
Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei

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ABSTRACT

We have developed a procedure for preparing extracts from nuclei of human tissue culture cells that directs accurate transcription initiation in vitro from class II promoters. Conditions of extraction and assay have been optimized for maximum activity using the major late promoter of adenovirus 2. The extract also directs accurate transcription initiation from other adenovirus promoters and cellular promoters. The extract also directs accurate transcription initiation from class III promoters (tRNA and Ad 2 VA).

INTRODUCTION

In recent years there have been developed soluble cell-free systems which mediate the accurate transcription of purified genes by class I, II, and III RNA polymerases (reviewed in 1). These systems have provided the means for more definitive investigations of eukaryotic transcription mechanisms at both the protein (RNA polymerase and accessory transcription factor) and DNA level. In the case of class II genes accurate transcription of purified viral (2) and cellular (3) genes was first demonstrated with a system comprised of purified RNA polymerase II and a high speed supernatant fraction (S100) from cultured human cells. This system has been used to determine promoter sequences (4-6), and in this laboratory, for the isolation of factors that are necessary (along with RNA polymerase II) for transcription from the adenovirus 2 major late promoter (7). A second system consisting of a high salt extract of whole cells (and containing endogenous RNA polymerase II) has also been shown to mediate accurate transcription (8) and has been used to analyze promoter sequences (9-12) and the mechanism of action of a negative regulatory factor (13). However, neither of these systems takes advantage of the presumed nuclear localization of the transcription components since one is obtained from a soluble post-nuclear fraction at low ionic strength (2) while the other is derived from a high salt extraction of a whole cell homogenate (8). In addition, there is no indication that the conditions employed

for extraction in these studies were optimized for the selective extraction of the required components since the extracts in each case are prepared by methods which are only slight modifications of earlier procedures developed for different purposes (14,15). In the present studies, we have developed a simple procedure for the preparation of extracts from nuclei and have optimized both the conditions of extraction and assay. This method has potential advantages over other procedures (2,8) in that it utilizes the nuclear localization of the required components to achieve a significant initial removal of contaminating cytoplasmic and nuclear components that are not required for the in vitro initiation of transcription and it utilizes conditions that have been optimized for the extraction of the required components.

EXPERIMENTAL PROCEDURES

Assay Conditions - In the standard assay for specific transcription initiation up to 25 μ l of nuclear extract was assayed in a final volume of 50 μ l. Final concentrations for various components (including those contributed by the extract) were: 12 mM hydroxyethyl piperazineethanesulfonic acid (HEPES), (pH 7.9 at 25°), 12% (v/v) glycerol, 0.3 mM dithiothreitol (DTT), 0.12 mM ethylenediamine tetraacetate (EDTA), 60 mM KCl, 12 mM MgCl₂, 600 μ M each of the three unlabelled triphosphates (ATP, CTP and UTP), 25 μ M α -³²P-GTP (5 Ci/mMole), and 1 μ g of pSmaF DNA cleaved with SmaI. The standard incubation was for 60 min at 30°C. Conditions were varied for other templates and in various experiments as indicated. After extraction as described (2) the RNA samples were dissolved in 98% formamide and electrophoresed on 4% acrylamide gels containing 7 M urea; the running buffer was 0.09 M borate, 0.09 M Tris and 0.01 M EDTA. Electrophoresis was continued until the xylene cyanol tracking dye was 1 cm from the bottom of the gel, after which the gels were soaked for 20 min in distilled water, dried, and subjected to autoradiography as previously described (2). In some experiments the band representing the specific transcript was cut from the dried gel and counted in toluene based scintillation fluid; regions of equal area above and below the band were excised, counted and the average of these values was subtracted as a blank.

Buffers - Buffers used for extract preparation are designated as follows: buffer A contains 10 mM HEPES (pH 7.9 at 4°C), 1.5 mM MgCl₂, 10 mM KCl and 0.5 mM DTT; buffer B contains 0.3 M HEPES (pH 7.9), 1.4 M KCl and 0.03 M MgCl₂; buffer C contains 20 mM HEPES (pH 7.9), 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5 mM DTT; buffer D contains 20 mM HEPES (pH 7.9), 20% (v/v) glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT. DTT and PMSF were added

fresh to the buffers just before use.

Cells - HeLa cells (a line obtained from G. Attardi, California Institute of Technology) were grown in spinner flasks at 37° in Joklik's MEM containing 5% calf serum. They were grown to 4 to 6 x 10⁵ cells per ml prior to harvesting for extract preparation.

Standard Procedure for Extract Preparation - HeLa cells were harvested from cell culture media by centrifugation (at room temperature) for 10 min at 2000 rpm in a Sorvall HG4L rotor. Pelleted cells were then suspended in five volumes of 4°C phosphate buffered saline and collected by centrifugation as detailed above; subsequent steps were performed at 4°C. The cells were suspended in five packed cell pellet volumes of buffer A and allowed to stand for 10 min. The cells were collected by centrifugation as before and suspended in two packed cell pellet volumes (volume prior to the initial wash with buffer A) of buffer A and lysed by 10 strokes of a Kontes all glass Dounce homogenizer (B type pestle). The homogenate was checked microscopically for cell lysis and centrifuged for 10 min at 2000 rpm in a Sorvall HG4L rotor to pellet nuclei. The supernatant was carefully decanted, mixed with 0.11 volumes of buffer B, and centrifuged for 60 min at 100,000 g_{av} (Beckman Type 42 rotor). The high speed supernatant from this step was dialyzed five to eight hours against 20 volumes of buffer D and is designated the S100 fraction.

The nuclear extract was prepared as follows. The pellet obtained from the low speed centrifugation of the homogenate was subjected to a second centrifugation for 20 min at 25,000 g_{av} (Sorvall SS34 rotor), to remove residual cytoplasmic material and this pellet was designated as crude nuclei. These crude nuclei were resuspended in 3 ml of buffer C per 10⁹ cells with a Kontes all glass Dounce homogenizer (10 strokes with a type B pestle). The resulting suspension was stirred gently with a magnetic stirring bar for 30 min and then centrifuged for 30 min at 25,000 g_{av} (Sorval SS34 rotor). The resulting clear supernatant was dialyzed against 50 volumes of buffer D for five hours. The dialysate was centrifuged at 25,000 g_{av} (Sorvall SS34 rotor) for 20 min and the resulting precipitate discarded. The supernatant, designated the nuclear extract, was frozen as aliquots in liquid nitrogen and stored at -80°. The protein concentration was usually 6 to 8 mg per ml and 15 to 20 mg of protein were obtained from 10⁹ cells.

RESULTS

To assess the activity of extracts prepared under various conditions for

accurate transcription initiation, we employed primarily the major late promoter of adenovirus 2. When a clone containing this viral promoter (pSmaF) is cleaved with the restriction enzyme SmaI, accurate transcription initiation generates a 536 nucleotide run-off transcript which corresponds in length to the distance from the transcription start site to an SmaI restriction site downstream from the promoter (2). DNA templates containing other viral or cellular genes were employed in a similar fashion by cleaving the DNA downstream from the promoter with an appropriate restriction enzyme.

Salt Optimum for Extraction - Since the transcription components for RNA polymerase II are presumably concentrated within the nucleus, we sought to take advantage of this in our isolation procedure. Hence, the first step after cell lysis is the isolation of a crude nuclear fraction. To achieve a selective enrichment of the required transcription components, we examined several NaCl concentrations for extraction of the nuclei. The optimum NaCl concentration was determined by suspending separate aliquots of crude nuclei in buffer C containing NaCl concentrations ranging from 0.2 M to 0.5 M. After preparation of extracts as described in Methods, specific transcription initiation assays were performed using the Ad 2 major late promoter as a template. As Figure 1 demonstrates, extracts prepared at 0.2 M (lanes 1 and 2) or 0.3 M NaCl (lane 3) were only slightly active while extracts prepared at salt concentrations of 0.35 (lane 4), 0.42 (lane 5) or 0.5 (lane 6) M NaCl were quite active. However, extracts prepared at 0.6 M NaCl were substantially less active and those prepared at 0.75 M and 1.0 M NaCl were inactive (data not shown). When nuclei that were previously used to prepare the 0.2 M NaCl extract (lanes 1 and 2) were subsequently treated with 0.5 M NaCl, the resulting extract was only slightly active (lanes 7 and 8). However, a combination of equal amounts of the primary 0.2 M and secondary 0.5 M NaCl extracts was active (lane 9), indicating that essential factors show a differential solubility. Since the higher salt concentrations (>0.6 M NaCl) extract some inhibitory material, we chose 0.42 M NaCl for subsequent experiments. The optimal salt concentration for extraction of factors required for transcription of a human H4 histone gene (pH4A, ref. 16) and a mouse β globin gene (pMGS-1, ref 3) was the same as that observed for the major late promoter of adenovirus 2 (data not shown). The S100 fraction derived during the cellular fractionation used for the preparation of the nuclear extracts (see Materials and Methods) was inactive for the transcription of adenovirus 2 major late (see below).

pH Optimum for Extraction - Extracts were prepared from nuclei at the follow-

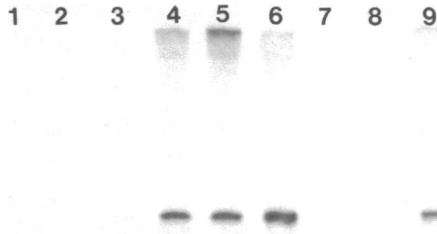


Fig. 1. Transcription at the Ad2 major late promoter with extracts prepared from nuclei extracted at different NaCl concentrations. Extracts were prepared using the standard procedure except that the NaCl concentration for extraction was varied. Extracts were assayed under standard conditions. Lanes 1 and 2 show assays which contained, respectively, 12.5 μ l (50 μ g protein) and 25 μ l (100 μ g protein) of the 0.2 M NaCl extract. Lanes 3 through 6 show, respectively, assays of the 0.3 M NaCl extract (25 μ l, 120 μ g protein), the 0.42 M NaCl extract (25 μ l, 140 μ g protein), and the 0.5 M NaCl extract (25 μ l, 150 μ g protein). Lanes 7 and 8 show assays which contained, respectively, 25 μ l (80 μ g protein) and 12.5 μ l (40 μ g protein) of the 0.5 M NaCl extract of nuclei previously extracted with 0.2 M NaCl. Lane 9 shows an assay which contained a mixture (12.5 μ l each) of the 0.2 M NaCl extract and the 0.5 M NaCl extract of nuclei previously extracted with 0.2 M NaCl.

ing pH values: 6.5, 7.0, 7.5, 8.0 and 8.5. The only modification made was in buffer C in which 20 mM piperazinebis-ethanesulfonic acid (PIPES) was used at pH 6.5 and 7.0 and 20 mM hydroxyethyl-piperazine-propane sulfonic acid (HEPPS) was used at pH 8.5; the other conditions of extraction were as described in Materials and Methods. The pH optimum for extraction appears to be quite broad with the extract prepared at pH 8.0 being only slightly more active than the extracts prepared at other pH values (data not shown). In our standard procedure we have employed pH 7.9.

Effect of Protease Inhibitors - Since cellular proteases could pose a serious problem during the preparation of crude extracts and the isolation of proteins, we tested the effect of several protease inhibitors to determine if they could enhance the activity of the nuclear extracts for transcription.

The compounds phenylmethylsulfonyl fluoride (0.5 mM), soybean trypsin inhibitor (100 $\mu\text{g/ml}$), leupeptin (10 μM), leustatin (10 μM), and antipain (10 μM) were tested by including these compounds at the indicated concentrations in the buffers used for cell lysis, extraction of the nuclei and dialysis of the extract. None of the extracts prepared with these inhibitors was appreciably more active than the control extract (data not shown). However, since proteases could have effects that are not immediately apparent in a crude extract, but which become apparent during protein purification, we have routinely used PMSF during the preparation of the extracts.

Effect of the Addition of S100 and RNA Polymerase II - Since components might partition differentially between the S100 and the nuclear extract we examined the effect of combinations of these two fractions, prepared from the same cells, on specific transcription from the major adenovirus late promoter (Fig. 2). The S100 is inactive when assayed alone (data not shown) or in the presence of purified calf thymus RNA polymerase II (lanes 3 and 4). As shown above, the nuclear extract is active without additional factors (lanes 1 and

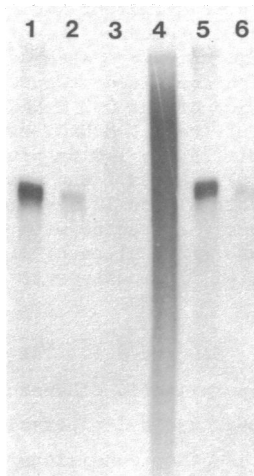


Fig. 2. Effect of the addition of S100 to a nuclear extract on the transcription initiation at the Ad2 major late promoter. Extracts prepared by the standard procedure were assayed under standard conditions. Lanes 1 and 2 show assays containing, respectively, 25 μl (150 μg protein) and 12.5 μl (75 μg protein) of nuclear extract alone. Lanes 3 and 4 show assays containing, respectively, 25 μl (250 μg protein) and 12.5 μl (125 μg protein) of S100 with 100 units of added calf thymus RNA polymerase II (ref. 17), Lane 5 shows an assay containing 25 μl (150 μg protein) of nuclear extract and 5 μl (50 μg protein) of S100. Lane 6 shows an assay containing 12.5 μl (75 μg protein) of nuclear extract and 12.5 μl (125 μg protein) of S100.

2) and the signal is not significantly enhanced with the addition of the S100 fraction (lanes 5 and 6). Although 90 percent of the extracts were active, the addition of the S100 to approximately 25 percent of the extracts suppressed a background of random transcription (data not shown). The addition of more calf thymus RNA polymerase II to the nuclear extract does not stimulate specific transcription and serves only to increase the background of random transcription (data not shown). Calf thymus RNA polymerase II was earlier shown to function in conjunction with human cell-derived transcription factors (2).

Optimum KCl and Mg^{++} Concentrations - Using an extract prepared under standard conditions, the optimum KCl concentration for transcription from the adenovirus major late promoter was found to be 60 mM (data not shown); this is the same as that observed with the previously described S100 extract in the presence of exogenous RNA polymerase II (2). Similar KCl optima (60 to 70 mM) were observed when mouse β -globin (pMGS-1) and human H4 histone (pH4A) templates were employed. However, as shown in Figure 3, the Mg^{++} optimum for

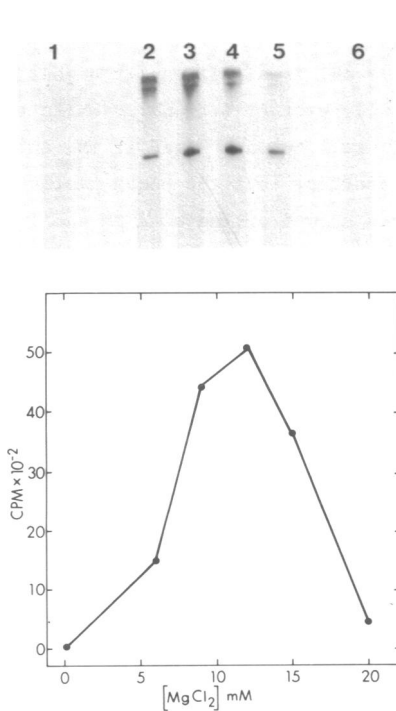


Fig. 3. Mg^{++} optimum for transcription initiation at the Ad2 major late promoter. Extract (25 μ l, 150 μ g protein) prepared by the standard procedure was assayed in duplicate under standard conditions except that Mg^{++} concentration was varied. Assays in lanes 1 through 6 contained, respectively, 0, 6, 9, 12, 15 and 20 mM Mg^{++} . After autoradiography, the bands were cut from the gels and counted as described in Methods.

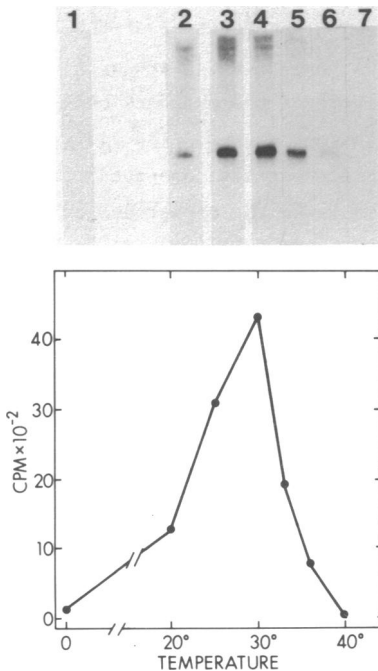


Fig. 4. Temperature optimum for transcription initiation at the Ad2 major late promoter. Extract (25 μ l, 150 μ g protein) prepared by the standard procedure was assayed in duplicate under standard conditions except that the temperature was varied. Assays in lanes 1-7 were incubated, respectively, at 0°, 20°, 25°, 30°, 32°, 36° and 40°. After autoradiography, bands were cut from the gel and counted as described in Methods.

the adenovirus major late promoter (10 to 12 mM) is significantly higher than that previously reported for the original S100 extract (7.5 mM). While the Mg^{++} optima for different DNA templates all fall between 8 and 12 mM, they appear to differ reproducibly from one to another; thus the human histone H4 (pH4A) and mouse β globin (pMGS-1) templates showed optima of 8 mM and 10 mM, respectively, which differ reproducibly (albeit slightly) from the Mg^{++} optimum for the pSmaF template. These results indicate that the Mg^{++} optimum should be determined for each template.

Temperature and pH Optima - The pH and temperature optima were determined using a standard extract assayed with the Ad2 major late promoter template as described in Materials and Methods. As shown in Figure 4, there is a sharp temperature optimum at 30°. As shown in Figure 5, the reaction has a broad pH optimum between 7.5 and 8.5.

Template Concentration Optima - Several templates have been examined with respect to optimal concentrations for specific initiation. While most promoters are active at DNA concentrations between 10 and 20 μ g/ml, different promoters exhibit different DNA optima for a given extract. In addition, the optimum for a given template can vary from extract to extract. Thus, while the basis for this variation in template optima is not entirely clear, ex-

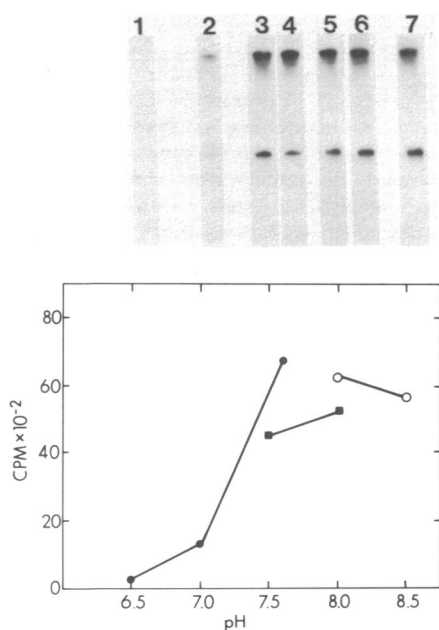


Fig. 5. pH optimum for transcription initiation at the Ad2 major late promoter. Aliquots of the standard extract were dialyzed against buffer D containing 20 mM PIPES (O—O), 20 mM HEPES ([]—[]), or 20 mM HEPPS (O—O). The pH indicated is that observed for the complete reaction mixture at 30°. Dialyzed extracts (25 μ l, 150 μ g protein) were assayed in duplicate under standard conditions except that pH was varied. Assays in lanes 1-7 were incubated, respectively, at pH 6.5, 7.0, 7.6, 7.5, 8.0, 8.0 and 8.5. After autoradiography, bands were cut from the gels and counted as described in Methods.

tracts should, for optimal activity, be assayed at several template concentrations to establish the DNA optimum both for a specific template and a given extract. In addition, when an equivalent amount of DNA (1 μ g) lacking a eukaryotic promoter (e.g. PRB322) is added to the reaction, the amount of plasmid DNA carrying Ad2 major late promoter can be reduced five-fold (to 0.2 μ g) without changing the intensity of the signal; without the addition of the PBR322 DNA, the signal from 0.2 μ g of the major late template is hardly detectable (data not shown).

Time Course of Synthesis and Stability of the Product - The time course of synthesis of the specific transcript from the Ad2 major late promoter is shown in Figure 6. Under optimal conditions of salt, pH, and temperature, incorporation of radioactivity into the specific run-off transcript continues linearly for at least 50 min after a short lag. When α -amanitin (at a concentration that specifically inhibits RNA polymerase II) is added at 30 or 60 min after the start of the reaction and the samples incubated an additional 30 min, little or no loss of radioactivity in the transcript is observed (compare lanes 8 and 9 with 6 and 7, respectively).

Transcription of Cellular and Viral Templates - To assess the utility of the extract for transcription from different eukaryotic promoters we examined several genes whose transcription had been previously characterized (see

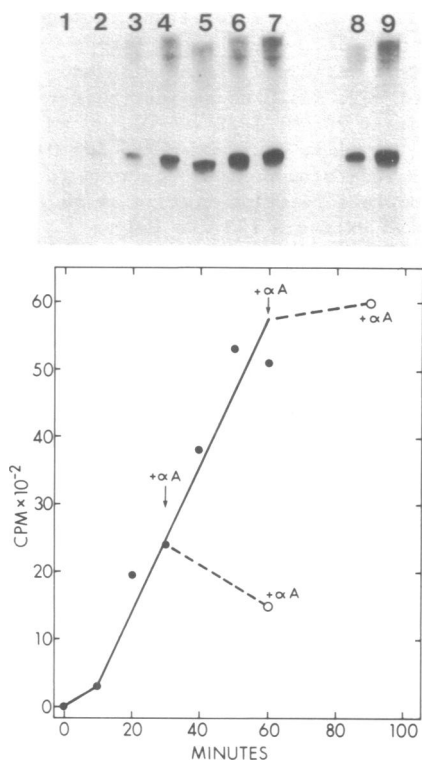


Fig. 6. Time course of synthesis for transcription initiation at the Ad2 major late promoter. Extract (25 μ l, 150 μ g protein) prepared by the standard procedure was assayed in duplicate under standard conditions except that the incubation time was varied. Assays in lanes 1-7 were incubated, respectively, for 0, 10, 20, 30, 40, 50 and 60 min. At 30 and 60 min (lanes 8 and 9, respectively) α -amanitin was added at 1 μ g/ml (a concentration that inhibits RNA polymerase II completely) to parallel tubes, and the samples were incubated an additional 30 min. After autoradiography, bands were cut from the gel and counted as described in Methods.

refs. 6, 7 and 14). Figure 7 shows the results of these experiments in which the transcripts were analyzed in the standard electrophoresis system (urea gels). In the cases examined the templates employed generated transcripts of the expected size. Thus, when pSmaF is cleaved with SmaI, the Ad2 major late promoter directs the synthesis of an RNA (see arrow) whose estimated size of 550 nucleotides is in good agreement with the size of 536 predicted from sequence analysis of this gene. When cleaved with Hind III pSmaF also directs the synthesis of a 200 nucleotide RNA which is close to the expected size of 197 nucleotides (data not shown). A clone containing the adenovirus EIV promoter (Ad2 pEcoRIc) directs the synthesis of two transcripts when the DNA is cleaved with Hind III (lane 2, Fig. 7). The more prominent band of about 700 nucleotides (arrow) corresponds to the transcript expected (673 nucleotides) for initiation at the EIV promoter. Two transcripts were observed with this template in an earlier study from this laboratory (18), but only the 700 nucleotide transcript could be identified as the EIV transcript. Only one transcript of approximately 300 nucleotides (284 nucleotides is the expected

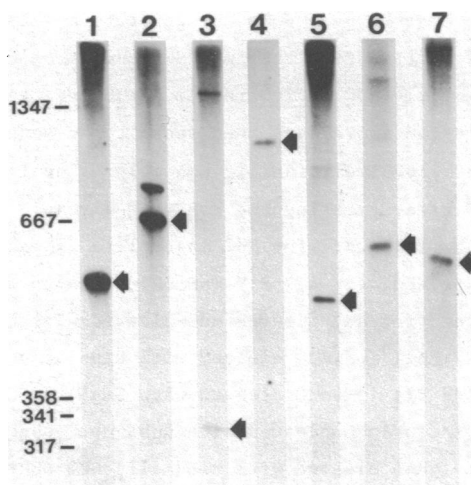


Fig. 7. Size analysis of in vitro synthesized transcripts obtained with several promoters. The standard extract (25 μ l, 150 μ g protein) was assayed under standard conditions except that the Mg^{++} concentration was changed as indicated for each template. The assays shown contained: Lane 1, Ad2 major late (pSmaF cleaved with Hind III), 12 mM Mg^{++} ; Lane 2, Ad2 EIV (pEcoR1C cleaved with Hind III), 10 mM Mg^{++} ; Lane 3, Ad2 EIB (pHindG, cleaved with Kpn I) 10 mM Mg^{++} ; Lane 4, Ad2 EIII (pHindH cleaved with Hind III), 10 mM $MgCl_2$; Lane 5, Ad2 EIA (pHindG cleaved with SmaI), 10 mM $MgCl_2$; Lane 6, Ad2 ppIX (pHindC cleaved with Hph I), 10 mM $MgCl_2$; Lane 7, human H4 histone (pH4a cleaved with Hind III), 8 mM $MgCl_2$. The plasmids employed are described elsewhere (18). The arrows indicate the α -amanitin sensitive transcripts discussed in the text.

size) is observed when this template is cleaved with HpaI (data not shown), an observation that is in accord with earlier work (18). Figure 7 also shows that appropriately cleaved plasmids (see Figure 7 legend) containing the adenovirus 2 E1b (lane 3), EIII (lane 4), E1a (lane 5), and polypeptide IX (lane 6) promoters generate transcripts (indicated by arrows) of approximately 330, 1050, 540 and 600 nucleotides, respectively. The sizes of these RNAs are in agreement with those of the 347, 1045, 510 and 604 nucleotide transcripts expected (from the sequence data) for accurate initiation at the respective promoters (18).

Transcription of a cleaved plasmid containing a human H4 histone gene (pH4A, ref. 13) is shown in lane 7 of Figure 7. The size of the transcript generated (600 nucleotides) corresponds to that expected for accurate initiation on this gene (determined by S1 mapping of *in vivo* RNA, N. Heintz and R. Roeder, unpublished) and termination at the downstream restriction site. The

transcription of the histone gene does not generate a transcript that corresponds to the size of a transcript that would result from correct transcription termination (approximately 400 nucleotides). In other experiments not shown, the transcripts indicated above have been shown to be sensitive to α -amanitin concentrations which selectively inhibit RNA polymerase II and their sizes have been determined after treating the RNA with glyoxal. The observed and expected sizes were as follows: for Ad2 major late (pSmaF cleaved with SmaI), 570 observed, 536 expected; for Ad2 EIV (pEcoRIc cleaved with Hind III) 680 observed, 673 expected; for Ad2 EIb (pHindG cleaved with Kpn I) 350 observed, 347 expected; for Ad2 EIII (pHindH cleaved with Hind III) 1000 observed, 1050 expected; for Ad2 EIa (pHindG cleaved with SmaI) 520 observed, 510 expected; for Ad2 ppIX (pHindC cleaved with HphI) 630 observed, 600 expected; for human H4 histone (pH4A cleaved with Hind III) 600 observed, approximately 600 expected.

Transcription of Class III Genes - The nuclear extract is active for the transcription of Ad2 VA and tRNA genes, but shows very little capacity for 5S gene transcription (P. Martin and R. Roeder, unpublished observations). The absence of 5S transcription apparently results from the absence (or reduced levels) of transcription factor IIIA in the nuclear extract; thus, when partially purified factor IIIA (23) is added to the nuclear extract, 5S genes are actively transcribed (P. Martin and R. Roeder, unpublished observations). The S100 fraction prepared in conjunction with the nuclear extract is nearly as active as the originally described S100 fractions (22) in transcribing the 5S, tRNA and VA RNA genes, indicating that it contains the 5S specific TFIIIA factor, as well as substantial levels of the other pol III factors (data not shown).

DISCUSSION

We have described a simple procedure for preparing cultured cell-derived extracts that are active for the in vitro transcription of purified cellular and viral class II genes. This procedure takes advantage of selective extraction of the transcription components from nuclei isolated at low ionic strength. It is noteworthy that partial resolution of some components can be achieved by sequential extraction of nuclei with buffers containing increasing NaCl concentrations (see Fig. 1). This observation is in accord with an earlier report from our laboratory (7) that the transcription of the adenovirus major late promoter requires multiple components in addition to RNA polymerase II. The primary advantage of this nuclear extract is that it

achieves a substantial separation of the required transcription components from contaminating cytoplasmic and nuclear material but, as with whole cell extracts (8), still contains endogenous RNA polymerase II.

While we have not completed extensive mapping studies of all the transcripts generated by the various class II gene templates analyzed in this system, the sizes of the run-off transcripts are in each case strongly indicative of accurate initiation events. In the case of the adenovirus 2 major late promoter (pSmaF template) and the mouse β -chain promoter (pMGS-1 template, ref. 3) the 5' termini of the in vitro transcripts have been shown to be indistinguishable from those of the corresponding in vivo transcripts when compared by primer extension analysis with reverse transcriptase (ref. 19 and D. Luse, personal communication). In addition, transcripts generated in the present system with a plasmid containing the adenovirus 2 EIIA-early promoter appear, by S1 nuclease mapping, to have 5' termini identical to those of the corresponding in vivo RNAs (D. H. Huang and R. G. Roeder). Moreover, while transcription from the EIIA-early promoter is difficult to detect with a template containing both the EIIA-early and EIII promoters (ref. 18 and Figure 7), a substantial and readily detectable level of accurate initiation is observed with a template containing only the EIIA promoter (D. H. Huang and R. G. Roeder, unpublished observation). This may be of significance in view of the fact that the major EIIA-early promoter does not contain a canonical TATA box and that this promoter is recognized only at a very low level in the other polymerase II transcription systems (20,21). Thus, the nuclear system could be more active in recognizing a broader group of class II promoters with differing requirements for polymerase II factors, although this important point remains to be further investigated.

Transcription of class II genes in vitro has proved to be quite inefficient with respect to the number of transcripts synthesized per DNA template (2,8). Under our standard conditions of assay, only about 0.03 transcripts are synthesized per template (on average) when the adenovirus 2 major late gene is employed, a result that is in accord with the observations of others (2,8). However, if vector DNA (pBR322) lacking eukaryotic promoters is added at the standard DNA concentration (20 μ g/ml), a five-fold reduction in the concentration of the major late promoter gives the same signal as when the major late template is used alone at its optimal concentration; thus, while the amount of specific transcript does not increase, the efficiency with which the template is transcribed can be increased five-fold. It is also noteworthy that our transcription experiments are usually performed at GTP concentrations

that are considerably below the K_m of RNA polymerase II for this nucleotide (40 μM to 60 μM). In accord with this fact we have observed that increasing the GTP concentration from 25 μM to 100 μM increases transcription of the adenovirus 2 major late promoter about four-fold. Thus, while in vitro transcription systems for class II genes appear to be inefficient, this problem may be partly overcome by altering the conditions of assay.

The nuclear extract described here also supports the accurate transcription of tRNA and adenovirus VA genes, but is inactive for 5S gene transcription unless the S100 fraction or a partially purified preparation of TFIIIA is added. While the S100 obtained during the preparation of the nuclear extract is active in tRNA and VA transcription, it appears that a significant fraction of the activity for these genes remains in the nuclear extract. Although we do not as yet understand the basis for the partitioning of the 5S gene-specific component TFIIIA into the S100 fraction, it should prove advantageous for the purification of this protein from mammalian cells.

In conclusion, the nuclear-derived transcription system described here mediates the accurate transcription of a broad spectrum of class II (and class III) genes and appears promising as a system for the further analysis of eukaryotic transcription controls, including the isolation and characterization of various transcription factors.

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