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Fine Mapping of the Immediate-Early Gene of the Indiana-Funkhauser Strain of Pseudorabies Virus

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The immediate-early gene transcript of pseudorabies virus was found to be 5.6 kilobases and colinear with the DNA genome. Transcription initiation started in the *Bam*HI E fragment, 45 nucleotides from the *Bam*HI-E/*Bam*HI-I junction. The first AUG codon was located in *Bam*HI-I, 260 nucleotides from the mRNA initiation site, and a potential TGA termination codon was located in *Bam*HI-J, 161 nucleotides from the *Bam*HI-I/*Bam*HI-J junction. The AATAAA polyadenylation signal was 30 bases from the stop codon, while the actual polyadenylation site was 18 bases further downstream.

The expression of viral genes in pseudorabies virus (PRV) is coordinately regulated, and the genes are sequentially transcribed (6, 8, 12, 18). Three classes of mRNA are synthesized during a productive infection: (i) immediate-early (IE) mRNA is synthesized before protein synthesis; (ii) early mRNA is synthesized before viral DNA replication; and (iii) late mRNA is synthesized after viral protein translation and DNA replication. In the presence of cycloheximide (an inhibitor of protein synthesis), one IE gene is predominantly expressed, and the general location of this gene has been described (13, 18). The nucleotide sequences of the 5′ end of the IE gene and its immediate upstream region, which showed similarities to the enhancers of human and murine cytomegaloviruses, were also reported (3). Synthesis of early and late PRV mRNAs is dependent upon the function(s) of this IE gene product. In heterologous systems, the PRV IE protein can activate the transcription of adenovirus and cellular genes (7, 10).

Presumably, gene activation in both the homologous and the heterologous systems is due to specific protein-nucleic acid interactions between the PRV IE protein and the respective promoters. Recently the IE protein was shown to bind to single-stranded DNA (5); however, its single-stranded-DNA-binding property in vitro has not been correlated with the observed *trans* activation activities.

As part of a study to examine the structure and function(s) of the PRV IE gene in detail, the exact location of the gene has been determined. The experiments described in this report were performed to determine the direction of transcription and RNA processing and the 5′ initiation and 3′ termination sites of the IE transcript in the Indiana-Funkhauser (In-Fh) strain of PRV.

The IE gene was localized to the repeated sequences of the PRV genome by hybridization studies. A [^{32}P]cDNA probe was made by reverse transcribing selected poly(A)$^+$ RNAs (1, 4, 9) isolated from PRV In-Fh (16)-infected, cycloheximide-treated MDBK cells (17). The probe was incubated with Southern blots (21) of PRV In-Fh DNA restriction enzyme digests (0.5 μg per lane). Hybridization to *Bam*HI I and J, *Sal*I A and B, and *Kpn*I E and H fragments (Fig. 1b) was observed, indicating that a copy of the IE gene is present in each of the repeated sequences of the viral genome at 0.74 and 0.95 map units (Fig. 1a).

Current estimates of the IE mRNA molecular size range from 5.1 to 6.0 kilobases. Northern (RNA) blot analysis (22) with nick-translated probes (19) showed that the transcript

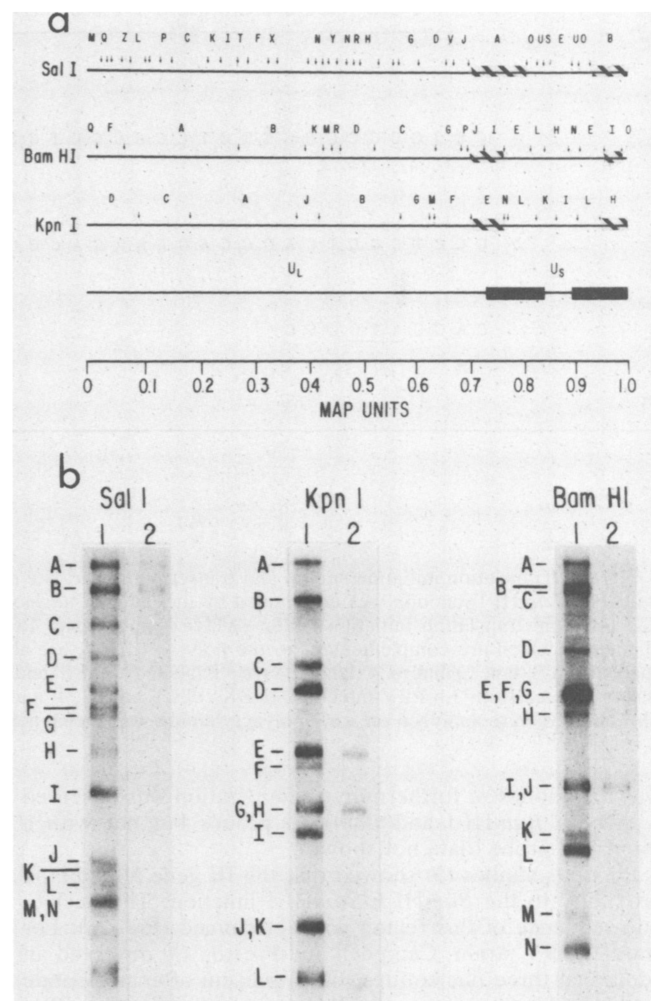

FIG. 1. Southern blot analysis. Uppercase letters indicate restriction enzyme fragments. (a) Schematic representation of the PRV genome and restriction enzyme patterns of *Sal*I, *Kpn*I, and *Bam*HI as described by Wathen and Wathen (23). Regions of hybridization are indicated (▲▲). (b) *Sal*I, *Kpn*I, and *Bam*HI Southern blots. The blots were hybridized with nick-translated PRV genomic DNA (lane 1) or with [^{32}P]cDNA prepared by reverse transcription of poly(A)$^+$ RNA isolated from cycloheximide-treated MDBK cells 3 h postinfection (lane 2).

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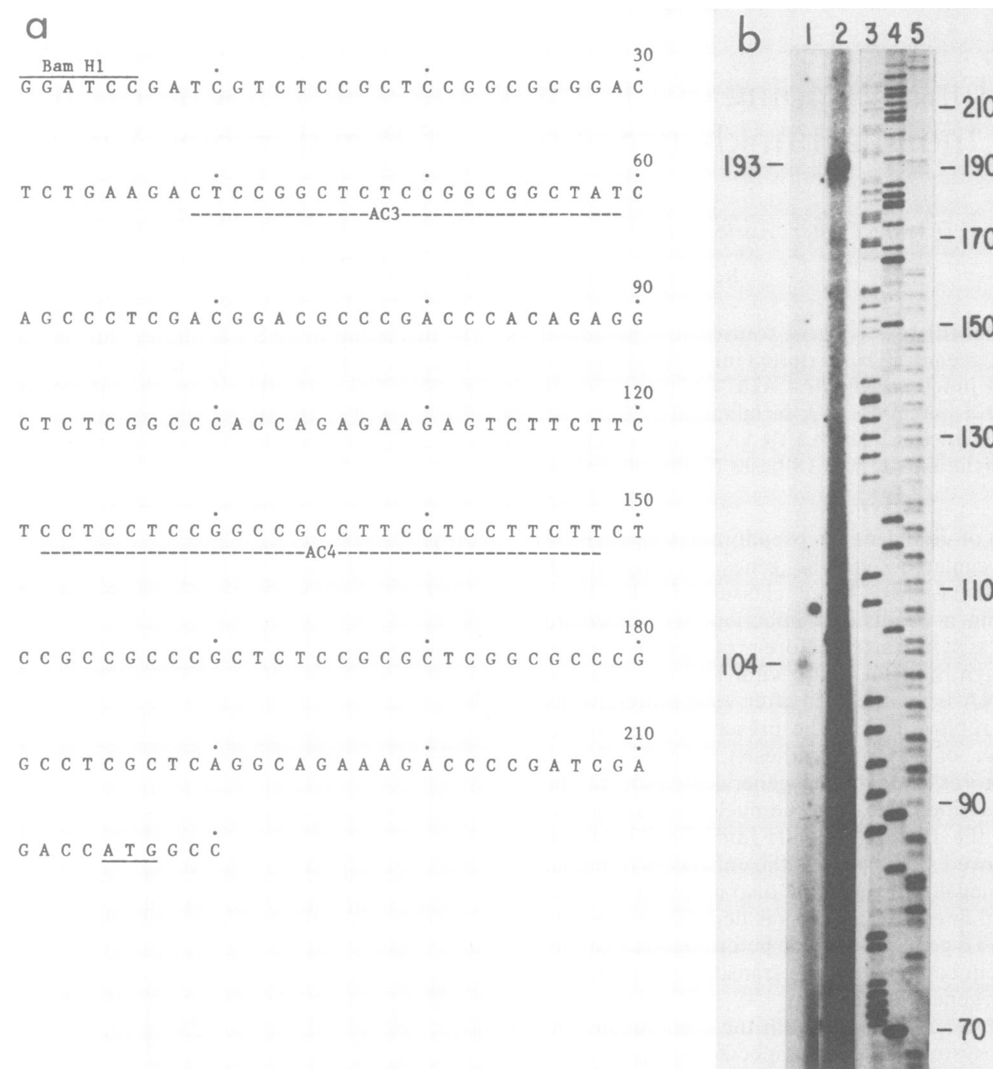

FIG. 2. Translation initiation site (a) and transcription start site mapping (b). (a) The nucleotide sequence of the *Bam*HI-I portion of the *Bam*HI-E/*Bam*HI-I junction was determined by the dideoxy-nucleotide chain termination method. Numbers indicate nucleotide positions. The potential translation initiation codon (ATG) is underlined. Positions of the 21 nucleotide (AC3) and 27-nucleotide (AC4) synthetic oligomers, which are complementary to the poly(A)$^+$ RNA, are also indicated. (b) Transcription start site mapping by primer extension analysis. AC3 (lane 1) and AC4 (lane 2) were ^{32}P labeled at the 5′ end (14) and used as primers. The experiment was carried out with poly(A)$^+$ RNAs isolated from 3-h PRV-infected MDBK cells. Lanes 3, 4, and 5 contain molecular weight markers from a known sequencing ladder. The samples were analyzed on a 6% polyacrylamide sequencing gel. Numbers indicate molecular sizes in nucleotides.

was 5.6 kilobases; furthermore, hybridization was observed with both *Bam*HI-I and *Bam*HI-J probes but not with a *Bam*HI-E probe (data not shown).

Previous studies (3) showed that the IE gene 5′ end is in proximity to the *Bam*HI-E/*Bam*HI-I junction. The nucleotide sequence of this region was determined (Fig. 2a). For another PRV strain, Campbell and Preston (3) observed an additional three nucleotides (CTT) present after nucleotide position 113. Therefore, the 5′ untranslated region in PRV In-Fh is 214 bases, whereas that of the other isolate is 217 bases. Three other nucleotide differences were also noted, at positions 9 (C instead of T), 86 (C instead of A), and 101 (G instead of A).

Two chemically synthesized oligomers, AC3 and AC4, were used in primer extension experiments (2) to determine the mRNA initiation site. Both primers terminated at exactly the same site, since AC3 (89 bases 5′ to AC4) and AC4 were extended to 104 and 193 nucleotides, respectively (Fig. 2b). Thus, the IE mRNA initiation site is in *Bam*HI-E, 45 bases 5′ of the *Bam*HI-I junction in both PRV isolates.

The 3′ end of the IE gene was established by cDNA cloning into the λgt10 (11) vector system and sequenced by the dideoxy method (20) (Fig. 3). In addition, the 26-nucleotide synthetic oligomer AC2 hybridized to the *Bam*HI J fragment and not to the *Bam*HI I fragment in Southern blot analysis (data not shown); thus, the 3′ end is in *Bam*HI-J. Further sequencing experiments on this portion of the IE gene confirmed that the genomic and cDNA sequences are identical and colinear. A potential stop codon (TGA) was located at 161 bases from the *Bam*HI-I/*Bam*HI-J juncture. The consensus polyadenylation signal AATAAA was located at 191 bases from this junction, while the poly(A) addition site is another 18 bases further downstream.

The IE mRNA has been assumed to be a nonspliced

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Bam H1                                                   30
G G A T C C C C C G G C C G G A G G T G G C T G C G G C G G

                                                         60
C G G C G G A G G T G G C G G C G G T G G T G G A A G C G G

                                                         90
C G G C G G C G G C C G C G G C G G A G G G C T C G G C G G

                                                        120
C C G A G G A T C G T C C C G G T C C C C T T C T C C T C C
                                    ---------------AC2------

                                                        150
C G C C G G T C C C C C G G T C C C C T T C T C C T C C T T C
--------------------

                                                        180
T C C C A T C G G G T G A G A A A A G A G T T T A T T T T C

                                                        210
A G A G T G A G A A A A T A A A G T T T G T G C T G T A T T

T T C T -- A A A . . .
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FIG. 3. 3′ end of the IE gene. The nucleotide sequence of the *Bam*HI-J portion of the *Bam*HI-I/*Bam*HI-J junction representing the 3′ end of the IE gene was determined by the dideoxy-nucleotide chain termination method. Numbers indicate nucleotide positions. The potential translation termination codon (TGA) and poly(A) addition signal (AATAAA) are underlined. A 26-nucleotide synthetic oligomer (AC2), the restriction enzyme recognition site for *Bam*HI, and the poly(A) tail (--AAA . . .) are also indicated.

message; however, no direct evidence of this is available. S1 nuclease protection experiments were performed to determine whether RNA splicing occurs. The entire *Bam*HI I genomic DNA fragment (4.8 kilobases) was inserted at the *Bam*HI site of the Bluescript plasmid (Stratagene, San Diego, Calif.), and a [^{32}P]UTP-labeled RNA probe was generated from the T7 promoter. This probe has polarity opposite to that of the IE gene and is flanked by nucleotide sequences of bacterial origin (approximately 50 nucleotides on the 5′ end and 30 nucleotides on the 3′ end). Hybridization was carried out according to the method of Zinn et al. (24). The RNA probe was protected from S1 nuclease digestion in the presence of the IE gene mRNA (Fig. 4). The samples shown in Fig. 4a were treated with glyoxal at 50°C for 1 h and then analyzed on a 1% agarose gel (15). Under these conditions, the RNA · RNA duplexes were not denatured and appeared as a higher-molecular-weight species (lane 3) than the input RNA probe (lane 1). The samples shown in Fig. 4b were treated for an additional 5 min at 100°C before electrophoresis. Upon heat treatment, the RNA · RNA duplex denatured and the protected probe migrated similarly to the input probe. These data demonstrated that the PRV IE mRNA is colinear with the genomic DNA sequences.

In summary, the 5′ untranslated sequences of the two isolates compared were very well conserved but contained differences such as base changes, deletions, or additions. The 3′ untranslated region was at least 30 bases and was followed by the consensus polyadenylation signal (AATAAA). The PRV In-Fh IE mRNA was 5.6 kilobases and colinear with the viral genome.

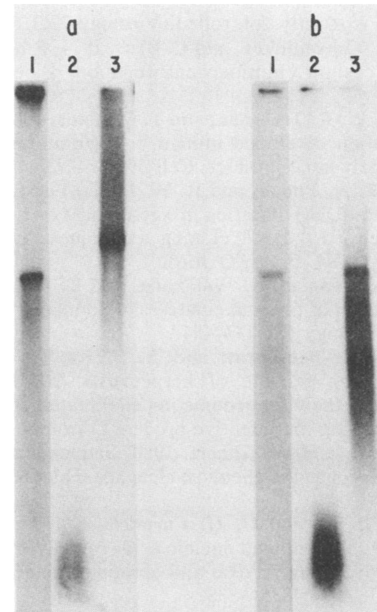

FIG. 4. Colinearity of IE mRNA and viral DNA. An S1 nuclease mapping experiment was carried out as described in the text (a). Samples in panel b were subjected to an additional denaturation step, 5 min at 100°C, before electrophoresis. Lanes: 1, input RNA probe; 2 and 3, poly(A)$^+$ RNAs isolated from mock-infected and 3-h PRV (In-Fh)-infected cycloheximide-treated MDBK cells, respectively.

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