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Regulation of Simian Virus 40 Gene Expression in *Xenopus laevis* Oocytes

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Expression of the simian virus 40 (SV40) early and late regions was examined in *Xenopus laevis* oocytes microinjected with viral DNA. In contrast to the situation in monkey cells, both late-strand-specific (L-strand) RNA and early-strand-specific (E-strand) RNA could be detected as early as 2 h after injection. At all time points tested thereafter, L-strand RNA was synthesized in excess over E-strand RNA. Significantly greater quantities of L-strand, relative to E-strand, RNA were detected over a 100-fold range of DNA concentrations injected. Analysis of the subcellular distribution of [^{35}S]methionine-labeled viral proteins revealed that while the majority of the VP-1 and all detectable small t antigen were found in the oocyte cytoplasm, most of the large T antigen was located in the oocyte nucleus. The presence of the large T antigen in the nucleus led us to investigate whether this viral product influences the relative synthesis of late or early RNA in the oocyte as it does in infected monkey cells. Microinjection of either mutant C6 SV40 DNA, which encodes a large T antigen unable to bind specifically to viral regulatory sequences, or deleted viral DNA lacking part of the large T antigen coding sequences yielded ratios of L-strand to E-strand RNA that were similar to those observed with wild-type SV40 DNA. Taken together, these observations suggest that the regulation of SV40 RNA synthesis in *X. laevis* oocytes occurs by a fundamentally different mechanism than that observed in infected monkey cells. This notion was further supported by the observation that the major 5′ ends of L-strand RNA synthesized in oocytes were different from those detected in infected cells. Furthermore, only a subset of those L-strand RNAs were polyadenylated.

The permissive host cell for simian virus 40 (SV40) is the kidney cell of the African green monkey. Primary cultures and established cell lines from this tissue respond to viral infection in a temporally regulated manner. The early phase of infection is characterized by transcription of RNA from the early strand (E-strand) of the viral genome, and these RNAs are processed and translated to the large T (L-T) and small t (S-T) tumor antigens. The transition to the late phase is coincident with the onset of viral DNA replication. During the late phase, L-T and S-T antigens continue to be synthesized, but the majority of the viral RNA is transcribed from the opposite late strand (L-strand) of the viral genome. This RNA is processed and translated to the major (VP-1) and minor (VP-2 and VP-3) capsid proteins (for a review see reference 39). Although very small amounts of late RNA have been detected early in lytic (3, 23) or abortive (7) infection, the great increase in this RNA synthesized late in lytic infection is most likely due to more than one factor. First, the number of templates for transcription, arising from the L-T antigen-mediated induction of viral DNA synthesis, increases vastly late in infection. The high ratio of L- to E-strand-specific RNA, then, is most likely due to the well-characterized autoregulatory function of L-T antigen that results in its specific repression of E-strand RNA synthesis (16, 23, 34, 35). However, recent studies have provided evidence that a separate L-T antigen activity that is independent of its function in viral DNA replication is required for the activation of L-strand RNA synthesis (4, 22). Moreover, as this activation seems to be specific for permissive cells where the majority of the DNA is unintegrated, it may well be that, in addition to these factors, efficient late-promoter function may require some structural feature of the template that results from replication of the viral minichromosome.

Xenopus laevis oocytes do not replicate SV40 DNA (17), but nonetheless synthesize relatively large quantities of SV40 L-strand RNA. Introduction of viral DNA into the oocyte results in the synthesis of not only substantial amounts of both late RNA and proteins, but also lesser amounts of early RNA and proteins (9, 30, 36, 41). Microinjection of DNA into the oocyte nucleus has several technical advantages, including the ability to control, rather precisely, both the amount of material introduced into the nucleus and the time of its introduction. We have therefore undertaken an analysis of the expression of the early and late SV40 transcription units in *X. laevis* oocytes to investigate further the extent to which viral gene expression in this system resembles that in the infected monkey cell and to gain insight into the mechanism(s) by which the late promoter can be activated.

MATERIALS AND METHODS

Materials. SV40 DNA I was purchased from Bethesda Research Laboratories, Inc. Recombinant plasmids containing wild-type and C6 SV40 DNA, pKI and pC6KI (11), respectively, were a kind gift from Y. Gluzman, and the clone pVAp (20) was generously provided by J. Manley. Restriction enzymes were purchased from New England BioLabs, Inc. *X. laevis* mature females were obtained from Nasco. Anti-SV40-capsid antiserum was a very generous gift from H. Ozer. Antitumor antiserum was prepared from hamsters bearing tumors 4 to 6 weeks after inoculation with 10^6 hamster cells transformed with SV40 (HSV line). T4 DNA ligase, calf intestinal phosphatase, T4 polynucleotide kinase, SI nuclease, and α-amanitin were obtained from Boehringer Mannheim Biochemicals.

Preparation of DNA for injection. SV40 DNA I was diluted

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with water immediately before injection. To delete plasmid sequences from recombinant plasmids and to generate circular viral DNA, pKI and pC6KI were cleaved with *Eco*RI and then ligated in a dilute solution (2 μg/ml) with T4 DNA ligase to 90 to 100% form II, as judged by agarose gel electrophoresis. After concentration with isobutanol and chloroform extraction, DNA was precipitated in ethanol and then digested with *Fnu*DII, which has no restriction sites in SV40 DNA and multiple sites on pmK16#6 (the pKI and pC6KI plasmid vector). DNA was treated with phenol, chloroform, and isoamyl alcohol and then precipitated in ethanol. The final estimation of DNA concentrations was achieved by comparison to known quantities of SV40 DNA form II (generated by a mild DNase I digestion of SV40 DNA I) electrophoresed in parallel on an agarose gel. To delete the SV40 *Nde*I B fragment, SV40 DNA form I was cleaved with *Nde*I and recircularized as described above. The excised *Nde*I B fragment was linearized by *Bst*XI digestion.

Injection into oocyte nuclei. Excised ovaries of *X. laevis* mature females were incubated in OR-2 medium (6) containing 0.15% collagenase (Worthington Diagnostics) at 20°C until the ovarian tissue was dissociated and follicle cells were digested. The defolliculated oocytes were maintained in modified Barth's solution (MB) (14). To microinject accurately into the oocyte nucleus, a modification of procedures described by Kressman et al. (24) was used. Oocytes were placed in 35-mm petri dishes in 3 ml of MB and centrifuged in an IEC clinical centrifuge rotor 215-369 at 800 × *g* for 12 min. This causes the germinal vesicle to rise to the surface of the animal pole, into which microinjection can be directed. Micropipettes were pulled from glass capillary tubes to tips 10 to 20 μm in diameter using a Leitz microforge. The micropipettes were calibrated to deliver 20 sequential microinjections of 20 nl each.

Labeling, extraction, and immunoprecipitation of oocyte proteins. Groups of 20 oocytes were labeled with 200 μCi of [^{35}S]methionine (Amersham Corp.) in MB 24 to 48 h after injection. The surviving oocytes (80 to 100%) were rinsed with distilled water and stored at −80°C.

When necessary, the cytoplasm and nucleus were separated by manual dissection after the injection and labeling period. A small hole was poked in the center of the animal pole, and the nucleus was extruded from the oocyte with a pair of fine forceps. Cytoplasmic and nuclear fractions were frozen immediately in dry ice in minimal amounts of MB.

For extraction of viral proteins, the oocytes were disrupted in a small Dounce homogenizer in 1 ml of buffer containing 0.1 M Tris hydrochloride (pH 8.6), 0.1 M NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.7 mM Na_2HPO_4, and 0.5% sodium dodecyl sulfate (SDS). The homogenate was sonicated for 1 min in an Ultrasonic W-220 sonicator, made 0.5% with Nonidet P-40, and then centrifuged at 15,000 × *g* for 15 min. Usually 200 μl of extract was used for immunoprecipitation, although the exact quantity was normalized to contain equal numbers of trichloroacetic acid-precipitable counts.

Immunoprecipitation of capsid proteins was performed as described by Prives et al. (33), and immunoprecipitation of tumor antigens was performed as described by Fradin et al. (9). All immunoprecipitations were performed in antibody excess. Some batches of antitumor antisera differed in their immunoreactivity to S-T antigen. Immunoprecipitated polypeptides were analyzed by electrophoresis through 12.5% polyacrylamide gels followed by autoradiography.

RNA preparation. When required, RNA was labeled by nuclear injection of 2 μCi of [α-^{32}P]GTP (ICN Pharmaceuticals Inc.) in 20 nl of water per oocyte. Groups of 20 oocytes were solubilized in 0.4 ml of 10 mM Tris hydrochloride (pH 7.5)–10 mM NaCl–1 mM $MgCl_2$–2% SDS. Proteinase K was added to a final concentration of 1 mg/ml, and samples were incubated at room temperature for 30 min, followed by phenol, chloroform, and isoamyl alcohol extraction and ethanol precipitation. DNase I digestion of RNA preparations was carried out in 200 μl of 5 mM $MgCl_2$–1.5 mM dithiothreitol–DNase I (50 μg/ml) and 100 U of RNAsin. Samples were incubated at 37°C for 15 min and subsequently extracted with phenol, chloroform, and isoamyl alcohol and then ethanol precipitated. Selection of polyadenylated RNA was performed by oligodeoxythymidylate-cellulose chromatography as described by Favaloro et al. (8).

Hybridization of labeled RNA to SV40 early and late separated strands. SV40 DNA I was cleaved with *Bam*HI and *Hpa*II, denatured, and subjected to neutral gel electrophoresis as described by Hayward (19). The DNA was transferred to nitrocellulose by the Southern technique (38). To assure that hybridizations were in DNA excess, 0.5-cm strips containing 1 μg of SV40 DNA each were used. Hybridization was performed in a siliconized glass tube which was rocked gently. Equal numbers of ^{32}P-labeled acid-precipitable RNA were added to filters (ca. the amount of RNA in one oocyte). Hybridization was performed in a solution containing 0.3 ml of 50% deionized formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 2× Denhardt reagent (0.1% each of Ficoll, polyvinylpyrrolidone, and bovine serum albumin), 20 mM sodium phosphate, 0.06% sodium pyrophosphate, 250 μg of sheared herring sperm DNA per ml, and 0.5% SDS. Nucleic acids were first denatured in the above buffer by incubation at 80°C for 10 min and then cooled and added to filters for a 24-h incubation at 37°C. Filters were washed three times with 2× SSC–0.5% SDS at 37°C and at 68°C, and one time with 0.2× SSC–0.5% SDS at 37°C and at 68°C. The filters were then dried and autoradiographed. In some cases the relative quantities of different RNA species were determined by densitometry.

SI nuclease mapping of spliced RNA species or 5′ ends of L-strand RNA. SI nuclease mapping was performed by the method of Berk and Sharp (2) as modified by Weaver and Weissmann (40). For analysis of spliced viral RNA species, SV40 DNA I was cut with *Bam*HI, treated with calf intestinal phosphatase, and labeled with [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase to a specific activity of $>10^7$ cpm/μg. Typically, RNA of 0.8 oocyte equivalent or polyadenylated RNA of two oocyte equivalents was used with 30 ng of DNA probe per hybridization, which was carried out for 6 h at 48°C in 80% formamide–0.4 M NaCl–0.04 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) [pH 6.5]–1 mM EDTA, followed by digestion with SI nuclease (4,000 U/ml) for 30 min at 45°C. Before incubation at the hybridization temperature, nucleic acids were denatured at 80°C for 10 min in the buffer described above. To map L-strand RNA 5′ ends, a single-stranded *Hpa*II-*Taq*I fragment was used. For this purpose, SV40 DNA was cleaved with *Hpa*II and *Taq*I and treated further as described above. After labeling, the *Hpa*II-*Taq*I fragment was denatured, and the DNA strands were separated on a 4% polyacrylamide gel. SI analysis was performed as described above except that hybridization was carried out at 37°C in 50% formamide for 12 h followed by SI nuclease digestion at 37°C. SI nuclease-resistant fragments were analyzed by denaturing glyoxal-agarose or urea-acrylamide gel electrophoresis.

RESULTS

Time course of early and late gene expression in *X. laevis* oocytes. Viral gene expression is temporally regulated in SV40-infected monkey cells so that within approximately the first 18 to 20 h after infection, the majority of the viral RNA and proteins are the product of the E-strand-specific transcription unit. The onset of viral DNA replication that occurs after this time interval marks the beginning of the late phase in which the L-strand-specific transcription unit is activated, resulting in the synthesis of a 20 to 100-fold excess of L-strand RNA over E-strand RNA (and a 5,000-fold excess over the L-strand RNA that can be detected early in infection) (27, 39). Although no SV40 DNA replication has been detected in oocytes (17), a similar high ratio of L-strand to E-strand RNA has been observed 24 to 48 h after injection of viral DNA (29). Viral RNA synthesis in oocytes has been detected as early as 2 h postinjection, although the ratio of L-strand- to E-strand-specific transcripts was not determined (30). It was therefore of interest to analyze whether, as is the case in infected cells, there is a substantial delay in the onset of synthesis of significant quantities of L-strand, relative to E-strand, RNA in oocytes. To test this, viral DNA was injected, and at different times RNA was labeled, extracted 2 h later, and hybridized to separated strands of viral DNA fragments containing primarily the early and late regions (Fig. 1A). Late RNA could be detected within 2 h following injection, and although early RNA was also detected, the ratio of L-strand to E-strand RNA (i.e., of RNAs that hybridized to the Bl and Ae strands, respectively) remained fairly constant over the time intervals examined (Fig. 1B). At no time was more E-strand than L-strand RNA detected, unlike the situation early in lytic infection. It should be noted that these determinations do not provide information about the size, processing, or cellular localization of the RNA in the oocyte. The sizeable quantity of RNA hybridizing to the early region containing L-strand DNA fragment, designated Al strand, is consistent with observations that L-strand RNAs of genome length or greater accumulate to a significant extent in oocytes (30, 41; T. Michaeli and C. Prives, unpublished data).

Despite the fact that viral RNA synthesis was detected as early as 2 h after injection, we found that viral proteins were not synthesized in significant quantities until the 6- to 8-h time interval (data not shown). Thus, there is a lag of approximately 2 to 4 h between the onset of viral transcription and detectable protein synthesis. However, after this lag, the synthesis of late and early region encoded proteins occurs concomitantly. We concluded from these results that the temporal regulation of viral L-strand RNA synthesis, and consequently of translation of L-strand mRNA, that is characteristic of SV40-infected monkey cells is not evident in *X. laevis* oocytes.

Analysis of early and late viral RNA and proteins after injection of different quantities of SV40 DNA. One factor that is likely to contribute to the induction of L-strand RNA synthesis in infected cells is the great increase in the number of viral DNA templates generated after DNA replication has initiated. We therefore analyzed the RNA synthesized 24 to 48 h after intranuclear injection of quantities of DNA ranging from 0.1 to 10 ng (approximately 10^7 to 10^9 molecules, respectively, of SV40 DNA) (Fig. 2). We found that it was not possible to detect viral RNA synthesis when DNA quantities below 0.1 ng were microinjected and that the total amount of stable L-strand and E-strand RNA increased only 2- to 5-fold over the 100-fold range of DNA concentrations

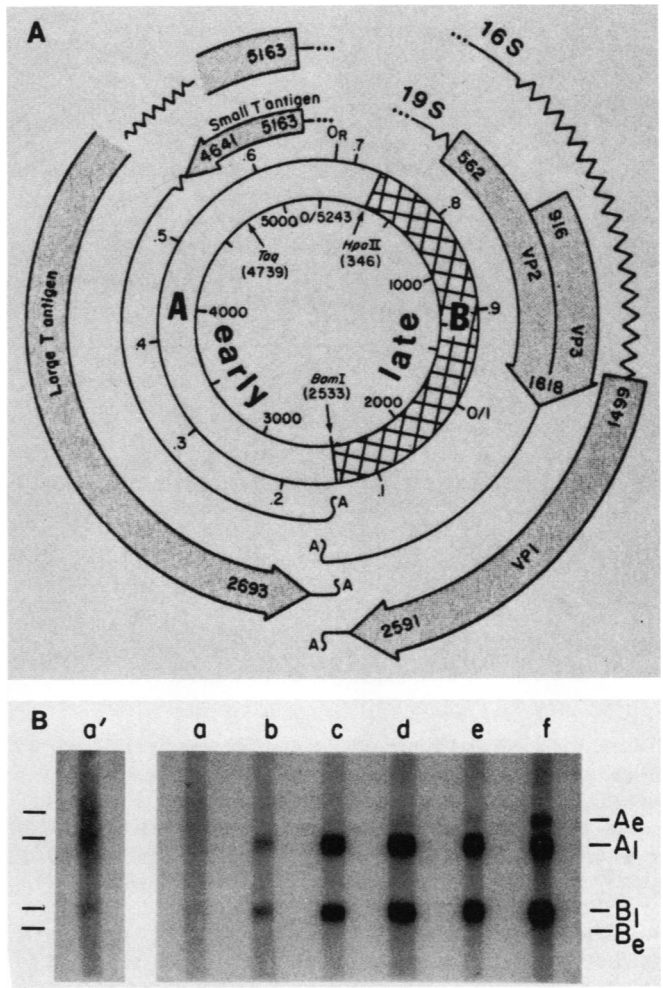

FIG. 1. (A) Map of the SV40 genome, major transcripts, and proteins. The inner circle represents the SV40 genome. Fractional map units and the origin of replication (OR) are indicated outside the circle. Nucleotide numbers and relevant restriction endonuclease cleavage sites are shown on the inside of the circle. The positions of the fragments generated by cleavage with *Bam*HI and *Hpa*II (fragment A, open; fragment B, cross-hatched) are shown, and the early and late regions of the genome are indicated. The outermost lines delineate the structures of viral mRNAs found in infected monkey cells. Dotted lines indicate the region of mRNA 5′ ends, wavy lines indicate sequences removed by splicing, and shaded areas indicate protein coding sequences. The two major late mRNAs, 19S and 16S, are marked. (B) Kinetics of SV40 E- and L-strand RNA synthesis in *X. laevis* oocytes. SV40 DNA I was injected into oocyte nuclei (2.5 ng per oocyte), and RNA was labeled for 2 h with [α-^{32}P]GTP by injection at different times after the initial DNA injection. (At time zero, DNA and label were coinjected.) After extraction, RNA samples were hybridized as described in the text to strips of nitrocellulose filters containing the separated strands of the A and B fragments described for panel A. RNA was labeled for 0 to 2 (a), 2 to 4 (b), 4 to 6 (c), 6 to 8 (d), 8 to 10 (e), and 10 to 12 (f) h after DNA injection. Filters were exposed for 16 h with (a′) and without (a to f) intensifying screens. Approximate genomic region (A, early; B, late) and strand identities (e, early; l, late) are marked on the right side of the autoradiogram, and their positions are indicated on both sides.

injected. Densitometry of autoradiograms of labeled RNA-DNA hybrids showed that at least 10-fold greater quantities of L-strand- than E-strand-specific RNA were synthesized in response to 0.1 ng of DNA. This ratio decreased with increasing quantities of injected DNA, so that at 10 ng of

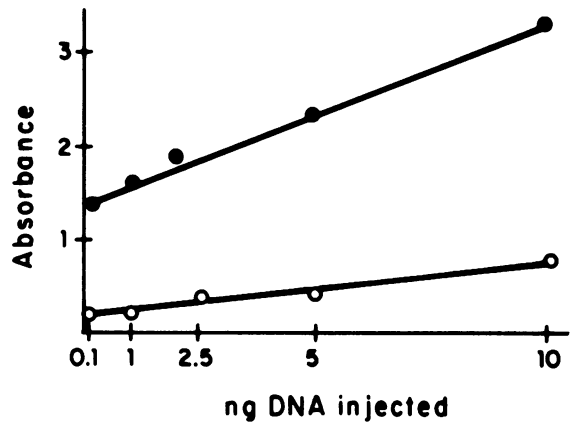

FIG. 2. Synthesis of viral RNA in *X. laevis* oocytes after microinjection of different quantities of SV40 DNA. SV40 DNA I was serially diluted, and the indicated quantities were injected into oocyte nuclei. [α-^{32}P]GTP was injected 24 h later, and RNA was labeled for an additional 24 h and subsequently extracted. RNA samples were hybridized as described in the text to nitrocellulose filters containing the separated strands of the A and B fragments described for Fig. 1, panel A. E- and L-strand RNAs (Ae and Bl, respectively) were quantitated by densitometry of the respective bands. Symbols: ○, E-strand RNA; ●, L-strand RNA.

DNA, the L-strand/E-strand RNA ratio was fivefold. However, at all DNA concentrations tested, L-strand RNA was synthesized in excess of E-strand RNA.

As there was some decrease in the relative ratio of L-strand to E-strand RNA at the higher DNA concentrations, even though the absolute amount of L-strand RNA was greater, we considered the possibility that L-T antigen-mediated repression of E-strand RNA synthesis may occur in the oocyte. Thus, quantities of L-T antigen sufficient to interact with viral chromatin and partially repress early transcription may be produced at lower DNA concentrations, but at higher DNA concentrations this amount of L-T antigen is insufficient to affect transcription to the same extent. The quantities of viral proteins synthesized after injection of different quantities of DNA were compared (Fig. 3). Both tumor antigens and the major capsid protein, VP-1, have been previously identified in SV40-injected oocytes by immunoprecipitation with specific antisera (9, 36). The amount of capsid proteins synthesized in response to quantities of microinjected DNA ranging from 1 to 10 ng was fairly constant, although there was some variation in the quantities of late proteins synthesized in different experiments. In contrast, we consistently observed that there was an optimal DNA concentration for synthesis of maximum quantities of the tumor antigens that varied somewhat between experiments but was usually 2.5 ng of injected DNA. At 10 ng of injected DNA, a considerable decrease in the amount of L-T antigen and a lesser decrease in S-T antigen quantities were consistently observed. The reasons for the decrease in quantities of tumor antigens at high DNA concentrations are not as yet clear. However, it should be noted that a significant fraction of the viral RNA remains in the nucleus, the majority of which is not polyadenylated (30, 41), and that the vast majority of early and late viral transcripts are spliced at all DNA concentrations that have been tested (Michaeli and Prives, unpublished data). Therefore, although increased quantities of E-strand RNA sequences were observed at higher DNA concentrations, it would be difficult to assess how this relates to the quantities of translatable viral mRNA in the oocyte cytoplasm. However, the fact that relatively reduced quantities of L-T antigen were synthesized at the highest DNA concentration injected raised the possibility that L-T antigen can autoregulate transcription in the oocyte. To examine further this possibility we first analyzed the subcellular localization of viral proteins synthesized in oocytes.

SV40 L-T antigen accumulates in the oocyte nucleus. *X.*

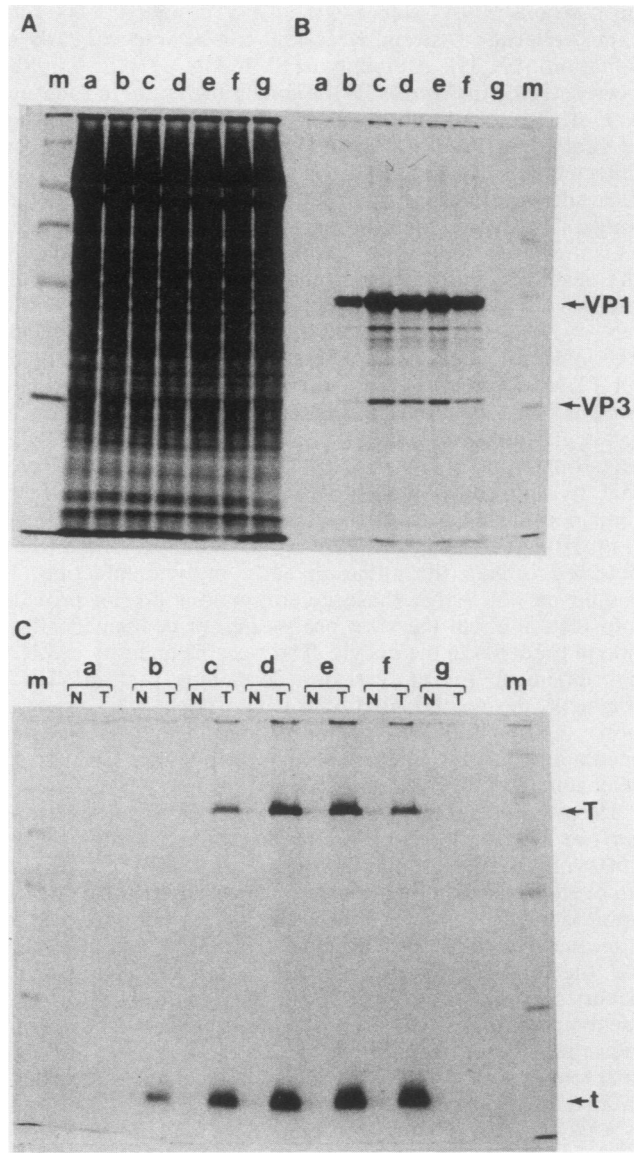

FIG. 3. Synthesis of viral proteins in *X. laevis* oocytes after microinjection of different quantities of SV40 DNA. SV40 DNA I was serially diluted, and 0.01 (a), 0.1 (b), 1 (c), 2.5 (d), 5 (e), 10 (f), and 0 (g) ng were injected into oocyte nuclei. Proteins were labeled with [^{35}S]methionine 24 to 48 h postinjection and subsequently extracted. Samples were immunoprecipitated with either anti-SV40 capsid antiserum (B) or antitumor antiserum (C), and polypeptides were separated by polyacrylamide gel electrophoresis. Aliquots of direct oocyte extracts were electrophoresed in parallel (A). In panel C, N indicates nonimmune serum and T indicates antitumor antiserum. Protein molecular weight markers (lane m) are (from the top) 200, 94, 67, 45, 30, and 12.3 kilodaltons.

laevis oocyte nuclei can be separated from the cytoplasm rather cleanly and efficiently by manual dissection (5). Analysis of [^{35}S]methionine-labeled proteins that accumulated in the two oocyte compartments was performed (Fig. 4). At least fivefold more labeled proteins were found in the cytoplasm than in the nucleus under the labeling and separation procedures utilized. The patterns (size and abundance) of labeled total polypeptides present in these fractions were significantly different, indicating the effectiveness of the separation procedure. Immunoprecipitation of the viral proteins showed that greater quantities of the capsid proteins, VP-1 and VP-3, were located in the oocyte cytoplasm than in the nucleus, in contrast to the infected monkey cell where the vast majority of VP-1 is found tightly associated with nuclear structures (1, 13). However, in oocytes both tumor antigens localized in a manner similar to that in transformed or infected cultured cells in that virtually all of the detectable S-T antigen was in the cytoplasmic portion, whereas the majority of the L-T antigen was found associated with the oocyte nuclei. It should be noted that quantities of S-T antigen varied between experiments due to differences in batches of antitumor antisera used. The presence of substantial quantities of labeled nuclear L-T antigen led us to test whether this product functions in viral gene regulation in oocytes.

L-T antigen is not required for activation of L-strand RNA or repression of E-strand RNA synthesis in *X. laevis* oocytes. Roles for L-T antigen in the regulation of early and late viral RNA synthesis have been demonstrated in infected monkey cells (4, 22, 23, 34) and in cell-free transcription systems (16, 35). The repression of E-strand RNA synthesis is mediated by its high-affinity binding to specific sites at the viral replication origin. In addition, studies on the activation of L-strand RNA have implicated the specific DNA binding function of L-T antigen (4, 22). To determine whether the L-T antigen synthesized in oocytes is involved in control of the relative amounts of L- and E-strand RNA, two mutants in which L-T antigen functions in RNA synthesis are defective were utilized. The first of these, C6, was previously shown to be unable to function in viral DNA replication (11), presumably because of its defect in sequence-specific viral DNA binding (37). The second mutant was constructed to lack the SV40 *Nde*I B fragment, deleting sequences in the L-T coding region from nucleotide 3808 to nucleotide 4826 (amino acid residues 83 to 337). The E-strand RNA transcript of this mutant lacks the 3′ common splice acceptor site for L-T and S-T antigens and therefore may well not synthesize any tumor antigen-related polypeptide at all. In any case, any L-T that might be synthesized (if a cryptic splice acceptor site was used in the oocyte) would lack sequences required for its high affinity DNA binding (32). In the course of these experiments it was discovered that SV40 DNA (wild type or mutant) in recombinant plasmids (pBR322 or other vectors) is very poorly, if at all, expressed in oocytes (T. Michaeli and C. Prives, unpublished data and manuscript in preparation). Therefore, viral DNA sequences were routinely excised from recombinant plasmids and ligated to circular form. Recircularized plasmid molecules were then linearized with restriction enzymes that do not cleave viral DNA. When C6 or *Nde*I mutant DNAs were injected into oocyte nuclei, large quantities of L-strand RNA relative to E-strand RNA were synthesized, as was consistently observed in all our experiments with wild-type DNA (Fig. 5). In the case of C6 DNA, similar quantities of viral late proteins were synthesized when compared to that of wild-type DNA, although somewhat less early proteins were detected (Fig. 6). Moreover, while comparable quantities of viral capsid proteins were synthesized upon injection of wild-type and *Nde*I mutant DNA, no tumor antigens were detected with the latter. These results suggest that T antigen, synthesized in oocytes, does not influence viral RNA synthesis, and that the relative quantities of L- and E-strand

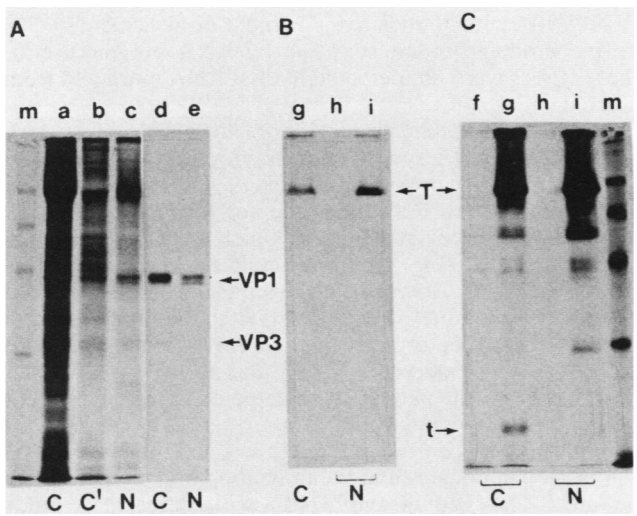

FIG. 4. Determination of cytoplasmic and nuclear localization of SV40-encoded proteins in *X. laevis* oocytes. Oocytes were intranuclearly injected with 2.5 ng of SV40 DNA, and 24 h later they were labeled with [^{35}S]methionine. After a 24-h labeling period, the cytoplasm and nuclei were manually separated and subsequently extracted. Direct cytoplasmic (a) and nuclear (c) extracts equivalent to 1/20 of an oocyte are shown, as well as 1/100 of an oocyte's cytoplasmic extract (b). Protein extracts equivalent to the cytoplasm and nucleus, respectively, of one oocyte were immunoprecipitated with anti-SV40 capsid antiserum (d, e), hamster antitumor antiserum (g, i), and hamster nonimmune serum (f, h). Immunoprecipitations of cytoplasmic extracts are shown in lanes d, f, and g, and of nuclear extracts in lanes e, h, and i. C indicates cytoplasm and N indicates nucleus at the bottom of the panels. Lane m contains protein molecular weight markers as described for Fig. 3. Lanes a to c were exposed for 24 h, lanes d and e for 1 h, panel B for 2 h, and panel C for 72 h.

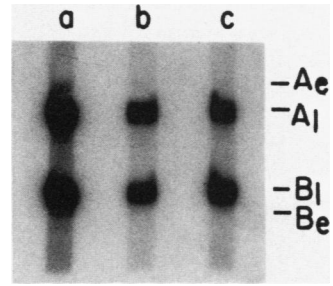

FIG. 5. SV40 RNA synthesis after microinjection of wild-type and mutant SV40 DNA. *X. laevis* oocytes were injected with 2.5 ng of circular wild-type (a) and mutant C6 (b) SV40 DNA the SV40 *Nde*I A fragment (c) prepared as described in the text. After 24 h [α-^{32}P]GTP was injected, and RNA was labeled for 24 h and extracted. RNA samples were hybridized as described in the text to nitrocellulose filters containing the separated strands of the A and B fragments described for Fig. 1, panel A. Approximate genomic regions (A, early; B, late) and strand identities (e, early; l, late) are marked on the right side of the autoradiogram.

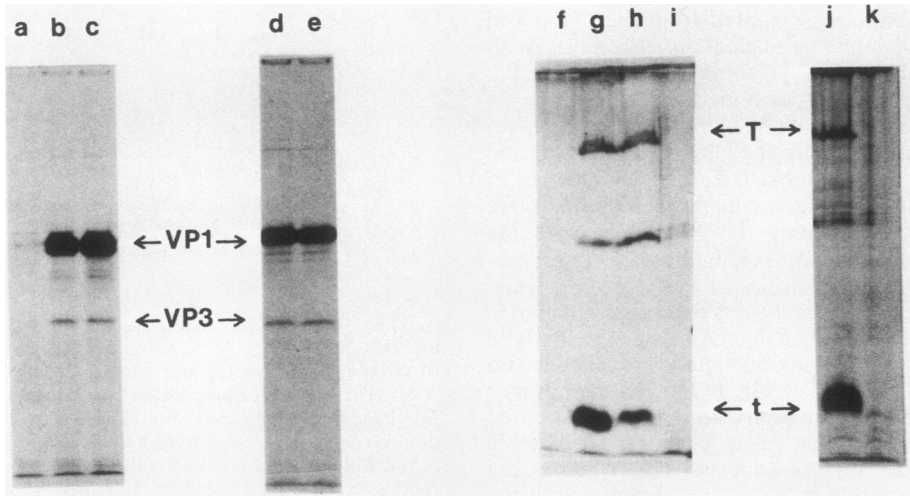

FIG. 6. Synthesis of viral proteins after injection of SV40 wild-type and mutant DNA into *X. laevis* oocytes. *X. laevis* oocytes were injected with 2.5 ng of wild-type (b, d, g, j) and mutant C6 (c, h) SV40 DNA the SV40 *Nde*I A fragment (e, i, k) prepared as described in the text. After 24 h, oocytes were labeled with [^{35}S]methionine for an additional 24 h and subsequently extracted. Protein extracts were immunoprecipitated with anti-SV40 capsid antiserum (a to e) and antitumor antiserum (f to k). Water-injected control sample immunoprecipitates are shown in lanes a and f. After polyacrylamide gel electrophoresis, gels were exposed for 1 day (a to e) and 2 days (f to k).

RNA result from factors that differ from those operating in infected or transformed cells in culture.

The major 5′ ends of SV40 L-strand RNA in oocytes are different from those detected in infected monkey cells and only a subset of the oocyte viral RNAs are polyadenylated. These experiments indicated that the factors controlling the production of SV40 RNA in the *X. laevis* oocyte differ from those in infected cells and suggested that the promoters for the viral transcription units may function differently. To determine whether this was reflected in the initiation sites of L-strand RNA, SI analysis of the 5′ ends of late RNA was performed. Comparison of the 5′ ends of RNA synthesized in oocytes 48 h after injection of viral DNA to those synthesized 48 h after infection of CV-1 cells showed striking differences (Fig. 7). First, little or no L-strand RNA initiating at or near nucleotide 325, the major species detected in infected cells (10, 31), was synthesized in oocytes. Rather, the majority of the RNAs were initiated upstream of that region. Some of these RNAs mapped farther upstream than the region of the origin of replication. Second, there seemed to be even greater heterogeneity of 5′ ends in injected oocytes than in infected cells. The pattern of 5′ ends was identical in all batches of oocytes analyzed, except for an occasional increase in the ratio of RNA initiated at or near nucleotide 300 to the rest of the 5′ ends. Third, a substantial amount of the RNA hybridized to the full length of the viral DNA probe, probably reflecting the previously noted (30, 41) existence of viral L-strand RNA that is longer than the length of the viral genome in injected oocytes. Perhaps the most striking observation made was a difference in the viral L-strand 5′ ends detected in total RNA extracted from oocytes when compared to oocyte RNA that was first bound to and then eluted from oligodeoxythymidylate-cellulose (Fig. 7, lanes d to g). This indicated that only a subset of viral RNAs initiated in oocytes becomes polyadenylated. One explanation for this unanticipated finding could have been that these transcripts were initiated by RNA polymerase III. However, comparison of the sensitivity of these different RNAs to varying quantities of α-amanitin, previously shown to affect RNA polymerase II but not RNA polymerase III (15), showed that all species of viral L-strand RNA were equally sensitive to low concentrations of the inhibitor. Control experiments of microinjection of pVAp, a plasmid encoding the adenovirus VA I gene (20), known to be transcribed by RNA polymerase III (39), showed that quantities of α-amanitin that were a factor of 10 greater than those that inhibited all of the SV40 RNA species did not significantly affect expression of VA I RNA (data not shown). Therefore, it is likely that this class of nonpolyadenylated SV40 RNAs with 5′ ends mapping near or at nucleotide 300 are the products of transcription by RNA polymerase II. These RNAs were not detected in total RNA extracted from infected cells.

Analysis of the splicing patterns of total and polyadenylated L-strand RNA synthesized in oocytes was performed by SI mapping. As previously shown (9, 41), the relative use of 19S compared to 16S splice acceptor sites in oocytes was found to be the reverse of that which occurs in infected monkey cells (see Fig. 1A for the map positions of the 19S and 16S mRNAs). However, no striking differences between the relative quantities of viral RNAs with the major 19S and 16S 3′ splice acceptor sites in total versus polyadenylated viral RNA was observed (Fig. 8), and no significant variations in the splicing pattern were detected between different batches of oocytes analyzed. Thus, there was no obvious relationship between the difference in the 5′ ends of RNAs synthesized in oocytes and the utilization of 3′ splice acceptor sites. The size of the RNAs with different 5′ ends detected in total and polyadenylated RNA in oocytes is currently being investigated to understand further the basis for the striking differences observed in the two classes of viral RNA. It is possible that the viral RNAs not found in polyadenylated RNA are considerably shorter than the other viral RNA species, perhaps resulting from premature termination or pausing by the RNA polymerase II, as postulated by others (18). These experiments further support the notion that the synthesis of SV40 L-strand RNAs in *X. laevis* oocytes is controlled by different mechanisms than it is in infected monkey cells.

DISCUSSION

We analyzed various aspects of viral RNA and protein synthesis after microinjection of SV40 DNA into the nuclei

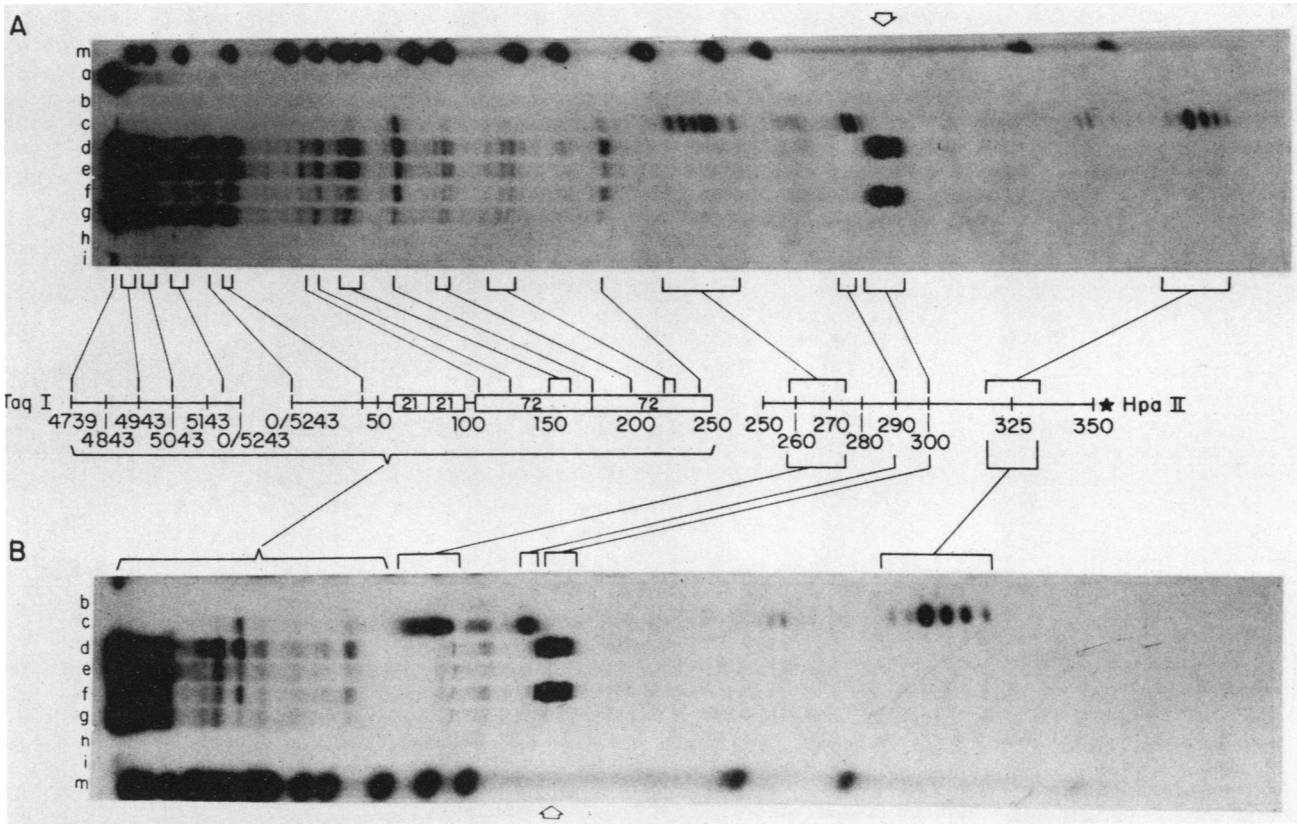

FIG. 7. Mapping the 5′ ends of SV40 L-strand RNA synthesized in *X. laevis* oocytes. *X. laevis* oocytes were injected with 2.5 (d, e) and 1 (f, g) ng of SV40 DNA I and H_2O (h, i). After a 48-h incubation, RNA was extracted and a fraction was purified by oligodeoxythymidylate-cellulose chromatography. Total RNA (d, f, h) and polyadenylated RNA (e, g, i) as well as RNA from uninfected (b) and SV40-infected (c) CVI cells were hybridized to 4 ng of a 5′-labeled single-stranded *Hpa*II-*Taq*I fragment (a) as described in the text. This amount of DNA probe is in hybridization excess because lower exposures of the autoradiogram show that only a portion of the probe was protected by hybridization to viral RNA from either monkey cells or oocytes. After hybridization and SI nuclease digestion, the protected fragments were subjected to denaturing 5% (A) and 12% (B) polyacrylamide gels containing urea. Lane a contains one-sixth of the quantity of probe used for hybridization. Molecular weight markers (lane m) are the fragments generated by *Hpa*II cleavage of pBR322 and are (from the top) 622, 527, 404, 309, 242, 238, 217, 201, 190, 180, 160, 147, 122, 110, 90, 76, 67, 34, 26, 15, and 9 nucleotides in length. A diagrammatic localization of L-strand RNA 5′ ends detected on gels is shown between autoradiograms including nucleotide numbers and positions of the 21- and 72-base-pair repeat sequences (39). This probe does not protect RNAs with a 5′ splice donor site at nucleotide 294.

of *X. laevis* oocytes. Our results suggest that there are several differences between these processes as they occur in the oocyte when compared to the infected monkey cell. In oocytes, L-strand and E-strand RNAs are synthesized concomitantly, and at all times and DNA concentrations tested there is considerably more of the former class of RNA than of the latter. Furthermore, consistent with the observations of Miller et al. (30), L-T antigen is required neither for the repression of E-strand RNA synthesis nor for the activation of L-strand RNA synthesis.

Among the mammalian cultured cell lines that have been infected with SV40, only cells derived from African green monkey kidney cells eventually synthesize a high ratio of L-strand to E-strand RNA. Although the validity of comparisons between *X. laevis* oocytes and somatic cells can be questioned, it should be noted that Graessman et al. (13) previously showed that when large, but not small, quantities of SV40 DNA were microinjected into the nuclei of nonpermissive 3T3 mouse cells, V antigen (presumably VP-1) could be detected by indirect immunofluorescence. This suggested that the expression of the late transcription unit is related to viral gene dosage, and it was proposed that a threshold level of L-T antigen must accumulate before late RNA can be synthesized. This might be relevant to our experiments in *X. laevis* oocytes in which relatively large numbers (10^7 to 10^9) of DNA molecules were injected into a single oocyte. However, the results of Graessman et al. (13) differed from ours in that the nonpermissive cells expressed T but not V antigen when low DNA concentrations were microinjected, whereas in the oocyte both late and early RNAs and proteins were detected at all DNA concentrations tested. In fact, the ratio of L- to E-strand RNAs actually decreased somewhat with increasing quantities of DNA. Furthermore, even nonpermissive 3T3 mouse cells injected with high concentrations of DNA exhibited a substantial time lag between the detection of T and V antigens (12). Therefore, the mechanism responsible for activating the late promoter in *X. laevis* oocytes is most likely different from that in injected nonpermissive cells.

We have observed that a single oocyte microinjected with 10^8 SV40 DNA molecules accumulates quantities of SV40 L-strand RNA equivalent to those detected from 10^6 monkey CV-1 cells late in lytic infection (Michaeli and Prives, unpublished data). As it has been estimated that a single infected cell accumulates approximately 10^5 SV40 DNA molecules (39), the viral RNA synthesized in oocytes there-

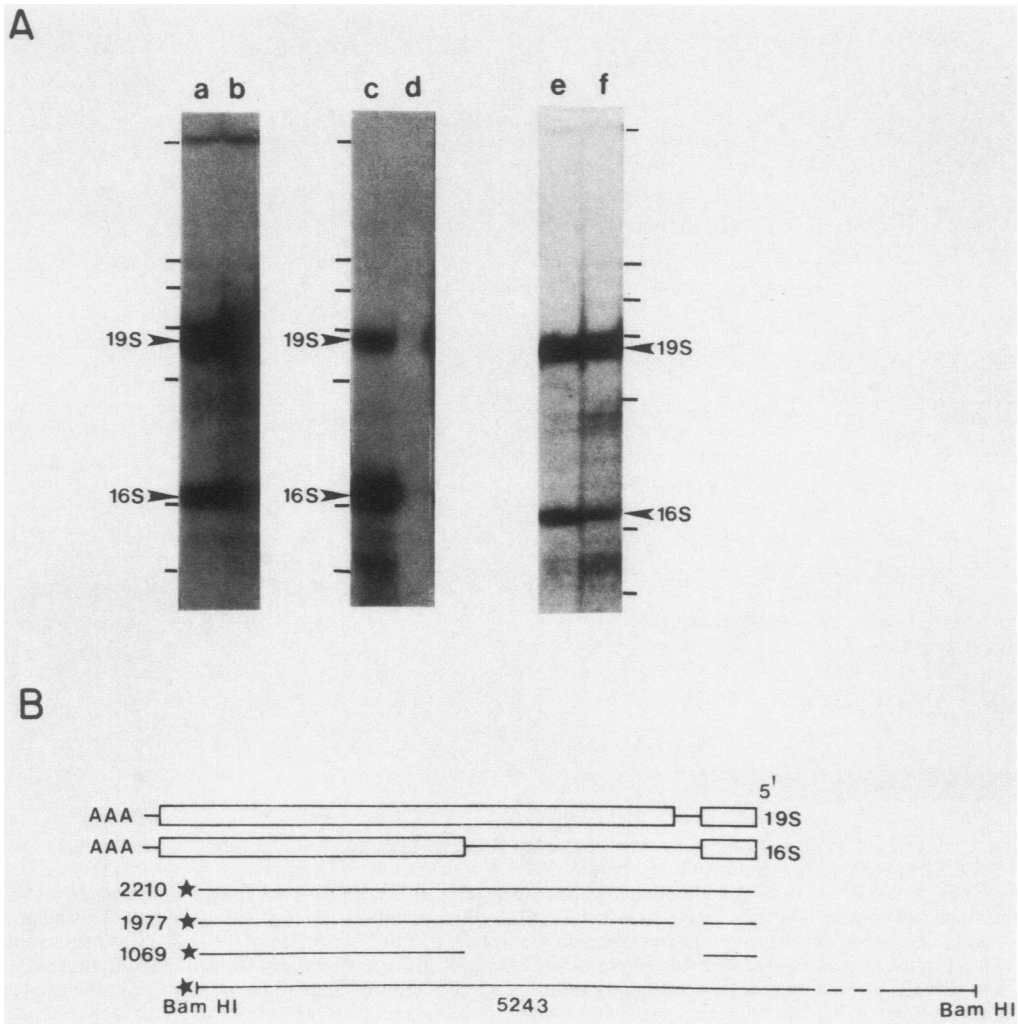

FIG. 8. SI analysis of total and polyadenylated L-strand SV40 RNA synthesized in *X. laevis* oocytes. *X. laevis* oocytes were injected with 1 (a, e) and 2.5 (b, f) ng of SV40 DNA I. After a 48-h incubation, RNA was extracted and a fraction was purified by oligodeoxythymidylate-cellulose chromatography. Total RNA (a, b) and polyadenylated RNA (e, f) as well as RNA from uninfected (d) and SV40-infected (c) CVI cells were hybridized to a 5'-labeled *Bam*HI-cleaved SV40 DNA probe. After hybridization and SI nuclease digestion, the protected fragments were subjected to 1.2% denaturing glyoxal-agarose gel electrophoresis. Total oocyte RNA and SV40-infected cells RNA (a to d) were analyzed separately from polyadenylated oocyte RNA (e, f). Location of the 19S and 16S spliced RNA protected fragments is indicated by arrows. Molecular weight markers are marked next to autoradiograms and are (from the top) 5243, 2672, 2362, 2036, 1651, 1050, and 766 nucleotides in length. Positions of L-strand spliced RNA species and unspliced RNA as they map in SV40 infected monkey cells, probe, and the expected protected fragments are depicted in panel B.

fore results from quantities of DNA a factor of 10^3 lower than those present in infected cells. Although it has not been satisfactorily determined in either case what proportion of the DNA templates are transcriptionally active, these numbers suggest that oocytes may be relatively much more efficient in synthesizing SV40 RNA. It should be noted, however, that over the 100-fold range of DNA concentrations microinjected, there was less than a 5-fold increase in viral RNA synthesis. This could reflect some limitations to SV40 RNA synthesis in oocytes, such as limiting quantities of factors required for formation of transcriptional complexes at higher DNA concentrations.

In infected cells the vast majority of both VP-1 and the L-T antigen are located in the nucleus, as determined both by indirect immunofluorescent antibody staining (12) and by immunoprecipitation of detergent extracts of the cytoplasmic and nuclear portions of radioactively labeled cells (1, 28). It was thus rather surprising that only a relatively small proportion of the VP-1 and VP-3 capsid proteins were found in the oocyte nucleus. Despite previous observations (25) of accurate posttranslational processes in this system, these proteins may be aberrantly modified in oocytes such that their transport or accumulation in the nucleus may be affected. Alternately, the intranuclear cytoskeletal structures postulated to be important in capsid protein transport in infected cells (28) may be absent or altered in the meiotically arrested oocyte nucleus. In addition, the nuclear localization of the capsid proteins may somehow require the presence of replicating viral DNA complexes that do not exist in oocytes. By contrast, the majority of the L-T antigen was found to be in the oocyte nucleus. This suggests that the sequences in L-T antigen that have recently been shown to mediate either the transport or the intranuclear accumulation of this product (21, 26) are most likely recognized in this cell. Our results therefore indicate that at least some of the factors responsible for the nuclear localization of the L-T

antigen differ from those responsible for the cellular sorting of the capsid proteins.

Despite the fact that L-T antigen enters and accumulates in the oocyte nucleus, its lack of effect on viral RNA synthesis indicates that it is not functionally associated with viral minichromosomes. Several possibilities exist to explain this lack of function. First, insufficient quantities of L-T antigen may be produced relative to the large number of SV40 DNA molecules (10^7 to 10^9) injected per oocyte. We are currently attempting to determine the approximate amount of L-T antigen synthesized by methods other than radiolabeling. Second, L-T antigen may be incorrectly modified in oocytes such that it cannot bind specifically to DNA. Third, as postulated by Miller et al. (30), a sufficiently abundant L-T antigen-like protein may already exist and function in oocytes such that the requirement for the viral product in SV40 gene expression is obviated. The existence of such a protein would be of considerable interest in understanding the origin and function of the papovavirus L-T antigens. Such a protein, however, lacks extensive cross-reactivity with any of the antitumor antisera that we have tested in uninjected oocytes, or else it was synthesized only before labeling. Fourth, Brady et al. (4) have suggested that late gene expression may be regulated in transformed or early infected cells by a repressor, the effect of which may be overcome when sufficient quantities of L-T antigen are present. The putative repressor would most likely not exist or function in oocytes, judging from the relatively large quantity of late RNA synthesized. The oocyte may therefore serve as an excellent test system for the identification of such a molecule, should sufficient quantities be produced in monkey cells. Fifth, differences between frog and monkey RNA polymerase II subunits or other transcriptional factors may result in SV40 gene expression that is independent of L-T antigen function. Alternately, RNA polymerase II in the oocyte may function differently with respect to viral promoters than its monkey cell counterpart because of differences in the structure of the minichromosome, particularly in transcription complexes. The absence of an effect of L-T antigen upon viral gene expression in oocytes may be related to a different chromosome structure in the region of the promoters for the early and late transcription units. A detailed analysis of the structure of the SV40 minichromosome in the *X. laevis* oocyte has not been reported and would very likely provide information relevant to the results we have described.

Our analysis of late RNA synthesized in oocytes revealed that while, as is the case in the infected monkey cell, there are numerous transcription starts, the position of the 5′ ends of L-strand RNAs are markedly different, being considerably upstream. In fact, a substantial proportion of these RNAs map to the early side of the origin of replication. This is consistent with the suggestion above that the polymerase functions differently in oocytes, possibly due to altered template structure. It is noteworthy that another unique characteristic of oocytes is that the relative efficiencies with which the late region (9, 41) and early region (9) splice acceptor sites are utilized are different from those observed in infected or transformed mammalian cells. Whether this is related to the difference in 5′ ends that we have noted in L-strand RNA remains to be determined. However, we observed a striking difference between the 5′ ends of oocyte RNAs that do and do not bind to oligodeoxythymidylate-cellulose. L-strand viral RNAs with 5′ ends mapping at or near nucleotide 300 were observed only in total and not in polyadenylated oocyte RNA. Their α-amanitin sensitivity was found to be similar to all other viral RNAs indicating that they are transcribed by RNA polymerase II. SI analysis revealed no striking differences in the proportions of total or polyadenylated SV40 RNAs with the 19S and the 16S 3′ splice acceptor sites, suggesting that these differences in the 5′ ends were not correlated with the cleavage of different splice acceptor sites in oocytes.

Several size classes of SV40 RNA have been detected in oocytes (30, 41). These include a class of RNAs greater in size than the viral genome, that may represent those RNAs detected by the 5′-end analysis that hybridized to the entire probe from SI digestion. In addition, an abundant 5,300-nucleotide transcript, a 2,200-nucleotide transcript that represents the authentic 19S RNA (41), and smaller RNAs including a 4S class (30) have been identified. A small proportion of the 5,300-nucleotide transcript and a large proportion of the 2,200-nucleotide transcript are polyadenylated (41). Experiments to characterize further the nonpolyadenylated L-strand RNAs with 5′ ends in the region of nucleotide 300 are under way. The existence of these RNAs is of interest because of the possibility that a particular RNA structure or sequence at the 5′ end may influence its subsequent processing or termination.

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