downstream of the cloning site in pSP6-R7 followed by a unique restriction site that can be used to linearize the template. Complete runoff transcripts of an insert can be selected from the products by chromatography on oligo(dT) cellulose columns. Development of such vectors should greatly facilitate the problem of obtaining large quantitities of RNA *in vitro* from defined genetic sequences.

Acknowledgments

Many of the methods described here have been worked out in collaboration with colleagues at Berkeley, both in our own and other laboratories. We are grateful for their contributions. We particularly thank Jon Narita and Daisy Roulland, for their studies on SP6 RNA polymerase, and Tom Kadesch and Steve Rosenberg for their work on the stability and DNA binding properties of *E. coli* RNA polymerase.

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[35] *In Vitro* Transcription: Whole-Cell Extract

By JAMES L. MANLEY, ANDREW FIRE, MARK SAMUELS, and PHILLIP A. SHARP

Three classes of nuclear DNA-dependent RNA polymerases have been identified in eukaryotic cells.[1] RNA polymerase I catalyzes the synthesis of rRNA precursors, RNA polymerase II transcribes primarily the genes that give rise to mRNA, and RNA polymerase III transcription results in the production of tRNAs, 5 S RNA, and several other RNAs of unknown function. It has been clear for many years that in order to study the mechanisms of transcription as well as to identify the factors and nucleotide sequences that control and regulate gene expression, cell free systems that accurately and specifically transcribe exogenously added DNA are required. Early attempts at achieving this aim, which utilized purified RNA polymerases, were unsuccessful. In the last several years, however, *in vitro* systems have been developed in which accurate transcription by all three types of RNA polymerases can be obtained. Two basic approaches have been successful. In one, purified RNA polymerase is supplemented with cell extracts that contain factors required for accurate transcription.[2] This method has been used primarily for RNA polymerase II-mediated transcription and is described in this volume [36]. The

[1] R. G. Roeder, *in* "RNA Polymerase" (R. Losick and M. Chamberlin, eds.), p. 285. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1976.
[2] P. A. Weil, D. S. Luse, J. Segall, and R. G. Roeder, *Cell* **18**, 469 (1979).

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other approach is to prepare concentrated cell lysates that contain not only the factors required for transcription, but also sufficient amounts of RNA polymerase so that addition of purified enzyme is not required. For RNA polymerase I[3] and III[4] such systems can be simply prepared from cytoplasmic extracts, because sufficient amounts of these polymerases and their required factors leak out of the nucleus at isotonic salt concentrations. RNA polymerase II, on the other hand, remains almost entirely within the nucleus. Thus, to obtain extracts containing this activity, a whole cell extract must be prepared.[5] We describe here the preparation and properties of such an extract, which contains all the factors and enzymic activities necessary for accurate and specific transcription, not only by RNA polymerase II, but also by RNA polymerases I and III.

Most experiments to date have utilized extracts prepared from human cells that grow in suspension culture (HeLa or KB). Such extracts show quite broad species specificities for RNA polymerases II and III. Polymerase III genes from virtually all higher eukaryotes that have been tested are accurately transcribed in HeLa lysates. Whole-cell extracts do not seem able to transcribe yeast polymerase II genes accurately, but have been shown to be capable of transcribing *Drosophila*[6] and chicken[7] polymerase II genes as well as many such genes from higher eukaryotes and their viruses.

Synthesis of mature RNA molecules requires additional enzymes and factors other than those needed to bring about accurate transcriptional initiation (e.g., processing enzymes). Soluble HeLa lysates appear to contain virtually all the enzymes required for tRNA processing, including the splicing enzymes.[8,9] Although work with RNA polymerase I systems is just beginning, soluble extracts appear to contain at least one processing enzyme.[10,11] RNA polymerase II transcripts synthesized *in vitro* are efficiently capped and methylated at their 5′ ends.[2,5] Although one report in the literature claims that an extract efficiently spliced RNA,[12] we have not

[3] I. Grummt, *Proc. Natl. Acad. Sci. U.S.A.* **78,** 727 (1981).

[4] G. J. Wu, *Proc. Natl. Acad. Sci. U.S.A.* **75,** 2175 (1978).

[5] J. L. Manley, A. Fire, A. Cano, P. A. Sharp, and M. L. Gefter, *Proc. Natl. Acad. Sci. U.S.A.* **77,** 3855 (1980).

[6] R. Morimoto, unpublished observations.

[7] B. Wasylyk, C. Kedinger, J. Corden, O. Brison, and P. Chambon, *Nature (London)* **285,** 366 (1980).

[8] D. N. Standring. A. Venegas, and W. J. Rutter, *Proc. Natl. Acad. Sci. U.S.A.* **78,** 5963 (1981).

[9] F. Laski, A. Fire, U. L. RajBhandary, and P. A. Sharp, unpublished observations.

[10] I. Grummt, E. Roth, and M. R. Paule, *Nature (London)* **296,** 173 (1982).

[11] K. G. Miller and B. Sollner-Webb, *Cell* **27,** 165 (1981).

[12] B. Weingartner and W. Keller, *Proc. Natl. Acad. Sci. U.S.A.* **78,** 4092 (1981).

observed in a variety of experiments either splicing or creation of polyadenylated 3′ termini in the cell-free system described here.

Methods

Preparation of Extract

Extracts are prepared by modification of a procedure originally described by Sugden and Keller,[13] who used the method as a first step in RNA polymerase purification. We have used HeLa cells almost exclusively. These cells are easy to obtain in large quantities, and the resultant extracts are relatively free of nuclease activity at 30°. Lysates with transcriptional activity have been prepared from a few other cells lines; most other cell lines and tissues, however, have yielded extracts without detectable levels of transcription or with high levels of nuclease. Cells are grown in suspension culture in Eagle's minimal essential medium supplemented with 5% horse serum to a density of 4 to 8 $\times$ 10^5 cells/ml. The cell density appears not to be crucial, although we have obtained slightly more active extracts with cells harvested at the lower end of the range indicated.

The following operations are carried out at 0–4°.

1. Cells are harvested by centrifugation and washed twice with phosphate-buffered saline.

2. The volume of the resultant cell pellet is determined, and the cells are resuspended in four packed-cell volumes (PCV) of 0.01 *M* Tris-HCl (pH 7.9), 0.001 *M* EDTA, and 0.005 *M* dithiothreitol (DTT) (6 $\times$ 10^8 cells yield approximately 2 ml of packed cells). At this point, the cells should visibly swell.

3. After 20 min, the cells are lysed by homogenization in a Dounce homogenizer with eight strokes using a "B" pestle.

4. Four PCV of 0.05 *M* Tris-HCl (ph 7.9), 0.01 *M* $MgCl_2$, 0.002 *M* DTT, 25% sucrose, and 50% glycerol are added, and the suspension is gently mixed. With continued gentle stirring, one PCV of saturated $(NH_4)_2SO_4$ is added dropwise. After this addition, the highly viscous lysate is gently stirred for an additional 30 min. Stirring must be very gentle to prevent shearing of the DNA, which would interfere with its removal in the next step. Nuclear lysis can be detected by increased viscosity after approximately half the $(NH_4)_2SO_4$ has been added. Occasionally, lysates appear clumpy and only slightly viscous, rather than extremely viscous

[13] B. Sugden and W. Keller, *J. Biol. Chem.* **248**, 3777 (1973).

and uniform as usually observed. We have obtained active extracts from both types of lysates, although more reproducibly from the latter.

5. The extract is carefully poured into polycarbonate tubes and centrifuged at 45,000 rpm in a SW 50.2 rotor for 3 hr.

6. The supernatant is decanted so as not to disrupt the pellet (the last 1 or 2 ml are left behind), and protein and nucleic acid are precipitated by addition of solid $(NH_4)_2SO_4$ (0.33 g/ml of solution). After the $(NH_4)_2SO_4$ is dissolved, 1 *N* NaOH [0.1 ml/10 g solid $(NH_4)_2SO_4$] is added and the suspension stirred for an additional 30 min.

7. The precipitate is collected by centrifugation at 15,000 *g* for 20 min (the supernatant should be completely drained off), and resuspended with 5% of the volume of the high-speed supernatant with 0.025 *M* HEPES (adjusted to pH 7.9 with NaOH), 0.1 *M* KCl, 0.012 *M* $MgCl_2$, 0.5 m*M* EDTA, 2 m*M* DTT, and 17% glycerol.

8. The suspension is dialyzed against two changes of 50–100 volumes each of the resuspension buffer for a total of 8–12 hr. The volume of the solution increases 30–50% during dialysis. The conductivity of a 1:1000 dilution of dialyzed extract into distilled H_2O (23°) should be 12–14 μmho.

9. The dialyzate is centrifuged at 10,000 *g* for 10 min to remove insoluble material. The supernatant is divided into small aliquots (0.2–0.5 ml), quick frozen in liquid nitrogen or powdered dry ice, and stored at −80°. Extract can be thawed and quick frozen several times without loss of activity and retains full activity at −80° for at least a year.

10. Lysates contain between 15 and 30 mg of protein per milliliter and up to 2 mg of nucleic acid per milliliter. We routinely start with 1–50 liters of cells. One liter of cells should yield about 2 ml of whole-cell extract (WCE), or enough for 100–400 assays. More concentrated extracts are desirable because with these the same optimal protein concentration can be obtained in reaction mixtures with a smaller volume of lysate. In this manner, the salt concentration in the *in vitro* reaction mixture can be lowered (high salt severely inhibits transcription; see below). Attempts to obtain more concentrated extracts by resuspending the pellet in a smaller volume after precipitation, or by tying the dialysis bag tightly (to reduce expansion during dialysis), have not been reproducibly successful owing to increased protein precipitation during dialysis. Likewise, dialysis against buffer containing lower salt concentrations results in less active lysates, again as a result of increased protein precipitation.

The Transcription Reaction

Reactions can be done in volumes of a few microliters or more. Analytical reactions are conveniently performed in 20 μl. A typical reaction mix might contain the following: 30–60% whole-cell extract in its dialysis

buffer, 0.2–1.5 μg of template DNA (see below for effects of DNA and extract concentrations), 50 μ*M* ATP, 50 μ*M* GTP, 50 μ*M* CTP, 5 μ*M* UTP, 5 m*M* creatine phosphate, and 10 μCi of [α-^{32}P]UTP [commercial preparations of aqueous nucleotides can be obtained at a high enough concentration (~10 mCi/ml) and a sufficient specific activity (>200 Ci/mmol) to be added directly to the transcription]. After incubation for 30–120 min, the reactions can be extracted directly or placed at −80° for up to a week before extraction.

Extraction of RNA and Resolution of Products by Gel Electrophoresis

To terminate transcription, 200 μl of stop buffer [7 *M* urea, 100 m*M* LiCl, 0.5% sodium dodecyl sulfate (SDS), 10 m*M* EDTA, 250 μg/ml tRNA, 10 m*M* Tris-HCl (pH 7.9)] and 300 μl of PCIA (phenol–chloroform–isoamyl alchohol, 1:1:0.05, water saturated and buffered with 20 m*M* Tris, pH 7.9) are added, the tubes are blended in a vortex mixer and centrifuged at 12,000 *g* for 15 min. The aqueous phase (discarding interface) is extracted once more with PCIA and once with chloroform and then pooled with 200 μl of 1.0 *M* ammonium acetate and precipitated with 900 μl of ethanol. The pellet is washed with ethanol and resuspended in 20 μl of 10 m*M* Na_2HPO_4 (pH 6.8)–1 m*M* EDTA; to this is added 50 μl of 1.4 *M* deionized glyoxal–70% dimethyl sulfoxide–10 m*M* Na_2HPO_4 (pH 6.8)–1 m*M* EDTA–0.04% bromophenol blue. After 1 hr at 50°, 25 μl of the samples are loaded on 1.4% agarose gels (run in 10 m*M* PO_4–1 m*M* EDTA).[14] This extraction procedure removes most of the free nucleotides from the RNA preparation.

For some techniques, larger reaction volumes are necessary. The above extraction protocol can be scaled up with modifications as follows: After removal of the first aqueous phase, three reaction volumes of stop buffer are added to the first organic phase, and the mixture is again homogenized and spun at 12,000 *g* (for 1 min). The organic phase is removed, and an equal volume of chloroform is added. After brief homogenization and centrifugation, the aqueous phase can be easily removed. The two aqueous phases are then pooled and reextracted once with PCIA, and twice with chloroform. Any precipitate at the interface of these extractions should be discarded. After the first ethanol precipitation, the pellet is resuspended in 200 μl of 0.2% SDS and 1 m*M* EDTA. An equal volume of 2 *M* ammonium acetate is added, and nucleic acid is reprecipitated with ethanol. The pellet is washed with ethanol and can be resuspended in the buffer of choice.

For analysis by hybridization and SI nuclease digestion,[15] it is impor-

[14] G. K. McMaster and G. C. Carmichael, *Proc. Natl. Acad. Sci. U.S.A.* **74,** 4835 (1977).
[15] P. A. Sharp, A. J. Berk, and S. M. Berget, this series, Vol. 65, p. 750.

tant first to remove the template DNA. The pellet is resuspended in 0.3 *M* sodium acetate (pH 5.2) and reprecipitated and washed with ethanol. The dried pellet is resuspended in 100 μl of 10 m*M* Tris-HCl (pH 7.5), and 100 m*M* NaCl. RNase-free DNase (treated with iodoacetate[16]) to 50 μg/ml, and $MgCl_2$ to 10 m*M* are added. After 5 min at 37°, 100 μl of 10 m*M* EDTA, 0.2% SDS, 150 m*M* NaCl are added, and the mixture is extracted with PCIA and chloroform. The final aqueous is reprecipitated with 0.25 ml of ethanol as above, and the pellet is resuspended in 60 μl of 0.2% Sarkosyl–1 m*M* EDTA (pH 8.0) and stored at −20°.

Sizing and Mapping *in Vitro* RNA

In general, RNA polymerase II does not terminate transcription *in vitro*. However, distinct length RNA products can be generated by the "runoff" assay. This method uses, as template, DNA molecules that have been cleaved by a restriction enzyme that cuts downstream from a putative transcription start site. RNA polymerases that transcribe this DNA will stop or fall off when they reach the end of the DNA. If a substantial number of enzymes initiate transcription at the same site, then a population of molecules of a discrete size will be produced; such a population will migrate as a band on gel electrophoresis. DNA segments that have been cleaved by different restriction enzymes are used as templates in separate reaction mixtures; the transcription start site can be deduced by comparison of the sizes of the RNAs produced. This technique has been widely used for promoter mapping with *in vitro* transcription systems.

An example of the technique is shown in Fig. 1. The RNAs were transcribed from recombinant plasmids containing the adenovirus late promoter and various segments of the long (30 kb) late transcription unit. Several points are exemplified by this experiment. The *in vitro* system is capable of synthesizing very long RNAs, up to 7–8 kb, and hence contains little nuclease activity. Also, the glyoxal method of analyzing RNA is sensitive over a wide range of sizes. Plots of log molecular weight vs migration are linear for transcripts from 0.2 kb to over 5 kb.

Analysis of runoff transcripts is a simple, sensitive, and accurate method for determining the structure of *in vitro* synthesized RNA. However, it does have some limitations. The WCE contains relatively high levels of nucleic acid. Since most of this is 18 S and 28 S rRNA, it is impossible to load more than 25–50% of the RNA obtained from a 20-μl reaction mix onto a standard size gel slot (6 mm × 3 mm) without producing severe overloading of the gel in the regions occupied by these

[16] S. B. Zimmerman and G. Sandeen, *Anal. Biochem.* **14**, 269 (1966).

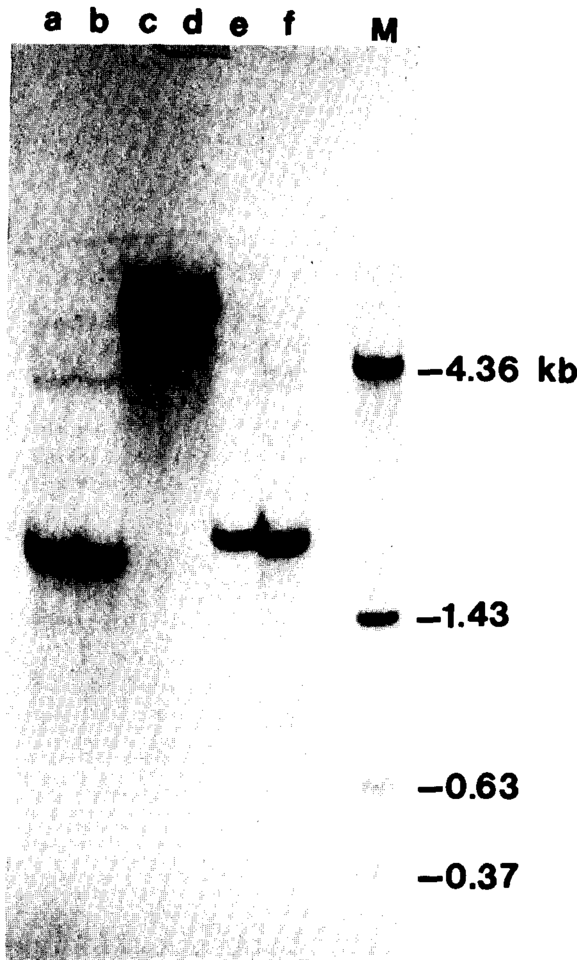

FIG. 1. Analysis of RNA runoff products by glyoxalation and agarose gel electrophoresis. Recombinant plasmids containing the adenovirus late promoter and various segments of the late transcription unit were constructed (R. Jove and J. L. Manley, unpublished), cleaved with restriction enzymes, and used as templates for *in vitro* transcription. DNAs were cleaved so that runoff transcripts of 1.8 kb (lanes a and b), 7.0 kb (lanes c and d), and 1.95 kb (lanes e and f) would be produced. Size markers (lane M) were also produced by *in vitro* transcription (see text).

RNAs. An additional problem is that specific transcripts produced by very weak promoters can sometimes be obscured by nonspecific initiation or termination, or by exogenous nucleic acids labeled by end labeling activity in the extract (particularly rRNA and its breakdown products). This

latter activity is insensitive to amanitin and actinomycin D and can thus be distinguished from *de novo* RNA synthesis. Use of radioactive GTP as tracer produces the least end labeling. Use of CTP or ATP produces high levels of tRNA labeling by enzymes exchanging the 3′-terminal CCA. A third limitation of analyzing runoff RNAs by denaturation and agarose gel electrophoresis is that end points of transcripts can be mapped only to within ~20 nucleotides at best. To map the 5′ ends of RNAs more precisely, short runoff transcripts can be analyzed on polyacrylamide sequencing gels.

The above analysis can be extended, and some of the problems circumvented, by using several variants on the technique of hybridization and SI nuclease digestion. By using labeled RNA and a nonradioactive DNA probe, the problem of spurious RNA labeling can be eliminated, since RNA not complementary to the DNA probe is destroyed by the nuclease. Use of nonradioactive RNA and a DNA probe 5′ end-labeled 50–200 nucleotides downstream from the promoter allows resolution of ± 1 nucleotide. The structure of the 5′ end of *in vitro* synthesized RNA can also be studied by classical RNA fingerprinting techniques.[2,5]

Transcriptional Activity

Extracts made from different cell preparations can vary in activity over a 5- to 10-fold range, with about two in three extracts exhibiting activity within 2-fold of the observed maximum. Extracts should be compared for their activity using a runoff assay from a standard polymerase II promoter, such as the major late promoter of adenovirus 2. With optimal DNA and extract concentrations, a good extract (20 μl) will yield 10^6 dpm, or 20 ng of a 2200 nucleotide runoff transcript from the Ad2 late promoter, in 1 hr ([α-^{32}P]UTP at 100 Ci/mmol). This represents the synthesis of one RNA molecule per 10 DNA template molecules present. However, the extract may actually be utilizing a smaller fraction of templates with multiple rounds of initiation per active template.

DNA and Extract Concentrations

Titrations both of DNA and of extract yield nonlinear responses. At a constant extract concentration, measuring runoff transcription as a function of DNA concentration yields (*a*) a threshold DNA concentration below which no transcription occurs; and (*b*) an inhibitory effect of high DNA concentration.[5] The requirement for a minimal DNA concentration is nonspecific; i.e., by using a concentration of a promoter specific DNA that is below the threshold, carrier DNA such as pBR322 or *Escherichia*

coli DNA can be added to stimulate specific transcription. The duplex alternating copolymers poly[d(I-C)]:poly[d(I-C)] and poly[d(A-T)]:poly [d(A-T)] will also act as carrier DNA, thereby demonstrating a total lack of sequence specificity in the bulk DNA requirement.[17] A further advantage of these copolymers as carrier DNA is that the transcribed RNA products of the carrier poly[d(I-C)]:poly[d(I-C)] and poly[d(A-T)]:poly[d(A-T)] contain only two nucleotides. Thus, poly[d(I-C)]:poly[d(I-C)] carrier in a reaction containing [α-^{32}P]UTP yields no radioactive background. The key aspect of bulk DNA dependence is that at a fixed total DNA concentration, the molar yield of transcripts per promoter is constant and independent of the source of carrier. In general, specific competition between promoter-containing fragments is not observed.

A critical dependence of transcription upon extract concentration is also observed.[5] In fact, DNA concentration dependence and extract protein concentration dependence are not independent.[18] Specific transcription can be obtained in a range of 4–18 mg of extract protein per milliliter. At low extract concentration the DNA optima tend to be much lower (in the range of 10 μg/ml). There is still a bulk DNA dependence, but it is less steep and the threshold concentrations are lower. At a high extract concentration the DNA titration becomes sharper, and the threshold becomes higher. Under such conditions it is often necessary to use 60 μg/ml of DNA in order to see any transcription. Thus, for each new extract it is necessary to do careful DNA and extract titrations, to determine optimal conditions.

For a given promoter, very short runoff transcripts ($<$300 n) have a higher optimum DNA concentration than longer runoff transcripts.[18] This effect can be taken into account by measuring the synthesis of different length runoff products from the same promoter. No length dependence has been observed with runoff products between 400 and 4000 nucleotides.

To further complicate matters, the ratio of activity from two promoters can vary as much as 20-fold over a range of DNA and extract concentration.[17–20] An example of this is shown in Fig. 2 where the relative activities of an early and a late Ad2 promoter are compared in an uninfected extract. The ratio of these activities varies 10-fold at different DNA concentrations. Comparison of promoter strengths in different extracts must thus be cautiously controlled and interpreted, a crucial point in assaying for regulatory phenomena.

[17] U. Hansen, D. J. Tenen, D. M. Livingston, and P. A. Sharp, *Cell* **27,** 603 (1981).
[18] A. Fire, C. C. Baker, J. L. Manley, E. B. Ziff, and P. A. Sharp, *J. Virol.* **40,** 703 (1981).
[19] D. Rio, A. Robbins, R. Myers, and R. Tjian, *Proc. Natl. Acad. Sci. U.S.A.* **77,** 5706 (1980).
[20] D. C. Lee and R. G. Roeder, *Mol. Cell. Biol.* **1,** 635 (1981).

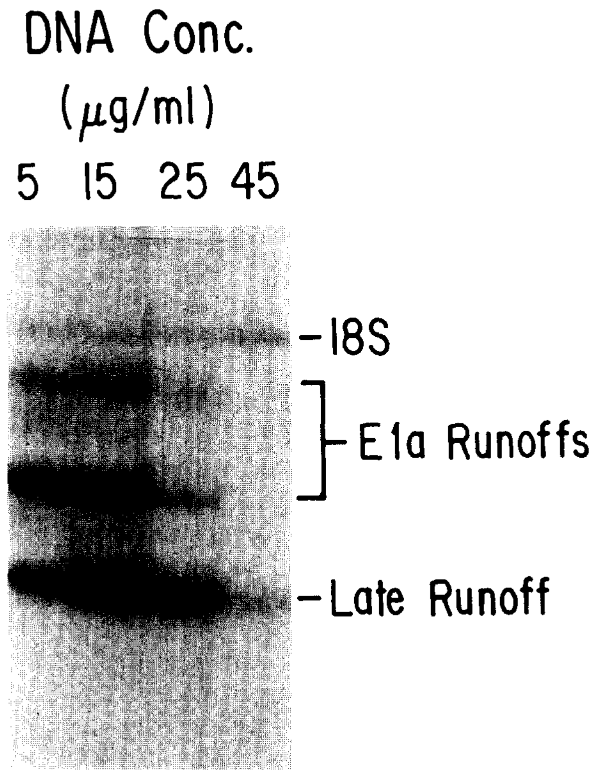

FIG. 2. Relative transcriptional activities of two adenovirus promoters as a function of bulk DNA concentration. Each reaction contained, per milliliter, 4 μg of a plasmid containing the adenovirus Ela promoter cleaved to give a 1220 n runoff and 1 μg of a plasmid containing the late promoter cleaved to give a 974 n runoff. Bulk DNA concentration was increased by addition of poly[d(I-C)]:poly[d(I-C)]. The transcription products were resolved on glyoxal gels as described above.

Reaction Conditions

One unusual feature of the WCE is the temperature dependence of the reaction. Transcription is routinely done at 30°, where the *in vitro* synthesized RNA product is stable for 8 hr. Increasing the temperature to 37° greatly enhances the rate of RNA degradation; RNA made at 30° is degraded within 10 min after shifting to 37°. Transcription assayed at 23° yields the expected Arrhenius effect.

Specific transcription in the WCE is highly sensitive to ionic strength. Concentrations of KCl and NaCl above 60 m*M* significantly inhibit the reaction; concentrations in the 30–40 m*M* range are optimal. Reactions can also be performed in 15–30 m*M* $(NH_4)_2SO_4$. The divalent cations

Ca^{2+}, Zn^{2+} and Mn^{2+} inhibit transcription and 0.5 mM EDTA is added to control their effect and the effect of other heavy metal contaminants. Reactions are done at pH 7.9, which is optimal for purified RNA polymerase II.[1]

Even after extensive dialysis, most WCEs seem to contain a free pool of 1 μM nucleotides.[18] The extract also contains creatine kinase (CPK) and other kinases and phosphatases so that the β and γ phosphates in nucleotides are labile.[21] For example, [γ-^{32}P]ATP will rapidly exchange label with other triphosphates. Label at the α position of the nucleotide triphosphates does not exchange in the WCE, thus allowing RNA to be uniquely labeled with ^{32}P in the α position of each triphosphate. Addition of 5 mM creatine phosphate to the reaction mix ensures charging of the triphosphates and allows reduction in triphosphate concentrations, thus permitting the use of higher specific activities.[21] Extracts from some cell lines tested lack CPK activity and the enzyme must be added exogenously to maintain nucleotide concentrations.[22] One must also recall that $(NH_4)_2SO_4$ inhibits creatine kinase. In the presence of creatine phosphate, concentrations of UTP, CTP, and GTP as low as 5 μM saturate specific transcription; higher concentrations (up to 500 μM) do not inhibit specific transcription.[21] Because of endogenous pools, the transcription reaction is not fully dependent on addition of these three nucleotides.[18] A higher concentration of ATP is required for optimal activity (50 μM); ATP concentrations above 500 μM inhibit the reaction.[21]

The dialyzed extract also contains sufficient *S*-adenosylmethionine (SAM) to methylate the 5′ ends of the *in vitro* transcripts.[5] Internal methylation has not been studied, however. Addition of exogenous SAM does not affect the reaction.[18,23]

Time Course

The rate of elongation in the WCE is approximately 300 nucleotides/min.[23] The rate *in vivo* is 10-fold higher, but one must recall the difference of 7° in temperature between the two. After DNA template, nucleotides, and extract are mixed, there is about a 5-min lag before specific transcription commences. The lag cannot be eliminated by preincubation of extract alone or with nucleotides, but is eliminated by preincubation of extract together with DNA (in the absence of nucleotides).[24] This suggests that the lag represents the time required for assembly on the DNA of fac-

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[22] N. Crawford, unpublished results.

[23] R. Jove, unpublished results.

[24] M. Samuels and A. Fire, unpublished results.

tors required for initiation. The rate of accumulation of runoff transcripts is approximately linear for over an hour after the initial lag period.[5]

Some Other Properties of the WCE

The preparation procedure for the WCE was originally designed for solubilization of RNA polymerase II from mammalian cells. A standard 20 μl reaction mix typically contains 2–3 units of RNA polymerase II.[5] Under optimal conditions, at most one in ten polymerase II molecules gives rise to a specific transcript in a 1-hr reaction. Supplementation with excess purified polymerase has no significant effect on the WCE.[22] Endogenous RNA polymerase II in the WCE is inhibited by α-amanitin at 0.5 μg/ml; addition of a purified mutant enzyme resistant to α-amanitin reconstitutes specific transcription.[6,24,25]

RNA polymerase II preferentially initiates transcription at the termini of DNA fragments and at internal nicks.[26] The enzyme is also capable of end labeling DNA fragments with [α-^{32}P]NTPs to yield full-length labeled molecules, which are resistant to RNase digestion.[24] These reactions are each sensitive to α-amanitin (0.5 μg/ml), and are suppressed *in vitro* by the addition of a 110,000-dalton ADP-ribosyltransferase that may blockade nicks and ends.[27] This 110,000 dalton protein is present in large quantities in the WCE (up to 0.1% of total WCE protein).

The WCE should contain most of the soluble proteins in the cell. Most of these are of no concern; however, some can interfere with interesting experiments. Most extracts have high levels of topoisomerase type I and II activities as well as DNA ligase. Thus DNA topologies can change rapidly in the reaction mix, preventing, for instance, studies of supercoiled DNA. The extract also contains RNA polymerases I and III.[13] Their contribution to the background pattern can be assessed with α-amanitin. Most template DNAs do not contain promoters for these enzymes, and their contribution to background incorporation is small. Some genomic clones contain dispersed repetitive elements, which often contain polymerase III genes.

Partial Fractionation of the WCE

A number of inhibitory activities can be removed by fractionation on phosphocellulose, yielding a more efficient transcription extract.[24,28]

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[26] M. K. Lewis and R. R. Burgess, *J. Biol. Chem.* **255**, 4928 (1980).
[27] E. Slattery, J. D. Dignam, and R. G. Roeder, unpublished results.
[28] T. Matsui, J. Segall, P. A. Weil, and R. G. Roeder, *J. Biol. Chem.* **255**, 11992 (1980).

In Vitro TRANSCRIPTION STUDIES USING A WHOLE-CELL EXTRACT (WCE)

Template	Comments	References*
Adenovirus	Almost all *in vivo* promoters are recognized *in vitro*	2, 5, 7, 12, 18, 20
	Detailed 5′ terminal analysis, dependence on upstream sequences	18, 20, *a–d*
	Inactivation of transcription in WCEs of poliovirus-infected cells	*e*
	Changes in transcriptional pattern in WCEs of adenovirus-infected cells	18
Globins	α-Globin and β-globin genes are recognized *in vitro*, dependence on upstream sequences	*f–i*
	Mutant α- and β-thalassemia globin genes are transcribed *in vitro*	*g*, *j–l*
SV40	Early and late promoters are recognized *in vitro*	19, 21
	Detailed 5′-terminal analysis of RNA from early promoter	17, *m*, *n*
	In vitro inhibition of transcription by T antigen	17, 19, *o*
	Cell-free translation of *in vitro* synthesized RNA	*p*
Conalbumin and ovalbumin	Promoters are recognized *in vitro*, dependence on upstream sequences, effects of altering TATA box	7, *a*, *q*, *r*
	Transcription in a homologous system	29
Type C retroviruses	Promoters in the long terminal repeat (LTR) of several RNA tumor viruses are recognized *in vitro*, dependence on upstream sequences	*s–u*
Fibroin	Promoter is recognized *in vitro* by WCEs of HeLa cells and of silk worm glands, dependence on upstream sequence in both extracts	*v*, *w*
Herpes simplex virus	Early promoters are recognized *in vitro* in uninfected cell WCEs	*x*

Template	Comments	References*
Histone H2A	Promoter is recognized *in vitro*, dependence on upstream sequences using linear or circular DNA template	*y*
Adeno-associated virus	Identification of a new promoter, detailed 5′ terminal analysis	*z*

* *Key to references:* Numbers refer to text footnotes. Letters refer to the following.

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e. N. Crawford, A. Fire, M. Samuels, P. A. Sharp, and D. Baltimore, *Cell* **27**, 555 (1981).

f. D. S. Luse and R. G. Roeder, *Cell* **20**, 691 (1980).

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After dialysis to remove Mg^{2+} and dilution to 40 m*M* KCl, a WCE is chromatographed on phosphocellulose (Whatman P-11) yielding a breakthrough fraction, and two higher salt washes (0.35 and 1.0 *M* KCl). Reconstitution of the breakthrough and the dialyzed 1.0 *M* wash with purified RNA polymerase II in optimal ratios yields a mixture capable of specifically transcribing DNA at 10 times the efficiency of the original WCE. Tsai *et al.* have used a similar protocol to remove inhibitors from an extract of hen oviduct.[29]

Summary of Results

Since the first demonstration of transcription by RNA polymerase II in a soluble system and development of the WCE procedure a number of investigators have studied transcription of promoters using these systems. The preceding table represents a moderately comprehensive listing of such studies.

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[36] Eukaryotic Gene Transcription with Purified Components

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In eukaryotic organisms the large ribosomal RNAs, messenger RNAs, and some low molecular weight RNAs (5 S RNA, tRNA, and some small viral RNAs) are transcribed by three structurally and functionally distinct enzymes, RNA polymerases I, II, and III, respectively (see reviews [1-3]). Although these enzymes are structurally complex, highly purified preparations of RNA polymerases I, II, and III are unable to execute accurate

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