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# 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

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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 $10^5$ 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 $10^9$ 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 $10^9$ cells.

## RESULTS

To assess the activity of extracts prepared under various conditions for

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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 µl (50 µg protein) and 25 µl (100 µg protein) of the 0.2 M NaCl extract. Lanes 3 through 6 show, respectively, assays of the 0.3 M NaCl extract (25 µl, 120 µg protein), the 0.42 M NaCl extract (25 µl, 140 µg protein), and the 0.5 M NaCl extract (25 µl, 150 µg protein). Lanes 7 and 8 show assays which contained, respectively, 25 µl (80 µg protein) and 12.5 µl (40 µg 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 µl 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.

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

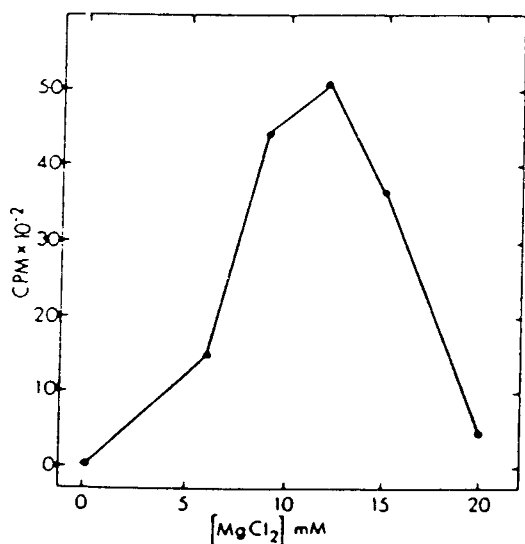


Fig. 3. $Mg^{++}$ optimum for transcription initiation at the Ad2 major late promoter. Extract (25 μl, 150 μg protein) prepared by the standard procedure was assayed in duplicate under standard conditions except that $Mg^{++}$ concentration was varied. Assays in lanes 1 through 6 contained, respectively,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

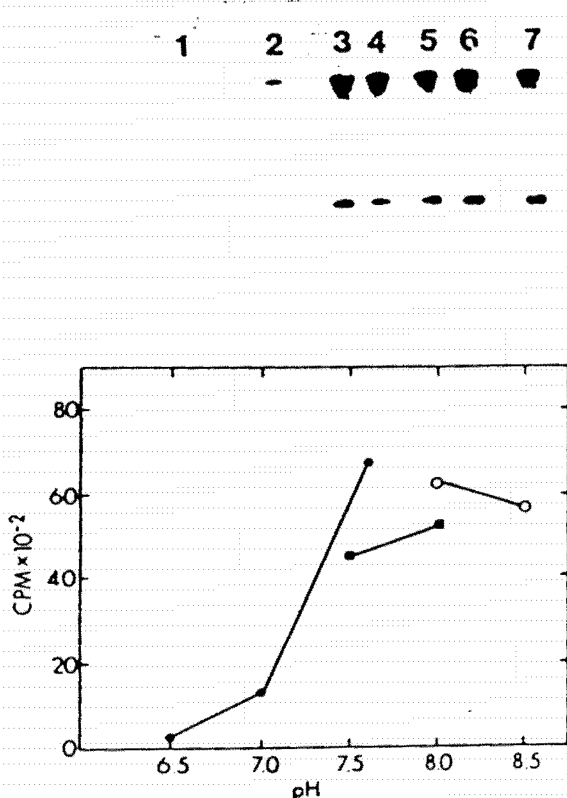


Fig. 5. pH optimum for transcription initiation at the Ad2 major late promoter. Aliquots of the standard extract were dialyzed against buffer D containing 20 mM PIPES (O—O), 20 mM HEPES ([]—[]), or 20 mM HEPPS (O—O). The pH indicated is that observed for the complete reaction mixture at 30°. Dialyzed extracts (25 μl, 150 μg 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 μg) 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 μg) without changing the intensity of the signal; without the addition of the PBR322 DNA, the signal from 0.2 μg 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

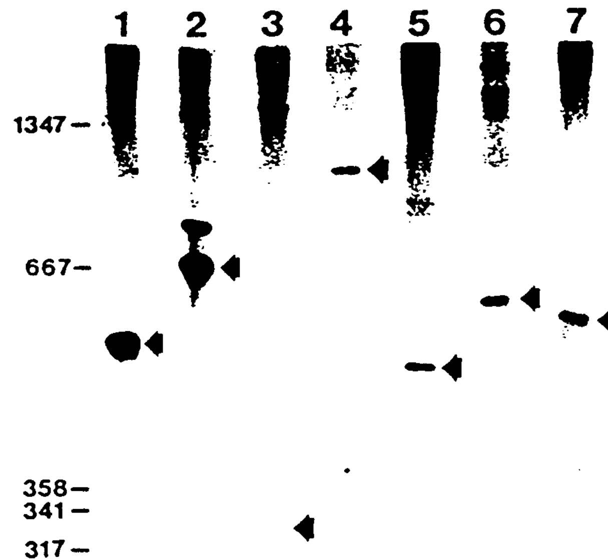


Fig. 7. Size analysis of in vitro synthesized transcripts obtained with several promoters. The standard extract (25 μl, 150 μg protein) was assayed under standard conditions except that the $Mg^{++}$ concentration was changed as indicated for each template. The assays shown contained: Lane 1, Ad2 major 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 Elb (lane 3), EIII (lane 4), Ela (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

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 μg/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

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