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trans Activation of an Immediate-Early Frog Virus 3 Promoter by a Virion Protein

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We investigated the protein and DNA sequence requirements for the expression of an immediate-early frog virus 3 (FV3) gene, infected-cell RNA (ICR) 169. We used a plasmid containing the 78 nucleotides 5′ to the transcription start site of ICR-169 placed upstream from the coding sequence for the bacterial enzyme chloramphenicol acetyltransferase (CAT). This construction, when introduced by $CaPO_4$-mediated transfection into various eucaryotic cell lines, promoted CAT synthesis only if the transfected cells were subsequently infected with FV3. Dot-blot hybridization of RNA extracted from transfected, FV3-infected cells with a radioactive CAT probe showed that the induction of CAT synthesis by FV3 was at the level of transcription. When transfected cells were infected with FV3 in the presence of cycloheximide, induction of CAT-specific RNA still occurred, demonstrating that a virion protein was responsible for the *trans* activation. FV3-induced CAT synthesis was inhibited by α-amanitin in wild-type Chinese hamster ovary (CHO) cells but not in CHO cells with an α-amanitin-resistant RNA polymerase II. The results suggest that a virion protein alters either the DNA template or the host polymerase to allow transcription from immediate-early FV3 promoters.

Gene expression in cells infected with *Iridovirus* frog virus 3 (FV3) is sequentially ordered and coordinately regulated to produce distinct classes of transcripts at different stages of infection (32). The immediate-early genes are transcribed from parent templates even in the presence of cycloheximide; the lack of a requirement for de novo protein synthesis indicates either that this stage of transcription is performed by the host DNA-dependent RNA polymerase (10) or that the virion itself possesses transcriptase activity (16). Using an α-amanitin-resistant Chinese hamster ovary (CHO) cell line, Goorha et al. (13) demonstrated conclusively that early FV3 RNA synthesis depends on a functional host RNA polymerase II. However, transcription also appears to require a protein component of the virion, as purified FV3 DNA (uninfectious by itself) can be nongenetically reactivated by UV-inactivated virions (34). In addition, the random distribution of immediate-early genes along the genome (33; A. Aubertin, personal communication) suggests that these genes have separate but similar promoter regulatory sequences that respond to the same *trans*-acting factor, thereby permitting simultaneous transcription of all immediate-early FV3 genes.

Our overall goal is to identify the specific DNA sequences responsible for the coordinate induction of temporal classes of FV3 mRNA, as well as the factors and enzymes that interact with these sequences. The major immediate-early gene of FV3 is transcribed into infected-cell RNA (ICR) 169, which codes for an 18-kilodalton protein of unknown function. The coding region plus 78 nucleotides 5′ to the start site of transcription have been sequenced (30). The 5′ flanking sequence contains an A+T-rich region at position −30, an appropriate location for a TATA box. In the hope that this 78-nucleotide sequence would be sufficient for regulating transcription of ICR-169, we placed it in a plasmid in the proper orientation upstream from the gene coding for the bacterial enzyme choramphenicol acetyltransferase (CAT). This chimeric plasmid, p169PCAT, was then introduced via $CaPO_4$-mediated transfection into eucaryotic cells, and the 78-base-pair (bp) fragment was assayed for promoter function (i.e., CAT activity) in uninfected versus FV3-infected cells. We found that this 78-bp sequence contained all the information necessary for transcription but only when the cells were subsequently infected by FV3. Moreover, the results substantiated our hypothesis that a virion protein, acting in *trans*, interacts with the promoter sequences to permit transcription by the host RNA polymerase II.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* LE392 was used for the growth of all plasmids; strain JM103 was used for the replication of M13 bacteriophages. Plasmids used for the constructions described in this paper include pKSV-10 (Pharmacia Inc), which contains the plasmid pKB111 origin of DNA replication and ampicillinase gene joined to a simian virus 40 (SV40) transcriptional unit, and pCM4 (Pharmacia), which contains the bacterial CAT gene flanked by *Bam*HI restriction sites. Plasmid and bacteriophage replicative-form DNA were isolated by alkaline lysis and cesium chloride centrifugation (19). The identity of all cloned DNA was confirmed by restriction endonuclease digestion and agarose gel electrophoresis. Recombinant DNA manipulations were done essentially as outlined by Maniatis et al. (21).

Construction of chimeric plasmids. The steps followed in the construction of the p169CAT plasmid are outlined in Fig. 1. The vector, pKSV-10, contains the SV40 enhancer and early promoter region followed by a *Bgl*II site, as well as SV40 splice sequences and poly(A) termination signals. The entire immediate-early FV3 gene for ICR-169 had been previously cloned for dideoxy sequencing in the M13mp10 cloning vector (30). The 2-kilobase-pair FV3 insert was excised from the clone with *Hin*dIII and *Eco*RI and purified by sucrose-ethidium bromide centrifugation (27). There is a unique *Aha*III site (TTTAAA; Fig. 2) at the transcriptional start site, and this enzyme was used to separate the 5′ flanking 78 bp from the coding sequence. *Bam*HI linkers were ligated to the blunt *Aha*III ends, and the entire mixture was then ligated, along with the *Bam*HI CAT cartridge, into

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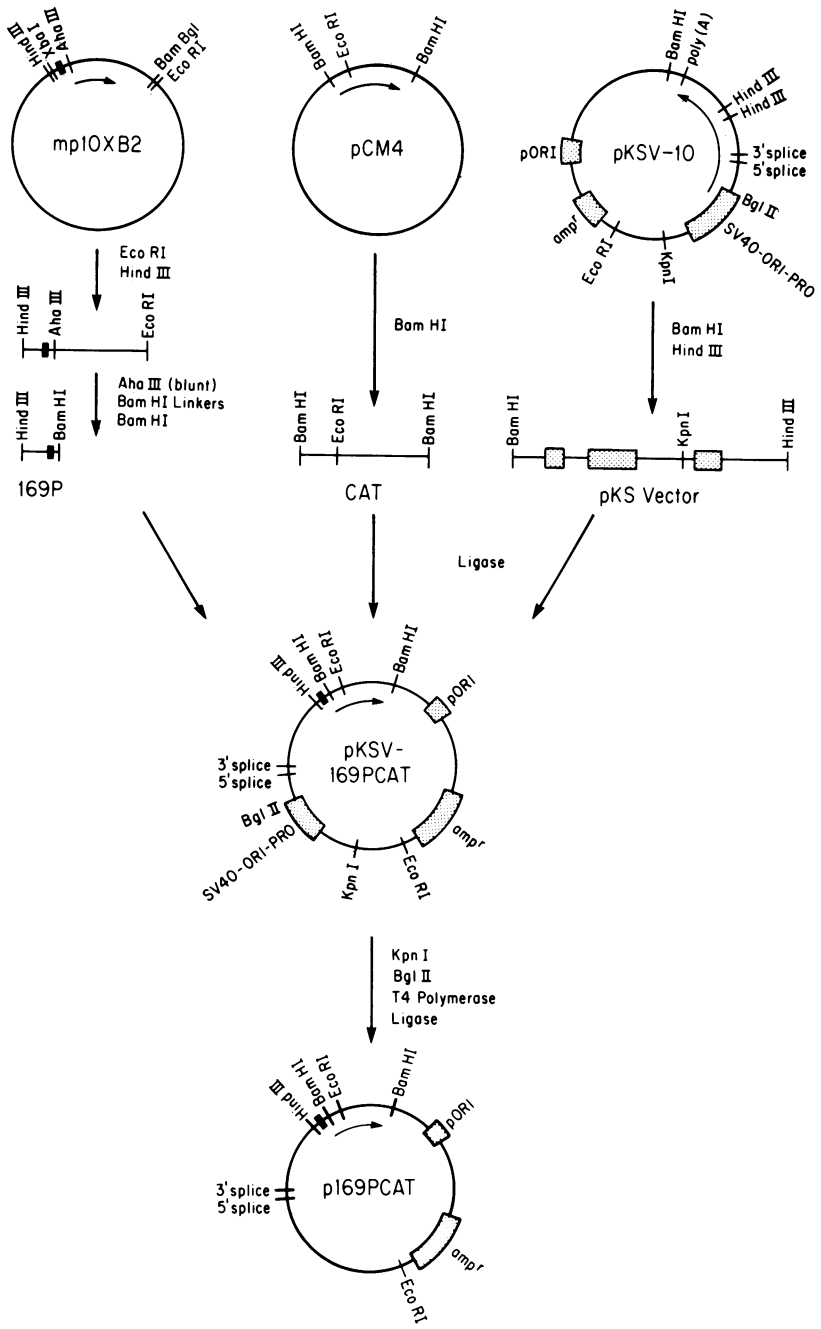

FIG. 1. Construction of the p169PCAT plasmid. A plasmid containing 78 bp of the 5′ flanking region of immediate-early FV3 gene ICR-169 placed in the proper orientation in front of the bacterial CAT gene was constructed as described in Materials and Methods. The maps are not drawn to scale. The arrows show the direction of transcription.

the *Hin*dIII-*Bam*HI sites of plasmid pKSV-10. The transformed colonies were screened by hybridization to nick-translated (25) ICR-169 promoter or CAT probes. Colonies positive for both inserts were further analyzed for orientation by restriction enzyme digestion with *Eco*RI and *Hin*dIII. The SV40 enhancer-promoter region was eliminated by digesting with *Kpn*I and *Bgl*II, filling in the ends with T4 polymerase, and religating. Note that the resulting plasmid, p169PCAT, also lacks the SV40 poly(A) termination signal (Fig. 1). Control plasmids were devised that placed the CAT gene in the *Bgl*II site of pKSV-10 under the control of the SV40 promoter (pSV40CAT) or in the *Bam*HI site, away from any promoter (p0PCAT).

Cells and virus. Fathead minnow (FHM) cells were propagated at 33°C as monolayers in roller bottles or tissue culture dishes (94 mm) in Eagle minimal essential medium containing 5% fetal calf serum (MEM-5). A clonal isolate of FV3 was used to prepare virus stocks at 25°C; virus was harvested and plaque assayed as previously described (23). Wild-type α-amanitin-sensitive CHO cells and a mutant

FIG. 2. The 5′ flanking sequence of the gene coding for ICR-169. The start site of transcription is position 1; two possible translational start sites are underlined. Brackets encompass the putative Goldberg-Hogness box at position −29.

CHO line with a well-characterized α-amanitin-resistant RNA polymerase II (5) were maintained as monolayers at 37°C in α-medium with 10% fetal calf serum.

For heat-inactivation studies, FV3 was heated in 5-ml portions in MEM-5 for 15 or 30 min. UV irradiation was performed in an open tissue culture dish with a 15-W germicidal lamp at a distance of 19 cm. UV inactivation for 2 min or heat inactivation for 15 min reduced the titer to $<10^2$ PFU/ml (18, 34).

Enzymes and reagents. Restriction enzymes, T4 ligase, T4 DNA polymerase, and S1 nuclease were purchased from New England BioLabs, Inc., or Bethesda Research Laboratories, Inc., and used according to the manufacturer's directions. Boehringer Mannheim Biochemicals supplied the calf alkaline phosphatase and T4 polynucleotide kinase. [^{14}C]chloramphenicol, [γ-^{32}P]ATP, and Gene-Screen-Plus nylon membranes were obtained from New England Nuclear Corp.

Transfection of eucaryotic cells. Cells were seeded in 94-mm tissue culture dishes at approximately 40% confluency and incubated at their optimum temperature for 16 to 18 h. Three hours before the addition of DNA, the overlying medium was removed and replaced with fresh medium. Plasmid DNA was coprecipitated with $CaPO_4$ as described by Graham and van der Eb (15), and 10 μg of DNA per dish was dropped directly into the medium. After 3 h of incubation, the cells were subjected to a 2-min 10% glycerol shock and incubated for a further 18 h at their optimum temperature (33°C for FHM, 37°C for mammalian cells) before infection with FV3. Transfected cells were harvested for CAT assay or RNA purification as described below.

Assay for CAT. Cell extracts were prepared by three freeze-thaw cycles in dry ice-acetone of a cell pellet from one 94-mm dish in 100 μl of 0.25 M Tris hydrochloride (pH 7.8). After the insoluble debris was removed by centrifugation, 50 μl of the supernatant was assayed for CAT by the method of Gorman et al. (14), with 0.2 μCi of [^{14}C]chloramphenicol per reaction. The final reaction products, in 10 μl of ethyl acetate, were spotted on silica gel thin-layer plates and run by ascending chromatography in $CHCl_3$-methanol (95:5). The separated acetylated forms were identified by autoradiography, by using spray En-^{3}Hance (New England Nuclear) and Kodak XAR-5 X-ray film. For quantitation, radioactive spots were cut out and counted in a liquid scintillation system.

Isolation of mRNA, S1 nuclease mapping, and dot-blot analysis. Cytoplasmic RNA used in S1 nuclease mapping was isolated from FV3-infected cells as previously described (35). This RNA was hybridized with a 5′-labeled 355-bp *Xba*I-*Hpa*II fragment specific for ICR-169 (30) under the conditions described by Berk and Sharp (3). Digestion of the RNA-DNA complex with S1 nuclease resulted in a labeled DNA fragment 275 bp in size.

Extraction of total RNA for dot-blot analysis was accomplished by dissolving cells in cold guanidine-HCl buffer, placing the lysate over a 5.7 M CsCl cushion, and centrifuging at 150,000 × *g* in a Beckman SW41 rotor for 22 h (21). The resulting RNA pellet was dissolved in 10 μl of 0.01 M Tris (pH 7.5) and spotted onto a sheet of Gene-Screen-Plus by using a Bio-Rad dot-blot apparatus. After drying, the membrane was hybridized (16 h, 65°C) to a nick-translated and denatured probe of the CAT gene-coding sequences. The absence of DNA in the RNA pellet was confirmed by spotting a parallel sample boiled 5 min in 0.3 N NaOH.

RESULTS

CAT synthesis directed by the FV3 ICR-169 promoter requires a *trans*-acting viral protein. Three plasmid constructions were used to transfer the CAT gene into recipient FHM cells. In one, the CAT gene was under the control of the SV40 promoter (pSV40PCAT); in another, it was under the control of the presumed FV3 ICR-169 promoter (p169PCAT); and in the third, it was without a promoter (p0PCAT). Approximately 18 h after transfection, the cells were either mock infected or infected with FV3 and transferred to 30°C, which is the optimum temperature for virus production (17). Extracts were assayed for CAT after 4 h, the time when the virus is beginning late protein synthesis (35); the results are shown in Fig. 3. The pSV40PCAT construct was actively transcribed in FHM cells, whether they were infected with FV3 or not. When the CAT gene was removed from SV40 control (p0PCAT), no CAT was synthesized under either condition. This assay also showed that FV3 in itself does not code for a protein with CAT activity. The CAT gene placed downstream from the 78-bp ICR-169 promoter sequence (p169PCAT) did not direct the synthesis of CAT unless the cells were subsequently infected with FV3. This result strongly suggests that a virus-specific product is required for the activation of the ICR-169 promoter. As a sidelight, it also shows that, at least in FV3-infected cells, poly(A) addition signals are not required for the synthesis and transport of mRNA. We do not know whether this observation is related to the lack of poly(A) on authentic FV3 mRNA (31).

Kinetics of ICR-169 promoter-directed CAT synthesis are the same as those of the FV3 18K protein. The infected-cell protein (ICP) coded for by ICR-169 is an 18,000-dalton (18K) protein of unknown function designated ICP-18 (35). Its rate of synthesis peaks at 3 to 4 h and declines thereafter, although the rate of synthesis of ICR-169 continues at a

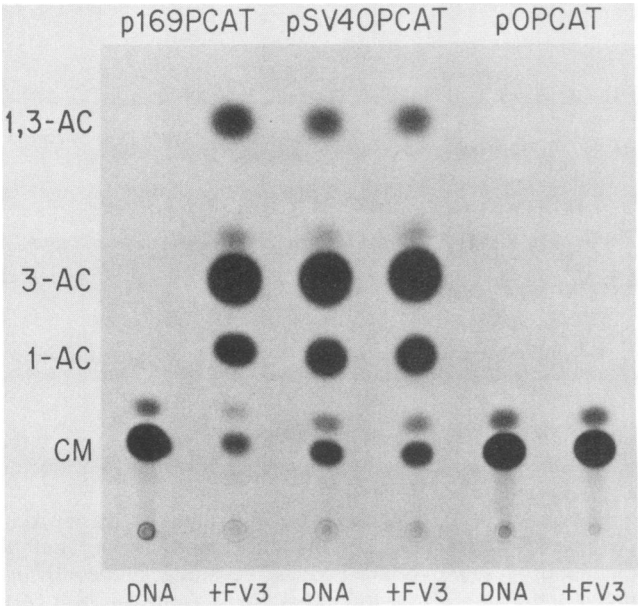

FIG. 3. *trans*-Acting FV3 protein induces CAT synthesis from the ICR-169 promoter. FHM cells were transfected with either p169CAT, pSV40PCAT, or p0PCAT plasmids as described in the text. After 24 h, one dish from each set was infected with 20 PFU of FV3 per cell. Four hours later, extracts were prepared for CAT assay by thin-layer chromatography. The degree of CAT activity is proportional to the amount of [^{14}C]chloramphenicol converted to acetylated forms. CM, Unacetylated chloramphenicol; AC, acetylated chloramphenicol.

relatively high rate. To ascertain whether the isolated ICR-169 promoter attached to the CAT gene was subject to the same kinetic parameters, transfected FV3-infected FHM cells were assayed for CAT activity at 0, 1, 2, 4, and 6 h postinfection. CAT activity peaked at 4 h, with a slight decline at 6 h, similar to what would be expected if the plasmidborne promoter acted as the natural one (Fig. 4). As the assay measures cumulative synthesis rather than rate, we did not expect to see a sharp decline at 6 h; the consistently observed decrease probably indicates that CAT does not continue to accumulate after this time. Whether this is due to the translational block of immediate-early protein synthesis seen in a normal infection (12) was not investigated.

Functional host RNA polymerase II is required for FV3 induction of p169PCAT. Previous work by Goorha (10) had shown that the production of infectious virus, as well as the production of all virus-specific macromolecules, was severely inhibited by α-amanitin in sensitive CHO cells but not in cells characterized as having an α-amanitin-resistant polymerase II. The drug had to be added to the cells at least 1 h before infection for the inhibitory effect to be displayed, and Goorha postulated that the host enzyme took part in early but not late viral mRNA synthesis. However, the level of detection was not sensitive enough to ensure that a small amount of viral mRNA or protein was not synthesized in the amanitin-sensitive cells. To establish that host RNA polymerase II is also involved in the FV3-induced transcription of p169PCAT, we performed transfection-infection experiments in α-amanitin-sensitive and -resistant CHO cells. The addition of 5 μg of α-amanitin per ml 12 h before infection with FV3 substantially reduced FV3-induced CAT synthesis in sensitive but not in resistant cells (Fig. 5). Increasing the concentration of α-amanitin to 50 μg/ml and adding it 1 h before infection totally abolished the induction of CAT by FV3 (data not shown). Therefore, the host RNA polymerase II, as well as a virus factor, was involved in transcription from the ICR-169 promoter, either directly or through a short-lived intermediate.

***trans*-Acting FV3 factor destroyed by heat inactivation but not UV inactivation.** If the virus-specific component of ICR-169 promoter transcriptional activation is a virion-associated protein rather than an immediate early protein, one would expect that heat-inactivated virus would not be able to activate this promoter, whereas UV-inactivated virus would. FV3 inactivated by heat (56°C, 30 min) retains its ability to switch off host cell macromolecular synthesis (12), but even 15 min at 56°C completely abolished the ability of the virions to induce CAT synthesis from the 169P promoter (Fig. 6). This result was not due to a failure to release DNA from heated virions, because previous studies have shown that heat-inactivated FV3 can provide a template for transcription, if active virion proteins are also present (18, 34). FV3 subject to 2 min of UV irradiation is no longer able to replicate (18) but retains the power to induce ample levels of CAT from the ICR-169 promoter (Fig. 6). Increasing the time of UV irradiation did decrease the amount of CAT that the virions were capable of inducing; but after 16 min of irradiation, even the activity of the FV3 switch-off protein is affected (6).

The ability of UV-inactivated virions to induce the synthesis of CAT from the ICR-169 promoter caused us to reanalyze our earlier assumption that UV-inactivated virus was incapable of being transcribed (16, 34). When we measured the levels of genuine ICR-169 transcription by S1 nuclease mapping after the exposure of FHM cells to UV-inactivated virus, we found that it took over 16 min of irradiation to eliminate synthesis of this RNA completely (Fig. 7). This length of time is comparable to that required to inactivate the factor that stimulates transcription from a

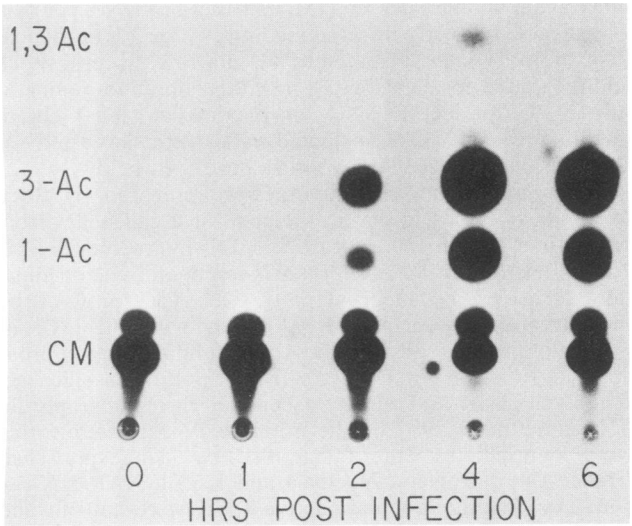

FIG. 4. Kinetics of FV3-induced CAT synthesis. FHM cells were transfected with p169PCAT DNA and infected 24 h later as described in Materials and Methods. Zero time was after 1 h of virus adsorption at 25°C; additional samples were processed for CAT assay after the indicated number of hours of incubation at 30°C. CM, Unacetylated chloramphenicol; Ac, acetylated chloramphenicol.

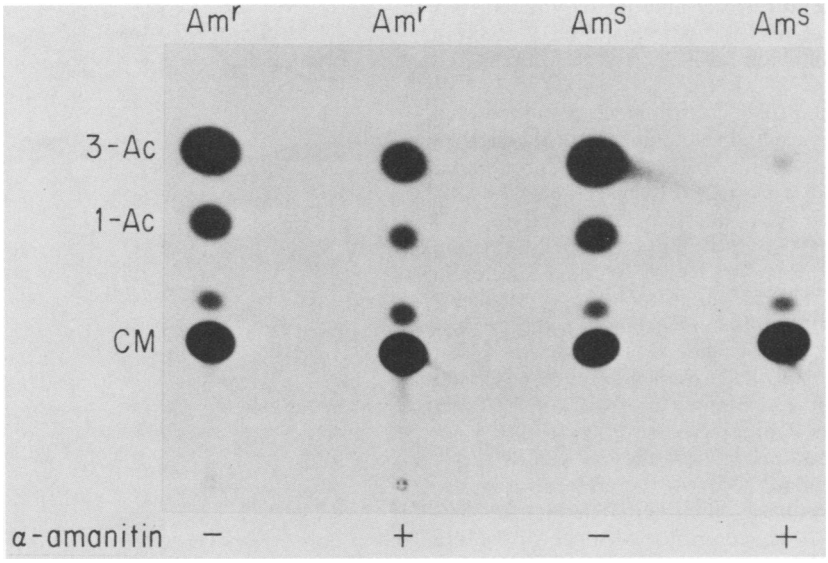

FIG. 5. Functional RNA polymerase II is required for FV3-induced CAT synthesis promoted by p169PCAT. CHO cells with an α-amanitin-resistant RNA polymerase II (Am^r) and wild-type α-amanitin-sensitive CHO cells (Am^s) were transfected with p169PCAT DNA, and half of each set of cultures was incubated at 37°C overnight in the presence of 5 μg of α-amanitin per ml. The next day, all cultures were infected with 20 PFU of FV3 per cell at 30°C and assayed for CAT activity after 4 h. CM, Unacetylated chloramphenicol; Ac, acetylated chloramphenicol.

nonirradiated promoter (Fig. 6) and indicates that both phenomena could be due to the same mechanism, i.e., UV inactivation of a virion protein. These data also support the observation of Martin et al. (22) that expression of immediate-early genes is relatively resistant to UV irradiation.

Because ICR-169 and possibly other immediate early mRNAs could be transcribed from UV-irradiated template DNA, we could not be certain that the *trans*-acting protein was not one of these early gene products rather than a viral structural protein. Therefore, we decided to measure FV3-induced CAT-specific RNA made in the absence of protein synthesis.

FV3-induced activation of p169PCAT is at the level of transcription. Previous investigations using the CAT assay for the measurement of *trans* activation indicated that such induction takes place at the level of transcription (24, 26). Nevertheless, we wanted to confirm this in our system and at the same time establish whether de novo viral protein synthesis is necessary for the induction of ICR-169 promoter-directed CAT synthesis. p169PCAT-transfected cells were mock infected or infected with FV3 in the presence or absence of cycloheximide (Fig. 8). In contrast to many other virus-cell systems, protein synthesis is irreversibly inhibited >99% by cycloheximide in FV3-infected FHM cells, and

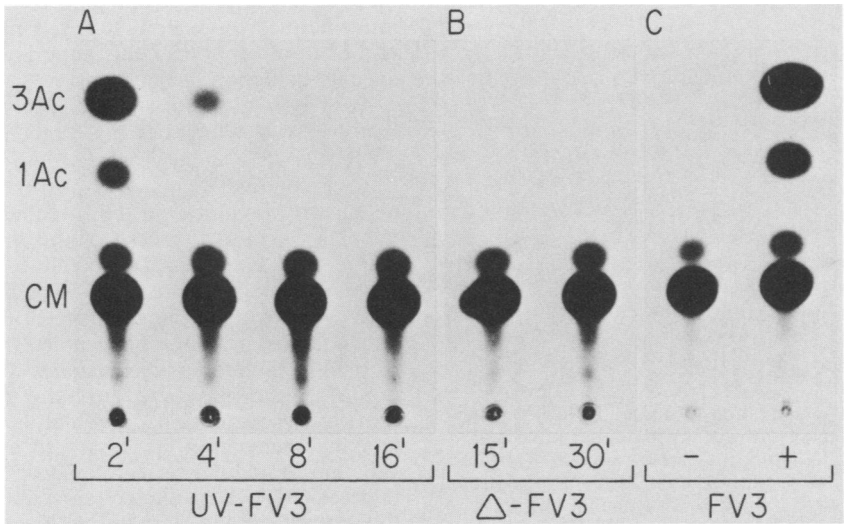

FIG. 6. Effect of heat or UV irradiation on FV3-induced CAT synthesis from p169PCAT. Virus suspensions were inactivated as described in Materials and Methods; the numbers under each lane represent the minutes of exposure to UV or heat (56°C). FHM cells were transfected with p169PCAT DNA and infected 24 h later with active FV3 or equivalent amounts of the inactivated viral suspensions. Cell extracts were prepared and assayed for CAT activity after 4 h. CM, Unacetylated chloramphenicol; Ac, acetylated chloramphenicol.

there is a marked reduction in the amount of viral mRNA synthesized in the presence of both this drug and more potent inhibitors of protein synthesis such as puromycin and anisomycin (32).

Total RNA was isolated from the transfected and infected cells, dotted on a nylon membrane, and hybridized to a radioactive probe corresponding to the CAT coding region. The results show that little or no CAT-specific mRNA was synthesized from the p169CAT template in the absence of FV3 infection. Moreover, FV3 induction of transcription took place even in the absence of protein synthesis, although the relative amount of CAT-specific mRNA was increased if protein synthesis was permitted. The increase in FV3-induced CAT mRNA synthesis when protein synthesis was allowed versus that seen in the presence of cycloheximide corresponded well with the similar increase in ICR-169 synthesis observed under comparable conditions (32). By 4 h after infection, substantial amounts of late (as well as early) viral proteins are being made (35), so the amount of *trans*-acting virion protein is probably amplified. The fact that 169 PCAT mRNA synthesis was seen in the absence of protein synthesis, combined with our earlier data that viral DNA is not infectious (34), argues strongly that the *trans*-acting protein is a component of the virion. We conclude that a virion protein is responsible for the *trans* activation of the ICR-169 promoter.

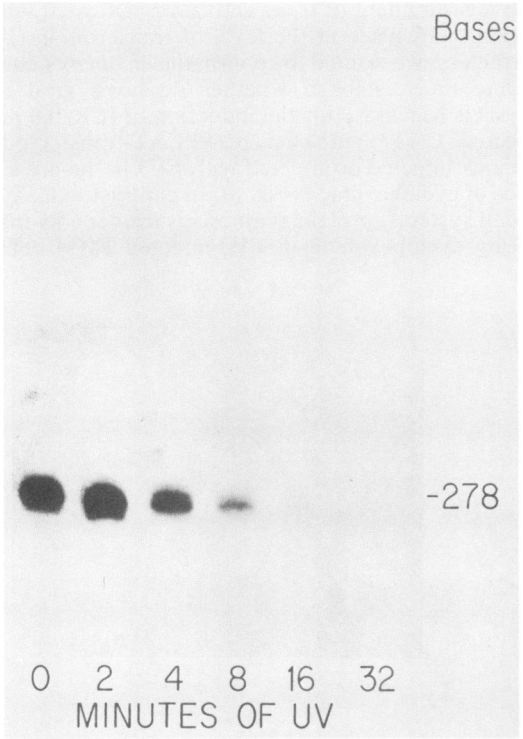

FIG. 7. Kinetics of UV inactivation of authentic ICR-169 synthesis. FHM cells were infected with FV3 that had been exposed to UV inactivation for the length of time indicated. After 4 h, RNA was extracted from the cytoplasm and quantitated by S1 nuclease mapping. The S1 digests were extracted with phenol and $CHCl_3$, dissolved in formamide sample buffer, heated to 90°C for 5 min, and subjected to electrophoresis on a 6% polyacrylamide–6 M urea gel. The location of radioactive bands was determined by autoradiography.

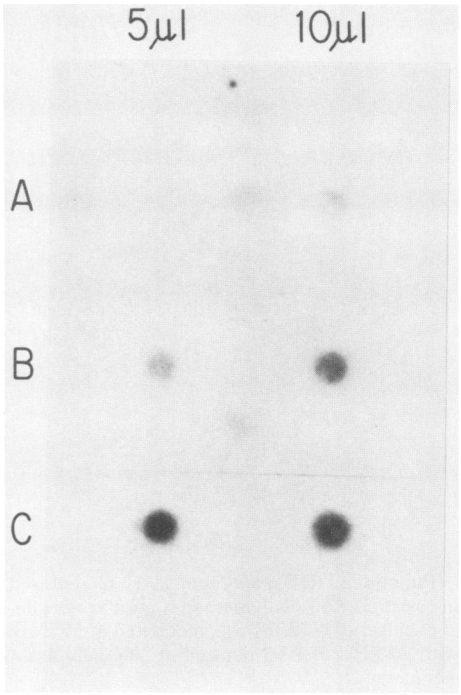

FIG. 8. The *trans*-acting protein is a viral structural protein. FHM cells were transfected with p169PCAT DNA; 24 h later, the cells were (A) mock infected, (B) infected with FV3 in the presence of 50 μg of cycloheximide per ml, or (C) infected with FV3 in the absence of drug. Total RNA was extracted after 4 h of infection, and 5 or 10 μl was spotted onto a nylon membrane as described in Materials and Methods. The membrane was hybridized overnight at 65°C to a ^{32}P-labeled probe corresponding to the CAT coding sequence. The washed and dried membrane was exposed to Kodak XAR-5 film for 3 days with a Dupont Cronex intensifying screen. Control dots of alkaline-hydrolyzed RNA gave no detectable hybridization.

DISCUSSION

We showed that an FV3 virion protein acts in *trans* to induce transcription from the promoter of the major FV3 immediate-early gene, ICR-169. Only 78 bp 5′ to the start site of transcription of this gene is required for this virus-specific induction to occur.

trans-Acting factors interacting with promoter or enhancer DNA sequences have recently been described for SV40 (9, 28), adenovirus (2, 29), herpesvirus (24), and retrovirus (26) systems. It is postulated that similar mechanisms are involved in both cellular differentiation and neoplasia (7). In the case of the viral systems mentioned above, the enhancer DNAs all have a short consensus sequence, usually more than 150 nucleotides from the TATA box, that positions the start site of transcription; the *trans*-acting factors are interchangeable (8). That only 78 bp of the 5′ ICR-169 sequence was required for FV3-specific regulation of transcription was surprising but resembles the situation with vaccinia virus, in which less than 100 bp of the 5′ flanking sequence is necessary to induce transcription (4). We plan to make a series of site-directed mutants to determine exactly which nucleotide sequences are necessary for this regulation and, in addition, sequence additional immediate-early FV3 genes to learn whether similar regulatory sequences are found in the same position.

The mechanism of FV3 *trans* activation is unknown. One

possibility is that the virion protein interacts with the host polymerase in a manner analogous to the bacteriophage sigma factors (20) and that the modified enzyme recognizes virus specific DNA sequences. A more easily testable hypothesis is that the virion protein binds to the regulatory DNA sequence in such a way as to enhance the binding of RNA polymerase II. Several FV3 proteins are known to bind to DNA (1, 11). If we can identify the one that binds specifically to the ICR-169 promoter, we can then isolate and purify it for testing in an in vitro transcription system.

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