Structure and Regulation of the Immediate-Early Frog Virus 3 Gene That Encodes ICR489

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To test whether the promoters of two immediate-early genes from frog virus 3 were similar in nucleotide sequence, we have cloned and sequenced an immediate-early gene encoding an infected-cell mRNA of 489 kilodaltons (ICR489) and have shown that the protein product of this gene is approximately 46 kilodaltons. The 5' and 3' ends of the transcripts from this gene, as determined by mung bean nuclease analysis, were microheterogeneous. The promoter region was subcloned upstream from a promoterless chloramphenicol acetyltransferase gene, forming the recombinant plasmid pBS489CAT. As with the previously sequenced frog virus 3 immediate-early gene encoding ICR169, expression of chloramphenicol acetyltransferase in transfected cells required activation by a virion-associated protein. Although the promoter region of the gene encoding ICR489 contained TATA, CAAT, and GC motifs similar to those of typical eucaryotic promoters, it showed no significant homology to the ICR169 promoter, indicating that the concomitant temporal expression of these two genes is not due to similar promoter sequences.

Frog virus 3 (FV3), a large icosahedral DNA virus containing a linear, double-stranded genome of 170 kilobase pairs, belongs to the genus *Ranavirus* of the family *Iridoviridae* (35). Although transcription of viral mRNA takes place in the nucleus and requires the host RNA polymerase II (9), the mature virus particles are assembled in the cytoplasm (for a review, see reference 35). FV3 provides an excellent model system for examining virus-specific macromolecular synthesis, since infection rapidly shuts down host-cell synthesis, making it possible to incorporate radioisotopes predominantly into viral protein, DNA, or RNA (1, 16). By examining the kinetics of [³H]uridine-labeled RNA synthesis in FV3-infected cells, Willis et al. (37) found that FV3 mRNA could be subdivided into three temporal classes: immediate early, delayed early, and late. The immediate-early class contains those RNAs synthesized in the presence of cycloheximide (39). The delayed-early class contains the immediate-early RNA plus additional RNAs formed in the presence of the amino acid analog fluorophenylalanine, and the late class contains the full array of viral RNA formed in the absence of inhibitors (39). The ability to distinguish these three classes with drugs that prevent (cycloheximide) or restrict (fluorophenylalanine) protein synthesis suggests that at least two distinct proteins are needed to temporally regulate FV3 transcription (11).

As a step towards further understanding of transcriptional regulation in FV3, we have begun to examine the structure and regulation of representative members of the immediate-early, delayed-early, and late gene classes. Here we report the cloning, sequencing, and initial promoter analysis of the gene for the immediate-early infected-cell RNA (ICR489) that encodes an infected-cell protein of approximately 46 kilodaltons (kDa) (ICP46). This gene is of particular interest because its mRNA is overexpressed in the presence of cycloheximide, suggesting that its expression may normally be controlled by an immediate-early repressor protein (39). In addition, it is the second of two immediate-early genes

sequenced in this laboratory; the first encoded ICR169 (33). The results reported in this paper demonstrate that the concomitant temporal regulation of these two genes is not associated with similar promoter sequences.

MATERIALS AND METHODS

Cells and virus. Fathead minnow (FHM) cells were propagated at 33°C as monolayers in roller bottles or 100-mm-diameter tissue culture dishes with Eagle minimal essential medium containing 5% fetal calf serum. A clonal isolate of FV3 was used to prepare virus stocks by infecting cells at a multiplicity of 1 PFU per cell. Virus was harvested and assayed as previously described (26). Published procedures for virus inactivation by UV and heat (14) were followed.

Bacteria, plasmids, and bacteriophages. The single-stranded M13 bacteriophages mp10 and mp11 and the host *Escherichia coli* JM101 and JM103 were purchased from Pharmacia Fine Chemicals. The vector pUC13 was from Bethesda Research Laboratories, Inc., the Bluescript (BS) M13 plasmid vector was from Stratagene Cloning Systems, and pMBV17 was a gift from J. Corden.

RNA probes. Viral mRNA was labeled in vivo with ³²P_i and purified by the procedures of Willis et al. (33). Viral RNA species were separated by electrophoresis in acid urea-agarose gels (20), and the desired bands of labeled RNA were eluted from the gels and purified by the procedure of Landridge et al. (18).

R-loop analysis. Immediate-early FV3 mRNA was partially purified by hybridization to and elution from the *Xba*I F fragment of FV3 DNA immobilized on nitrocellulose filters. This RNA was hybridized to purified *Xba*I F fragments by using the conditions described for S1 nuclease mapping by Berk and Sharp (3). The hybridization mixture was diluted and spread for electron microscopy by the technique of Chow et al. (6).

Plasmid constructions. The highly methylated DNA of FV3 was refractory to cloning, and therefore viral DNA was isolated from a DNA methyltransferase-negative mutant of

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FIG. 1. DNA restriction maps showing the location of ICR489. (A) *XbaI* sites in the total FV3 genome. Although the restriction map is circular (10, 19), it is represented here as linear for convenience. (B) Enlargement of the *XbaI* F fragment showing *PstI* sites used for subcloning. (C) Enlargement of the *XbaI*-*PstI* fragment which contained the gene encoding ICR489. The fragments shown below the map are the 360-bp *AccI*-*Sau3A* fragment and the 174-bp *XhoII*-*XbaI* fragment used in mung bean nuclease mapping of the termini of the transcript and the 486-bp *HpaII* fragment subcloned to form pBS489CAT for promoter analysis. The wavy arrow shows the direction of transcription of ICR489. Kb, Kilobase.

FV3 (36). The *XbaI* F fragment (Fig. 1B) was cloned into the *XbaI* site of pMBV17, a pBR322 derivative. An *XbaI*-*PstI* fragment of 1,640 base pairs (bp) (Fig. 1C) was subcloned into M13 vectors mp10 and mp11 for sequencing. Hybridization of labeled ICR489 to Southern blots containing the *XbaI*-*PstI* fragment ensured that the correct fragment was subcloned.

To construct pBS489PCAT, the promoterless chloramphenicol acetyltransferase (CAT) gene (*cat*) from pCM4 (Pharmacia) was first inserted into the *Bam*HI site of the cloning/sequencing vector Bluescript M13 (Stratagene), forming pBS-CAT. The promoter region of the gene encoding ICR489 was excised with the restriction enzyme *HpaII* (Fig. 1C). The 486-bp *HpaII* fragment was cloned upstream from the *cat* gene in two steps. First, the *HpaII* fragment was inserted into the pUC13 *AccI* site, which allowed selective isolation of Lac⁻ *E. coli* JM101 transformants containing the insert; neither the *AccI* nor the *HpaII* site was regenerated. In the second step, the fragment was excised with *PstI* and *SmaI*, which closely flank the *AccI* site of the pUC13 cloning cartridge. As a result, the fragment inserted in the promoter cloning vector pBS-CAT to form pBS489PCAT contained some pUC13 sequences: 3 bases at the 5' (or *PstI*) end and 18 bases at the 3' (or *SmaI*) end. Orientation of the 489 promoter in relation to *cat* was confirmed by restriction analysis.

DNA sequence analysis. Sequencing was by the dideoxy chain termination method (31) with the 1,640-bp *XbaI*-*PstI* fragment cloned into M13mp10 and M13mp11. The sense strand was sequenced by using the mp11 clone as the template; the anti-sense strand was cloned by using mp10.

For the sense strand, overlapping deletions were generated by using the cyclone system (International Biotechnologies, Inc.), and each deletion was sequenced from the same universal primer by the quasi end-labeling technique (4). For the anti-sense strand, the known complementary sequence was used to select a set of synthetic primers at appropriate distances from each other. The overlaps were identified by computer analysis. Analysis of DNA sequences was done with the Pustell Sequence Analysis Program purchased from International Biotechnologies, Inc.

In vitro translation of FV3 mRNA. The conditions for translation of FV3 mRNA in micrococcal nuclease-treated reticulocyte lysates (Promega Biotec) were as described by Willis et al. (33). Hybrid-arrested translation (5) was done by hybridizing (65°C, 10 min) FV3 mRNA to single-stranded cloned DNA in a solution containing 10 mM Tris (pH 7.5), 50 mM potassium acetate, and 5 mM MgCl₂. The samples were ethanol precipitated and were then used to program the reticulocyte lysate for FV3 protein synthesis.

Single-strand nuclease analysis. Mung bean nuclease (15) was used to determine the 5' and 3' ends of ICR489. For the 5' end, total infected-cell RNA, prepared as described by Tham et al. (32), was hybridized to a 360-bp *AccI*-*Sau3A* fragment containing the 5' region and end labeled at the *Sau3A* site (Fig. 1C). Hybridization proceeded under the conditions described by Berk and Sharp (3) for 3 h at 47°C. Subsequent treatment of the samples with mung bean nuclease and electrophoresis of the digestion products on polyacrylamide gels in parallel with a sequencing ladder was performed as described by Willis et al. (33). For the 3' end, the same procedure was done, except the RNA was hybridized to a 174-bp *XhoII*-*XbaI* fragment containing the 3' region and end-labeled at the *XhoII* site (Fig. 1C).

Transfection of eucaryotic cells and CAT assay. Published procedures for transfection of eucaryotic cells (13) were followed. Four hours after transfection, FHM cells were glycerol shocked (28) and incubated for an additional 18 h at 33°C before infection with FV3. Cells were infected (or mock infected) with 10 to 20 PFU per cell and were incubated 4 h at the optimum temperature for FV3 replication, 30°C. Transfected cells were harvested for the CAT assay by the method of Gorman et al. (12) with 0.2 µCi of [¹⁴C]chloramphenicol per reaction.

RESULTS

The gene encoding ICR489 located near the end of the *XbaI* F fragment. Figure 1A shows the *XbaI* restriction map of FV3 DNA. Only the *XbaI* F fragment hybridized to ³²P-labeled ICR489 (results not shown), indicating that this fragment contained all of the corresponding gene. R-loop analysis (6) of the hybridization product of *XbaI*-F with partially purified ICR489 showed that the gene was located at one end of the *XbaI* F fragment (Fig. 2). In addition, since only one R loop was detected and its size (~1,300 nucleotides) approximated that of ICR489 measured in denaturing polyacrylamide gels (39), splicing was either absent or very limited. The appropriate end of the *XbaI* F fragment was separated from *PstI* digests of *XbaI* F (Fig. 1B) by agarose gel electrophoresis. In Southern blots of these digests, ³²P-labeled ICR489 hybridized only to the *XbaI*-*PstI* 1,640-bp fragment (Fig. 1C). This fragment was eluted from agarose gels (18) and was cloned into M13mp10 and M13mp11, which allowed sequencing of both strands.

Sequence of the gene that encodes ICR489. The sequence of the entire *XbaI*-*PstI* fragment is shown in Fig. 3. The

FIG. 2. Location of ICR489 on the *XbaI* F fragment. Partially purified ICR489 was hybridized to the double-stranded *XbaI* F fragment, and the mixture was spread for electron microscopy. The arrowhead shows the displaced single-stranded DNA (R loop), indicating hybridization at one end of the fragment.

direction of transcription was from the *PstI* toward the *XbaI* site, since ICR489 hybridized to the single-stranded DNA insert in M13mp11 where the orientation was such that, during sequencing reactions, nucleotide polymerization proceeded from the *PstI* site to the *XbaI* site (results not shown). ICR489 did not hybridize to the complementary strand present in M13mp10. This direction is the same as that found for the gene encoding ICR169, an unlinked immediate-early gene located on the *XbaI* K fragment (33). A presumptive TATA box was located at bases -41 to -35 from the major start site. Further upstream, at bases -103 to -97, was a presumptive CAAT box which could function in the recognition of transcription factors, as has been found for certain established eucaryotic promoters (2, 7). At bases -181 to -176, there was a CCGCCC box which could also be a potential binding site for transcription factors. This GC-rich region is clearly involved in binding the transcription factor Sp1 to an upstream region of the simian virus 40 early promoter (8) and probably plays a similar role for a number of genes, such as the herpesvirus *tk* gene (23). The sense strand contained an open reading frame beginning with an ATG start codon 343 nucleotides from the *PstI* site and extending 1,182 nucleotides to a TAA stop codon. Translation in this frame would result in a protein of 394 amino acids. The computer-derived molecular weight was 45,787, in agreement with estimates from polyacrylamide gels which have ranged from 42 (21) to 50 (29) kDa.

Hybrid-arrested translation of the protein encoded by ICR489. To confirm that the *XbaI-PstI* fragment encoded a protein of ~46 kDa, a reticulocyte lysate was programmed with FV3 immediate-early mRNA (those RNAs formed in the presence of cycloheximide) that had been hybridized to the *XbaI-PstI* strand complementary to ICR489. Figure 4 shows that this hybridization inhibited production of a 46-kDa protein, whereas a heterologous fragment (the internal 2-kb *PstI* fragment from *XbaI* F [Fig. 1B]) had no effect on synthesis of this protein. We concluded that the inhibited protein, ICP46, was encoded by ICR489. Note that the 46-kDa protein was a major translation product. This finding is consistent with the *in vitro* results of Raghov et al. (29) who associated ICP46 (their 50-kDa protein) with ICR489 on

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-311 CTGCAGGACCCCCACCCATCCGGATCCACCAATTACGGTAGACTGACCAACGCCAGCCTT
-251 AACGTCACCTGTCCGCTGAGGCCACACCGGCCGCCGAGGAGGTGGAGGTAAACAACCTG
-191 GGGTACACCAACCGCCCAAAAGTACGCCCTCATCGTTCTGGCCATCAACCAACAATTATC

-131 CGCATCATGAACGGCTCGATGGGATTCCTTCTGTAAGAGTATTTTCAGCGCAAAAG
-71 TCTTTTCCGTCATGGGTCCCTCATGATGGAATATAAAATCATGAAGTGTCCGTTTGTGCAAA
+1 MANFVT
-11 AACGGGTCTTTTGGAGTCACTTGTCTGACAAATCTTAACATGGCAAACTTTGTGACA
DSRNLGTITSCAPQDQSHLHP
50 GACTCTCGCAATGGGCTCACCATCTCTTGGCTCTCAGGATCAGTCTCACCTGCACCCC
TIRALLVMEGDSVIFRGLPHF
110 ACAATCAGGGCTCTGGTTATGGAGGGTATTCTGTAATCTTTAGAGGACTGCCACATCCA
DIHREAPPAGLRLKDKCLVYD
170 GACATTCACCGGAGGGCTCTCCCGCGGATTGAGGCTCAAGGACTGCTGGTGTACGAT
SYEGALVNVFWHGGQWFFCT
230 TCGTACGAGGGCCCTTGGTCAATGTCTTTTGGCACGGAGGGCAGTGGTGGTCTGCACC
NKKLSIDRASWSASPQSFKR
290 AACAGAAGCTGAGCATCGACGGGCTCTTGGAGCGCTCTCCCGGAGCTTCAAGAGA
AFVNCRLRKMWRDDRSWADLF
350 GCCTTCGTCAACTGCTGCGGAAAATGTGGAGGACGACAGGAGCTGGGCCGATCTCTTT
DRSYMPSFCDANLKDGLGYV
410 GACAGGAGCTACATGCCAGCTTTTGCAGCGCAATCTGGCAAGGACCTGGGATATGTT
FMVVFDPPEERIVCSDEQRLR
470 TTTATGGTCTTTGACCGGAGGAGCGCATCGTCTGCTCAGACCGAGCAGCTCTCCGT
LLATTFDRCTNSHSECSLTL
530 CTGCTGGCGACATTCGACAGGTGACCAACTCTCAGACTCAGAGTGTCTCTGACCCGT
TCGTEVEVPRPICLKNEREF
590 ACTTGTGGCAGGAAGTGGAGGTGCCAGGCCAATCTGCCTCAAGAAGCAGAGAGAGTTT
LLHLRSQDPCRVAGVVLIDA
650 CTCTTGACCTGAGATCTCAGGATCTTCAAGGTGCGCCGGCTGGTCTGATGACGCT
LDIHYKILPSEYTKVLDARG
710 CTGGACATTCACACAAGATTCTCCCTCTGAGTACACAAGGTGCTCGACGCGAGGGGA
EQPRLNLNRMFQLMEMGPGE
770 GAGCAGCCGACGCTGCTCAACAGAATGTCCAGCTGATGGAGATGGGTCCAGAGGGAGAG
AHIEVLCRYFSDAARVAMERA
830 GCCACATCGAAGTCTGTGCCGCTACTTTCAGACGCGAGGTGGCATGGAGAGGGCG
WDVREIRIVQCYLDDLTEPDS
890 TGGGACGTCAGGAGAGGATAGTCCAGTGTCTGATCTGACTGAGCCAGACTCTGAG
PQVWMTRRLMEIVRGCRPT
950 CCTCAGGCTGGATGACAAGGAGGCTGATGAGATTGTGCGCGGTTGACAGACCGGAAACC
ERTMIDEFLRTMTTGQQRKR
1010 GAGAGGACCATGATTGACGAGTCTTGCAGGACCATGACCCAGGACAGAGAAGCGTTT
TRSTLPGRRQCLDFFRCSRH
1070 ACAAGAAGCAGCTGCTGGCCGAGGAGACAATGCCGTGATTCTTCAGATGCTCCCAT
QAAKTVDQLVDFDNDNCQDM
1130 CAAGCAGCAAGACTGTCCAGGATCTGTAGAGTTCCCGACGACAACGCCAGGACATG
DEL FVVRA
1190 GACGAGCTGTTTGTGGTCAGAGCTTAAGGAGGACACTGAGGTGAGTATGAGGTGAGTATG
1250 AGGTGAGTATAAAATGTAACACTGTGTTTAAACAGTATCTACATTGCATCGAGAGCTG
1310 TCTGATGGAAAATATCTAGA

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FIG. 3. DNA sequence of the *XbaI-PstI* fragment (sense strand) containing the gene encoding ICR489. Presumptive TATA, CAAT, and GC motifs are boxed. The major start site for transcription, as determined by mung bean nuclease mapping, is at +1. In-phase translational start and stop codons are underlined, and the deduced amino acid sequence for the coding region is shown above the DNA sequence. An arrowhead shows a major stop site for transcription. The numbers to the left of the figure indicate bases.

the basis of size and level of production. The function of ICP46 is unknown.

The start and endpoints of transcription. The absence of splicing in ICR489 allowed us to use mung bean nuclease mapping (15) to locate the termini of ICR489. Figure 5A and B show the 5' and 3' ends, respectively. At the 5' end, there was a major protected fragment starting 312 bases in from the *PstI* site (Fig. 3). A secondary site was found five bases downstream, and several minor bands were also observed. These bands may represent minor start sites or they may be artifacts caused by RNA degradation or nuclease nibbling (15). A presumptive TATA box was located at bases -41 to -35 from the major start site (Fig. 3). The sequence, AATAAAA, deviated from the consensus TATAAAA by one base. Whether this change is the cause of the minor start sites is not known, but lack of a classical TATA box has the potential to cause microheterogeneity at the 5' end of a message (27). At the 3' end, heterogeneity of the transcript was also detected. Major protected fragments were at 76, 78, and 80 bases downstream from the translation stop codon, and several minor bands were present (Fig. 5B). Interestingly, a classical Goldberg-Hogness box for promotion was found in this termination region (45 bases downstream from the stop codon), a situation similar to that seen in another FV3 immediate-early gene, that encoding ICR169 (see Fig.

FIG. 4. Hybrid-arrested translation of a 46-kDa protein by DNA complementary to ICR489. Reticulocyte lysates were programmed with RNA isolated from FHM cells infected with FV3 in the presence of cycloheximide (immediate-early RNA) or with this RNA hybridized to specific single-stranded DNA fragments. The [35 S]methionine-labeled proteins synthesized are shown separated on a 10% sodium dodecyl sulfate-polyacrylamide gel. Programmed in lane 1, immediate-early RNA (unhybridized); lane 2, RNA hybridized to the 1,640-base *XbaI-PstI* strand containing sequences complementary to ICR489; lane 3, RNA hybridized to DNA from a heterologous fragment (the internal 2-kb *PstI* fragment from *XbaI* F [Fig. 1B]). A 46-kDa protein (46 kd) is shown. Synthesis of this protein was inhibited when the RNA was hybridized to the *XbaI-PstI* fragment.

8). No AATAAA polyadenylation signal was found near the 3' terminus, as expected since FV3 mRNAs lack poly(A) (38).

Promoter function of the 5'-flanking sequences. To confirm that the 5'-flanking sequences of ICR489 could act as a promoter, a 486-bp *HpaII* fragment was inserted into a promoter assay vector 5' to the gene coding for the bacterial enzyme CAT. The inserted promoter fragment contained the 291 bases upstream from the major start point of transcription and extended 195 bases downstream from this site (Fig. 1C). The resulting plasmid construct, pBS489CAT (Fig. 6), was transfected into FHM cells, and the synthesis of CAT was measured as described in Materials and Methods. The results of the CAT assay are shown in Fig. 7. Cells transfected with the parental pBS-CAT alone or superinfected with FV3 did not synthesize significant amounts of CAT (lanes 5 and 6). Cells transfected with pBS489PCAT did not synthesize CAT (lane 4), but when superinfected with FV3 or UV-inactivated FV3 (lanes 1 and 2), pBS489PCAT-transfected cells did synthesize CAT. Cells treated with heat-inactivated FV3 (lane 3) did not synthesize CAT. These results suggest that the promoter for ICR489 was activated by a heat-labile, virion-associated protein. In addition, a second, negative effector was suggested by the amount of [14 C]chloramphenicol acetylated by extracts from cells superinfected with UV-inactivated FV3 (lane 2): 100%, compared with 30% for the active virus (lane 1). One possible explanation is that active virus produces a negative effector which controls expression of the ICR489 promoter, and this effector is not functional in cells treated with UV-inactivated virus. The UV-inactivation study of Martin et al. (21) indicated that, under conditions where delayed-early proteins could be specifically analyzed, the ICR489 gene product (their 42-kDa protein) had a relatively slow inactivation rate,

FIG. 5. The start and stop sites of transcription determined by mung bean nuclease mapping. (A) Start site. Lane 1, Nuclease-protected fragments of a 360-base *AccI-Sau3A* fragment (Fig. 1C), 5' labeled at the *Sau3A* end and hybridized to RNA from FV3-infected cells; lane GA, corresponding G+A sequencing (Maxam and Gilbert) ladder used to determine the size of the protected fragments. To the right of this ladder is the sequence of the anti-sense strand in the region of the start site. Large arrowheads and small arrows designate major and minor start sites, respectively. (B) Stop site. Lane descriptions are similar to those above, except the protected fragments were from a 174-base *XhoII-XbaI* fragment (Fig. 1C) 3' labeled at the *XhoII* end.

FIG. 6. pBS489PCAT. Construction was as described in Materials and Methods. Insertion of the *cat* gene inactivated *lacZ*. The presumptive promoter region (486-bp *HpaII* fragment [Fig. 1C]) for the gene encoding ICR489 (489P) is inserted in the proper orientation to drive CAT synthesis. The plasmid does not contain recognition sequences for poly(A) addition, but this has been found to be nonessential for FV3-induced CAT synthesis (34). Kb, Kilobase.

FIG. 7. Promotion of CAT synthesis from pBS489PCAT requires infection with FV3. FHM cells were transfected with pBS489PCAT or the parent plasmid pBS-CAT, which lacks an FV3 promoter region. After 24 h, some samples were infected with FV3 or inactivated FV3. Cells were harvested for the CAT assay 4 h after infection. Lane 1, pBS489PCAT (infected with FV3); lane 2, pBS489PCAT (treated with UV-inactivated FV3); lane 3, pBS489PCAT (treated with heat-inactivated FV3); lane 4, pBS489PCAT (uninfected); lane 5, pBS-CAT (uninfected); lane 6, pBS-CAT (infected with FV3). Quantitation of CAT synthesis was done by cutting out the spots corresponding to [14 C]chloramphenicol (CM) or its acetylated derivatives (AC) and counting in a liquid scintillation system.

and the researchers suggested that overexpression of a gene normally controlled by at least one of the early products could account for the results. In addition, the explanation is consistent with ICR489 overproduction in cells infected in the presence of cycloheximide (39), suggesting negative regulation at the level of transcription.

Comparison of the promoters from genes encoding ICR489 and ICR169. Two immediate-early FV3 genes that are activated by a virion-associated protein(s) have been sequenced to date: the present one encoding ICR489 and another encoding ICR169 (33). The similar mode of activation suggests that the two promoters might have similar sequences that respond to the same virion *trans*-acting protein. A 21-base sequence 5' to the coding region for ICR169 (Fig. 8) has been shown, by mutational analysis, to be important for promoter activity; a deletion of the region caused a 98.5% reduction in CAT activity (34). This 21-bp *cis*-responsive region had no significant homology (less than 50%) to the promoter region of the gene encoding ICR489 (Fig. 8). Therefore, the concomitant temporal regulation of these two promoters was not reflected in the sequences immediately upstream from the start point of transcription.

DISCUSSION

A common theme present in the lytic infection of eucaryotic cells by large DNA viruses is the tightly regulated expression of viral genes. Infection of susceptible cells with the nuclear DNA herpes simplex virus 1 induces the synthesis of three general classes of mRNA, α , β , and γ (30). The division of these three classes is based on differences in their order of expression and in the viral gene products necessary for their synthesis. The cytoplasmic DNA vaccinia virus also has three major classes of transcripts but carries within its

virion all the enzymes necessary to transcribe early viral mRNA in the cytoplasm (25).

Transcription of FV3 in infected cells also proceeds in a highly controlled fashion, with three classes of transcripts—immediate-early, delayed-early, and late transcripts—whose synthesis is dependent on the cascade production of specific viral proteins (11, 39). Although FV3 virions are assembled in the cytoplasm, like vaccinia virus (for a review, see reference 35), early viral mRNA is synthesized in the nucleus by the cooperative interaction of host-cell RNA polymerase II (9) and a virion-associated protein (41). In this respect, FV3 more closely resembles herpes simplex virus 1, in which a virion protein has been shown to greatly increase transcription of the α genes (24). However, in contrast to herpes simplex virus 1, and similar to vaccinia virus, purified FV3 DNA is not infectious (40). Therefore, the requirement for the FV3 virion protein(s) to initiate immediate-early transcription is absolute.

Although several viral and cellular positive regulatory sequences from many systems have been identified, relatively few well-defined negative regulatory functions have been characterized. Because the FV3 gene ICR489 had been previously demonstrated to have a negative regulatory aspect (39), we undertook to clone and sequence this gene and to compare the sequence of its promoter with that of another immediate early FV3 gene (ICR169), which was only regulated in a positive manner (37).

The sequence of the gene for ICR489 revealed several interesting features. Heterogeneity was noted at both the 5' and 3' ends of ICR489, as analyzed by mung bean nuclease analysis (15). At the 5' end, this heterogeneity could be explained by the lack of a classical TATA box, a situation that has been shown to cause aberrations at the 5' ends of transcripts from simian virus 40 (22). At the 3' end, the lack of the poly(A) addition signal site, AATAAAA, may have contributed to the fact that the signal here was different from that for the cellular poly(A) polymerase, since vaccinia virus uses a virus-specified enzyme. Another noteworthy feature of the ICR489 sequence was the occurrence of a perfect TATA box 31 bases upstream from the transcriptional ter-

FIG. 8. Sequences flanking the coding regions for ICR489 and ICR169. (A) In the 5' region, presumptive TATA, CAAT, and GC motifs are boxed, and the major start site for transcription is designated as 1. In the 3' region, an arrow shows a major transcription stop site, and a broken-line box shows a TATA motif located just upstream from the stop site. In-phase translational start and stop codons are underlined. (B) Similar to above, except the broken-line box shows the 21-base region essential for ICR169 promoter activity (34).

mination site. Whether this sequence has any bearing on termination is not known, but it is interesting that a similar sequence was found at the termination site of ICR169 (Fig. 8) (33).

The genes encoding ICR489 and ICR169 are both immediate early, and both appear to be activated by a virion-associated protein(s) (41) (Fig. 7), but their promoters showed no significant homology in DNA sequence. It is possible that the gene encoding ICR489 is an exception among the immediate-early genes. In the laboratory of one of us (A.M.A.), another immediate-early gene has been examined; a sequence that does have significant homology to the promoter from the gene encoding ICR169 is present. However, it is positioned at a greater distance from the 5' end of the message, as determined by S1 nuclease mapping. In addition, ICR489 is overproduced in extracts from cells grown in the presence of cycloheximide (39). The most likely explanation is that a protein having negative control over ICR489 production is not made in the presence of cycloheximide. Consistent with this are the CAT assay results showing overexpression from the ICR489 promoter in cells infected with UV-inactivated virus. This finding would be expected if a negative effector were not functional under these conditions. Overproduction has not been reported for other immediate-early FV3 transcripts, which supports the possibility that the regulation of ICR489 is exceptional among immediate-early transcripts. The question remains of how the temporal expression of two genes with diverse promoters is concomitantly regulated. One possibility consistent with the difference in promoter sequences is that the virion proteins needed to activate each of these genes are not identical and therefore have different recognition sites. This hypothesis may be testable by separating and purifying virion proteins and introducing them into transfected cells by microinjection (17). A mutational analysis of the specific sequences required for ICR489 promoter function is currently under way in our laboratory (D.W.).

As more FV3 genes are sequenced, the significance of promoter sequence and structure on temporal expression should become clearer. In particular, we will know whether the promoter for ICR489 is an exceptional case among the immediate-early genes and whether the delayed-early and late gene promoters have marked sequence distinctions from the immediate-early promoter sequences. Finally, the finding that the genes for both ICR489 and ICR169 required activation by an FV3 protein suggests that *trans*-acting proteins play an important role in FV3 regulation.

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