The virus-associated (VA) RNA genes in human adenovirus 2 DNA have been shown by Wu (Wu, G. J. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 2175-2179) to be transcribed by RNA polymerase II in a human KB cell-free extract. In the present report we have examined the fidelity of transcription of adenovirus 2 DNA and *Xenopus* oocyte 5 S DNA templates by RNA polymerase III. The extracts derived from cultured human, murine, and amphibian kidney cells. Size and sequence analysis of the discrete transcripts synthesized in these homologous and heterologous systems indicate that they result from accurate transcription of the corresponding genes. The specific transcripts identified include both the adenovirus VA RNA, and VA RNA, *Xenopus* 5 S RNA, and VA RNAI and 5 S RNA species with elongated 3' termini. The extracts derived from the various cell types differ in their ability to discriminate between the two VA RNA genes or between the heterogeneous 5 S RNA genes in the cloned DNA fragment. Whereas the human cell extracts transcribe the VA RNA, and VA RNAI genes of adenovirus at a relative frequency close to that observed in isolated nuclei, the amphibian cell extract appears to transcribe only the VA RNA, gene. The amphibian cell extract transcribes primarily that 5 S RNA gene (within 5 S DNA) which encodes the dominant oocyte 5 S RNA, whereas the human cell extract transcribes at least two distinct 5 S RNA genes. Additionally, it is shown that the VA RNA, and VA RNAI genes have separate promoter sites. The kinetics of the transcription reactions have been examined and conditions optimal for specific transcription have been established by examining the effects of salt, metal ion, and template concentrations on both total and specific RNA synthesis. It is also shown that components in the cell-free extract (from human cells) are active in directing the accurate transcription of adenovirus DNA by purified RNA polymerase II.

Genes which are transcribed by class III RNA polymerases in eukaryotic cells (reviewed in Ref. 1) include the reiterated 5 S and tRNA genes (2, 3), genes encoding some small nuclear RNAs (4), and two adenovirus VA RNA genes expressed in productively infected human cells (5-7). Of these, the 5 S RNA genes expressed in *Xenopus* oocytes (8) and the adenovirus VA RNA genes have been extensively characterized and provide attractive systems for investigating the mechanism of selective gene transcription by RNA polymerase III. *Xenopus* oocyte 5 S DNAs (from both *X. laevis* and *X. borealis*) have been cloned and sequenced, providing detailed information regarding gene-spacer arrangements and presumptive promoter and terminator sequences (9, 10). Similarly the adenovirus 2 VA RNA gene and flanking regions have been sequenced (11, 12) and the VA RNAI gene has been shown to lie about 75 nucleotides upstream from the VA RNA, gene (13). Both the mature 5 S RNAs (14, 15) and the VA RNAs (13, 16, 17) are simple and well characterized and appear to be derived from primary gene transcripts without processing of the 5' termini. (Although the possibility of processing at the 3' termini is suggested by the demonstration of distinct 5 S RNA gene (14, 15) and VA RNA, gene (18) transcripts with extended 3' termini, it has not yet been demonstrated that these transcripts are obligatory precursors to the corresponding mature RNAs.)

The class III RNA polymerases from mature *X. laevis* oocytes (19-21) and from both uninfected and adenovirus 2-infected human KB cells (22) have been isolated and characterized. However, these enzymes show no transcriptional specificity with respect to the 5 S or VA RNA genes when the corresponding purified DNAs are used as templates. In contrast, the 5 S RNA genes in chromatin from immature oocytes and the VA RNA, gene in nuclear templates from adenovirus-infected cells are accurately and selectively transcribed by purified class III RNA polymerases but not by bacterial or eukaryotic class I or II RNA polymerases (21, 22-26). These findings have led to the conclusion that both RNA polymerase III and chromatin or nuclear template-associated components are necessary for the faithful transcription of these genes.

Further attempts to determine the nature and mechanism of action of the various transcription components necessary for selective transcription have emphasized the development of systems in which purified DNA templates can be accurately transcribed. In the case of the amphibian 5 S genes, it has been shown that both genomic and cloned (15, 27) 5 S DNA will direct the accurate synthesis of 5 S RNA when microinjected into oocytes, thereby establishing the functionality of these purified DNAs. Very recently the cloned 5 S genes have been shown to be accurately transcribed by RNA polymerase
III present in extracts derived either from oocyte nuclei or from whole oocytes (28, 29). In the latter case an RNA polymerase-free fraction from immature oocytes was also shown to direct the accurate transcription by purified RNA polymerase III of 5 S RNA genes in a cloned 5 S DNA fragment. Similarly, the VA RNA gene in purified adenovirus DNA has been shown to be transcribed by an RNA polymerase III in extracts derived from cultured KB cells (30).

In the present report we have further analyzed the transcription by cell-free extracts of the two adenovirus 2 VA RNA genes and the Xenopus oocyte 5 S RNA genes in purified DNA templates. We show that these genes (including both VA RNA genes) are accurately transcribed by class III RNA polymerases in crude systems derived from a variety of homologous and heterologous cell types. Additionally the reaction conditions essential for the demonstration of specific transcription are defined.

EXPERIMENTAL PROCEDURES

Cell Culture, Virus Infection and DNA Purification—Mouse plasmacytoma 460 cells were grown in suspension culture as described (24). Conditions for growth of KB cells, infection with adenovirus 2, and extraction of viral DNA replication were as described previously (25). An X. laevis cell line, derived originally from adult kidney tissue (31) was grown in glass roller bottles in Lebovitz L-15 medium (diluted with an equal volume of water) supplemented with 10% fetal calf serum, penicillin and streptomycin.

Plasmid DNA Propagation and Purification—The recombinant DNA plasmids used contain HindIII fragments of Xenopus 5 S DNA inserted at the HindIII site in the plasmid pMB9 (10, 15, 20). The plasmid pXbo3 contains a 2700 base pair fragment of X. borealis oocyte type (Xbo) 5 S DNA with six 5 S RNA genes. The other plasmid used (pMB9XIS6) contains a four repeat unit fragment of X. laevis oocyte type (Xlo) 5 S DNA. These recombinant DNA plasmids were obtained from D. D. Brown (Carnegie Institution of Washington, Baltimore). Growth and handling of plasmid DNA has been carried out according to National Institutes of Health guidelines (P2 EK1). Supercoiled plasmid DNAs were purified as described (33) and pXbo3 DNA was provided by Dr. C. Parker of this institution.

RNA Polymerase III Isolation—KB cell RNA polymerase III (a mixture of forms IIIa and IIIb) was partially purified by a modification of the procedure described previously (20). One unit of activity catalyzes the incorporation of 1 pmol of UMP into RNA in 20 min under optimal conditions (34) using commercial calf thymus DNA as template.

Preparation of Cell Extracts—Crude cellular extracts from cultured human KB, MOPC 480, and X. laevis kidney cells were prepared essentially as described (35) with slight modifications as indicated. Cells were harvested and washed with phosphate-buffered saline, and the packed cell volume was measured after centrifugation at 800 g for 5 min. The cells then were washed with hypotonic buffer (10 mM Hepes (pH 7.9), 1.4 mM MgCl₂, 0.5 mM dithiothreitol), and the cell pellet was resuspended in twice the packed cell volume of hypotonic buffer. The cells were allowed to swell 10 min on ice and then were disrupted in a Kontes all glass Dounce homogenizer by five strokes of the D Pestle. The extent of cell lysis was generally 90 to 95% as monitored by light microscopy after staining with toluidine blue. Nuclear lysis was minimal. One-tenth volume of a solution containing 0.3 M Hepes (pH 7.9), 1.4 M KCl, 0.03 M MgCl₂, was added and the lysate was centrifuged at 100,000 × g for 40 min.

The supernatant from this centrifugation, containing approximately 7.5 × 10⁶ cell equivalents/ml of extract, was used for all experiments. The mammalian cell extracts contained about 600 units of RNA polymerase III activity/ml (measured under standard conditions with a calf thymus DNA template). This level of activity approximates the previously determined levels of RNA polymerase III measured in mammalian cell lines (32, 34). The protein concentration in these extracts averaged about 7 mg/ml.

In Vitro Synthesis and Electrophoretic Analyses of RNA—The final volume of the standard RNA synthesis reaction was 50 μl and contained: 25 μl of either [α-32P]GTP or [γ-32P]UTP (2.5 to 12.5 Ci/mmol), a 600 μM concentration of each of the three unlabeled ribonucleoside triphosphates, 10 mM Hepes (pH 7.9), 0.5 mM dithiothreitol, and the concentrations of KCl, MgCl₂, DNA, and cell-free extract indicated below. Reactions were initiated by the addition of the extract and were incubated at 30°C for 60 min. For adenovirus 2 DNA directed in vitro transcription, the standard reaction contained 65 to 80 mM KCl, 5.0 to 7.5 mM MgCl₂, 30 to 40 μg of DNA/ml, and 15 to 20 μl of extract. With pXbo3 plasmid DNA as template the standard reaction contained 65 to 80 mM KCl, 2.5 mM MgCl₂, 17 to 20 μg of DNA/ml, and 15 to 25 μl of extract.

Preparative scale reactions for fingerprint analyses were performed as described above except that the reaction volumes were 1 ml and the specific activity of the labeled ribonucleoside triphosphate was 25 Ci/mmol. In each case the reaction was terminated by treatment with electrophoretically purified DNAase I (100 μg/ml) for 5 min at 30°C. Following the addition of sodium dodecyl sulfate to 0.5% and carrier tRNA to 500 μg/ml small aliquots of the reaction were spotted on DE-81 filter discs (19) to determine total RNA synthesis. RNA was extracted by the phenol/chloroform method of Palmer (36) and precipitated from the final aqueous phase with ethanol (37). Recoveries of RNA averaged 70 to 100%.

Following precipitation with ethanol, RNA were dissolved in 50% formamide and heated for 5 min at 80°C. After the addition of sucrose and bromophenol blue the samples were subjected to electrophoresis on 12% polyacrylamide (crosslinked with N,N'-methylenebisacrylamide) slab gels (6). Analytical gels were dried under vacuum and subjected to autoradiography after staining with ethidium bromide followed by X-ray film. Autoradiographs were developed using Fuji-Rapid Lightning-Plus intensifying screens. To determine the radioactivity in specific RNA species, the autoradiographs were used as templates to cut out the appropriate gel bands. The gel slices then were reconstituted with 200 to 300 μl of H₂O and radioactivity was measured by Cerenkov counting. With preparative gels, the RNA species were visualized by autoradiography of the wet slabs covered with Saran wrap.

Fingerprint Analysis of RNAs—RNAs were eluted from gel slices, purified on DEAE-cellulose columns, and digested with T. ribonuclease (38). Oligonucleotides were fractionated by ionophoresis on cellulose acetate (first dimension) and DEAE-cellulose (second dimension) by the method of Sanger and Brownlee as described elsewhere (18).

All oligonucleotides were identified by position only.

RESULTS

Synthesis of Distinct RNA Species by RNA Polymerase III in Response to Purified DNA Templates

In the transcription experiments reported here we have used as templates purified adenovirus 2 DNA and a bacterial plasmid (pXbo3) containing inserts of X. borealis oocyte-type 5 S DNA. The latter template contains several 5 S RNA genes and is described in more detail in a subsequent section. The cell-free extracts employed for these experiments were derived by high speed centrifugation (100,000 × g) of a cytoplasmic fraction prepared from cultured cells essentially according to the method of Wu and Zubay (35). These extracts contain significant levels of RNA polymerase III activity (see "Experimental Procedures") consistent with the previous demonstration that cytoplasmic fractions prepared by a variety of means from mammalian tissue or cultured cells contain the bulk (65 to 90%) of the cellular RNA polymerase III (24, 25, 34). As discussed previously (see also Ref. 1) much of the RNA polymerase III, and likely other components as well, may leak from the nucleus during cellular fractionation. In the following sections it is shown that such cell-free extracts prepared from cultured human KB, mouse plasmacytoma, and amphibian kidney cells actively and selectively transcribe specific genes within the adenovirus 2 and pXbo3 DNA templates. The RNA products of the transcription reactions carried out in the presence of [α-32P]GTP or [γ-32P]UTP were first analyzed by polyacrylamide gel electrophoresis and autoradiography to identify putative 5 S and VA RNA gene transcripts. The authenticity of these transcripts was then verified by fingerprint analysis.

Transcription in Extracts from Human KB Cells An electrophoretic analysis of the RNAs synthesized with extracts...
from human cells is shown in Fig. 1. Incubation with adenovirus 2 DNA (Lane 1) results in the synthesis of a major transcript designated VAI, which co-migrates with a VAI (VA RNA\textsubscript{r}) and VAI\textsubscript{i} (VA RNA\textsubscript{i}), as well as several other low molecular weight transcripts, are also resolved. (The VA RNA\textsubscript{r}, VA RNA\textsubscript{i}, and VA RNA\textsubscript{II} transcripts are more readily visualized in other experiments presented below.) The synthesis of lesser amounts of VA RNA\textsubscript{r} and VA RNA\textsubscript{i}, relative to visualized in other experiments presented below.) The synthesis of these RNA species is dependent upon the presence of the exogenous DNA templates (data not shown here; see Lane 1 in Fig. 10 below) and, in the presence of the template, is abolished by the addition of actinomycin D to 160 µg/ml (data not shown).

When reactions are performed in the presence of 1 µg of α-amanitin/ml, a concentration of toxin which specifically and completely inhibits RNA polymerase II activity (34), the extent and nature of RNA synthesis is essentially unchanged (Fig. 1, Lanes 2 and 5). However, when the reactions are performed in the presence of 200 µg of α-amanitin/ml, a concentration of toxin at which only RNA polymerase I remains active, the synthesis of the various RNA species is dramatically reduced (Fig. 1, Lanes 3 and 6). Hence, an RNA polymerase III activity present in the KB cell-free extracts is responsible for the synthesis of the VA RNA and 5 S RNA species. These results confirm and extend the studies by Wu (30) which demonstrated the synthesis of VA RNA, by RNA polymerase III on the VA RNA\textsubscript{r} and VA RNA\textsubscript{i} genes. Additionally they show that each gene has a separate entry site for the RNA polymerase.

Transcription in Extracts from Mouse Plasmacytoma Cells—The transcripts observed when the adenovirus 2 DNA and pXbo3 templates are incubated with extracts from cultured mouse plasmacytoma cells (MOPC 460) are shown in Fig. 1 (Lanes 7 to 13). In the experiment shown, a single discrete putative VA RNA\textsubscript{r} transcript is observed in response to adenovirus DNA (Lane 7), although putative VA RNA\textsubscript{r} and VA RNA\textsubscript{i} species have been observed in other experiments with the MOPC extracts. With the pXbo3 template a single discrete 5 S RNA species is observed (Lane 11), although minor species with mobilities slightly less than the 5 S RNA are observed (Lanes 12 and 13).
S RNA species (cf. results with KB extracts) have been observed in some experiments (data not shown). The synthesis of these RNA species is again dependent upon the exogenous DNA templates (Lane 10) and in all cases is mediated by an RNA polymerase III activity (Lanes 7 to 9 and 11 to 13). As is evident from the data in Fig. 1, the relative efficiencies of transcription of the adenovirus VA RNA genes and the pXbo3 5 S RNA genes differ for the extracts prepared from the human and the murine cell types. The possible significance of this is not yet understood, particularly since there is considerable variability in the quality of the MOPC extracts.

Transcription in Extracts from Amphibian Kidney Cells—The gel analyses presented in Fig. 3 show the transcripts synthesized when the adenovirus 2 DNA and pXbo3 templates are transcribed in extracts from a X. laevis kidney-derived cell line. Adenovirus DNA directs the synthesis of the VA RNA1 species. Repeated experiments have, however, failed to reveal synthesis of either VA RNA1 or VA RNAII. The pXbo3 template directs the synthesis of a 5 S RNA and a minor RNA with a reduced mobility. Similar results are observed with a plasmid containing a fragment (4 repeat units) of X. laevis oocyte 5 S DNA (Lane 7). In this case, however, two distinct species other than the 5 S species are observed. As described above for the KB and MOPC extracts, experiments with α-amanitin indicate that all of the discrete VA and 5 S RNA transcripts are also synthesized by RNA polymerase III in the amphibian extract (cf. Lanes 1 to 3, 4 to 6, and 7 to 9).

Additionally, none of the transcripts are synthesized in the absence of DNA (Lane 10) or in the presence of the plasmid vector alone (Lane 11).

Fingerprint Analysis of Adenovirus 2 DNA and pXbo3 Transcripts

To verify the identity of the presumptive VA RNAs and 5 S RNAs synthesized in the cell-free extracts, the transcripts have been further characterized by fingerprint analysis of RNase T1 digests. For these experiments the reaction was scaled up and the labeled transcripts were resolved on preparative polyacrylamide gels. The RNA species to be fingerprinted were eluted from the gels and digested with RNase T1, and the resulting oligonucleotides were subjected to a standard two-dimensional ionophoresis fractionation.

Adenovirus 2 Transcripts Synthesized in KB Cell Extracts—The VA RNAII, VA RNAI, and VA RNAI [α-32P]GTP-labeled transcripts synthesized in the KB cell-free extract are well resolved by preparative polyacrylamide gel electrophoresis, as shown in the autoradiograph presented in Fig. 4A (Lane 2). The oligonucleotide patterns for these RNAs are shown in Fig. 5. The fingerprint pattern of VA RNAI (Fig. 5, Panel A) is precisely that expected from the published patterns of the VA RNAI (5.5 S RNA) synthesized in intact cells (11, 13) and the VA RNAI (previously designated V&RNA) synthesized in nuclei from adenovirus-infected cells (18). All of the labeled oligonucleotides are present in the expected molar ratios (data not shown). This includes Spot 51 which is the first unique RNase T1 digestion product from the 5' terminus of VA RNAI. The 3'-terminal oligonucleotide (Spot 45) is not observed since it is not labeled with [α-32P]GTP. However, it is observed when the VA RNAI is labeled with [α-32P]CTP (data not shown).

The VA RNAII synthesized in nuclei from adenovirus infected cells has been shown to be a 3' extended transcript of the VA RNAI gene (18). The fingerprint pattern of VA RNAII synthesized in the cell-free extract is shown in Fig. 5B. Except for Spot 10′ and two additional spots (designated H1 and H2) near the origin of the second dimension, the pattern is, as expected, nearly identical to that of VA RNAI. H1 and H2 correspond, respectively, to Spots 1 and 4 in the published homochromatography patterns of VA RNAII (18). These represent the two uridylic acid-rich RNase T1 oligonucleotides resulting from read-through of the first 15 nucleotides beyond the expected termination site of VA RNAI. Spot 10′ represents the trinucleotide expected from read-through of sequences beyond those encoding H1 and H2. In addition, fingerprints of [α-32P]CTP-labeled VA RNAII synthesized in this system show the absence of the oligonucleotide 45, which is the 3'-terminal oligonucleotide in VA RNAI (data not shown). Hence, the

![Fig. 3](left). Polyacrylamide gel electrophoresis of RNAs synthesized by an X. laevis kidney cell-free extract with purified DNA templates. Reaction conditions were standard except that the α-amanitin concentrations were 0 (Lanes 1, 4, 7, and 10), 0.24 μg/ml (Lanes 2, 5, and 8), 0.40 μg/ml (Lanes 3, 6, and 9). The DNAs added were adenovirus 2 (Lanes 1 to 3), pXbo3 (Lanes 4 to 6), pMB9X3S (Lanes 7 to 9), pMB9 (Lane 10), or none (Lane 11). A 5 S RNA marker labeled in cultured kidney cells is shown in Lane 12. The specific activity of the [α-32P]CTP label was 11,820 cpm/pmol and the levels of total incorporation were 1.27, 0.92, 0.22, 0.89, 0.97, 0.26, 0.83, 0.72, 0.23, 0.21, and 0.07 pmoles for Lanes 1 to 11, respectively. Fig. 4 (right). Preparative polyacrylamide gel electrophoresis of RNAs synthesized in cell-free extracts for fingerprint analyses. RNAs were synthesized under standard conditions in scaled up reactions in the presence of [α-32P]GTP and subjected to electrophoresis as described under “Experimental Procedures.” Panel A, RNAs synthesized in a KB cell-free extract with either pXbo3 (Lane 1) or adenovirus 2 DNA (Lane 2) as templates. Panel B, RNAs synthesized in the amphibian cell-free extract with pXbo3 DNA as template.
Fingerprint analyses of RNAs synthesized in a KB cell-free extract in response to adenovirus 2 DNA. The RNAs analyzed in Panels A, B, and C correspond, respectively, to the VA, VA, and VA RNAs indicated in Panel A, Lane 2, of Fig. 4. Analyses were performed as described under "Results" and "Experimental Procedures."

The fingerprint pattern of the putative VA RNA synthesized in the KB cell-free extract is identical to that of the VA RNA (formerly designated VZOO) species synthesized in isolated nuclei (18). Since the electrophoretic mobilities of these two RNA species are identical, it is probable that the molecule synthesized in the present system also results from transcription termination within the stretch of six deoxythymidylic acid moieties located 40 nucleotides beyond the expected termination site of VA RNA (18).

The RNA eluted from the VA RNA band (Fig. 4A) was rerun on a formamide gel under denaturing conditions. The VA RNA migrated as a single electrophoretically pure species in this system (18). The fingerprint analysis of this RNA, shown in Fig. 5C, is almost identical to that published recently for the VA RNA synthesized in vivo (13). The only difference is the altered mobility of Spot 19 (ApApApCpApGp). Most likely, the composition of the Spot 19 in our fingerprint is identical to that reported by Mathews and Pettersson (13) since, in our hands, some lots of DEAE-cellulose cause a slight retardation in the mobility of larger T1 digest products composed only of A, C, and G. However, we have not yet ruled out the unlikely possibility that the Spot 19 observed here actually contains an additional one or two adenyllic acid moieties.

pXbo3 Transcripts Synthesized in KB Cell Extracts—The Xbo 5 S DNA fragment present in pXbo3 is 2700 base pairs in length and contains six gene sequences (15, 32). This particular fragment has not yet been sequenced but another cloned fragment (Xbol) of Xbo 5 S DNA has been shown to contain one gene whose sequence corresponds exactly to that of the major oocyte 5 S RNA and two additional genes which differ in sequence by 2 and 15 nucleotides, respectively (10). Similar heterogeneity in genomic (uncloned) 5 S gene sequences has been demonstrated (10). At least some of the heterogeneous Xbo gene sequences are transcribed both in intact cells and in cell-free systems (10, 39).

The oligonucleotide patterns of the major 5 S and minor 5 S' RNA species (Lane 1 in Fig. 4A) synthesized in the KB cell-free extract are shown, respectively, in Panels A and B of Fig. 6. Each of these patterns shows all of the oligonucleotides present in fingerprints of the dominant Xbo 5 S RNA synthesized in oocytes. These are denoted with numbers according to the nomenclature used by Brown and Gurdon (27). Only one oligonucleotide, Spot 15, is present in a molar ratio much less than that predicted from the known sequence of the Xbo 5 S RNA (39). This could be due to the marked susceptibility of this oligonucleotide to overdigestion, especially when in vitro transcripts are analyzed (27).

The 5'-terminal nucleotide (pppGp) expected in the primary transcript (40) has not been identified in these experiments. Presumably it would have migrated off the cellulose acetate strip in the first dimension under the conditions employed. However, trace levels of pGp, the major 5'-terminal nucleotide in stable oocyte 5 S RNA, is detected in most cases (see spot just above and to left of Spot 8 in Fig. 6B). The unique RNAse T1 oligonucleotides closest to the 5' terminus (Spot 13, residues 2 to 7) and to the 3' terminus (Spot 10, residues 114 to 116) are present in the expected stoichiometries in both the 5 S and the 5 S' RNA fingerprints. However, the 5 S' pattern shows an additional uridylic acid-rich oligonucleotide at the origin of the second dimension (denoted by the arrow in Fig. 6B). Most likely the occurrence of this oligonucleotide is indicative of read-through of the expected 3' termination site and a continuation of transcription down to the second cluster of four deoxynucleotides (10, 27).

Both the 5 S and the 5 S' RNA fingerprint patterns exhibit a subset of oligonucleotide spots which do not correspond to the expected sequence of the dominant Xbo 5 S RNA. These oligonucleotides are considerably more prevalent in the fingerprint pattern of the major (5 S) transcript (Fig. 6A) and are denoted by capital letters. Most of these additional oligonucleotides appear to correspond to the unique sequences found in the third 5 S RNA gene of the cloned Xbo1 DNA fragment (10; see discussion above). These data suggest that the predominant 5 S band is composed of a minimum of two different transcripts of the six potential 5 S DNA sequences...

B. Harris, unpublished observation.
Faithful In Vitro Transcription by RNA Polymerase III

FIG. 6. Fingerprint analyses of RNAs synthesized in KB and amphibian cell-free extracts in response to pXbo3. The RNAs analyzed in Panels A and B correspond, respectively, to the 5 S RNA and 5’ S RNA synthesized in the KB cell-free extract (Lane 1 of Panel A in Fig. 4). The RNA analyzed in Panel C corresponds to the 5 S RNA synthesized in the amphibian cell-free extract (Panel B in Fig. 4).

present in the cloned Xbo3 fragment. The majority of these transcripts originate from 5 S sequences which are very similar or identical to the dominant oocytic type 5 S RNA, whereas approximately 30% of the transcripts appear to originate from the divergent 5 S DNA sequence(s) described earlier. It is interesting that the minor 5 S’ RNA species contains considerably lower molar yields of these additional oligonucleotides, suggesting either a more efficient termination at the end of the more divergent sequence 5 S gene(s) or a more efficient processing of transcripts of this gene(s).

Adenovirus 2 DNA and pXbo3 Transcripts Synthesized in MOPC Cell Extracts—The putative VA RNA, VA RNA₂, and VA RNA₃ species synthesized in the presence of adenovirus 2 DNA in MOPC cell-free extracts have also been subjected to fingerprint analysis. In each case the oligonucleotide patterns were found to be the same as those for the corresponding RNA species synthesized in the KB cell-free extracts (data not shown). Thus, the VA RNA genes also appear to be accurately transcribed in the MOPC extracts. The 5 S RNA species synthesized in response to pXbo3 have not yet been analyzed.

pXbo3 and Adenovirus 2 Transcripts Synthesized in Amphibian Cell Extracts—The major pXbo3 transcript synthesized in the homologous amphibian system is equivalent in size to the naturally occurring oocyte 5 S RNA (Figs. 3 and 4), although a minor transcript with a slower electrophoretic mobility is occasionally (but not always) observed (compare Figs. 3 and 4B). A fingerprint analysis of the predominant pXbo3 transcript (Fig. 4B) is shown in Fig. 6C. Except for the greatly reduced level of oligonucleotide 15 (see discussion in preceding section) this fingerprint is very similar to that of the natural dominant Xbo 5 S RNA (cf. Ref. 27). The major two qualitative differences between this fingerprint and those of the RNAs synthesized in the heterologous (KB cell) system described above are the vastly reduced levels of oligonucleotides thought to be derived from the divergent 5 S gene sequence(s) and the absence of the uridylic acid-rich oligonucleotide present in the 5 S’ product. From these observations we suggest that in the homologous amphibian transcription system there is a reduced relative level of initiation at the divergent 5 S gene sequence and either a reduced level of transcription beyond the normal termination site or an increased level of processing of the “read-through” product. The minor pXbo3 (and pX10) transcripts seen occasionally in the amphibian system (Fig. 3, Lane 4) are presumably analogous to the minor 5 S’ transcript seen in the heterologous system, although they have not yet been analyzed directly.

Fingerprint analysis of the putative VA RNA₁ species synthesized in the amphibian cell extract in the presence of adenovirus 2 DNA (Fig. 3, Lane 1) has also demonstrated that this species is an accurate transcript of the VA RNA₁ gene (data not shown).

Properties of the Endogenous RNA Polymerase III Transcription Reactions

Previous studies have shown that purified class III RNA polymerase (assayed with exogenous templates) and endogenous (nuclear) class III RNA polymerase activities exhibit characteristic properties, which in many cases distinguish them from the corresponding class I and II RNA polymerase activities (reviewed in Ref. 1). For comparison, and in order to establish optimal conditions for faithful gene transcription, we examined various characteristics of the endogenous class III RNA polymerase activities in the present systems, both with respect to total RNA synthesis and specific (5 S, VA) RNA synthesis. In the following, we describe studies of adenovirus DNA and pXbo3 transcription in the human KB cell extract. Although not presented, analogous studies have been performed with these same templates in the amphibian system with results very similar to those presented for the KB cell system. Additionally, the transcription of a plasmid containing X. lavies oocyte 5 S DNA has been examined with results (not shown) similar to those reported with pXbo3. In the experiments presented below, total RNA synthesis was measured by spotting aliquots of the reaction mixtures on DEAE-paper (see “Experimental Procedures”). The synthesis of specific RNA species was monitored by first resolving the RNA species in the remainder of the sample on polyacrylamide gels and
subsequently excising and measuring the Cerenkov radioactivity in appropriate gel regions. Since the DEAE-paper binding method scores oligonucleotides as short as 10 nucleotides, the levels of specific synthesis calculated from the graphical data is underestimated, relative to the levels estimated by direct visualization of the gels. For comparison, in many cases where the distribution of radioactivity throughout a gel slot was determined, more than 90% of the radioactivity was in the major RNA band (cf. Fig. 4).

**Divalent Metal Ion and Ionic Strength Optima**—The results presented above have shown the accurate transcription of the VA RNA genes in adenovirus 2 DNA and the 5 S RNA gene(s) in pXbo3 in the presence of Mg"" as the sole divalent cation. As revealed by electrophoretic resolution of the corresponding RNA species, these same genes are also accurately transcribed when Mg"" is replaced with Mn"" (Fig. 7, Lanes 1 and 2). The data in Fig. 8 show quantitatively the effects of variable concentrations of Mg"" and Mn"" on transcription. In the presence of Mg"" maximal levels of total RNA synthesis are observed at 5 to 7 mM with an adenovirus 2 DNA template (Fig. 8, Panel A, open circles) and at 2.5 mM with the pXbo3 template (Fig. 8, Panel C, open circles). This contrasts with the broad optimum of 4 to 16 mM Mg"" observed with a purified mammalian RNA polymerase III (34). In the presence of Mn"" maximal levels of RNA synthesis are observed at 2.5 mM with both adenovirus 2 (Fig. 8, Panel B, open circles) and pXbo3 (Fig. 8, Panel D, open circles) templates. This metal ion optimum is similar to that reported for the purified RNA polymerase III (34).

Significantly, the metal ion activation profiles for the specific synthesis of VA RNA (Fig. 8, Panels A and B, closed circles) and for the specific synthesis of 5 S RNA (Fig. 8, Panels C and D, closed circles) closely parallel those determined for total RNA synthesis with the corresponding adenovirus DNA and pXbo3 templates. The levels of total RNA synthesis and specific RNA synthesis with the two templates are calculated to be, respectively, 1.5- to 1.8-fold and 2.1- to 2.7-fold higher with Mg"" than with Mn"" (at the optimum concentrations). This contrasts somewhat with the results reported for the purified RNA polymerase III which usually exhibits a 2-fold greater activity with Mn"" than with Mg"".

Although KCl has been used for the experiments presented in this manuscript, an accurate transcription of the VA RNA and the 5 S RNA genes is also observed in the presence of ammonium sulfate, as revealed by electrophoretic resolution of the corresponding VA and 5 S RNA species (Fig. 7, Lanes 3 and 4). The data in Fig. 9 show quantitatively the effects of variable KCl and ammonium sulfate concentrations on transcription in the presence of an optimal concentration of Mg"". Total RNA synthesis with either adenovirus DNA (Fig. 9, Panel A) or pXbo3 DNA (Fig. 9, Panel B) is optimal at 60 to 100 mM KCl (open circles) and at 20 to 50 mM ammonium sulfate (open squares). For comparison, the rate of transcription of purified adenovirus DNA with purified RNA polymerase III is optimal at 60 mM KCl and at 40 mM ammonium sulfate (41). Overall, the salt activation profiles for the specific synthesis of VA RNA and of 5 S RNA (Fig. 9, Panels A and B, solid symbols) parallel those for total RNA synthesis with the respective adenovirus DNA and pXbo3 templates (Fig. 9, Panels A and B, open symbols).

**Effects of Template Concentration**—The effects of increasing template concentrations on total and specific RNA synthesis are shown in Fig. 10. As the concentration of either adenovirus DNA (Panel A) or pXbo3 DNA (Panel B) in the transcription reaction is increased, total RNA synthesis (open

![Fig. 7. Polyacrylamide gel electrophoresis of RNAs synthesized in a KB cell-free extract in the presence of Mn"" or in the presence of ammonium sulfate. Transcription reactions were carried out as described in the legends to Figs. 8 and 9, with the following template, divalent metal, and salt conditions: Lane 1, adenovirus 2 DNA, 2 mM Mn"", 75 mM KCl; Lane 2, pXbo3, 1.5 mM Mn"", 75 mM KCl; Lane 3, adenovirus 2, 7.5 mM Mg"", 45 mM ammonium sulfate; Lane 4, pXbo3, 2.5 mM Mg"", 40 mM ammonium sulfate.](http://www.jbc.org/)

![Fig. 8. Effect of divalent metal ion concentrations on total and specific RNA synthesis in a KB cell-free extract. An aliquot of an extract prepared from KB cells was dialyzed into buffer containing 20 mM Heps (pH 7.9), 150 mM KCl, and 0.5 mM dithiothreitol. Standard reactions contained adenovirus DNA (A and B) or pXbo3 DNA (C and D) and the indicated concentrations of MgCl₂ (A and C) or MnCl₂ (B and D). Total RNA synthesis (O—O) and radioactivity in VA RNA or 5 S RNA (■—■) were determined. Maximal activities were: A, 6.75 pmol of UMP (7,100 cpm/pmol) incorporated into total RNA and 6,140 cpm into VA RNA; B, 10.5 pmol of GMP (3,089 cpm/pmol) incorporated into total RNA and 2,980 cpm into VA RNA; C, 3.07 pmol of GMP (9,440 cpm/pmol) incorporated into total RNA and 3,180 cpm into 5 S RNA; and D, 1.79 pmol of GMP (6,440 cpm/pmol) incorporated into total RNA and 1,660 cpm into 5 S RNA.](http://www.jbc.org/)
higher molecular weight RNA which does not enter the gel.

The polyacrylamide gel analyses of the reaction products at various DNA concentrations are shown in the insets of Fig. 10. These gels show that, as the transcription of the genes for VA RNA, (Fig. 10, Panel A) or 5 S RNA (Fig. 10, Panel B) decreases, there is a concomitant increase in the synthesis of higher molecular weight RNA which does not enter the gel. This high molecular weight RNA presumably results from random transcription of the respective templates, although it has not yet been excluded that it results from the aberrant termination of accurate initiation reactions. The sequence content of the high molecular weight RNA has not been analyzed. Possible explanations for the effects of high DNA concentrations on specific RNA synthesis are presented under "Discussion."

Kinetics of Transcription—The kinetics of RNA synthesis in the human cell extract are shown in Fig. 11 with both the adenovirus DNA (Panel A) and pXbo3 (Panel B) templates. In each case total RNA synthesis (open circles) begins rapidly and is linear for at least 1 h. Moreover, the extract continues to actively synthesize RNA for at least 4 h. The RNA products of reactions incubated for various times were analyzed by polyacrylamide slab gel electrophoresis. As seen in the insets of Fig. 11, both the VA RNA (Panel A) and the 5 S RNA (Panel B) are synthesized within the first 4 to 6 min of the reaction. Furthermore, the radioactivity incorporated into VA RNA (closed symbols of Panel A) and 5 S RNA (closed symbols of Panel B) increases nearly in parallel with total RNA synthesis from 10 min to 4 h. These results are in accord with previous studies showing that the endogenous RNA polymerase III reactions in isolated nuclei continue for extended periods of time (cf. Ref. 6).

Effects of Exogenous RNA Polymerase III on Transcription

RNA polymerase III purified from uninfected KB cells has been previously shown to accurately transcribe the VA RNA1 gene in nuclear viral templates, but not in purified DNA. The effects of the addition of this enzyme to the present system have been investigated. When the cell-free extract is employed at standard concentrations, the addition of exogenous RNA polymerase III has no effect on the transcription of the VA RNA1 gene in adenovirus DNA (data not shown). However, as shown in Fig. 12, if the amount of cell-free extract present in the standard reaction is markedly reduced there is a concomitant reduction in the levels of total RNA synthesis and VA RNA1 synthesis (Fig. 12, Lanes A and B). These
levels can now be greatly increased by the addition of exogenous RNA polymerase III (Fig. 12, Lanes B and C). In the experiment shown, the nonsaturating amount of RNA polymerase III added enhanced total and VA RNA1 synthesis by 5.9- and 3.3-fold, respectively. For comparison, Lane D of Fig. 12 shows an analysis of the products obtained when the purified DNA is transcribed in the presence of the RNA polymerase III. The specific activity of the labeled nucleoside triphosphate used for these experiments was 12,350 cpm/pmol of GTP.

**DISCUSSION**

A most significant development for the further investigation and understanding of transcriptional controls has been the establishment of cell-free systems in which specific genes in purified DNA templates are accurately transcibed (see the introduction to the text). In the present report we have used human, murine, and amphibian systems similar to that described by Wu (30) to further investigate transcription of human adenovirus VA RNA1 and VA RNA11 genes and Xenopus oocyte 5 S RNA genes.

**Transcription of the Adenovirus 2 Genome**—The combined size (electrophoretic) and sequence (fingerprint) analyses of the adenovirus DNA-directed transcripts clearly show that the VA RNA1 and the VA RNA11 genes are accurately and selectively transcribed by RNA polymerase III in cell-free extracts. These data confirm and extend the report by Wu (30) who detected only the VA RNA1 gene product by size and hybridization analyses. Additionally we observe the synthesis of an extended VA RNA1 species (VA RNA1), previously shown by us to be synthesized in nuclei from virus-infected cells. The significance of this extended product is unclear. However it can be processed to VA RNA1 in vitro and could represent a natural (but not necessarily an obligatory) precursor to VA RNA1 (see “Discussion” in Ref. 18).

The transcription studies with restriction endonuclease fragments of adenovirus DNA clearly demonstrate separate promoter sites for the VA RNA1 and VA RNA11 genes. Thus, like the short spacer regions (approximately 80 nucleotides) which separate Xbo 5 S genes (10), the short region (approximately 75 nucleotides) which separates the VA RNA1 genes (13) also contains the sequences necessary for initiation by RNA polymerase III. The relative rates of synthesis of VA RNA1 and VA RNA11 observed in isolated nuclei or intact cells (6, 7, 18) are also maintained in the cell-free extract. This suggests that viral promoter sequences and host transcription components are sufficient for regulating the rates of transcription of these genes. A comparison of the respective promoters (cf. Ref. 10) may help identify those sequences which determine promoter strength.

**Transcription of Xenopus Oocyte 5 S DNA**—The combined size and sequence analyses of the Xbo 5 S DNA-directed transcripts also suggest a selective and accurate transcription of the 5 S RNA genes. In this case the predominant transcripts are 5 S in size although discrete RNA species with slightly

---

(B) Harris and R. Roeder, unpublished observation.

---

![Fig. 11. Kinetics of total and specific RNA synthesis by a KB cell-free extract with adenovirus 2 DNA or pXbo3 as template. Standard reactions containing extracts prepared from KB cells and adenovirus 2 DNA (A) or pXbo3 DNA (B) were started by the addition of the extract to a mixture of the other reaction components. Both components had been separately prewarmed to 30°C. At the indicated times, the reactions were terminated by rapid chilling and the addition of sarkosyl to 0.5%. Total RNA synthesis in the presence (C—O) or absence (C—C) of DNA was determined and the RNA products were analyzed on a 12% polyacrylamide slab gel. Radioactivity in the VA, RNA and 5 S RNA bands (C—O) was determined as described under "Experimental Procedures." The inset shows the autoradiographs of those lanes of the gel containing RNA extracted from reactions after 2, 4, 6, 8, and 10 min of synthesis. The specific activity of the labeled nucleoside triphosphate used for these experiments was 12,350 cpm/pmol of GTP.

![Fig. 12. Electrophoretic analysis of RNA synthesized by a KB cell-free extract supplemented with purified KB RNA polymerase III. Reaction conditions in the presence of adenovirus DNA were standard, except that the following amounts of KB cell-free extract and purified KB RNA polymerase III were used: A, 15 μl of extract; B, 2 μl of extract; C, 2 μl of extract and 31.3 units of RNA polymerase III; D, no extract and 31.3 units of RNA polymerase III. The specific activity of the labeled GTP used was 4260 cpm/pmol. [32P]RNA was extracted and subjected to electrophoresis on a 12% polyacrylamide slab gel. The autoradiograph of the gel is shown. The amounts of radioactivity incorporated were: Lane A, 72,400 cpm; Lane B, 2,980 cpm; Lane C, 17,380 cpm; Lane D, 13,280 cpm. The VA RNA1 band of Lanes B and C contained 324 and 1083 cpm, respectively.)
decreased electrophoretic mobilities are also observed in some cases. Extended 5 S RNA species analogous to the latter transcripts have been observed in cultured oocytes (14) and in other 5 S DNA-directed transcription systems (10, 15). They appear to result either from read through of primary termination sites (15) or possibly from the transcription of altered 5 S gene sequences (10). As discussed under “Results,” a strict interpretation of the present data is complicated by the multiplicity and probable heterogeneity of the 5 S genes present in the pXbo template employed here. Despite this uncertainty, there is little doubt that at least some of the 5 S genes present in the plasmid are selectively and accurately transcribed in the cell-free systems employed here (see also following section).

Transcription of Specific Genes in Homologous Versus Heterologous Cell Types—Both VA RNA genes are transcribed in extracts from uninfected KB cells and in extracts from murine cells. While these observations do not rule out the involvement of virus-induced components in the regulation of these genes in vivo, they are consistent with the reported synthesis of VA RNA1 and VA RNAII in virus-infected cells in the absence of (viral) protein synthesis (13). They are also consistent with our previous studies which failed to detect any structural modifications in the RNA polymerase III from virus-infected cells (22) and which showed that the structurally similar class III enzymes from infected KB cells, uninfected KB cells, murine plasmacytoma cells, and Xenopus cells are equally effective in transcribing the VA RNA1 gene in nuclear templates from virus-infected cells (25). In the present study we have observed VA RNA1 gene transcription, but failed to detect VA RNAII gene transcription, in the amphibian system. Apparently the VA RNAII promoter is not recognized, at a detectable level, by the heterologous Xenopus RNA polymerase III.

Xenopus oocyte-type 5 S genes present in pXbo are actively transcribed not only in the homologous amphibian cell extract but also in the mammalian cell extracts. As discussed under “Results,” however, the 5 S RNA transcripts generated in the homologous system are apparently more homogeneous and more closely resemble the dominant oocyte 5 S RNA than those generated in the heterologous mammalian extracts. Thus, with these cloned genes, the heterologous transcription system appears less able than the homologous system to discriminate between the heterogeneous 5 S genes. It is also significant that the 5 S RNA genes are transcribed in extracts from cells (cultured kidney cell line) which contain, but do not normally express, the oocyte type 5 S genes (see further discussion below).

General Properties of the Cell-free Transcription System—To facilitate further studies with the cell-free transcription systems employed here, the conditions optimal for specific gene transcription by RNA polymerase III have been carefully defined. Under optimal conditions comparable levels of specific transcription are observed with either Mn2+ or Mg2+ as the divalent cation and with either ammonium sulfate or potassium chloride. Moreover, specific RNA synthesis accounts for a large and rather constant fraction of total RNA synthesis at varying metal ion and salt concentrations. These results differ in part from those reported for the transcription of the 5 S DNA in Xenopus oocyte extracts, which effect specific gene transcription only in the presence of Mg2+ (29).

In agreement with the findings reported for the oocyte-derived transcription system, specific gene transcription is not observed in the present systems at elevated DNA concentrations, even though total RNA synthesis continues to increase (for further discussion, see below).

As reported by Wu (30), total RNA synthesis continues for extended periods of time in the cell-free system. The synthesis of specific RNA species, which accounts for a high proportion of total RNA synthesis at all times of the reaction, can be detected as early as 5 min. Thus, if modifications of the template, such as organization within a chromatin structure (see below), are prerequisites for specific transcription, they must occur relatively rapidly in the cell-free system.

Although the VA RNA and 5 S RNA genes are accurately transcribed in the present system, the overall efficiency of transcription is apparently low. For example, at the optimal adenovirus DNA concentration the average number of VA RNA1 transcripts generated/gene/h is about 3 to 4 while the approximate number of transcripts generated/RNA polymerase III molecule/h is about 6 to 8 (assuming all RNA polymerase III is recovered in the cell-free extract). It is presently unknown whether all the RNA polymerase III molecules are active or whether all the DNA molecules are functional templates. Neither the viral DNA nor the pXbo templates are significantly degraded in the extracts although the supercoiled pXbo molecules appear to be rapidly converted to the nonsupercoiled forms4.

Mechanism of Specific Gene Transcription and Regulation of Gene Activity—The present report demonstrates that the subcellular extracts employed contain, in a soluble form, components which are sufficient for the accurate transcription of adenovirus VA RNA and amphibian 5 S RNA genes. While the number and site(s) of action of the functional transcription component(s) (other than RNA polymerase III) present in the crude extracts are presently unknown, it is likely there is an effect of at least one component at the level of initiation as suggested by the following. First, purified class III RNA polymerases initiate transcription (apparently randomly) on both strands of 5 S and adenovirus DNA and in the latter cases at regions far to the left (upstream) of the VA RNA genes5 (21–23). Second, and in contrast, the 5 S and VA RNAs generated in the crude cell-free systems contain the same polyphosphate termini (29) found in the corresponding RNAs synthesized in vivo (14, 16), demonstrating accurate initiation at the corresponding genes and the absence of processing at 5′ termini. Thus, the observed specificity of synthesis cannot be due solely to an enhanced specificity of termination in the crude extracts, although it is possible that some circumstances could selectively block the action of termination functions in the crude systems, leading to the loss of discrete sized transcripts (see discussion under “Results”). Because sequence data suggest the presence of specific, recognizable termination sites in class III genes (10) and because of the observed kinetics of synthesis it also seems unlikely that the discrete class III transcripts are generated by processing from much larger precursors.

Under appropriate conditions transcription in the crude system can be significantly stimulated by exogenous RNA polymerase III, suggesting that the transcription factor(s) is present in excess relative to the RNA polymerase. This factor(s) appears to be a protein as indicated by its heat lability and sensitivity to inactivation by trypsin. Furthermore, it has been possible to isolate from the KB cell-free extract a partially purified fraction which is devoid of detectable RNA polymerase activity and which stimulates accurate transcription of the VA RNA1 gene by a purified RNA polymerase III. An RNA polymerase deficient fraction from immature oocytes has also been shown to direct the accurate transcription of 5 S RNA genes by purified RNA polymerase III (29). These results clearly show the presence of a transcription specificity.

4 P. A. Weil, unpublished observation
5 J. Segall and P. A. Weil, unpublished observations.
factor(s) which does not copurify, at least in an active form, with the RNA polymerase. The inhibitory effect of high DNA concentrations on specific transcription suggests that the factor(s) can interact with DNA, consistent with our previous demonstration of template associated specificity determinants (see the introduction to the text). It is reasonable to speculate that the factor(s) interacts directly with promoter or terminator sites on the DNA or, alternatively, that an RNA polymerase-factor complex recognizes these sequences. In either case increased levels of DNA could lead to the sequestration of the factor(s) at nonspecific sites, leading to a loss of specific transcription. It is also possible, but considered unlikely on the basis of preliminary purification results, that this factor(s) assembles the DNA into a chromatin structure (42), which is of itself sufficient to direct accurate transcription by RNA polymerase III. (The characterization of the functional template in the crude system is made difficult by the low efficiency of transcription, which indicates that a minor fraction of the input DNA could account for the observed transcription.)

One interesting aspect of the present study is that some genes are transcribed in extracts derived from cells in which they are not normally transcribed (e.g. oocyte 5 S RNA genes are transcribed in cultured kidney cell extracts). This suggests that some elements of in vivo control are not retained in the cell-free system. In intact cells nuclear DNA is present in a chromatin structure and chromatin structural modifications have been associated with actively transcribed genes (reviewed in Ref. 49). Thus, if DNA is, in fact, not completely assembled into chromatin in the cell-free systems employed here, the purified DNAs may represent artificially “dere-}

Achnowledgments—We thank Donald D. Brown for the recombinant DNA plasmids and Becky Tietze for expert technical assistance.

REFERENCES
Faithful transcription of eukaryotic genes by RNA polymerase III in systems reconstituted with purified DNA templates.

P A Weil, J Segall, B Harris, S Y Ng and R G Roeder


Access the most updated version of this article at http://www.jbc.org/content/254/13/6163

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/254/13/6163.full.html#ref-list-1