Template Structural Requirements for Transcription In Vivo by RNA Polymerase II

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Purified simian virus 40 (SV40) DNA is reconstituted into chromatin and transcribed by endogenous RNA polymerase II when microinjected into nuclei of *Xenopus laevis* oocytes. We have correlated the kinetics of chromatin reconstitution with that of accumulation of virus-specific RNA in this system. A delay of approximately 3 h was found in the appearance of appreciable numbers of both fully supercoiled molecules and transcriptionally active templates. SV40 mini-chromosomes, isolated from virus-infected monkey cells with 0.2 M NaCl, also exhibited this lag in onset of transcriptional activity when microinjected into oocytes. These findings indicate that neither purified SV40 DNA nor SV40 DNA containing a full complement of nucleosomes can function as a template for transcription in vivo before association with appropriate cellular nonhistone chromosomal factors has taken place. In addition, the gradual degradation of linear SV40 DNA in oocytes was not sufficient to account for the fact that it was much less transcriptionally active than circular SV40 DNA. Taken together, these results indicate that the conformational state of the DNA can affect its ability to function as a template for transcription in vivo by RNA polymerase II. In contrast, transcription by RNA polymerase III of purified, circularized cloned DNAs encoding genes for 5S rRNA was detectable long before the injected DNAs had time to reconstitute into chromatin. Therefore, the template structural requirements for transcription in vivo by RNA polymerases II and III are different.

Several types of systems have been developed for studying transcription of cloned eucaryotic genes: microinjection of DNAs into nuclei of *Xenopus* oocytes (see reference 11 for review); transcription in monkey cells of genes linked to simian virus 40 (SV40) vectors (12, 28); expression of DNAs stably introduced into mammalian cells in culture (see reference 29 for review); and in vitro transcription with cell-free extracts (1, 22, 38, 40). Together with the availability of DNA sequencing and in vitro mutagenesis techniques, these systems have enabled much progress to be made in the identification of elements within eucaryotic DNAs that are involved in regulating promotion and initiation of RNA synthesis. However, except for the elegant studies of Brown, Roeder, and their colleagues on control of transcription *Xenopus* 5S RNA genes (see reference 18 for review), only limited progress has been made to date concerning the roles played by either specific cellular chromosomal proteins or chromatin structure in regulating eucaryotic gene expression.

The *Xenopus* oocyte system has several advantages for studying the involvement of chromosomal factors in regulating RNA synthesis. First, the injected DNAs are transcribed efficiently with the appropriate RNA polymerase under normal physiological conditions (see reference 11 for review). Second, the oocytes reconstitute essentially all of the injected DNA into chromatin containing regularly spaced nucleosomes (24, 42). Third, components such as radiolabeled triphosphates, α -amanitin, and potential regulatory proteins can be readily added to the reaction at any appropriate time. Last, both the DNA, in association with chromosomal proteins if desired, and the RNA made from it can be isolated from the oocytes in quantities sufficient for analysis by a variety of procedures.

The template we have picked for our studies is the small double-stranded circular DNA genome of SV40 (see reference 36 for review). This well-characterized DNA is transcribed by the cellular RNA polymerase II and exists in vivo in association with cellular histone proteins. Many of the biological properties of SV40 DNA injected into nuclei of *Xenopus* oocytes have already been determined, including its reconstitution into

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chromatin (24, 42), its rates of synthesis of RNA (27), and the DNA sequences that are necessary and sufficient for its transcription (J. E. Mertz, L. A. Trimble, T. J. Miller, and G. Z. Hertz, manuscript in preparation). Furthermore, SV40 DNA can be isolated by a variety of procedures from virus-infected monkey cells in fairly large quantities as "minichromosomes," manipulated in vitro, and, subsequently, injected into oocytes to assay their transcriptional activity. Employing this procedure, one may successfully identify chromosomal factors necessary for transcription in vivo.

Here, we describe a series of experiments designed to determine some of the template structural requirements for transcription in vivo by RNA polymerase II. Correlations are made between the kinetics of chromatin reconstitution and transcription of a variety of templates injected into the nuclei of *Xenopus* oocytes. From these data, we conclude that (i) neither purified SV40 DNA nor SV40 DNA containing a full complement of nucleosomes can function as a template for transcription in vivo before associating with other necessary factors; (ii) the conformational state of a DNA molecule can affect its ability to be transcribed in vivo by RNA polymerase II; and (iii) the template structural requirements for transcription in vivo by RNA polymerase III differ from those stated above for RNA polymerase II.

MATERIALS AND METHODS

Cells, virus, DNAs, and enzymes. CV-1P is an established line of African green monkey kidney cells. These cells were grown as described previously (25), except that 5% fetal bovine serum was used in place of 10% calf serum. After infection with virus, they were maintained in medium containing 2% fetal bovine serum.

Our wild-type strains of SV40 are WT800 and WT830, plaque-purified derivatives of strains Rh911 and SV-S, respectively. Stocks of virus were made and titered as described previously (25).

Radiolabeled supercoiled (form I) SV40 DNA (specific activity 1×10^5 to 3×10^5 cpm/ μ g), containing less than 5% nicked circular molecules (form II), was prepared as follows. Confluent monolayers of CV-1P cells were infected with virus at a multiplicity of infection of approximately 40 PFU per cell. Twenty-four to 30 h later, the medium was replaced with fresh medium containing 2% dialyzed fetal bovine serum and 25 μ Ci of [methyl- 3 H]thymidine per ml (20 Ci/mmol). At 54 to 60 h postinfection, the cells were lysed with 0.6% sodium dodecyl sulfate, and the cellular DNA was precipitated by the addition of NaCl to 1 M (13). The supercoiled DNA remaining in the supernatant was purified by two cycles of equilibrium centrifugation in cesium chloride-ethidium bromide gradients (31). After removal of ethidium bromide by repeated extraction with isopropanol, the viral DNA was precipitated with NaCl and ethanol, sedimented in neutral

sucrose gradients, re-precipitated with ethanol, and suspended in 10 mM Tris-chloride (pH 7.4)–0.1 mM EDTA. These latter purification steps removed cesium that can be toxic to *Xenopus* oocytes.

DNA containing the *Drosophila melanogaster* gene for histone protein H2A (21) attached to pBR322 DNA via synthetic *Bam*HI linkers was obtained from Dani Canaani. Recombinant DNA consisting of the *Hind*III–C restriction fragment of SV40 strain WT830 joined to pBR322 was a gift from Suresh Subramani. Recombinant DNAs containing genes for somatic 5S ribosomal RNA of *Xenopus borealis* (pXbs1) and oocyte 5S ribosomal RNA of *Xenopus laevis* (pXlo8) (10) were generously supplied by Donald Brown. They were grown in *Escherichia coli* strain HB101 by the chloramphenicol amplification procedure of Clewell (4). To obtain radiolabeled DNA, [3 H]thymidine was included in the growth medium. The plasmid DNAs were isolated by the cleared lysate method of Katz et al. (17) and purified as described above for SV40 DNA.

Restriction endonucleases and T4 DNA ligase were obtained from commercial sources. They were used as indicated by the manufacturer.

ColE1 and related bacterial plasmid DNAs are transcribed efficiently in *Xenopus* oocytes (26). Consequently, read-through transcription from vector DNA into cloned sequences can occur (39; Mertz et al., manuscript in preparation). To avoid this problem, the eucaryotic DNAs were excised from their bacterial cloning vectors by digestion with the appropriate restriction endonucleases and were purified by electrophoresis in agarose gels with $4\times$ TA (0.16 M Tris-acetate [pH 8.3]–0.08 M sodium acetate–8 mM EDTA) as the running buffer. After electroelution from gel slices with $0.4\times$ TA buffer, the DNAs were subjected to sequential extractions with butanol, phenol-chloroform-isoamyl alcohol (25:25:1), chloroform-isoamyl alcohol (25:1), and ether and were precipitated with ethanol. Since linear DNAs are poor templates for transcription (see Results), they were next incubated with T4 DNA ligase at DNA concentrations of ≤ 2 μ g/ml to create predominantly monomer-length circular molecules. Finally, the DNAs were re-extracted with phenol, chloroform, and ether, precipitated with ethanol, and suspended in 10 mM Tris-chloride (pH 7.4)–0.1 mM EDTA before microinjection into oocytes.

Handling and injection of oocytes. Full-grown (stage 6) oocytes were isolated from adult female *X. laevis*, incubated at 19°C in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-modified Barth's solution, and injected intranuclearly with the indicated samples as described previously (27, 34). Newly synthesized RNA was radiolabeled by microinjection into the animal pole of 20 nl of injection medium containing 12 to 25 mCi of [8- 3 H]GTP (16 Ci/mmol) or [α - 32 P]GTP (approximately 25 or 350 Ci/mmol) per ml. In the experiments in which the sample and radioactivity were added concurrently, the GTP was dried in a siliconized tube, suspended directly in the sample, and co-injected intranuclearly.

Purification and analysis of radiolabeled RNAs and DNAs from injected oocytes. After incubation at 19°C in HEPES-modified Barth's solution for the indicated times, the reactions were terminated by homogenization of groups of 15 to 30 oocytes directly in proteinase K buffer (50 mM Tris-chloride [pH 7.4]–10 mM

EDTA-1% sodium dodecyl sulfate-750 μ g of proteinase K per ml; 50 μ l per oocyte) and incubated at 37°C for 1 to 2 h. RNA was purified from the oocytes as described previously (26, 27).

The amounts of radiolabeled SV40- and histone-specific RNA were determined by hybridization to nitrocellulose filters (6.5 mm diameter) containing the corresponding DNAs bound to them (see references 26 and 27 for details). The data were normalized to RNA per oocyte, although several oocytes' worth of RNA was used in each hybridization reaction. Corrections were made for machine backgrounds of approximately 10 and 16 cpm for ^3H and ^{32}P , respectively, but not for nonspecific binding of radioactivity to the filters (approximately one in 10^4 input cpm) or for the efficiency of hybridization (50 to 70%). Rates of RNA synthesis were calculated as described previously (27).

Synthesis of radiolabeled 5S RNA was analyzed by electrophoresis in 7.5% nondenaturing polyacrylamide gels with 40 mM sodium phosphate buffer as described previously (27). Endogenous 5S RNA synthesis, determined by analyzing RNA samples from oocytes injected in parallel with ^{32}P GTP only, was subtracted from the total radiolabeled 5S RNA made to obtain a measure of that directed by the injected DNA.

For the purification of DNA, the homogenized mixtures were extracted twice with 0.1 M Tris-chloride (pH 9.0)-saturated phenol-chloroform-isoamyl alcohol (25:25:1) and concentrated by precipitation with ethanol. To determine the structures and amounts of the radiolabeled plasmid DNAs present after incubation in oocytes, the samples were electrophoresed at 35 V for 14 to 20 h in 1.4% agarose gels (17 by 14 by 0.2 to 0.3 cm) submerged in $4\times$ TA buffer. Afterwards, the gels were incubated in En^3Hance (New England Nuclear Corp.) for 2 h, rinsed in 3% glycerol for 2 h, and dried in vacuo. Fluorography was performed at -70°C with Kodak XAR-5 X-ray film.

Preparation of SV40 minichromosomes for injection into oocytes. SV40 DNA containing chromosomal proteins was isolated by using several procedures. To obtain them from *Xenopus* oocytes, nuclei were manually dissected as described previously (27) from oocytes that had been injected 24 h earlier with ^3H -labeled SV40 DNA. The nuclei were homogenized in buffer A (10 mM HEPES [pH 7.8]-5 mM KCl-1 mM dithiothreitol-0.5 mM MgCl_2 -0.1 mM phenylmethylsulfonyl fluoride [PMSF]) by being passed 10 times through an Eppendorf tip. After centrifugation for 1 min in a Beckman microfuge, the material in the supernatant was collected. The amount of SV40 DNA in the supernatant was determined by electrophoresis in agarose gels alongside known standards. The concentration of SV40 DNA was adjusted by desiccation to approximately 25 $\mu\text{g}/\text{ml}$ before injection of 50 nl of the lysate per oocyte.

To prepare SV40 minichromosomes from monkey cells, CV-1P cells were infected with wild-type SV40 at a multiplicity of infection of approximately 50 PFU per cell. After incubation at 37°C for 24 to 30 h, the medium was replaced with fresh medium containing 2% dialyzed fetal bovine serum and 15 μCi of ^3H thymidine per ml. At 40 to 48 h postinfection, SV40 minichromosomes were extracted from the nuclei of the infected cells, using either the Triton-EDTA-0.2 M NaCl procedure of Green et al. (9) or the low-salt

(buffer A) method of Su and DePamphilis (35) as modified by Fanning and Baumgartner (7). Where indicated, α -amanitin (100 $\mu\text{g}/\text{ml}$) was added to the cells immediately before incubation of the nuclei at 0°C for 1 h with the extraction buffer. Afterwards, the nuclear material was pelleted by centrifugation at $10,000 \times g$ for 5 min. The SV40 minichromosomes in the supernatant were purified by sedimentation (36,000 rpm; 3 h; 4°C; SW41 rotor) in 5 to 30% sucrose gradients in 10 mM Tris-chloride (pH 7.9)-0.2 M NaCl-0.2 mM EDTA-0.1 mM PMSF or buffer A, respectively, dialyzed against 10 mM Tris-chloride (pH 7.9)-1 mM EDTA at 4°C, pelleted by centrifugation (55,000 rpm; 16 h; 4°C; SW60 rotor), and resuspended in 10 mM Tris-chloride (pH 7.4)-0.5 mM EDTA for microinjection. The material from mock-infected cells treated as described above that copurified with SV40 minichromosomes was mixed with SV40 DNA as a control. The concentration of viral DNA in each preparation of minichromosomes was determined from its absorbance assuming 1 unit of absorbance at 260 nm = 25 μg DNA; their specific activities were approximately 10^5 cpm/ μg .

RESULTS

Kinetics of accumulation of radiolabeled RNAs in DNA-injected oocytes. In the first experiments reporting that mRNA-encoding DNAs are transcribed efficiently after microinjection into the nuclei of *X. laevis* oocytes, the oocytes were

FIG. 1. Kinetics of accumulation of radiolabeled SV40 RNA in oocytes preinjected with SV40 DNA. Stage 6 oocytes were injected intranuclearly with 5 ng of supercoiled SV40 DNA. After 20 h, ^3H GTP was injected. At various times thereafter, groups of 30 oocytes were harvested by homogenization in proteinase K buffer. The amount of radiolabeled RNA present in each sample that could hybridize with SV40 DNA bound to nitrocellulose filters was determined as described in the text. The insert shows a magnified version of the earliest timepoints. Approximately 9% of the total radiolabeled RNA was SV40 specific in this experiment.

FIG. 2. Kinetics of accumulation of SV40 RNA in oocytes co-injected with $[^3\text{H}]\text{GTP}$ and 5 ng of supercoiled (○) or *TaqI* restriction endonuclease-linearized (●) SV40 DNA. At the times indicated, transcription was terminated by homogenization of groups of 20 oocytes in proteinase K buffer. The total radiolabeled RNA present in each sample from the supercoiled SV40 DNA-injected oocytes (Δ) was determined from the amount of radioactivity that bound to DE81 filters (see reference 27 for details). Incorporation of $[^3\text{H}]\text{GTP}$ into SV40-specific RNA was determined as described in the text. Unit-length linear SV40 DNA was made by cleavage of supercoiled SV40 DNA with the restriction endonuclease *TaqI*. The insert gives a magnified version of the earliest timepoints.

incubated with the injected DNAs for 1 or more days before the addition of radiolabeled precursor to RNA (26). This protocol was employed because it was assumed that the injected DNAs might need time to interact properly with factors present in nuclei before they could function as transcriptionally active templates. The experiments presented here were designed to test this hypothesis.

Figure 1 shows a typical example of the data obtained when transcription assays were performed by using oocytes injected 20 h earlier with 5 ng (approximately 10^9 molecules) of supercoiled SV40 DNA. Within 10 min after the addition of $[^3\text{H}]\text{GTP}$, radioactivity was found in SV40-specific RNA. Initially, virus-specific RNA accumulated without a detectable lag at a linear rate of approximately 0.2 pmol of GTP (1 ng) per oocyte per h. The gradual decrease with time in the rate of accumulation of radiolabeled SV40-specific RNA is due to the fact that most of it turns over in the nucleus with a half-life of 20 to 40 min (27). Results similar to these are

also seen with cloned DNA that encodes the sea urchin histone genes (30).

In contrast to the above results, a delay in the synthesis of SV40-specific RNA at appreciable rates was always seen when supercoiled SV40 DNA and radiolabeled GTP were co-injected into the nuclei of oocytes (Fig. 2). During the earliest time points, very little radiolabeled SV40-specific RNA was detected in the oocytes. After 3 h, virus-specific RNA began to accumulate in sizable amounts, but at a rate that was still only one-ninth of that seen when the viral DNA was injected one day before the radiolabeled GTP (Fig. 1). Thereafter, the rate at which SV40-specific RNA was synthesized gradually increased with time after injection of the viral DNA. (The rate of accumulation of radiolabeled SV40 RNA 16 to 22 h after injection was less than the initial rate of SV40 RNA synthesis seen in Fig. 1 because most of the viral RNA turns over with a short half-life [27].)

These same oocytes incorporated radioactivity into endogenous RNA at constant rates start-

FIG. 3. Kinetics of accumulation of SV40-specific RNA in oocytes co-injected with [³H]GTP and circularized DNA of the *Hind*III-C restriction fragment of SV40 (O). The *Hind*III-C restriction fragment of SV40, having been subcloned by using recombinant DNA techniques, was excised from pBR322 DNA by cleavage with the restriction endonuclease *Hind*III, purified as described in the text, and incubated with DNA ligase. One nanogram of this DNA, approximately 40% of which was in a circular form, was injected intranuclearly per oocyte together with [³H]GTP (O). As a control, oocytes obtained from the same frog were co-injected in parallel with [³H]GTP and 1 ng of circular wild-type SV40 DNA (●). The amount of radiolabeled SV40-specific RNA present in each sample was determined as described in the text. The total radiolabeled RNA present in the wild-type SV40 DNA-injected oocytes (▲) is shown for comparison.

ing immediately after injection (Fig. 2). This latter finding, as well as the data shown in Fig. 1, indicates that the delay seen in the synthesis of radiolabeled SV40-specific RNA at appreciable rates when the DNA and radioactivity were co-injected cannot be attributed to (i) the injections themselves having caused the transcriptional machinery of the oocyte to be disrupted temporarily; (ii) the injected GTP having needed time to equilibrate with the endogenous pool of GTP before it could serve as precursor to RNA; or (iii) the average rate at which RNA polymerase II molecules proceeded along DNA templates having changed with time. We therefore conclude that the rate at which SV40-specific RNA was being synthesized was probably a reflection of the number of these injected templates that were in a transcriptionally active state.

Several types of experiments were performed to test a variety of explanations for the lag seen in the appearance of appreciable numbers of transcriptionally active templates. Previous reports have shown that oocytes injected with wild-type SV40 DNA synthesize both "early" and "late" virus-coded proteins (5, 7a, 32, 33). Therefore, one possibility is that SV40 RNA synthesis begins shortly after injection, but at the low levels indicative of the "early" stage of the lytic cycle. This minute amount of RNA is then translated to produce a virus-coded protein such as large T antigen that, subsequently, in-

duces viral RNA synthesis to occur at high levels.

To test this possibility, we examined the kinetics of accumulation of SV40 RNA in mutants defective in the hypothetical inducing function. Data to be reported elsewhere (Mertz et al., manuscript in preparation) indicate that the 1,118-base-pair *Hind*III-C restriction fragment of SV40 (nucleotides 5,171 through 1,046 in the numbering system given in reference 36) is sufficient for synthesis of SV40-specific RNA in oocytes. This small region of the viral genome contains the presumed promoters for initiation of early and late strand viral RNA synthesis, but it does not encode any known viral proteins other than the 61-amino acid late leader protein (15, 16, 26a; P. Southern and P. Berg, personal communication; D. Margolskee and D. Nathans, personal communication). Therefore, we compared the kinetics of accumulation of SV40-specific RNA in oocytes co-injected with [³H]GTP and either wild-type SV40 DNA or purified, circularized SV40 *Hind*III-C DNA (Fig. 3). Within experimental error, the two sets of data were identical: both samples required an approximately 3-h incubation period before exhibiting appreciable numbers of transcriptionally active templates. Furthermore, a fragment of SV40 DNA containing only nucleotides 5,171 through 294, consequently encoding no known protein, also synthesizes appreciable amounts of

FIG. 4. Kinetics of accumulation of histone RNA (○) in oocytes co-injected with [³H]GTP and *D. melanogaster* histone protein H2A DNA. Stage 6 oocytes were injected intranuclearly with 4 ng of *Drosophila* DNA encoding histone protein H2A that had been purified away from its cloning vector as described in the text and incubated with DNA ligase. Approximately 50% of the resultant molecules were monomer-length circles; most of the rest were monomer-length linears. As a control, oocytes obtained from the same frog were co-injected in parallel with [³H]GTP and 1 ng of supercoiled SV40 DNA. The amount of radiolabeled histone-specific RNA and SV40-specific RNA (●) present in each sample was determined as described in the text.

viral RNA in oocytes (Mertz et al., manuscript in preparation). Therefore, we conclude that the lag is not due to the need for a known SV40-coded protein.

Further support for this conclusion comes from the finding that other types of mRNA-encoding DNAs also exhibit a delay in transcriptional activity after injection into oocytes. The data in Fig. 4 indicate a lag period with *D. melanogaster* histone protein H2A DNA of approximately 1 h. Although this lag was shorter than usual, the oocytes from this particular frog produced the same result with SV40 DNA (Fig. 4). This variation in the length of the lag period appeared to be oocyte, not template, specific. From analysis of experiments involving the injection of the same preparation of SV40 DNA into oocytes obtained from different frogs, we have noted lags ranging from 1 up to 4 h (compare Fig. 2 and 3 with Fig. 4 and 7, for example). Nevertheless, the length of the lag was quite reproducible, regardless of the template injected, when experiments were performed in parallel employing oocytes obtained from the same frog (see Fig. 3, 4, and 7, for example). Furthermore, a delay of approximately 3 h in the synthesis at appreciable rates of RNA from *Psammecinus miliaris* histone gene DNA and *Drosophila* heat-shock gene DNA injected into *Xenopus* oocytes has also been observed by R. Grosschedl (personal communication) and Voellmy and Rungger (37). Consequently, we

conclude that this lag is a general phenomenon common to at least some, and possibly all, DNAs transcribed by RNA polymerase II.

As a final control, we also examined the kinetics of accumulation of somatic 5S ribosomal RNA in oocytes injected with DNAs encoding genes for its synthesis. Gurdon and Brown (10) had shown previously that 5S ribosomal DNA from pXbs1, a recombinant DNA containing sequences from *X. borealis* that encode somatic 5S rRNA, is transcribed very efficiently within 30 min after injection into the nuclei of *X. laevis* oocytes. To demonstrate that this is also true in our hands, we repeated their experiment (data not shown). As expected, we found that within 20 min, severalfold more radiolabeled 5S RNA was present in oocytes injected with 4 ng of *X. borealis* ribosomal DNA than in those receiving only [³²P]GTP. By 40 min after injection, the amount of radiolabeled 5S RNA that had accumulated was already 140-fold above the level detected in the control. Thereafter, it continued to increase, reaching levels 500 to 1,000 times above that of endogenous 5S RNA synthesis by 20 h after injection. Injection of 5S ribosomal DNA excised from pXlo8, a plasmid containing genes for oocyte 5S rRNA of *X. laevis*, also resulted in increased levels of 5S rRNA synthesis within 20 min, although the stimulation effect in this latter case was only a few fold (data not shown). Furthermore, several investigators have reported that DNAs from a diversity of

FIG. 5. Time course for reconstitution of circular SV40 DNA into chromatin. Tritiated monomer SV40 DNA (>95% supercoiled) was injected intranuclearly into oocytes obtained from the same frog used for the transcription experiments performed in parallel for Fig. 2. At the times indicated, groups of 20 oocytes were homogenized in proteinase K buffer. The radiolabeled DNAs present in the samples were purified, and their structures were resolved by electrophoresis in a 1.4% agarose gel as described in the text. The fluorograph shown here was exposed for 7 weeks. Lane 1, uninjected SV40 DNA; lanes 2 to 9, SV40 DNA that had been incubated in oocytes for 2 min, 5 min, 10 min, 20 min, 40 min, 80 min, 2.5 h, and 6 h, respectively. The positions of fully supercoiled (I) and nicked and relaxed circular (II) SV40 DNA are indicated.

species that encode a variety of transfer RNAs function as templates for faithful transcription by RNA polymerase III within minutes after injection into oocytes (for examples see references 6, 19, and 23). These findings show that not all injected DNAs need time either to diffuse within the oocyte or to associate with appropriate cellular factors before they can serve as transcriptionally active templates. In addition, these data, taken together, indicate that the requirements for transcription of injected DNAs by RNA polymerases II and III are different.

Kinetics of chromatin reconstitution in oocytes. Wyllie et al. (42) have shown previously by a number of criteria, including the conversion of relaxed circular (form II) DNA into fully supercoiled (form I) molecules, that purified SV40 DNA reconstitutes into chromatin within 10 h after injection into the nuclei of oocytes. Consequently, one plausible hypothesis to explain the lag seen in the onset of mRNA synthesis is that the injected DNAs may need to reconstitute properly into chromatin before they can be used by RNA polymerase II for transcription. If so, one would expect to find a correlation between the appearance of transcriptionally active templates and the time needed for the injected DNAs to reconstitute into chromatin.

To look for such a correlation, we determined the time course for reconstitution of injected DNAs into chromatin as well as the kinetics of accumulation of RNA made from them. These experiments were performed in parallel, using oocytes obtained from the same frog. An example of the data obtained from one such experiment is shown in Fig. 5. A majority of fully supercoiled, radiolabeled SV40 DNA molecules were converted, within 2 min after injection, to relaxed circular ones (lane 2); the remainder had lost some, but not yet all, of their negative supertwists and, consequently, migrated in the gel at numerous intermediate rates. Not until 80 min after injection were any molecules present that migrated again in the position of fully supercoiled DNA (lane 7). With increasing incubation times, more of the DNA molecules became fully reconstituted into chromatin. By 6 h, reconstitution was essentially complete as measured by the reappearance of fully supercoiled DNA in amounts similar to that originally injected (compare lanes 1 and 9).

An unexpected observation was that by 10 min after injection, an appreciable amount of the SV40 DNA failed to enter the gel. The percentage of the total injected material that was in this "aggregated" form gradually increased and then

decreased in amount, peaking at approximately 40 min after injection (Fig. 5). Data presented elsewhere (J. E. Mertz and T. J. Miller, *Mol. Cell. Biol.*, in press) indicate that at least some of this aggregated material is catenated oligomers that form shortly after injection but gradually resolve back to monomer circles with further incubation in oocytes.

The delay in the appearance of sizable amounts of reconstituted chromatin correlated well with the lag observed before oocytes from this frog synthesized virus-specific RNA at appreciable rates after injection in a parallel experiment with [^3H]GTP and non-radiolabeled SV40 DNA (Fig. 2). Furthermore, oocytes that exhibited shorter or longer lags in the appearance of transcriptionally active templates (see above) also showed correspondingly altered rates at which they reconstituted DNA into chromatin (data not shown). We therefore conclude that SV40 DNA, and possibly other message-encoding DNAs, may need to associate with appropriate cellular chromosomal factors before they can function as transcriptionally active templates *in vivo*.

To compare the above results with the template requirements for transcription by RNA polymerase III, the time course for reconstitution into chromatin of somatic 5S ribosomal DNA injected into oocytes was also determined in conjunction with that for synthesis of RNA from it (Fig. 6). Since the 700-base-pair 5S ribosomal DNA was excised from its cloning vector and ligated *in vitro*, it was initially present as a mixture of relaxed circular (II) and unit-length linear molecules. However, within 5 min after injection, some of the DNA had already acquired one or more supertwists (Fig. 6, lane 1). With time, an increasingly larger percentage of the injected DNA became partially supertwisted. Nevertheless, as with SV40, the vast majority of the DNA molecules did not become fully supertwisted—i.e., fully reconstituted into chromatin—until 3 to 6 h after injection. (The existence of only a few discrete bands representing intermediate superhelical densities was due to the fact that this small DNA accommodates only four nucleosomes when fully reconstituted [14].) Taken together with the finding that this DNA was transcribed efficiently within 20 to 40 min after injection into oocytes obtained from the same frog (see above), these data indicate that 5S DNA is probably transcriptionally functional in the absence of a full complement of nucleosomes. Since the time courses for reconstitution of SV40 DNA and 5S ribosomal DNA are similar, the dramatic differences in their onsets of transcriptional activity may be a reflection of specific properties of these two

templates. However, a more likely hypothesis is that these findings result from differences in the template requirements for transcription by RNA polymerases II and III.

Which cellular chromosomal factors are necessary for transcriptional activity of SV40 DNA in oocytes? One way to approach the question of which factors are necessary for transcriptional activity is to assay the transcriptional activity of SV40 DNA already associated with the potentially important factors at the time of injection into the nuclei of *Xenopus* oocytes. Since these "minichromosomes" do not need to reconstitute into chromatin first, one might expect them to function immediately as templates for RNA synthesis.

We chose SV40-infected monkey cells as our source of viral minichromosomes. Using this

FIG. 6. Time course for reconstitution of somatic 5S ribosomal DNA into chromatin. The 700-base-pair restriction fragment of *X. borealis* DNA, obtained by cleaving tritiated pXbs1 DNA with the restriction endonuclease *Hind*III, was purified and incubated with DNA ligase. This DNA, consisting of approximately equimolar amounts of monomer-length circles and linears, was injected intranuclearly into oocytes obtained from the same frog used for the transcription experiments. At the times indicated, groups of 20 oocytes were homogenized in proteinase K buffer. The samples were processed as described in the legend to Fig. 5. Lanes 1 to 8, 5S ribosomal DNA that had been incubated in oocytes for 5 min, 10 min, 20 min, 40 min, 90 min, 3 h, 6 h, and 20 h, respectively. The positions of fully supercoiled (I), nicked and relaxed circular (II), and unit-length linear (L) DNA are indicated.

FIG. 7. Kinetics of accumulation of SV40 RNA in oocytes co-injected with [³²P]GTP and SV40 minichromosomes obtained from monkey cells (Δ). SV40 minichromosomes were isolated in the presence of α-amanitin from virus-infected monkey cells by the Triton-EDTA-0.2 M NaCl procedure and purified as described in the text. Then 10 nl of this material (SV40 DNA concentration, approximately 100 μg/ml) was injected intranuclearly per oocyte together with [³²P]GTP. At the times indicated, groups of 20 oocytes were harvested, and their contents of SV40-specific RNA were determined as described in the text. For controls, oocytes obtained from the same frog were co-injected in parallel with [³²P]GTP and either 1 ng of purified, supercoiled SV40 DNA (○) or 1 ng of supercoiled SV40 DNA that had been mixed before injection with the material obtained from mock-infected monkey cells that co-purified with SV40 minichromosomes (●). The total radiolabeled RNA present in each sample was determined from the amount of radioactivity that bound to DE81 filters. The data shown here for total radiolabeled RNA (□) were obtained from the oocytes co-injected with SV40 DNA plus mock-infected cell material; data obtained from the other oocytes were similar.

approach, one can readily obtain fairly large quantities of viral DNA associated with cellular factors derived from its natural host. Figure 7 shows the results obtained from one such experiment in which SV40 minichromosomes isolated and purified in 0.2 M NaCl were injected into oocytes along with [³²P]GTP. The kinetics of accumulation of SV40-specific RNA were similar to that seen when oocytes from the same frog were injected in parallel with either purified SV40 DNA or SV40 DNA that had been mixed together in vitro with cellular material obtained from mock-infected monkey cells that copurified with the SV40 minichromosomes (also Fig. 7). Nevertheless, as expected, these minichromosome-injected oocytes synthesized radiolabeled endogenous RNA without a measurable lag (Fig. 7).

The minichromosome preparations used in the

experiments described above had been treated during the isolation procedure with 100 μg of α-amanitin per ml to prevent preexisting transcriptional complexes from being responsible for the viral RNA synthesized after injection. The fact that a lag in the appearance of transcriptionally active templates was observed indicates that the drug treatment had successfully inactivated monkey RNA polymerase II molecules that may have been present but had not inhibited *Xenopus* RNA polymerase II molecules from working subsequently. Nevertheless, to eliminate the possibility that the treatment with α-amanitin was somehow responsible for the lag, the experiment was repeated with a preparation of SV40 minichromosomes that had been isolated and purified in parallel in an identical manner, except for the omission of the drug; the delay in the synthesis of radiolabeled virus-specific RNA at

FIG. 8. Superhelical density of the SV40 DNA from minichromosomes incubated in oocytes for various lengths of time. SV40 minichromosomes were purified in parallel with those used for the experiment in Fig. 7 from virus-infected monkey cells to which [^3H]thymidine had been added. Ten nanoliters of this material (SV40 DNA concentration ca. 100 $\mu\text{g}/\text{ml}$; specific activity ca. 5×10^5 cpm/ μg) was injected intranuclearly per oocyte. At the times indicated, groups of 20 oocytes were homogenized in proteinase K buffer. The samples were processed as described in the legend to Fig. 5. Lane 1, SV40 DNA purified from minichromosomes that had been incubated in vitro at 20°C for 15 min; lanes 2 to 9, SV40 DNA purified from minichromosomes that had been incubated in oocytes at 19°C for 5 min, 10 min, 20 min, 40 min, 90 min, 3 h, 6 h, and 20 h, respectively; lane 10, SV40 DNA purified from uninjected minichromosomes that had been kept at 4°C.

appreciable rates was still observed (data not shown).

Another plausible hypothesis is that the lag seen with the minichromosomes resulted from the loss of monkey nucleosomes and their gradual replacement by ones from frog oocytes. To test this possibility, the superhelical density of the DNA from minichromosomes incubated in oocytes for various lengths of time was also examined. As opposed to injection of purified circular DNAs, the DNA from minichromosomes remained fully supertwisted (Fig. 8). (Some of the relaxed circular DNA seen in Fig. 8 was present in the preparation before injection [lane 9]; the remainder of it plus the small amount of linear DNA probably resulted from gradual degradation that is known to occur when DNA is not properly injected into the nuclei of oocytes [20, 24, 41]. The material seen near the top of the gel probably resulted from catenation within the oocytes of the injected minichromosomes [Mertz and Miller, in press]). Furthermore, S. Weisbrod and J. B. Gurdon (personal communication) have shown that exchange between exogenous and endogenous histone proteins does not occur when SV40 chromatin that has been reconstituted in vitro with chicken histone proteins is incubated in *Xenopus* oocytes for 2 days. Taken together, these findings indicate that the presence of a full complement of nucleosomes is not sufficient to enable SV40 DNA to function as a transcriptionally active template in vivo. In addition, we also noted that the strand and region specificity of the viral RNA made from minichromosomes isolated from SV40-infected monkey cells was similar to

that synthesized from SV40 DNA reconstituted in vivo with *Xenopus* histone proteins (data not shown; see reference 27 for experimental protocol). Consequently, the source of the nucleosomes and other chromosomal factors that remain associated with the injected minichromosomes does not appear to affect the relative efficiency of usage for transcriptional initiation of the early versus late strand promoters of SV40.

Transcriptional activity of linear DNA. Linearization of mRNA-encoding DNAs from a variety of sources has been shown to reduce 10- to 100-fold their transcriptional activity in oocytes (19, 20, 26, 30). This statement also holds for SV40 DNA (Fig. 2). One hypothesis to explain this observation is that linear DNA molecules are degraded after injection into the nuclei of oocytes. To test this hypothesis, we determined by agarose gel electrophoresis the approximate rate of disappearance of the unit-length linear SV40 DNA injected into oocytes: approximately 40% of the injected DNA was either still intact or only slightly reduced in size after 8 h (data not shown; see reference 24). Therefore, degradation by itself cannot account for the low efficiency of transcription of the linear SV40 DNA in oocytes.

DISCUSSION

The data presented here show that SV40 DNA does not function as an efficient template for the synthesis of appreciable amounts of viral RNA in *Xenopus* oocytes until 1 to 4 h after injection (Fig. 2 to 4 and 7). A variety of controls were performed to rule out trivial explanations for this

finding (Fig. 1 to 4). Based upon the observation that the delay in the accumulation of virus-specific RNA at appreciable rates correlated reproducibly with the time needed for the injected DNA templates to reconstitute into chromatin (Fig. 2 versus Fig. 5), we conclude that the lag results from the need for the DNA to associate with appropriate cellular chromosomal factors before it can function as a template for proper transcription by RNA polymerase II. Further support for this conclusion comes from the findings that linearized SV40 DNA both functions very poorly as a template for transcription in oocytes (Fig. 2) and fails to reconstitute properly into chromatin (see accompanying paper [24]). The fact that cloned histone genes and *Drosophila* heat-shock genes behave similarly (37; R. Grosschedl, personal communication; Fig. 4) indicates that this may be a general phenomenon common to at least some, and possibly all, DNAs transcribed by RNA polymerase II.

Our preferred hypothesis to explain the findings given above is that message-encoding DNAs need to associate with appropriate cellular chromosomal factors before RNA polymerase II can recognize the signals on the template involved in promotion and initiation of RNA synthesis. Two alternative possibilities that might lead to similar findings are that, before associating with appropriate cellular factors, the injected templates (i) are transcriptionally active but, due to incorrect initiation, make products that are rapidly degraded; and (ii) initiate RNA synthesis correctly, but terminate transcription prematurely. Although we have not definitely eliminated these latter possibilities, we consider them to be unlikely because (i) unstable SV40 RNA, having a half-life of 20 to 40 min in oocytes (27), would have been readily detected; and (ii) *all* message-encoding DNAs tested to date exhibit this delay in functioning as an efficient template for RNA synthesis. Therefore, although SV40 may possess an "attenuator" that prematurely terminates transcription (12a, 27), there exists no evidence for this phenomenon occurring with histone genes.

Template structural requirements for transcription by RNA polymerases II and III are different. Neither linear DNAs nor purified, circular DNAs of SV40 or cloned histone genes are transcribed efficiently in oocytes. Nevertheless, all DNAs transcribed by RNA polymerase III that have so far been tested in oocytes exhibit neither of these restraints (for further examples see also references 3, 6, and 19). Not only is 5S DNA transcriptionally active both before it has reconstituted into chromatin (Fig. 6) and under topological conditions in which it probably cannot form proper chromatin (24), but Bogenhagen

et al. (2) and Gottesfeld and Bloomer (8) have recently reported that association with histone proteins may actually inhibit this DNA from being capable of forming active transcriptional complexes.

Second, in vitro transcription systems for RNA polymerase III (1, 40) were comparatively easier to develop than ones for RNA polymerase II (22, 38). One of the difficulties encountered with the latter systems was the critical dependence upon fairly precise extract and DNA concentrations, a finding indicative of the need for specific interactions between the template and factor(s) in the extract. Together, these sets of observations provide good support for the proposal that the template structure requirements differ for transcription by these two RNA polymerases.

Transcription of SV40 minichromosomes injected into oocytes. We originally expected SV40 minichromosomes isolated from monkey cells by the 0.2 M NaCl procedure of Green et al. (9) to synthesize virus-specific RNA within minutes after injection into oocytes. However, even though they possessed a full complement of nucleosomes (Fig. 8), they exhibited the same delay in transcriptional activity that purified SV40 DNA did (Fig. 7). A likely explanation for this finding is that the treatment with 0.2 M salt results in the elution from the minichromosomes of cellular nonhistone chromosomal factors that are necessary for transcriptional activity. To test this hypothesis, we repeated these experiments, using the low-salt minichromosome extraction procedure of Su and DePamphilis (35) (see Materials and Methods). This time, not only did the lag still occur, but the subsequent synthesis of SV40 RNA was reduced in rate as well (data not shown). Experiments performed in parallel in which the low-salt-extracted minichromosomes were mixed with purified SV40 DNA indicated that the inhibitory factor(s) was associated with the minichromosomes rather than fortuitously copurifying with them.

One method by which the lag was successfully eliminated involved the isolation of SV40 minichromosomes from oocytes injected the previous day with SV40 DNA (see Materials and Methods) and their subsequent re-injection into oocytes (data not shown). This experiment demonstrates that the lag need not always occur with SV40, but it suffers from the likelihood that RNA synthesis was probably just proceeding from pre-initiated complexes that were present in the minichromosome preparation at the time they were injected. Clearly, further studies along these lines need to be performed. Hopefully, conditions can be found for purifying minichromosomes that can initiate transcription shortly after injection into oocytes. If so, it

should be possible to begin trying to identify the chromosomal factors needed for transcriptional activity by analyzing how various treatments of these minichromosomes affect their ability to be transcribed shortly after being injected into oocytes.

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