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Regulation of Adenovirus Transcription by an E1a Gene in Microinjected *Xenopus laevis* Oocytes

N. C. JONES,* J. D. RICHTER,† D. L. WEEKS, AND L. D. SMITH
Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907

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The regulation of adenovirus type 5 gene expression by the E1a gene product was examined in microinjected *Xenopus laevis* oocytes. Chimeric genes were constructed which included the promoter region of early adenovirus type 5 gene 3 and the structural sequence which codes for the bacterial enzyme chloramphenicol-3-O-acetyltransferase (CAT). A plasmid containing this chimeric gene as well as plasmids containing the E1a gene were coinjected into oocyte nuclei. The presence of the E1a gene was shown to increase CAT activity by up to 8.5-fold over basal levels. Synthesis of the functional product from the E1a gene requires the removal of intron sequences by RNA splicing. The E1a gene and a derivative that precisely lacks the intron were equally effective in increasing CAT activity, suggesting that splicing of the primary E1a transcript is efficiently accomplished in the oocyte nucleus. This was confirmed by directly examining the E1a mRNAs by the S1 mapping procedure. A protein extract from adenovirus type 5-infected HeLa cells enriched for the E1a protein may supplant the E1a plasmid in enhancing CAT activity. Synthesis of the CAT enzyme after gene injection is invariant in oocytes from the same frog, but oocytes from different frogs show a high degree of variability in their ability to synthesize the CAT enzyme. Microinjected *X. laevis* oocytes appear to be an extremely useful system to study the effects of protein elements on transcription.

One of the initial viral genes to be expressed after infection of permissive cells by adenovirus type 5 (Ad5) is the E1a gene. The product of this gene is required to transform rat embryo cells in culture since mutants defective for the E1a gene do not lead to transformation after viral infection (9, 18, 21). Furthermore, cells infected with E1a mutants do not synthesize the products of the other early viral genes E1b, E2, E3, and E4 (4, 22). Thus, it appears that the E1a gene product is a regulator of these other early viral genes. Nevins (31) determined that the E1a gene product acts at the level of transcription since rates of early gene transcription were greatly reduced when E1a mutants, versus wild-type viruses, were used to infect cells. Using a different approach, Weeks and Jones (44) also demonstrated that the E1a gene product is a transcriptional regulator. In that study chimeric genes were constructed which were composed of the 5′ ends of the early Ad5 genes and the structural sequences of genes that encode readily assayable products. One such set of chimeric genes encoded the bacterial enzyme chloramphenicol-3-*O*-acetyltransferase (CAT). In a series of gene transfection studies it was shown that CAT activity was greatly enhanced by the E1a product, showing that E1a regulation acts at the transcriptional level and that the sequences upstream of the transcriptional starts of the early genes encode signals that confer E1a responsiveness. To examine further the molecular mechanisms by which E1a regulates transcription, an in vivo system more amenable to DNA and protein manipulation was required.

Microinjected *Xenopus laevis* oocytes have been used for several years to study translational regulation (20, 31, 37). Recently, they have been used to examine the mechanisms involved with the transcription of eucaryotic genes. For example, the injection of genes which code for sea urchin histones (15, 35), herpes simplex virus thymidine kinase (26), chicken ovalbumin (46), and the papovavirus early and late proteins (11, 40) results in the synthesis of not only these specific transcripts but their polypeptide products as well. In the case of the injected papovavirus T antigen and ovalbumin genes, the detection of their encoded polypeptides indicated that oocytes must have correctly processed (spliced) the primary transcripts, although the efficiency

† Present address: Department of Biochemistry, University of Tennessee, Knoxville, TN 37996-0840.

with which splicing took place in the injected oocytes was not clear. In addition, *X. laevis* oocytes have been used to determine the DNA sequences which might be required for transcription. Specific alterations of DNA sequences can be introduced into cloned genes in vitro, and the consequences of these changes on gene expression can be examined after the injection of the modified DNA into oocytes. This approach has been used successfully to identify DNA sequences required for efficient transcription of the herpes simplex virus thymidine kinase gene (25). However, except for the elegant studies of 5S gene transcription (7, 8) and a study that describes a chromosomal factor involved in transcription termination of a sea urchin histone gene (41), oocytes have not been exploited as a system to study the regulation of transcription by protein elements.

This study describes experiments in which *X. laevis* oocytes were used to examine the regulation of adenovirus gene transcription by the E1a gene product. Employing the chimeric adenovirus CAT gene described above and plasmids containing E1a genes, we demonstrate that in microinjected *X. laevis* oocytes, the E1a gene product acts as a specific positive regulator of an early adenovirus gene promoter. We further present data which indicate that the primary E1a transcripts which are synthesized are processed efficiently. Finally, we demonstrate that a protein fraction enriched for the E1a protein positively regulates an early adenovirus gene promoter in injected oocytes. The significance of the *X. laevis* oocyte system as an efficient surrogate to study transcriptional regulation by proteins is discussed.

MATERIALS AND METHODS

Plasmids. Table 1 summarizes the salient features of the plasmids used in this study.

The recombinant plasmid pSV2CAT was kindly provided by P. Gruss and has been described previously (17). The construction of plasmid pKCAT23, which contains the E3-CAT chimeric gene, is described elsewhere (44). Briefly, the CAT coding sequences were fused to a 623-base pair (bp) fragment from the Ad5 genome (extending from 75.1 to 76.9 map units) that contains the E2 and E3 control regions. The fusion was such that the E3 control region is adjacent to the CAT coding sequences and CAT expression is dependent upon transcription initiated from this region. Flanking the 3′ end of the CAT sequences are the simian virus 40 (SV40) small t antigen intron and early polyadenylation signals.

Plasmids pJOLC3, pJN20, and pJOAC all contain the Ad5 E1a gene. Details on their construction will be presented elsewhere. pJOLC3 was derived by insertion of the 0 to 7.8 map unit, left-hand end fragment of the Ad5 genome into the *Hin*dIII site of pBR322. This fragment contains the entire E1a gene and the 5′ half of the flanking E1b gene. pJN20 is a derivative of pJOLC3 and contains a deletion of 116 bp within the E1a gene. The deletion precisely removes the sequences that are spliced out of the E1a primary transcript during the synthesis of the E1a 13S mRNA. Upon transfection of HeLa cells with this plasmid, functional 13S mRNA is synthesized, indicating that splicing per se is not an obligatory step for production of this message (N. C. Jones, unpublished data). pJOAC is also a derivative of pJOLC3 and contains a small 11-bp deletion within the E1a gene extending from nucleotide position 1,106 to 1,116. The deletion thus removes the donor splice junction utilized for the synthesis of 13S mRNA. Upon transfection of HeLa cells with this plasmid no 13S mRNA is synthesized, but elevated levels of 12S mRNA, the synthesis of which requires the use of a different donor splice junction, are found (J. Overhauser and N. C. Jones, unpublished data).

Plasmids pJOLC3E and pJN20E are derivatives of pJOLC3 and pJN20, respectively, that contain an additional insert from the SV40 genome which encodes the 72-bp repeat, enhancer sequences (3, 19, 30). The insert is the *Hin*dIII C fragment from the SV40 genome and was inserted into the unique *Hin*dIII sites of the E1a-containing plasmids.

Collection and microinjection of *X. laevis* oocytes. Female *X. laevis* frogs (South African Snake Farms, Fish Hoek, South Africa) were anesthetized by hypothermia, and an ovarian fragment was excised. Individual stage VI oocytes (13) were isolated from the ovary by manual dissection. Solutions (10 nl) of DNA (see figure legends for amounts) were injected into

TABLE 1. Summary of plasmids used in this study

Plasmid	Salient features
pSV2CAT	Contains the CAT structural sequences under the control of the SV40 early gene promoter.
pKCAT23	Contains the CAT structural sequences under the control of the Ad5 region E3 promoter.
pJOLC3	Contains the left-hand end 0 to 7.8% fragment of the Ad5 genome, which contains all of the E1a genes and the 5′ half of the E1b genes.
pJOLC3E	Same as pJOLC3; also contains the *Hin*dIII fragment from the SV40 genome which encodes the 72-bp repeat, activator sequence.
pJN20	Same as pJOLC3, with a deletion of 116 bp within the E1a structural gene that precisely deletes the intron sequences removed from the precursor RNA during the biogenesis of the E1a 13S mRNA.
pJN20E	Same as pJN20 but containing the SV40 insert present in pJOLC3E.
pJOAC	Same as pJOLC3 but with an 11-bp deletion within the E1a sequences that removes the donor splice junction utilized for the synthesis of the 13S mRNA.

each oocyte, aiming for the oocyte nucleus (germinal vesicle [GV]). Groups of 30 to 60 oocytes then were cultured in the medium of Eppig and Dumont (14) for about 48 h at 20°C. Microinjected solutions were deposited into the GV at least 80% of the time as judged by the frequency in which dye was successfully injected into GVs in separate experiments.

CAT assay. Microinjected oocytes were washed briefly in distilled water, suspended in 100 μl of 0.25 M Tris-hydrochloride (pH 8.0), sonicated for 15 s, and centrifuged (12,000 × *g*, 15 min) to pellet insoluble material. The cytoplasmic extract was then removed carefully to avoid lipid contamination, and a 50-μl portion was incubated with a solution of water (50 μl), acetyl coenzyme A (5 μl of a stock of 0.33 mg/ml), and [^{14}C]chloramphenicol (2.5 μCi, ca. 40 mCi/mmol; New England Nuclear Corp.) for 30 to 60 min at 37°C. The chloramphenicol was then extracted with 1 ml of cold ethyl acetate; the ethyl acetate was evaporated, and the chloramphenicol was suspended in 20 μl of ethyl acetate, which was spotted onto silica gel (Eastman Kodak, no. 13179) and chromatographed in chloroform and methanol (95:5). After chromatography, the gel was exposed to X-ray film for autoradiography. Regions of the gel containing acetylated and nonacetylated [^{14}C]chloramphenicol were cut out, and radioactivity was determined by liquid scintillation spectrometry.

Preparation of protein extracts. A crude protein extract was prepared from Ad5-infected and uninfected HeLa cells in the following manner. Plates (100 mm) of almost confluent HeLa cells were infected with ca. 50 PFU of wild-type Ad5 per cell. At 1 h postinfection, 20 μg of cytosine arabinoside was added per ml of medium. The cells were incubated for 48 h with the cytosine arabinoside being replenished at 12-h intervals. The cells were harvested, washed, suspended in 10 mM Tris (pH 8.0)–50 mM NaCl, and sonicated to break open the cells. The sonic extract was spun for 1 h at 40,000 rpm at 4°C in an SW50.1 rotor. The supernatant was then used for microinjection studies.

RNA isolation. A 2-ng sample of Supercoiled pJN20 or pJOLC3E was injected into the GV of each oocyte. The oocytes were cultured in the medium of Eppig and Dumont (14) for ca. 26 h at 20°C, and the total cellular RNA was extracted as described previously (12).

S1 mapping. Total RNA (50 μg) isolated from microinjected oocytes was hybridized to 5 ng of M13-E1a single-stranded DNA labeled with ^{32}P to a specific activity of ca. 5×10^6 dpm/μg (32) for 60 min at 68°C in 30 μl of 1.0 M NaCl–10 mM Tris (pH 7.4)–1 mM EDTA. The hybrids were treated with 500 U of S1 endonuclease per reaction for 60 min at 37°C as described previously (5). S1-resistant fragments were denatured and separated by electrophoresis on 8 M urea–3.8% polyacrylamide gels at 150 V for 15 h. Radioactive bands were visualized by autoradiography at −70°C with an intensifying screen.

The 3′ end-labeled probe was prepared by digesting pJOLC3 DNA with *Cla*I and *Kpn*I, extending the *Cla*I-generated recessed 3′ ends with [α-^{32}P]dCTP and DNA polymerase I large fragment, and isolating the *Cla*I-*Kpn*I fragment extending from nucleotide 920 to 2,049 by agarose gel electrophoresis. The probe was hybridized to 50 μg of total oocyte RNA as described (5), and S1 nuclease-treated hybrids were separated on 8 M urea–8% polyacrylamide gels.

RESULTS

Expression of the CAT enzyme in DNA-injected *X. laevis* oocytes. The bacterial transposable element Tn*9* encodes the enzyme CAT. This gene has been cloned into the procaryotic-eucaryotic vector pSV2 (17), where it is flanked by the SV40 early gene promoter, the SV40 small t-antigen intron, and the SV40 early region polyadenylation site (Fig. 1). These SV40 sequences allow for the expression of the CAT gene in eucaryotic cells (17). We have used plasmid pSV2CAT to test whether the CAT enzyme could be detected in *X. laevis* oocytes after DNA injection into oocyte GVs. Superhelical pSV2CAT DNA was injected into oocyte GVs, an oocyte extract was prepared and then incubated with [^{14}C]chloramphenicol and acetyl-coenzyme A, and the resulting acetylated chloramphenicol was detected by thin-layer chromatography and autoradiography. An extract prepared from pSV2CAT-injected oocytes contained readily detectable amounts of the CAT enzyme (Fig. 2). In contrast, no CAT enzyme could be detected in noninjected oocytes, confirming the observation (17) that eucaryotic cells possess no endogenous CAT activity. For comparison, CAT activity in HeLa cells after transfection with pSV2CAT also is demonstrated (Fig. 2).

In subsequent experiments, it became clear that there was considerable variability in the expression of injected CAT DNA in oocytes from different frogs. In light of these observations, as well as those by others who demonstrated an animal variability with respect to nuclear transplantation and gene reactivation (23), we performed experiments to assess the degree of variation in the expression of the CAT enzyme after DNA injection into oocytes of different females. Oocytes, all of which were classified morphologically as "good," were collected from five females selected at random and were injected with pSV2CAT. The resulting CAT enzyme activities are shown in Fig. 3. The levels of CAT activity in injected oocytes of different females varied by almost 30-fold. The oocytes from each individual female always gave a consistent response from experiment to experiment, indicating that the source of the variation was intrinsic to the oocytes and not the injection or assay procedures. The basis for this variability is not known. For consistency, therefore, frogs were first screened for those containing oocytes which efficiently expressed injected pSV2CAT DNA before they were used for subsequent experiments.

Regulation of E3-CAT chimeric gene transcription by E1a. We have constructed a chimeric gene in which the CAT coding sequence is flanked at its 5′ end by the Ad5 early gene 3 (E3)

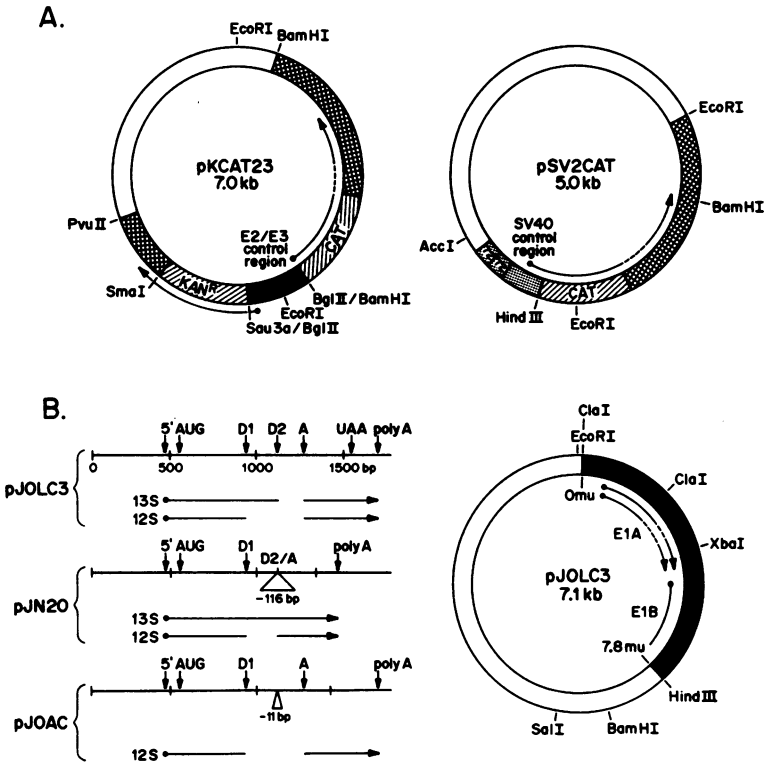

FIG. 1. Diagrammatic representation of the plasmid DNAs used in this study. (A) Structure of the plasmids containing the E3-CAT and SV40-CAT chimeric genes. Plasmid pKCAT23 (44) contains two chimeric genes, namely, the E3-CAT gene, where expression of the CAT coding sequences depends upon the activity of the Ad5 E3 control region, and the E2-Kan gene, where the sequences coding for the enzyme neomycin phosphotransferase II are fused to and under the control of the Ad5 E2 control region. The open portion represents pBR322 sequences, the hatched portion represents sequences containing necessary 3' control signals taken from either the SV40 early gene (flanking the CAT sequences) or the herpes simplex virus thymidine kinase gene (flanking the Kan^r sequences), and the striped portion represents sequences that encode either the CAT or the neomycin phosphotransferase II enzyme. The Ad5 insert containing the E2 and E3 control regions is represented by the solid portion. The structure of plasmid pSV2CAT has been described previously (17). The CAT coding sequences (striped) are flanked at the 5' end by a fragment from the 5' end of the SV40 early gene (stippled) and at the 3' end by SV40 sequences encoding the small t-antigen intron and early gene polyadenylation signals (hatched). (B) Structure of the E1a-containing plasmids. pJOLC3 contains an insert of the 0- to 7.8-map-unit fragment of the Ad5 genome (solid) into pBR322 sequences (open). The entire E1a gene is encoded within this insert. Expression of the E1a gene gives rise to 13S and 12S mRNAs through differential processing of a primary transcript. The 13S mRNA results from the removal of sequences from D2 to A, and the 12S mRNA results from the removal of sequences from D1 to A. The structure of the mutant E1a genes in pJN20 and pJOAC, both derivatives of pJOLC3, is also depicted. In pJN20 a deletion of 116 bp between D2 and A is present; 13S and 12S mRNAs can be synthesized, but synthesis of the 13S mRNA does not require a splicing step. In pJOAC, a deletion of 11 bp is present that removes the D2 splice junction. As a result, 13S mRNA cannot be synthesized, whereas 12S mRNA production can proceed normally.

upstream regulatory sequences and at its 3' end by the SV40 small intron and polyadenylation site (44). This gene is contained in plasmid pKCAT23 (Fig. 1) and is hereafter referred to as E3-CAT. When this plasmid is cotransfected into HeLa cells with the E1a gene-containing plasmid pJOLC3 (Fig. 1), CAT activity is increased ca. 40-fold (44). We have determined whether a similar phenomenon can occur in microinjected *X. laevis* oocytes.

The E3-CAT gene was injected into oocyte GVs with either pBR322 or a plasmid containing the E1a gene (pJOLC3E). This plasmid increased CAT activity by almost fivefold (Fig. 4A). To show that E1a acts specifically on the E3 regulatory sequences, control experiments were performed in which E1a-containing plasmid pJOLC3E was coinjected with pSV2CAT (Fig. 4A). In this case, a minimal increase (ca. 1.6-fold) of CAT activity by E1a was observed.

In other experiments no increase was observed. Thus, the increase of CAT activity by E1a is specific for the E3 regulatory sequence.

Biogenesis of the E1a 13S mRNA from pJOLC3E in eucaryotic cells involves the excision of a 116-bp intron (6, 10, 34). If oocytes are inefficient in splicing, then the increase of E3-CAT transcription by the E1a gene product might be stimulated even more if the splicing step was eliminated. To test this possibility, we constructed a second E1a gene which contains no intron. This gene, contained in plasmid pJN20, is similar to that in pJOLC3 except that the 116-bp intron has been deleted; this deletion has no effect in the generation of the E1a 13S mRNA in infected HeLa cells (Overhauser and Jones, unpublished data). As a result of the deletion, synthesis of 13S mRNA requires no splicing step. The results of coinjections of E3-CAT and pJN20 also are shown in Fig. 4A. The intronless E1a mutant increased CAT activity by an amount similar to that observed with the intron-containing E1a gene (pJOLC3E). The degree of CAT stimulation by the two E1a plasmids is shown by a time course on the CAT assay (Fig. 4B). By comparing the initial rates of enzyme activity, it is seen that both E1a plasmids stimulated CAT activity by an almost identical 8.5-fold. These data therefore suggest that splicing per se does not significantly alter levels of functional mRNA (see below).

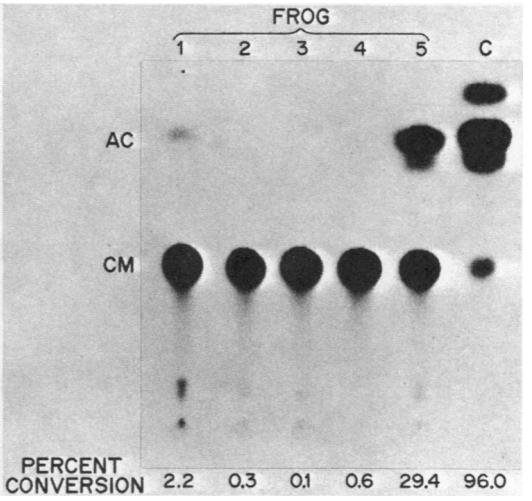

FIG. 3. Activities of CAT in oocytes of different frogs after DNA injection. Morphologically good oocytes were collected from five females and injected with 2 ng of pSV2CAT. Oocytes then were cultured for ca. 48 h, and the resulting CAT activities were detected as described for Fig. 2. The acetylation of [^{14}C]chloramphenicol by commercial CAT also is shown.

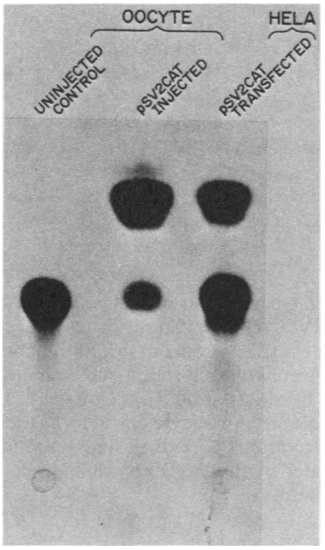

FIG. 2. Activities of the CAT enzyme in oocytes after DNA injection. Oocytes were injected with 2 ng of pSV2CAT and cultured for ca. 48 h. Injected and noninjected oocytes were then processed as described in the text, and CAT enzyme activity in the oocyte extract was detected by in vitro acetylation of [^{14}C]chloramphenicol, thin-layer chromatography, and autoradiography. The level of CAT activity also is shown for HeLa transfected with the same plasmid (10 μg) and incubated for 48 h before preparation of a cell extract.

In addition to the Ad5 sequences, pJOLC3E also contains an insert of SV40 DNA (Fig. 1). Within this insert is a 72-bp repeat sequence which has been shown previously to be a transcriptional enhancer (3, 19, 30). This sequence enhances the expression of linked genes regardless of its orientation with respect to, or its distance from, the 5′ end of the gene. We inserted this SV40 enhancer sequence in pJOLC3E, reasoning that expression of the linked E1a gene might be elevated, although no evidence existed as to whether this enhancer was functional in injected *X. laevis* oocytes. To compare directly the wild-type (pJOLC3E) and intronless (pJN20) E1a genes in increasing the expression of E3-CAT, we inserted the same 72-bp SV40 sequence into pJN20 (now called pJN20E). These plasmids then were injected into oocyte GVs, and the level of CAT activity was determined (Fig. 4A). The presence of the enhancer sequence in pJN20E only slightly increased the ability of E1a to elevate CAT activity relative to that observed with pJN20. Similar relative levels were observed throughout a time course on the CAT assay of the pJN20 and pJN20E extracts.

Increase of CAT gene expression is due to a

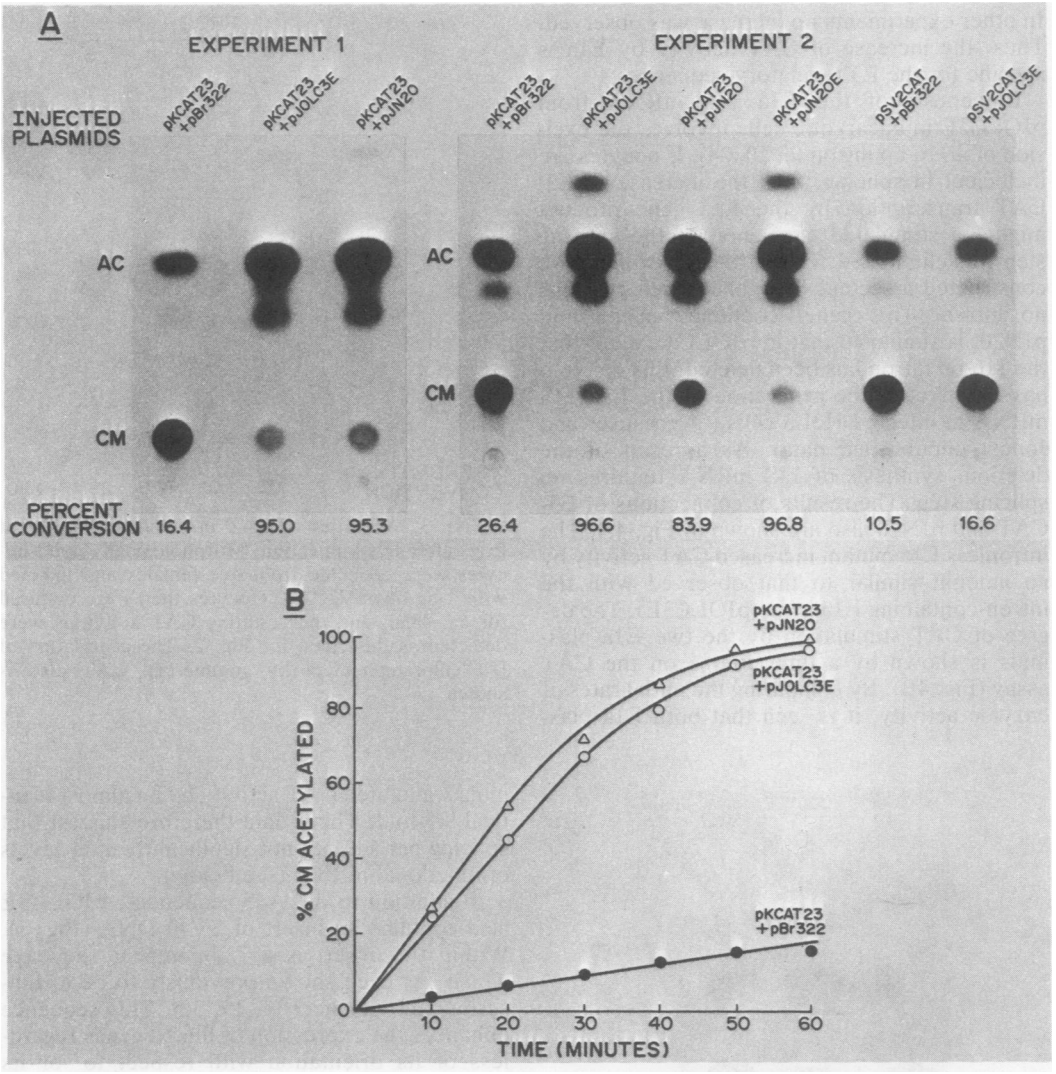

FIG. 4. Regulation of CAT transcription by E1a in oocytes injected with two plasmids. (A) Oocytes were injected simultaneously with 0.5 ng of pKCAT23 (E3-CAT) or pSV2CAT and 5.0 ng of pBR322, pJOLC3E (containing the wild-type E1a gene), pJN20 (containing an intronless E1a gene), or pJN20E (containing an intronless E1a gene and an SV40 activator sequence). Oocytes then were cultured for ca. 48 h, and the resulting CAT activities were determined as described for Fig. 2. (B) Oocytes injected with 0.5 ng of pKCAT23 (E3-CAT) and 5.0 ng of pJN20, pJN20E, or pBR322 were cultured as described above. An oocyte extract was then prepared and incubated with [^{14}C]chloramphenicol and acetyl coenzyme A for various periods of time up to 60 min. The resulting acetylated and nonacetylated chloramphenicols were separated by thin-layer chromatography, detected by autoradiography, and quantitated by liquid scintillation spectrometry.

product encoded by the E1a 13S mRNA. Isolation and characterization of mutants of adenovirus type 2 (Ad2) or Ad5 that differentially alter products of the E1a 12S and 13S mRNAs have shown that the regulation of adenovirus early gene expression is primarily due to a product encoded by the 13S mRNA (9, 29, 36). The increase of E3-CAT gene expression in *X. laevis* oocytes was also shown to be due to a 13S-encoded product. The E3-CAT gene was injected into oocytes together with an E1a gene containing a deletion of 11 bp from nucleotides 1,106 to 1,116 (present in pJOAC). This deletion removes the donor splice junction at nucleotide position 1,112 required for biogenesis of the 13S mRNA. Upon transfection of HeLa cells with pJOAC, only the 12S E1a mRNA can be detected (Overhauser and Jones, unpublished data). The result of the coinjection of E3-CAT and pJOAC into oocyte GVs is shown in Fig. 5.

pJOAC failed to stimulate expression of the E3-CAT gene, suggesting that synthesis of the 13S mRNA was critical for the activation effect.

E1a transcripts are efficiently spliced in *X. laevis* oocytes. Plasmids pJOLC3E and pJN20 both were capable of elevating the level of E3-CAT expression to the same extent. However, the E1a gene in pJOLC3E must be both transcribed and spliced, whereas the E1a gene in pJN20 need only be transcribed to produce 13S mRNA that encodes the regulatory product. The efficiency of transcription and processing of the injected plasmids was visualized directly by the hybridization-S1 nuclease technique of Berk and Sharp (5). Three different DNA probes were used for these experiments. Probe A was a ^{32}P-labeled, single-stranded M13 clone of the coding strand of E1a and part of the flanking E1b gene. The Ad5 sequences began ca. 100 nucleotides upstream of the major E1a transcription initiation site, which is at nucleotide position 499. Four DNA fragments are protected by hybridization of this probe to early cytoplasmic RNA isolated 6 h postinfection from HeLa cells infected with Ad5 (Fig. 6, lane 1). The 346-nucleotide fragment corresponds to sequences from the 5′ end of E1b mRNAs. The other three fragments result from hybridization to E1a mRNAs; the 613-, 475-, and 403-nucleotide fragments correspond to the 5′ exon of the 13S message, the 5′ exon of the 12S message, and the 3′ exon of both the 13S and 12S messages, respectively. Hybridization of this probe to total RNA isolated from oocytes injected with pJOLC3E DNA resulted in prominent S1-resistant bands of 830, 780, 720, 550, and 403 nucleotides (Fig. 6, lane 2). The presence of the 403-nucleotide band suggests that a significant proportion of the stable E1a transcripts in the oocytes were processed at the acceptor splice sites at nucleotide position 1,229 and had the correct 3′ terminus. Conversely, no 475-nucleotide band and only a faint 613-nucleotide band were observed, suggesting either that the 12S and 13S donor splice sites were not utilized or that transcription initiated at sites upstream from the normal position. It appears that the latter is correct. Efficient use of the 13S donor splice site was clearly seen when the RNA from pJOLC3E-injected oocytes was hybridized to an Ad5 DNA fragment 3′ end labeled at nucleotide position 920 (probe B; Fig. 6, lanes 4 and 5). Hybridization of this probe to early RNA from Ad5-infected cells gave two major bands 55 and 192 nucleotides long, corresponding to hybridization to the 12S and 13S mRNAs, respectively. However, hybridization to the oocyte RNA resulted in protection of the 192-nucleotide band only, showing that the 13S donor splice site was utilized in the oocytes during the processing of E1a RNA and that the 12S donor site was utilized very inefficiently, if at all. The lack of a 613-nucleotide band after hybridization of probe A to the oocyte RNA (which corresponds to the 5′ exon of the 13S message starting at the normal initiation site) is not therefore due to a failure in utilization of the 13S donor site, so it must be due to upstream transcription initiation. Minor upstream start sites are utilized at early and, more prominently, late times of infection of HeLa cells by Ad2 or Ad5 (33; Overhauser and Jones, unpublished data). Transcripts initiated at these upstream sites and subsequently processed at the 13S donor splice site give rise to an S1-protected band of 720 nucleotides when hybridized to probe A (Overhauser and Jones, unpublished data). A band of this size was observed when the probe was hybridized to RNA from pJOLC3E-injected oocytes.

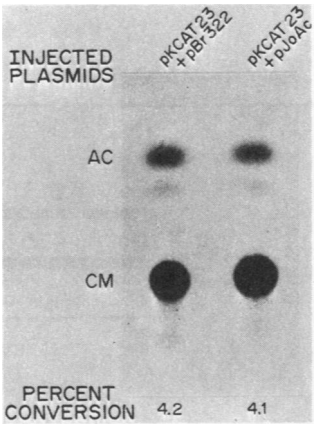

FIG. 5. Regulation of CAT transcription by an E1a mutant which lacks a donor splice site in oocytes injected with two plasmids. Ooctyes were injected with 0.5 ng of pKCAT23 (containing E3-CAT) and 5.0 ng of pJOAC (containing an E1a gene lacking donor splice site D2). Ooctyes then were cultured, and the resulting CAT activities were detected as described for Fig. 2.

To confirm that transcription of E1a genes in oocytes was initiated at specific upstream positions relative to the normal initiation site, RNA from pJOLC3E-injected oocytes was hybridized to a third probe. Probe C was an M13 clone containing sequences from the left-hand end of the Ad5 genome extending from nucleotides 1 to 2,049 with an internal deletion from nucleotides 919 to 1,140. Hybridization of early RNA from Ad5-infected cells to this probe gives three prominent bands of 420, 403, and 346 nucleotides (Fig. 6, lane 7). The 346-nucleotide band results from hybridization to E1b mRNA, the 403-nucleotide band results from hybridization to the 3′ exon of the E1a 12S and 13S mRNAs, and the 420-nucleotide band results from hybridization to the 5′ exon of the 12S and 13S

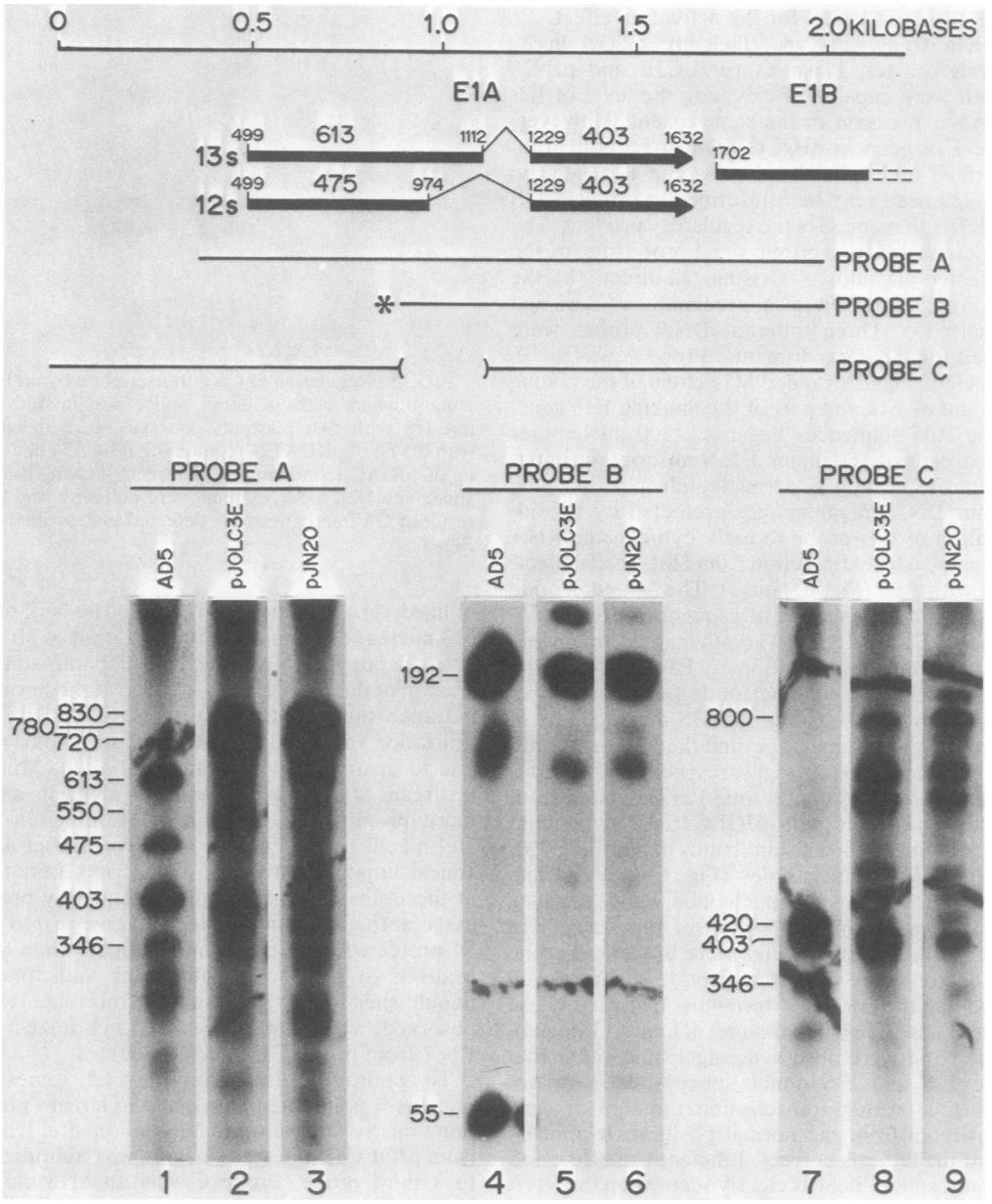

FIG. 6. S1 mapping of RNA from oocytes injected with pJOLC3E or pJN20 DNA. The line at the top of the figure represents the left-hand end 2 kb of the Ad5 genome. The structures of the two major early E1a mRNAs and the 5' portion of the E1b mRNAs are shown below the line. The exact positions of the major transcriptional start sites, splice junctions, and termination sites are indicated, together with the size in nucleotides of the exons fused to give the 12S and 13S E1a mRNAs. The sequences removed by splicing are indicated by carets. Also depicted are the E1 sequences present in the three DNA probes used for this study. Each probe was hybridized to 50 μg of total RNA from oocytes injected with pJOLC3E DNA (lanes 2, 5, and 8) or pJN20 DNA (lanes 3, 6, and 9) or to 100 μg of cytoplasmic RNA isolated from HeLa cells infected with Ad5 virus at 8 h postinfection (lanes 1, 4, and 7). The autoradiograph showing the results of the S1 mapping with probe B was overexposed to detect low levels of the 55-nucleotide fragment that may have been present in lanes 5 and 6.

mRNAs. Transcripts initiated at upstream positions generate protected fragments larger than 420 nucleotides. Fragments ranging in size from 450 to 800 nucleotides were detected when probe C was hybridized to RNA from oocytes injected with pJOLC3E (Fig. 6, lane 8). Thus in the oocyte, transcription of the E1a gene began at discrete, upstream sites. Transcription from the normal site was rare as evidenced by the faintness of the 420-nucleotide band.

At present we do not know the origin of the other S1-protected bands seen after hybridization of the oocyte RNA to probe A. However, it is possible that the 830-nucleotide band derives from E1a transcripts processed at the acceptor site but terminating at some point in the E1b or plasmid sequences.

Hybridization of all three probes to RNA from oocytes injected with pJN20 (Fig. 6, lanes 3, 6, and 9) gave a pattern of S1-resistant fragments similar to that obtained with RNA from oocytes injected with pJOLC3E. Some differences were evident with probe A, notably a lack of the 780 band and a much weaker 403 band.

We found very little evidence for the accumulation of unspliced E1a mRNA, suggesting either that splicing of the primary transcripts is very efficient or that the unspliced RNAs are too unstable to accumulate. Hybridization of RNA from oocytes injected with either pJOLC3E or pJN20 to probe B produced a 192-nucleotide fragment of equal intensity, even though pJN20 does not need to splice its primary transcript. If, as expected, transcriptional efficiency is about the same for these two plasmids, then the majority of the pJOLC3E primary transcripts must be spliced.

Increase of E3-CAT gene expression by a protein extract from wild-type Ad5-infected HeLa cells. Using two injected plasmids, we have shown that the gene product of the first (E1a) increases the transcription of the second (E3-CAT). In a final series of experiments, we asked whether the E1a plasmid may be supplanted by an E1a-containing protein extract. Although the E1a protein does not accumulate to a large extent in HeLa cells after normal infection by Ad5, levels of the protein are significantly higher when infected cells are cultured for 48 h in the presence of the DNA synthesis inhibitor cytosine arabinoside (16). We therefore cultured Ad5-infected and mock-infected cells by this regime to enhance the level of E1a. Cellular extracts were prepared and injected into the cytoplasm of oocytes previously injected with E3-CAT. The protein extract from cells infected with wild-type virus consistently increased CAT activity by two- to fourfold (Fig. 7). The protein extract prepared from mock-infected cells, however, actually elicited a slight decrease in CAT

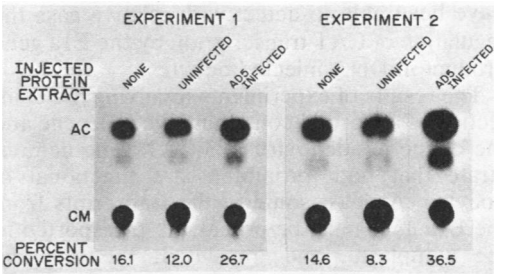

FIG. 7. Regulation of CAT transcription by a protein extract containing E1a. Oocytes were injected with 0.5 ng of pKCAT23 and 100 ng of protein extracted from either wild-type Ad5-infected or mock-infected HeLa cells. Oocytes were injected a second time 24 h later with 100 ng of the same protein extract. After another 24 h of culture, the oocytes were processed to determine the level of CAT activity.

activity. We were concerned that the detected stimulation by the infected-cell extract might be due to the presence in the extract of E1a containing DNA or RNA sequences, rather than to the presence of protein. To rule out this possibility, the extract was treated repeatedly with phenol and chloroform-isoamyl alcohol before injection into oocytes previously injected with E3-CAT DNA. When equivalent volumes of the treated and untreated extracts were injected, stimulation of E3-CAT gene expression was not detected after injection of the phenol-treated extract (data not shown), indicating that protein but not DNA or RNA is the biological enhancer molecule of E3-CAT transcription. The injection of the active protein extract into oocytes previously injected with pSV2CAT did not result in increased levels of CAT (data not shown). Thus, although Ad5-infected HeLa cell polyadenylated RNA is known to stimulate oocyte protein synthesis after injection (38), RNA in the crude protein extract used here did not.

DISCUSSION

The observation that *X. laevis* oocytes injected with heterologous molecules are capable of performing the myriad of events from chromatin assembly (27, 47) and transcription (7, 8, 11, 15, 20, 25, 26, 28, 35, 40) to protein secretion (24) suggests that they might be useful for studies on the regulation of gene expression. Indeed, they have served as an efficient test system to identify DNA sequences involved in the initiation of transcription (25). In the experiments reported here, we show that injected oocytes can be used to investigate the control of adenovirus early gene transcription by the product of the viral E1a gene. By constructing chimeric genes which contain viral regulatory sequences and the structural element from the bacterial CAT gene, we

have been able to detect with relative ease the regulation of CAT transcription by the E1a gene product in DNA-injected oocytes.

The results of experiments involving the coinjection of a plasmid containing the E1a gene and the chimeric adenovirus E3-CAT gene demonstrate that both promoters are functional in oocytes. At least some of the transcripts from the E1a gene must be processed, transported to the cytoplasm, and translated; the resulting polypeptide is then transported back to the nucleus for interaction with the E3-CAT gene. The E1a peptide appears to act specifically on the E3 promoter since the SV40 promoter present in another plasmid (pSV2CAT) failed to respond to E1a stimulation.

The analysis of Ad5 mutants that differentially affect the products of the E1a 12S and 13S mRNA species indicates that the 13S mRNA-encoded product is the positive regulator of early viral gene expression (9, 29, 36). The results obtained with the mutant E1a gene in pJOAC, which can be expressed to give a normal 12S mRNA but no 13S mRNA, show that this is also the case in oocytes. The observation that the wild-type E1a gene can increase E3-CAT gene expression suggests that splicing of the primary transcript can proceed in oocytes. The existence of mRNA splicing activity in oocytes has been inferred from other studies including: (i) the synthesis of proteins from injected genes that contain introns (40, 46) and (ii) the direct analysis of the structure of SV40-specific RNA in injected oocytes (28, 45). These studies showed that although splicing can occur it is not necessarily efficient, as evidenced by detection of unspliced transcripts in the cytoplasm (45). Analysis of stable E1a mRNAs in oocytes after injection of pJOLC3E DNA revealed efficient processing of E1a primary transcripts. However, two unusual features of processing and transcription of this gene were observed. First, the E1a primary transcript contains two different donor splice sites. During Ad5 infection of HeLa cells both sites are used, giving rise to a 12S and 13S mRNA. Only the 13S donor site is utilized in oocytes. No additional viral products are required for the use of the 12S site, as evidenced by the similar ratios of 12S and 13S mRNAs in pJOLC3E-transfected HeLa cells and in Ad5-infected HeLa cells (Overhauser and Jones, unpublished data). Specific cellular environments (i.e., *X. laevis* oocyte versus HeLa cell) may therefore modulate the selection and efficiency of different splice sites. Second, the predominant initiation site for E1a transcription is located 499 nucleotides away from the left-hand end of the Ad5 genome (2). Transcription of E1a in oocytes rarely started at this site but rather began at discrete sites from −30 to −380 nucleotides upstream. Use of upstream starts in E1a has been observed under two other conditions. They are found as a set of minor starts at late times of infection of HeLa cells with wild-type Ad2 or Ad5 (33; Overhauser and Jones, unpublished data). They are also observed at early times after the infection of HeLa cells with mutants of Ad5 that either fail to synthesize or synthesize an abnormal E1a 13S gene product (J. Overhauser and N. Jones, manuscript in preparation). This latter observation has led us to speculate that the 13S-encoded product not only regulates the level of E1a transcription (4; Overhauser and Jones, manuscript in preparation) but also the frequency of initiation at the 499-nucleotide site. At late times of infection there is a substantial increase in the ratio between E1a gene copy number and product; the steady-state level of E1a mRNA increases ca. fivefold, whereas the number of viral DNA molecules increases by a factor of 10^4 to 10^5. We suggest that this imbalance results in a failure of the E1a product to regulate expression of all of the E1a genes, resulting in an increase in the frequency of upstream transcriptional starts. A similar argument can be made for the situation in microinjected oocytes, where the ratio of E1a gene copy number to product is likely to be high.

It was somewhat surprising to find that the E3 promoter is very active in oocytes even in the absence of E1a. The E3 promoter is only marginally active in transfected HeLa cells (44). The reason for the high basal level of E3 promotion in oocytes is not known, but the adenovirus E3 promoter is not unique in this respect. Similar high basal level expression from other regulated adenovirus promoters, including those of the E2 and IVa_2 genes, has been observed (1; K. Haley and N. Jones, unpublished data). In the studies described here, the observation that an 8-fold increase of E3-CAT by E1a in injected oocytes is less than the 40-fold increase in transfected HeLa cells could be a result of the already high basal activity of the E3 promoter in oocytes.

It has been assumed, but not shown directly, that the E1a effector molecule is a polypeptide rather than an mRNA. We took advantage of the oocyte system to demonstrate that a protein, and not a nucleic acid, is responsible for stimulation of the E3 promoter. Since the protein fraction which contained the E3-CAT stimulating activity was prepared specifically so that the E1a protein would accumulate (16), it is reasonable to conclude that the E1a protein is indeed the effector molecule. The increase of CAT activity by the E1a-containing protein extract was about one-half of that observed with the injected E1a gene. There are several possible explanations for this: the concentration of the E1a protein in the total protein extract may have

been too low for maximal enhancement, the E1a protein might be relatively unstable in oocytes, or the transport of the E1a protein from the cytoplasm to the nucleus might be inefficient. Clearly, pure E1a protein is required to examine these possibilities as well as to study further the molecular mechanism of adenovirus gene expression in injected oocytes. In this regard, the E1a 13S gene product has been overproduced in *Escherichia coli* and purified (B. Ferguson, N. C. Jones, J. D. Richter, and M. Rosenberg, manuscript in preparation). Injection of this protein into the cytoplasm of oocytes previously injected with E3-CAT DNA gave an 8- to 10-fold stimulation in expression of the CAT sequences. These results add credence to our conclusion that the increase in CAT activity observed with the crude E1a protein-containing extract described above is significant and a result of the E1a protein itself.

A final interesting observation is the variation in the ability of oocytes from different frogs to synthesize CAT. After DNA injection, good oocytes will accumulate about 16 ng each of CAT in 48 h (based on the specific activity of commercial CAT enzyme). A fully grown oocyte synthesizes protein at an average rate of 17 ng/h (42). Therefore, if all of the CAT enzyme synthesized in each oocyte is stable for the duration of the experiment, it would account for about 2% of the rate of total protein synthesis. However, "bad" oocytes do not synthesize detectable levels of CAT, but probably synthesize protein at approximately the same rate as good oocytes. We are currently attempting to define the molecular lesion of bad oocytes. As far as we are aware, this represents the first clear demonstration of good and bad oocytes with respect to the expression of a microinjected, RNA polymerase II-transcribed gene. However, Korn et al. (23) have noticed that oocytes of only certain frogs reactivate oocyte-type 5S DNA, a gene transcribed by RNA polymerase III, after transplantation of erythroid cell nuclei into oocytes. These authors suggested that "nonreactivators" lacked a sufficient amount of an unknown factor essential for oocyte-type 5S DNA transcription in transplanted nuclei. Thus, by determining what transcription factor is present in good oocytes but lacking in bad oocytes, a greater understanding of the transcriptional regulation of this gene will be accomplished. It is possible that good and bad oocytes might also prove to be useful in elucidating factors previously unknown which are required for the expression of genes transcribed by RNA polymerase II.

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