pBR322 DNA inhibits simian virus 40 gene expression in *Xenopus laevis* oocytes

Tamar Michaeli¹ and Carol Prives*

Department of Biological Sciences, Columbia University, New York, NY 10027, USA

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ABSTRACT

SV40 DNA form I is expressed efficiently after its injection into the nuclei of *Xenopus laevis* oocytes, resulting in the synthesis of RNA and protein products of both viral late and early transcription units. However it was observed that injection of SV40 genes cloned into pBR322 or related plasmids yielded vastly reduced quantities of viral DNA and proteins. If SV40 DNA was cleaved from the plasmid, and then recircularized prior to microinjection, viral expression was regained. The inhibition by plasmid DNA was not confined to an effect *in cis* because coinjection of circular pBR322 DNA along with SV40 DNA, as separate entities, also blocked viral RNA and protein synthesis. As circular but not linear pBR322 DNA was actively transcribed by polymerase II in oocytes, even in the presence of SV40 DNA, it is likely that pBR322 competes for transcription factors required for viral gene expression. Injection of pBR322 as early as two hours after injection of SV40 DNA into the oocyte nucleus did not inhibit SV40 RNA synthesis, indicating that once initiated, SV40 transcription is stable and insensitive to the competition by plasmid DNA. A plasmid vector was developed that allows expression of SV40 DNA in *Xenopus laevis* oocytes.

INTRODUCTION

In order to gain insight into the regulation of RNA polymerase II promoters, the expression of SV40 DNA has been studied in a variety of cell types. Among these the *Xenopus laevis* oocyte has provided unique advantages, including the ability to microinject precise quantities of DNA into the nucleus of a single oocyte and to analyze its expression very early thereafter. Among somatic cell strains and lines growing in culture, while a large variety will synthesize RNA encoded by the SV40 early transcription unit, only primate cells such as those derived from African Green monkey kidneys will actively transcribe RNA from the viral late region as well (for review see ref. 1). Furthermore, the majority of late region transcription in cells requires that viral DNA replication from the viral origin has initiated. The *Xenopus laevis* oocyte is the only other cell type reported to synthesize substantial quantities of late RNA and proteins, as well as early region products (2-6). Both similarities and differences in the mode of viral RNA synthesis and processing in oocytes and cultured cells have been noted. Further analysis of viral expression in these two cell types may provide insight into cell specific differences in gene regulation.

However, the detailed analysis of gene expression often requires the construction and analysis of both deletion and point mutants in order to identify sequences involved in transcription initiation, RNA processing, and termination. The creation and propagation of mutants has been vastly aided by mani-

pulations of DNA cloned into bacterial plasmids. The introduction of recombinant DNA molecules containing both viral and plasmid sequences into somatic cells in culture by either transfection or microinjection, has not met with serious difficulties. However, while injection of SV40 DNA form I into *Xenopus laevis* oocyte nuclei results in synthesis of the products of both late and early viral transcription units, the microinjection of similar quantities of viral DNA as recombinant plasmids yielded very little or no detectable viral early or late gene products. This failure to obtain expression of viral genes from recombinant plasmids prompted an analysis of the effect of plasmid DNA on viral RNA and protein synthesis.

MATERIALS AND METHODS

Materials

SV40 DNA I was purchased from Bethesda Research Laboratories. *Xenopus laevis* mature females were obtained from Nasco. Recombinant plasmids pSVRI (7), p ϕ 4SVA (7), pSVod (8), p730 (9), and the replicative form of MP8 and MP9 were a kind gift from J. Manley. The recombinant plasmid pKI (10) was obtained from Y. Gluzman. Restriction enzymes, T4 DNA ligase, T4 DNA polymerase, and the large fragment of DNA polymerase I were purchased from New England Biolabs. Anti SV40 capsid antiserum was a generous gift from H. Ozer. Anti-tumor antiserum was prepared from hamsters bearing tumors 4 to 6 weeks after inoculation with 10^6 hamster cells transformed with SV40 (HSV line). DNase I, proteinase K, SI nuclease, T4 polynucleotide kinase and α -amanitin were obtained from Boehringer-Mannheim.

DNA preparation

pSVBcl was constructed by insertion of SV40 DNA, cleaved with the restriction endonuclease BclI, into the BamHI site in pBR322. pBR322 subfragments were generated by either (a) cleavage with the restriction enzymes PvuII and HincII, gel purification and recircularization of individual fragments or (b) cleavage with one of the restriction enzymes AatII, or SspI. In order to generate blunt ends, AatII cleaved DNA was treated with T4 DNA polymerase. Recircularization with T4 DNA ligase was performed as described (11) except at DNA concentrations of 5 μ g/ml. pXLO was constructed by addition of EcoRI linkers to the PvuII-AatII fragment (2066–4286). pOSRI was constructed by insertion of SV40 DNA into the EcoRI site of pXLO.

Injection into Oocyte Nuclei

Excised ovaries of *Xenopus laevis* mature females were incubated in OR-2 medium (12) containing 0.15% collagenase (Worthington) at 20°C until the ovarian tissue was dissociated and follicle cells were digested. The defolliculated oocytes were maintained in modified Barth's (MB) solution (13). In order to microinject accurately into the oocyte nucleus, a modification of procedures described by Kressman et al. (14) was used. Oocytes were placed in a 35 mm petri dish in 3 ml MB and centrifuged in an IEC clinical centrifuge rotor 215-369 at 800g for 12 minutes. 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 10-20 μ m 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 twenty oocytes were labeled with 200 μCi [^{35}S]-methionine (Amersham) in MB 24-48 hours after injection. The surviving oocytes (80-100%) were rinsed with distilled water and stored at -80°C . For extraction of viral proteins the oocytes were disrupted in a small Dounce homogenizer in 1 ml of buffer containing 0.1M Tris $\cdot\text{Cl}$, pH 8.6, 0.1M NaCl, 5mM KCl, 1mM CaCl_2 , 0.5mM MgCl_2 , 0.7mM Na_2HPO_4 , and 0.5% sodium dodecyl sulfate (SDS). The homogenate was sonicated for 1 minute in an Ultrasonic W-220 sonicator, made 0.5% with Nonidet P-40 (NP40) and then centrifuged at 15,000g for 15 minutes. Usually 200 μl 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. (15), and tumor antigens immunoprecipitated as described by Fradin et al. (6). All immunoprecipitations were performed in antibody excess. 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 [$\alpha\text{-}^{32}\text{P}$] GTP (ICN Pharmaceuticals) in 20 nl water per oocyte. Groups of 20 oocytes were solubilized in 0.4 ml of buffer containing 10mM Tris $\cdot\text{Cl}$, pH 7.5, 10mM NaCl, 1mM 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 minutes, followed by phenol, chloroform and isoamylalcohol extraction and ethanol precipitation. DNase I digestion of RNA preparations was carried out in 200 μl of 5 mM MgCl_2 , 1.5mM DTT, 1 mg DNase I/ml and 100u RNasin. Samples were incubated at 37°C for 15 minutes and subsequently extracted with phenol, chloroform and isoamylalcohol and ethanol precipitated.

SI nuclease mapping of the 5' ends of late RNA

SI nuclease digestion was performed by the method of Berk and Sharp (16) as modified by Weaver and Weissman (17). To map the 5' ends of late RNA, SV40 DNA was cleaved with the restriction endonucleases NaeI and BstXI, or with HpaII and TaqI, treated with calf intestinal phosphatase and labeled with [$\gamma\text{-}^{32}\text{P}$]ATP (Amersham) and T4 polynucleotide kinase to a specific activity of $>10^7$ cpm/ μg . The strand complementary to the 5' portion of late RNA was isolated from a 4% polyacrylamide gel and used as probe. Typically, RNA of 0.4 oocyte equivalent was hybridized to 2 ng of the probe in a buffer containing 50% formamide, 0.4M NaCl, 0.04M piperazine-N,N'-bis(2-ethanesulfonic acid), pH 6.5 and 1 mM EDTA. Hybridization was carried out for 6 hours at 37°C , and was followed by digestion with SI nuclease (4,000 U/ml) for 30 minutes at 37°C . SI nuclease resistant fragments were analyzed on a denaturing 6.5% polyacrylamide-urea gel.

Dot blot analysis

RNA samples were thermally denatured at 65°C for 10 minutes, and made 3X SSC (1xSSC=0.15M NaCl and 0.015M NaCitrate). One oocyte's worth of RNA was applied to a nitrocellulose filter placed on a dot blot apparatus with vacuum, followed by baking the filter in an

80°C vacuum oven for two hours. Hybridization to nick translated SV40 or pBR322 DNA probes were performed as described by Wahl et al. (18). Filters were washed three times, each time with 2x SSC, 0.5% SDS for 15 minutes at 37°C and then for 15 minutes at 68°C, and subsequently washed with 0.2XSSC, and 0.5% SDS for 15 minutes at 37°C and then for 15 minutes at 68°C. The filters were then dried and autoradiographed.

Hybrid selection of SV40 and pBR322 RNAs

SV40 and pBR322 were prepared by a modification of the method described by Gurney et al. (19). Briefly, DNA samples were treated with SI nuclease and proteinase K, denatured, then applied to nitrocellulose filters. Small circles, each containing 1 µg DNA, were cut and used in hybridizations. Equal numbers of [³²P]-labeled RNA acid-precipitable counts were added to filters (about one oocyte's worth of RNA). Each sample was hybridized to at least two filters. Hybridization was performed in 50 µl of buffer containing 50% deionized formamide, 5XSSC, 250µg/ml tRNA, and 0.5% SDS. RNA samples were denatured in the above buffer by incubation at 80°C for 10 minutes, cooled and added to filters for a twenty-four hour incubation at 37°C. The filters were washed as described above. RNA was eluted by boiling the filters three times in 50 µl water for 10 minutes and counted by liquid scintillation.

RESULTS

Recombinant plasmids bearing SV40 genes are not actively expressed in *Xenopus laevis* oocytes

When SV40 DNA (forms I and II), purified from virions or infected cells, is microinjected into the nuclei of *Xenopus laevis* oocytes, both late and early transcription units are expressed (2-6). Therefore, factors necessary for SV40 gene expression exist and function in oocytes. In particular, viral late region transcription is very active, yielding large quantities of late RNA and protein. However when similar quantities of recombinant plasmids bearing either intact viral late (pSVBclI) or early (pSVRI) regions were microinjected intranuclearly, only trace amounts (<5%) of the major capsid protein, VP1, or virtually undetectable quantities of the large or small tumor antigens, respectively, were observed (Fig. 1A). Microinjection of a plasmid (pφ4SVA) bearing the adenovirus 2 major late promoter ligated to the protein coding portion of the SV40 early region, previously known to be actively expressed after transfection into human cells growing in culture (20), also yielded greatly reduced quantities of SV40 T antigen, although the inhibitory effect was somewhat reduced, compared to pSVRI. Not all adenovirus transcription units are inhibited by the presence of pBR322 DNA, because, consistent with studies published by Asselbergs *et al.* (9), microinjection of a plasmid bearing the E2 gene encoding the 72 Kd viral protein yielded large quantities of the 72 Kd protein product (data not shown).

It was possible to regain expression of viral DNA from recombinant plasmids by cleaving the viral DNA from pBR322, purifying the viral fragment, and circularizing it with T4 DNA ligase (Fig. 1B). This demonstrated that the inhibition of SV40 DNA expression from a recombinant plasmid is not due to a modification of the DNA that occurred during its amplification in *E. coli*. It also provides a means, albeit rather cumbersome, of analyzing the expression of various SV40 mutants propagated as recombinant plasmids. We have recently analyzed the effects of a series of mutations that delete se-

Figure 1. Viral proteins synthesized upon injection of SV40 DNA I and cloned SV40 genes into oocytes. Panel A. Oocytes were intranuclearly injected with 2.5 ng SV40 DNA I (b, d, f, g), pSVBclI (c), pSVRI (e), p4SVA (h, i) and water (a). Proteins were labeled with [35 S]-methionine 24 to 48 hours postinjection and subsequently extracted. Samples were immunoprecipitated with anti-SV40 capsid antiserum (a-c), hamster anti-SV40 tumor antiserum (d, e, g, i) and hamster nonimmune serum (f, h). Polypeptides were separated by polyacrylamide gel electrophoresis and autoradiographed. The locations of the major capsid proteins VP1 and VP3, and the large and small tumor antigens (T and t respectively), are indicated on the sides of the autoradiograms. Panel B. Oocytes were microinjected with water (a), 2.5 ng SV40 DNA I (b) and the SV40 portion of pSVRI that was gel purified and recircularized (c). Following incubation with [35 S]-methionine 24 to 48 hours after injection, proteins were extracted and immunoprecipitated with anti-SV40 capsid antiserum. The location of the major capsid protein VP1 is indicated.

quences required for viral DNA replication upon late gene expression by a similar related procedure (21).

The greatly reduced quantities of viral proteins synthesized after injection of recombinant plasmids led us to analyze the viral RNA transcribed from these cloned DNAs. Viral late region RNAs synthesized after injection of circular SV40 or pSVRI DNAs were compared by hybridizing RNAs synthesized in oocytes after injection of similar quantities of either DNA, to a 5' end labeled single stranded NaeI-BstXI SV40 DNA fragment that spans the region between nts 4759 and 345 on the viral genome. This probe detects the majority of 5' ends of late region-specific RNA in oocytes. As noted

previously (11) the pattern of late 5' ends in oocytes after SV40 DNA I injection is very heterogeneous and differs from that detected in monkey cells such that RNAs map more upstream, although differences tend to be quantitative rather than qualitative. Injection of similar quantities of pSVRI resulted in greatly reduced amounts of late RNA analyzed in this fashion. It is of interest that not only the amounts but also the relative abundance of the late region 5' ends were affected by the presence of plasmid DNA.

Co-injection of circular but not linear plasmid DNA inhibits SV40 gene expression in oocytes

It has been reported that some prokaryotic DNAs are transcribed in *Xenopus laevis* oocytes (22,26-28). This raised the possibility that competition between pBR322 and SV40 DNAs results in the reduced viral expression in these cells. To test this varying amounts of pBR322 DNA along with a fixed quantity (2-5 ng) of SV40 DNA were injected into oocyte nuclei (Fig. 3). While co-injection of 0.1 ng of circular pBR322 DNA was only slightly inhibitory to the capsid or tumor antigen products of 2.5 ng of SV40 DNA (lanes c,c'), 1.0 ng of pBR322 abolished SV40 protein synthesis (lanes d,d'). As one tenth of either late or early region encoded viral proteins would be easily detected in these experiments, it appears that the effect upon expression of increasing quantities of pBR322 is not one of direct proportionality and that there may be limiting quantities of a factor (or factors) that have a considerably greater affinity for pBR322 DNA in oocytes.

As we and others (11, 22) have previously observed that increasing the concentration of SV40 DNA injected into oocytes to quantities greater than 10 ng did not decrease the amounts of viral expression increasing the total amount of DNA (pBR322 plus SV40) to 5 ng should not be inhibitory *per se*. Plasmids related to pBR322 such as pmK 16#6 and pUC8 were also inhibitory when injected, as well as pSVod, a plasmid that lacks the "poison" sequences (23), previously reported to interfere with efficient SV40 DNA replication in infected cells (data not shown). Preliminary experiments have also indicated that the replicative form but not the single stranded form of M13 DNA is inhibitory to SV40 gene expression in oocytes.

If 2.5 ng of pBR322 DNA that had been cleaved first with either FnuDII which cuts pBR322 into several fragments (lanes, h,h'), or with Sal I which cleaves it only once (lanes g,g'), were coinjected with 2.5 ng of circular SV40 DNA, little or no inhibition of viral protein synthesis was observed. This further supports the suggestion that pBR322 competes with SV40 for transcription in oocytes because it has been shown that cleaving circular DNA abolishes its transcription in these cells (24, 25).

pBR322 DNA competes with SV40 DNA for RNA polymerase II transcription

To test whether pBR322 exerts its inhibitory effect on SV40 gene expression by affecting viral transcription we analyzed RNAs synthesized in the injected oocyte by dot blot hybridization. As seen in Figure 4, SV40 RNA accumulation was virtually abolished when 2.5 ng of pBR322 DNA were coinjected with 2.5 ng of SV40 DNA. Consistent with the data shown in Fig. 3, cleaving pBR322 with Sal I prior to microinjection, did not appreciably inhibit SV40 RNA synthesis. When the amounts of SV40 and pBR322 DNAs were reduced by an order of magnitude, the inhibition of the former by the latter was greatly reduced, suggesting, again, that the factor(s) competed for by both DNAs are

Figure 2. 5' ends of late viral RNA synthesized upon injection of SV40 DNA I and cloned SV40 DNA. Oocytes were injected with 2.5 ng SV40 DNA I (SV40 I), pSVRI (pSVRI) and water (cntrl). Following a 48 hour incubation period RNA was extracted and hybridized to the 5' labeled single-stranded NaeI - BstXI SV40 DNA fragment. Samples were subsequently digested with SI nuclease, and the protected fragments were resolved on a denaturing polyacrylamide-urea gel. RNA from SV40 infected monkey cells was analyzed as well (Inf). One-fiftieth the amount of the probe used in analysis is depicted (probe). Molecular weight markers (M) are the fragments generated by HpaII 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 and 15 nucleotides in length. A diagrammatic representation of the late RNA 5' ends detected is shown including nucleotide numbers and positions of the 21 and 72 base pair repeat sequences.

limited in quantity. Taking this observation together with that shown in Fig. 3 it appears that the lowest quantity of pBR322 that inhibits SV40 expression is between 0.1 and 0.25 ng. Dot blot analysis of pBR322 RNA accumulation in oocytes showed that, as predicted, this prokaryotic DNA is transcribed and furthermore, that quantities of α -amanitin previously shown to inhibit RNA polymerase II but not RNA polymerase III, abolish its transcription (26). This strongly indicated that RNA polymerase II transcribes pBR322 DNA in oocytes, consistent with previously reported observations with related plasmids (27, 28). Cleaving pBR322 with Sal I markedly inhibited its transcription. Whether the small amount of RNA observed in this case was the result of inefficient cleavage by the restriction enzyme, or the result of a low level of transcriptional activity resulting from a linear template is not known at present. pSVRI yielded RNA hybridizing to both SV40 and pBR322 probes. It is likely that at least some RNA hybridizing to the SV40 probe initiated in the pBR322 portion of the recombinant plasmid. As the template strands, the initiation sites, and RNA species directed by pBR322 DNA in oocytes have not yet been identified, it is not possible to speculate on the relative contributions of SV40 and pBR322 templates in these experiments. However, the data in Fig. 2 in which the 5' ends of late viral RNAs expressed after microinjection of pSVRI are dramatically reduced indicate that late promoter function is greatly inhibited by pBR322 in *cis* as well as in *trans*. Comparing the RNA that accumulated when 2.5 ng of circular SV40 and pBR322 DNAs were injected separately (Fig. 4b and f) or together (Fig. 4c) it can be seen that the inhibition by pBR322 is largely non-reciprocal, although pBR322 RNA accumulation was somewhat reduced.

Figure 3. Synthesis of viral proteins after co-injection of SV40 and pBR322 DNAs into oocytes. Oocytes were co-injected with 2.5 ng SV40 DNA I (a-e, g, h) and with 0 (a), 0.01 (b), 0.1 (c) 1 (d), 2.5 (e) ng pBR322 DNA, or with 2.5 ng pBR322 DNA that was cleaved by the restriction endonucleases SalI (g) and FnuDII (h). After 24 hours, oocytes were labeled with [35 S]-methionine for an additional 24 hours period and subsequently extracted. Protein extracts were immunoprecipitated with anti-SV40 capsid antiserum (a-h) and hamster anti-SV40 tumor antiserum (a'-h'). Water injected control sample immunoprecipitates are shown in lanes f and f'. Protein molecular weight markers (m) are (from the top) 200, 94, 67, 45, 30 and 12.3 kilodaltons. The positions of VP-1 and VP-3 are indicated on left side and of T antigen on right side.

As pBR322 specific RNA accumulated when SV40 and pBR322 DNAs were injected together, while no SV40 specific RNA was detectable, the quantities of RNA synthesized from the two templates were compared by hybrid selection of labeled RNA to DNA filters (Table 1). pBR322 DNA transcription by RNA polymerase II in this system is more efficient than SV40 DNA, since pBR322 specific RNA amounted to 18% of the stable RNA synthesized during the labeling period, while SV40 specific RNA amounted only to 8%. Taken together, these observations support the notion that pBR322 DNA is a better template than SV40 for RNA polymerase II transcription in *Xenopus laevis* oocytes.

Once initiated, SV40 transcription is resistant to pBR322 DNA competition

To further characterize the competition between SV40 and pBR322 DNAs, we tested whether pBR322 DNA inhibits SV40 transcription once the latter process has initiated in oocytes. It has been reported that a lag of one to two hours occurs before SV40 transcription is detected in oocytes (3) indi-

Table 1. The relative quantities of SV40 and pBR322 RNA synthesized in oocytes.^a

DNA injected ^b	% labeled RNA ^c	
	SV40 RNA	pBR322 RNA
--	0.05	0.05
SV40	7.8	0.1
SV40 + pBR322	0.4	12.3
SV40 + linear pBR322	7.1	0.8
pBR322	0.1	18.0
pBR322 + α -amanitin ^d	-	0.02

^a RNA was labeled with [α -³²P]GTP, 24-48 hours following injection.

^b 2.5 ng of each of the DNA types were injected.

^c SV40 and pBR322 RNAs were selected by hybridization to the appropriate DNA filters as described in text.

^d α -amanitin concentration was calibrated to 0.1 μ g/ml.

cating a temporal requirement for either assembly of chromatin or transcription complexes or both.

SV40 DNA was injected into oocyte nuclei, and then at various times thereafter a second injection of pBR322 DNA was performed (Fig. 5). Oocytes were then incubated either for an additional two hours or until the twentieth hour following the initial DNA injection. Dot blot analysis of SV40 and pBR322 RNA revealed that, once initiated, SV40 transcription is resistant to pBR322 competition. SV40 RNA synthesis persisted both during the first two hours of pBR322 incubation and the time period until the twentieth hour following the initial DNA injection. pBR322 RNA was detected at all times. Thus, the factor(s) required for SV40 RNA synthesis may be stably associated with SV40 transcription complexes, or else may be necessary only during the initial assembly process. The same factor(s) may not be essential for pBR322 RNA synthesis or else, once dissociated from SV40 transcription complexes, may become associated with pBR322 DNA, as the reciprocal depletion of those factor(s) by the pre-injected SV40 DNA was not detected.

Construction of a pBR322 variant that allows expression of SV40 DNA

A goal of these studies has been to develop a plasmid vector that will allow expression of SV40 (and other) genes in *Xenopus laevis* oocytes. Initial studies have attempted to define the regions of pBR322 DNA that are transcribed by RNA polymerase II and that inhibit SV40 gene expression. In preliminary experiments it was found that when pBR322 was cleaved with PvuII and HincII, two of the three resulting fragments generated inhibited SV40 gene expression (see Fig. 6). This indicated that more than one region of the plasmid contains sequences that lead to reduced viral expression. The third fragment, spanning nucleotides 2066 to 3907 was less inhibitory. We therefore attempted to ex-

Figure 4. SV40 and pBR322 RNA synthesis in injected oocytes. Oocytes were injected with water (a); 2.5 ng SV40 DNA I alone (b), or either with 2.5 ng pBR322 DNA (c), or with 2.5 ng pBR322 DNA that was cleaved with the restriction endonuclease Sall (d); 2.5 ng pSVRI (e); 2.5 ng pBR322 alone (f) or with α -amanitin ca. to a final concentration of 0.1 μ g/ml (g); 0.25 ng SV40 DNA I alone (h) or with 0.25 ng pBR322 DNA (i). Following a 48 hour incubation period RNA was extracted and one oocyte's worth of RNA was spotted onto duplicated nitrocellulose filters. Filters were hybridized to either [32 P] labeled SV40 (S) or pBR322 (P) DNA.

tend the boundaries of the non-inhibitory region to include the Ampicillin resistance gene. For this purpose pBR322 DNA was cleaved with AatII and then with PvuII. The individual fragments were recircularized and then cleaved again with restriction enzymes that rendered only one fragment circular (BamHI and PstI). After coinjection with similar concentrations of SV40 into oocytes, RNA was extracted and hybridized to either [32 P] labeled pBR322 or SV40 DNA probes by dot blot analysis. The relative amounts of either pBR322 or SV40 specific RNAs that accumulated in oocytes were quantitated by densitometry. It was observed that the AatII-PvuII fragment spanning from nucleotide 2066 to the vicinity of nucleotide 4286 yielded a ten-fold reduction in pBR322 RNA and a correspondingly small (20%) inhibition of SV40 RNA production. This AatII-PvuII fragment was then used to construct a plasmid vector (pXLO) by addition of EcoRI linkers followed by recircularization and propagation in *E. coli* under Ampicillin selection. SV40 DNA cleaved at the EcoRI site was then cloned into pXLO. Analysis of the late region 5' ends of viral RNA synthesized after intranuclear injection into oocytes, by hybridization to a 5' end labeled single stranded HpaII-TaqI SV40 DNA fragment spanning nucleotides 4739 and 345, showed that the quantities of late RNA were greatly increased compared to those detected upon injection of pSVRI, approximating in abundance those amounts detected after injection of similar concentrations of SV40 DNA I. While a small number of the 5' ends resembled, in their relative abundance, those appearing after injection of pSVRI, the majority of the 5' ends were similar to those detected after injection of authentic SV40 DNA. Thus the pXLO vector provides the possibility of propagating recombinant SV40 genes that are expressed in oocytes. The same vector also resulted in recovery of SV40 early gene expression such that quantities of the tumor antigens syn-

Figure 5. SV40 and pBR322 RNA synthesis after sequential injections of SV40 and pBR322 DNAs. Oocytes were injected with 2.5 ng SV40 DNA I and incubated for 2 hours (a1,a2,a3), 4 hours (b1, b2, b3), 8 hours (c1, c2, c3) and 20 hours (d1). Following this initial incubation period oocytes were either frozen (a1, b1, c1, d1) or injected with 2.5 ng pBR322 DNA and incubated for an additional 2 hour period (a2, b2, c2) or until the twentieth hour following the initial DNA injection (a3, b3, c3). In addition, SV40 DNA I and pBR322 DNA (2.5 ng each) were co-injected and oocytes were incubated for 20 hours (d3). RNA was subsequently extracted and one oocyte's worth of RNA was spotted onto duplicated nitrocellulose filters. Filters were hybridized to either SV40(S) or pBR322 (P) [32 P]-labeled nick-translated DNA probes.

thesized after injection of similar quantities of SV40 DNA I were readily detected by immunoprecipitation (data not shown). It is estimated that at least 50% of viral expression detected in the absence of plasmid sequences was obtained with pXLO. As the original pSVRI consistently yielded undetectable quantities of the early region proteins in oocytes, the pOSRI represents a dramatic improvement over intact pBR322 - SV40 recombinant DNAs, and should provide a suitable vector for analysis of viral gene expression. Deletion of additional fragments from pXLO may not be possible as most of its genome is comprised of the essential origin of replication and the Ampicillin resistance gene that serves as a selectable marker. Thus, the vector developed, pXLO, markedly increased SV40 gene expression from recombinant plasmids.

DISCUSSION

The microinjected *Xenopus laevis* oocyte is a system used for studying various aspects of eukaryotic gene expression (29, 30). The efficiency and accuracy of RNA synthesized in the oocyte vary with the RNA polymerase involved and with the injected gene (30). Polymerase III transcription of injected

Figure 6. Effect of fragments of pBR322 upon SV40 expression in oocytes. The relative positions of the Ampicillin resistance gene (Ap), the Tetracycline resistance gene (Tc) and the origin of replication (ori) are shown. The locations of the sites cleaved by the restriction endonucleases AatII (nucleotide 4286), HincII (nucleotides 651 and 3907), and PvuII (nucleotide 2066) are indicated. pBR322 DNA was cleaved with restriction endonucleases Pvu II and either Hinc II or Aat II. Following cleavage with the restriction enzyme Aat II, the DNA was treated with T4 DNA polymerase. Individual fragments were gel purified, circularized, and injected along with SV40 DNA I into oocytes. SV40 expression was measured either by viral protein synthesis (a) or by RNA dot blot analysis (b). To construct the vector pXLO, EcoR I linkers were added to the fragment spanning nucleotides 2066-4286 (Pvu II-Aat II). pOSRI was constructed by insertion of EcoR I cleaved SV40 DNA into the EcoR I site of pXLO.

cloned 5S rRNA (26, 31) and tRNA (14, 32) genes yields large quantities of faithfully synthesized RNA. Transcription of cloned rRNA genes by polymerase I in *Xenopus laevis* and *Xenopus borealis* oocytes (33, 34) is also probably efficient, considering the amplification of endogenous rRNA genes. In contrast, the abundance and authenticity of RNA transcribed by RNA polymerase II varies considerably with the injected gene. As most of the genes analyzed have been cloned into plasmids, transcription of plasmid DNAs by polymerase II in oocytes ((27, 28), this study) may be related to these variations. Transcription of cloned genes by RNA polymerases I and III may well be less adversely affected by the presence of bacterial sequences. For example RNA polymerase III transcription of cloned 5S rDNA results in a very high ratio of gene to plasmid specific RNA (27). Several well characterized polymerase II genes cloned into plasmids have provided examples of templates that are accurately and efficiently transcribed in oocytes. These include DNA encoding U1 and U2 snRNA (35, 36), histone H4 (37), and human c-myc (38) among cellular genes, and Adenovirus 2 E2 and E3 (9, 39), and Herpes Simplex virus thymidine kinase (40), among viral genes. However, other cloned polymerase II genes such as chicken ovalbumin (28), various sea urchin histone genes (41), and others (38,42,43) have been reported to be inefficiently expressed in oocytes. In addition, cloned human β -globin gene while actively transcribed, is not accurately initiated in this system (44). The poor expression of any cloned gene can be the result of different causes related to the presence of plasmid sequences. Accumulation of readthrough RNA initiated within plasmid sequences (28) or anti sense RNA (28,41), can all affect the final products of transcription and/or translation.

Figure 7. 5' ends of late viral RNA synthesized upon injection of SV40 DNA I and SV40 genes cloned in pXLO. Oocytes were injected with 2.5 ng SV40 DNA I (SV40 I), pSVRI (pSVRI), SV40 DNA cloned in pXLO (pOSRI) and water (cntrl). Following a 48 hour incubation period, RNA was extracted and 0.1 oocyte equivalent was hybridized to the 5' labeled single stranded HpaII - TaqI SV40 DNA fragment. Samples were subsequently digested with SI nuclease and the protected fragments resolved on a denaturing polyacrylamide-urea gel. RNA from SV40 infected monkey cells was analyzed as well (Inf). One-tenth the amount of probe used in analysis is depicted (probe). Molecular weight markers (m) are the fragments generated by the HpaII cleavage of pBR322 as described in the legend to Figure 2. A diagram of the DNA probe, and the approximate location of prominent 5' ends is shown below the autoradiogram.

We have found in this study, that pBR322 DNA, either in *cis* or in *trans*, inhibits SV40 gene expression in *Xenopus laevis* oocytes. As SV40 by itself is transcribed efficiently, the poor expression of cloned SV40 genes is intriguing. The experimental results obtained indicate that pBR322 may compete for factor(s) required to establish stable transcription complexes. The nature of these common factor(s) is unknown. As the RNA polymerase itself is not stably bound to transcription complexes (45-47), and as active SV40 transcription complexes are stable and insensitive to pBR322 DNA competition, RNA polymerase II itself is probably not depleted by pBR322 DNA. A single oocyte has been reported to contain quantities of histones sufficient for assembly of a least an order of magnitude more DNA than the combined quantities of the injected SV40 and pBR322 DNAs (37). Therefore, it is unlikely that depletion of histones by pBR322 accounts for the inhibitory effect on SV40 expression. However, as only some of the circular subfragments of pBR322 are inhibitory, sequestering of sequence specific DNA binding factor(s) by pBR322 may account for its inhibitory effects on SV40 RNA synthesis. Identification and characterization of the factors that preferentially associate with or transcribe pBR322 in oocytes would be a valuable extension of these studies.

The complete inhibitory effect of pBR322 on SV40 gene expression for *trans* proved valuable in assessing the stability of SV40 transcription complexes by sequential injection of SV40 and pBR322 DNAs. SV40 complexes are stable, if allowed to assemble and initiate transcription for two hours or longer and then presented with competitor pBR322 DNA. The stability of SV40 transcription complexes may be indicative of irreversible conformational changes that generate stable transcriptionally active chromatin structure. Similar irreversibility of chromatin assembly processes has been previously observed for 5S rRNA, histone H4 and U2 snRNA genes (36,37). Interestingly, deletion of sequences located upstream of the U2 snRNA gene induced reversibility of the assembled chromatin structure, indicating that these sequences play a role in the formation of stable complexes (36). pBR322 transcrip-

tion complexes exhibited an unusual stability. Simultaneous injections of SV40 and pBR322 DNAs yielded virtually only pBR322 RNA, and even preassembly of transcribing SV40 complexes did not reciprocally inhibit assembly of active pBR322 chromatin, indicating differences in the mechanism by which SV40 and pBR322 transcription complexes are established. SV40 active chromatin assembly requires common factor(s) that pBR322 may deplete very efficiently. However, these common factor(s) are not essential for pBR322 transcription initiation, unless they are only initially associated with SV40 complexes, and then become available for pBR322 complexes.

The observation that pBR322 DNA is inhibitory to SV40 gene expression only at the outset, can provide a useful basis for future studies. For example, in order to study either the influence of other genes on SV40 expression, or conversely of SV40 products such as large T antigen on expression of cloned genes, it may be possible to overcome the plasmid inhibition problem by first injecting SV40 DNA and then, as early as two hours later, injecting the cloned gene of choice.

The identification of pBR322 fragments that inhibit SV40 expression to varying extents, led to the development of a vector, pXLO, that allows at least partial recovery of SV40 gene expression. Sassone-Corsi *et al* (48) have identified two "TATA"-like sequences on pBR322 DNA that promote transcription by RNA polymerase II *in vitro*. One of these, in the vicinity of nucleotide 4320, is not found in pXLO, however the second one (around nucleotide 2608) is within the pBR322 origin of replication and therefore can be deleted only with considerable difficulty. Thus it may not be possible to remove all the sequences in pBR322 or related plasmids that affect SV40 gene expression. Nevertheless, this vector allowed considerable expression of both late and early region genes, and led to the formation of the various 5' ends of late RNA in a manner similar to that observed upon injection of SV40 DNA I. Therefore, this vector can be used for the analysis of the mechanisms and sequence requirements of SV40 RNA synthesis and processing in *Xenopus laevis* oocytes. As mentioned above several cloned viral and cellular genes have been shown to be inefficiently or inaccurately expressed in oocytes. It has also been reported that prokaryotic vector sequences depress β -globin or growth hormone genes in transgenic mice after their introduction into fertilized eggs (49-51). Although it has yet to be determined, this vector may be useful for studying the expression of other cloned polymerase II genes in these and other cells.

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Present address: ¹Cold Spring Harbor Laboratories, Cold Spring Harbor, New York

*To whom reprint requests may be addressed

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