated briefly at 30° in the absence (lanes 1 and 2) or in the presence (lanes 3 and 4) of a low concentration of N-ethylmaleimide. The preincubated nuclei were then washed and incubated under RNA synthesis conditions in the absence (lanes 1 and 3) or in the presence (lanes 2 and 4) of KB RNA polymerase III. Preincubation of the nuclei in the presence of N-ethylmaleimide results in an irreversible inhibition of 5.5 S RNA synthesis by the endogenous RNA polymerase III (lane 3) whereas nuclei preincubated in the absence of N-ethylmaleimide retain the capacity for 5.5 S RNA synthesis (lane 1) at a level comparable to that observed for non-preincubated nuclei. The addition of exogenous KB RNA polymerase III markedly stimulates 5.5 S RNA synthesis in nuclei preincubated either in the absence (lane 2) or in the presence (lane 4) of N-ethylmaleimide. Stimulation of the V<sub>200</sub> species by exogenous RNA polymerase III is also observed in each case.

A quantitative analysis of the experiment shown in Fig. 5 is presented in Fig. 6. The endogenous synthesis of 5.5 S RNA in N-ethylmaleimide-treated nuclei was reduced by greater than 90%. Exogenous RNA polymerase III apparently stimulated 5.5 S RNA synthesis about 3- and 10-fold, respectively, in the control and N-ethylmaleimide-treated nuclei. The degree of stimulation observed in the N-ethylmaleimide-treated nuclei varies somewhat among different experiments, depending on the efficiency of removal of the endogenous RNA polymerase III; in some experiments values as high as 30-fold have been observed (see also below). In the presence of exogenous RNA polymerase III, the maximum level of synthesis observed in N-ethylmaleimide-treated nuclei is always significantly less than that observed with control nuclei, suggesting that some nuclear transcription elements other than RNA polymerase III are damaged by N-ethylmaleimide.

In the experiments presented above, exogenous RNA polymerase III<sub>4</sub> from uninfected cells was shown to transcribe the viral 5.5 S RNA gene in N-ethylmaleimide-treated nuclei. Other studies have shown RNA polymerases III<sub>4</sub> and III<sub>3</sub> from uninfected KB cells and RNA polymerases III<sub>4</sub> and III<sub>3</sub> from virus-infected cells to be equally effective in transcribing the viral 5.5 S RNA gene in N-ethylmaleimide-treated nuclei (data not shown, see Ref. 29; see also Figs. 8 and 9 below). In the experiments reported here, saturating amounts of the class III RNA polymerases have been employed. A representative saturation curve with enzyme form III<sub>4</sub> from uninfected KB cells is shown in Fig. 7.

The kinetics of viral 5.5 S RNA synthesis in N-ethylmaleimide-treated nuclei in the presence and absence of exogenous KB RNA polymerase III (form III<sub>4</sub> from uninfected cells) are shown in Fig. 8. In the presence of the exogenous RNA polymerase, synthesis continues in an apparently linear fashion for at least 80 min, again raising the possibility of continued reinitiation by the exogenous RNA polymerase in this system.

**Transcription of Specific Viral Genes in N-Ethylmaleimide-treated Nuclei by Heterologous RNA Polymerases**—The effects of heterologous RNA polymerases on the synthesis of viral 5.5 S RNA in nuclei with high levels of endogenous RNA polymerases were described above. Similar experiments have been performed with the N-ethylmaleimide-treated nuclei in order to facilitate a clearer evaluation of the ability of the heterologous enzymes to transcribe the viral 5.5 S gene. In the experiment shown in Fig. 9, virtually no 5.5 S RNA synthesis was observed in N-ethylmaleimide-treated nuclei in the absence of exogenous RNA polymerases (lanes 1 and 2). In confirmation of results cited above, RNA polymerases III<sub>4</sub> from uninfected KB cells (lane 3) and from virus-infected cells (lane 4) markedly stimulated the synthesis of 5.5 S RNA. In addition, RNA polymerase III<sub>4</sub> from mouse plasmacytoma cells (lane 5) and RNA polymerase III from X. laevis oocytes (lane 6) also stimulated 5.5 S RNA synthesis as previously (27).

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1 J. A. Jaehning, unpublished observations.
FIG. 5. Polyacrylamide gel analysis of RNA from N-ethylmaleimide-treated nuclei. Nuclei were prepared from adenovirus-2-infected KB cells and were treated with N-ethylmaleimide as described under "Experimental Procedures." 2.4 × 10⁷ nuclei which were preincubated without N-ethylmaleimide (lanes 1 and 2) and 2.2 × 10⁷ nuclei preincubated with 1 mM N-ethylmaleimide (lanes 3 and 4) were incubated as described with [α-³²P]UTP (2 Climmol) and 0.25 μg of α-amanitin per ml for 60 min at 30°C. Products were analyzed as described in the legend to Fig. 1. Lanes 1 and 3, RNA synthesized by endogenous RNA polymerase activity. Lanes 2 and 4, RNA synthesized in the presence of 130 units of exogenous RNA polymerase III from uninfected KB cells. Efficiently as did the KB class III enzymes. All these class III enzymes (lanes 3 to 6) also stimulated the synthesis of material (apparently ≥8 S in size) which does not enter the gel. The nature of this RNA has not been examined further.

In contrast to the results with class III enzymes and in agreement with studies described earlier, RNA polymerase II from virus-infected KB cells had no noticeable effect on the synthesis of viral 5.5 S RNA (compare lanes 1 and 8 in Fig. 9) although total RNA synthesis was increased about 2- to 3-fold. These assays (analyzed in lanes 7 and 8) were performed in the absence of α-amanitin. In addition, the effects of E. coli RNA polymerase were monitored. This enzyme markedly stimulates transcription when incubated with N-ethylmaleimide-treated nuclei. However, as shown in Fig. 9 (lanes 7) a very hetero dispersal array of transcripts is generated. When the gel analyzed in Fig. 9 was subjected to a shorter autoradiographic exposure, no 5.5 S RNA band was evident (data not shown). Moreover, no 5.5 S RNA band was evident when the gels were sliced and analyzed by liquid scintillation counting.

Hybridization of Nuclear Transcripts to Adenovirus DNA — As an independent measure of viral 5.5 S RNA synthesis in these reconstructed transcription systems, the in vitro transcripts have been characterized by hybridization to adenovirus DNA. In the experiment shown in Table II, RNA synthesized in N-ethylmaleimide-treated nuclei was hybridized to adenovirus DNA in the presence and absence of a nonradioactive 5.5 S RNA competitor. The total radioactivity in 5.5 S RNA was calculated as D/E, where D equals the difference between the total amount of radioactive RNA hybridized to adenovirus DNA and the amount hybridized in the presence of the 5.5 S RNA competitor, and E equals the fractional efficiency of hybridization of an ³H-labeled 5.5 S RNA internal standard. In the experiments shown in Table II, only a very small and possibly insignificant amount of 5.5 S RNA was synthesized by the endogenous RNA polymerase. In contrast, exogenous KB class III RNA polymerases from uninfected KB cells and from X. laevis oocytes markedly stimulated (at least 30- to 40-fold) the synthesis of 5.5 S RNA. The stimulation is almost completely inhibited by α-amanitin at a concentration (160 μg/ml) which inhibits RNA polymerase III activity about 90% (14). In addition, 5.5 S RNA sequences
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Fig. 7. Stimulation of 5.5 S RNA synthesis in N-ethylmaleimide-treated nuclei by limiting concentrations of exogenous RNA polymerase. RNA was synthesized in $3.4 \times 10^7$ N-ethylmaleimide-treated nuclei from adenovirus 2-infected KB cells in the presence of the indicated amounts of exogenous RNA polymerase III from uninfected KB cells. All reactions contained 0.25 μg of α-amanitin per ml and [α-32P]UTP (2 Ci/mmol) and were incubated at 30°C for 60 min. The products were subjected to electrophoresis on a 12% polyacrylamide gel slab. The region of the gel containing the 5.5 S RNA was cut out and the radioactivity was determined as described previously (27).

Fig. 8. Kinetics of 5.5 S RNA synthesis in N-ethylmaleimide-treated nuclei. $3.4 \times 10^7$ N-ethylmaleimide-treated nuclei from adenovirus 2-infected KB cells were incubated for the indicated times at 30°C in the presence of 0.25 μg of α-amanitin per ml and [α-32P]UTP (5 Ci/mmol). Products were subjected to electrophoresis on a 12% polyacrylamide gel slab. The region of the gel containing the 5.5 S RNA was cut out and the radioactivity was determined as described previously (27). O --- O, synthesis in the presence of 110 units of RNA polymerase III from adenovirus-infected KB cells. A background of 46 cpm has been subtracted from all points.

Fig. 9. Comparison of heterologous RNA polymerases. RNA was synthesized in $3.4 \times 10^7$ N-ethylmaleimide-treated nuclei from adenovirus 2-infected KB cells in the presence of [α-32P]UTP (2 Ci/mmol). Nuclei were incubated for 60 min at 30°C in the absence of α-amanitin (lanes 1 and 8) or in the presence of 0.25 μg of α-amanitin per ml (lanes 2 to 7). The various RNA polymerases added to the nuclear reactions were: lanes 1 and 2, endogenous RNA polymerase alone; lane 3, uninfected KB III, 160 units; lane 4, infected KB III, 130 units; lanes 5, MOPC III, 290 units; lane 6, Xenopus laevis III, 290 units; lane 7, Escherichia coli, 640 units; lane 8, infected KB II, 290 units. The arrow indicates the position of the 5.5 S RNA band.

Again, a high level of 5.5 S RNA synthesis is observed and the level is about 40-fold greater than that observed in the comparable N-ethylmaleimide-treated nuclei.

DISCUSSION

Faithful Transcription of Specific Viral Genes in a Reconstructed System—Previous studies (see introduction to text) have shown that nuclei isolated from adenovirus-infected cells actively synthesize several discrete low molecular weight virus-coded RNAs, the most prominent of which is the 5.5 S RNA. The synthesis of these RNAs is mediated by an endogenous RNA polymerase III, which appears to initiate and terminate synthesis in vitro with the same specificity observed in the intact infected cells. Comparative fingerprint analyses of 5.5 S RNAs synthesized in vitro and in isolated nuclei have confirmed this latter hypothesis.

The present studies confirm that nuclei isolated from adenovirus-infected cells are active in RNA synthesis and that 5.5 S RNA is the major product of the endogenous RNA polymerase III. In addition, the ability of exogenous class III RNA polymerases to significantly stimulate nuclear RNA synthesis has been demonstrated. The overall 2- to 3-fold

accounted for about 30% of the total RNA synthesized. The RNA which hybridizes to adenovirus DNA in the presence of competitor 5.5 S RNA has not been further characterized, but probably includes some of the other known RNA polymerase III transcripts of the viral genome (15). Table II also shows an analysis of the RNAs synthesized by the endogenous RNA polymerase in nuclei not treated with N-ethylmaleimide.

3 B. Harris and R. G. Roeder, unpublished observations.
stimulation reflects predominantly an increased rate of synthesis (3- to 5-fold) of discrete low molecular weight RNAs, including the viral 5.5 S RNA and the viral V₃₉ RNA. The ability of exogenous class III RNA polymerases to stimulate nuclear RNA synthesis might reflect the replacement of enzyme(s) lost during preparation of the nuclear templates. Thus, only a small fraction of the total cellular RNA polymerase III was found localized in isolated nuclei, the remainder being in the cytosol fraction. Studies of the class III RNA polymerases in other mammalian cells (e.g. Refs. 28, 31, and 32) also suggest that these enzymes may leak from the nucleus during some aqueous nuclear isolation techniques. In the reconstructed system described here, the final concentration of added RNA polymerase III which gives maximal stimulation (about 4,000 units/ml) is similar to the intranuclear concentration expected if all the enzyme is localized within the nucleus in the intact cell.

In a further extension of these studies, it was found that the endogenous RNA polymerase III activity could be irreversibly inactivated with N-ethylmaleimide, under conditions which did not abolish the ability of exogenous RNA polymerases to selectively transcribe the endogenous viral templates. With these nuclear templates the exogenous class III enzymes stimulated transcription of the 5.5 S RNA gene up to 30-fold. This was demonstrated both by hybridization-competition analysis and by direct analysis of discrete 5.5 S RNA transcripts on polyacrylamide gels. In all these reconstructed systems the generation of discrete 5.5 S RNA transcripts implies that both initiation and termination signals are being read properly by the exogenous RNA polymerases. A continued initiation by the class III RNA polymerases is also indicated by the prolonged synthesis of 5.5 S RNA synthesis at a linear rate and by quantitation of the 5.5 S RNA transcripts. For example, during a 40-min reaction approximately 150,000 5.5 S RNA molecules are synthesized per nucleus (N-ethylmaleimide-treated) in response to exogenous RNA polymerase III. Fourteen hours after infection when these nuclei are isolated, there are approximately 10,000 to 100,000 viral genomes per nucleus (33, 34), suggesting a minimal value of 2 to 20 5.5 S RNA transcripts per gene. The actual number is probably much higher since not all viral DNA molecules are likely to be involved in transcription complexes (27).

The novel feature of these studies is the demonstration that purified class III RNA polymerases accurately initiate and terminate synthesis of viral RNAs from crude nuclear templates. In addition, however, they confirm a recent report (9) that these class III enzymes also enhance significantly the transcription of cellular (host) 5 S RNA and 4.5 S RNA (pre-tRNA) genes in isolated nuclei. Some viral-induced alteration in the relative rates of synthesis of 5 S RNA and 4.5 S RNA is suggested by the present experiments, although this question requires further analysis.

### Table II

| RNA polymerase          | α-Amanitin Input | cpm hybridized | 5.5 S RNA hybridization efficiency | Total cpm in 5.5 S RNA | Fraction of total endogenous % input |
|-------------------------|------------------|----------------|-----------------------------------|------------------------|--------------------------------------|
| Endogenous reaction     | 0.25             | 4,575          | 576                               | 321                    | 28                                  |
| + KB III₉               | 0.25             | 12,400         | 1,655                             | 891                    | 19                                  |
| + KB III₁₀              | 180              | 5,400          | 410                               | 360                    | 27                                  |
| + X. laevis III         | 0.25             | 12,925         | 2,503                             | 1,267                  | 36                                  |
| Untreated nuclei endogenous reaction | 0.25 | 18,175 | 2,658 | 1,264 | 22 |

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#### Function of Different Class III RNA Polymerases—The Transcription of Specific Cellular and Viral Genes—The availability of template systems which respond in a specific manner to exogenous RNA polymerases has allowed an investigation of the RNA polymerase III requirement for viral 5.5 S RNA synthesis. The most definitive studies reported are those which have employed N-ethylmaleimide-treated nuclei although similar results have been obtained with untreated nuclei.

Two chromatographic forms of class III RNA polymerases exist in adenovirus 2-infected KB cells (27). These enzymes probably correspond to the murine III₁₉ and III₁₀ described by Sklar and Roeder (9), which differ only in the size of one of the ten subunits. Since a number of cellular and viral genes are transcribed by class III RNA polymerases in infected cells, it seemed possible that the two enzyme forms might transcribe distinct subsets of genes. This does not appear to be the case since both forms were equally capable of transcribing the genes for all of the small RNAs analyzed in this study (see also Ref. 9). However, these observations do not rule out interconversion of the enzymes after incubation with the nuclear template nor do they exclude other possible functional roles not detectable by this assay.

An equally important question is whether host RNA polymerases are modified during virus infection and whether such modifications, if they exist, are of any consequence for cellular or viral gene transcription. The present studies demonstrate that the class III RNA polymerases from uninfected KB cells are as effective in stimulating viral and cellular gene transcription as are the RNA polymerases from virus-infected cells, suggesting that RNA polymerase modifications are not essential for transcription of the genes encoding the low molecular weight viral RNAs. This conclusion is supported by the observation that the highly purified class III RNA polymerases from uninfected and from adenovirus-infected cells have identical subunit structures (21). However, these studies do not exclude the possibility that the host enzymes undergo minor modifications during the course of lytic infection or during incubation in the crude template systems (for further discussion see Ref. 21). Analysis of these questions must await further refinement of the template system.
It is also significant that the heterologous X. laevis oocyte transcribe the viral 5.5 S gene as efficiently and apparently as accurately as do the homologous KB enzymes. While this is somewhat unexpected in view of the diverse tissues from which these class III enzymes are isolated, the KB, murine plasmacytoma, and X. laevis class III RNA polymerases have remarkably similar subunit structures (21-23). The fact that plasmacytoma, and X. laevis class III RNA polymerases have selection of specific transcription events from more defined components.

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REFERENCES
1. Roeder, R. G. (1976) in RNA Polymerases (Losick, R., and Chamberlin, M., eds) pp. 285-329, Cold Spring Harbor Press, Cold Spring Harbor, New York
2. Axel, R., Cedar, H., and Felsenfeld, G. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 2029-2032
3. Gilmour, R. S., and Paul, J. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 3440-3442
4. Tsai, M. J., Towlie, H. C., Harris, S. E., and O'Malley, B. W. (1976) J. Biol. Chem. 251, 1969-1968
5. Weintraub, H., and Groudine, M. (1976) Science 193, 848-856
6. Garel, A., and Axel, R. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 3966-3970
7. Parker, C. S., and Roeder, R. G. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 44-48
8. Parker, C. S., Ng, S. Y., and Roeder, R. G. (1976 in Molecular Mechanisms in the Control of Gene Expression (Nierlich, D. P., Rutter, W. J., and Fox, C. F., eds) pp. 223-247, Academic Press, New York
9. Sklar, V. E. F., and Roeder, R. G. (1977) Cell 10, 405-414
10. Flint, J. (1977) Cell 10, 153-166
11. Wallace, R. D., and Kates, J. (1972) J. Virol. 9, 627-635
12. Price, R., and Penman, S. (1972) J. Virol. 9, 621-626
13. Price, R., and Penman, S. (1972) J. Mol. Biol. 70, 435-450
14. Weinmann, R., Raskas, H. J., and Roeder, R. G. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 3426-3430
15. Weinmann, R., Brendler, T. G., Raskas, H. J., and Roeder, R. G. (1976) Cell 7, 557-566
16. Matthews, M. B. (1975) Cell 6, 223-229
17. Reich, P. R. Forgett, B. G., Weissman, S. M., and Rose, J. A. (1966) J. Mol. Biol. 17, 426-429
18. Ohe, K., and Weissman, S. M. (1971) J. Biol. Chem. 246, 6991-7009
19. Craig, E. A., and Raskas, H. J. (1974) J. Virol. 14, 751-757
20. Craig, E. A., Zimmer, S., and Raskas, H. J. (1975) J. Virol. 15, 1905-1913
21. Jaehning, J. A., Woods, P. S., and Roeder, R. G. (1977) J. Biol. Chem. 252, 8762-8771
22. Sklar, V. E. F., and Roeder, R. G. (1976) J. Biol. Chem. 251, 1064-1073
23. Sklar, V. E. F., Schwartz, L. B., and Roeder, R. G. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 348-352
24. Burgess, R. R., and Jendrisak, J. J. (1975) Biochemistry 14, 4634-4638
25. Jaehning, J. A., Steward, C. C., and Roeder, R. G. (1975) Cell 4, 51-57
26. Palmiter, R. D. (1974) Biochemistry 13, 3606-3615
27. Weinmann, R., Jaehning, J. A., Raskas, H. J., and Roeder, R. G. (1976) J. Virol. 17, 114-126
28. Schwartz, L. B., Sklar, V. E. F., Jaehning, J. A., Weinmann, R., and Roeder, R. G. (1974) J. Biol. Chem. 219, 5889-5897
29. Jaehning, J. A. (1977) Ph.D. thesis, Washington University, St. Louis, Mo.
30. Howk, R. E., Williams, D. R., Heberman, A. B., Parke, W. P., and Sechnick, E. M. (1974) Cell 3, 15-22
31. Seiffart, K. H., and Benecke, B. J. (1975) Eur. J. Biochem. 53, 205-208
32. Weil, P. A., and Blatti, S. P. (1976) Biochemistry 15, 1500-1509
33. Green, M., and Daesch, G. (1961) Virology 13, 169-176
34. Wilhelm, J., Brison, O., Rediger, C., and Champon, P. (1976) J. Virol. 19, 61-81
35. Jaehning, J. A., Weinmann, R., Breindler, T. G., Raskas, H. J., and Roeder, R. G. (1976) in RNA Polymerases (Losick, R., and Chamberlin, M., eds), pp. 819-834, Cold Spring Harbor Press, Cold Spring Harbor, New York
36. Austin, G. E., Belo, L. J., and Furth, J. J. (1976) Arch. Biochem. Biophys. 177, 346-354
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J. Biol. Chem. 1977, 252:8753-8761.

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