Mapping of the Transcriptional Initiation Site of the Herpes Simplex Virus Type 1 ICP8 Gene in Infected and Transfected Cells

LISHAN SU AND DAVID M. KNIPE*

Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

Received 18 June 1986/Accepted 8 October 1986

The initiation site for transcription of the herpes simplex virus type 1 (HSV-1) gene encoding the major DNA-binding protein, ICP8, was mapped by nuclease S1 analysis of RNA-DNA hybrids. When RNA isolated from cells infected with HSV-1 was used, one major start site of ICP8 gene transcription was mapped at 89 base pairs to the right of the *Bst*EII site at 0.409 map units. In cells transfected with a cloned ICP8 gene, the same major start site was detected either in the presence or absence of the immediate-early (alpha) genes encoding ICP4 or ICP0, which have been shown to stimulate ICP8 gene expression in transfected cells. Both ICP4 and ICP0 stimulated the accumulation of the ICP8 gene transcripts in the transient expression system, and their effects were synergistic. By comparison of the sequence of the putative promoter region of the ICP8 gene with the promoter of the HSV-1 TK gene, a significant similarity was detected between the three transcriptional regulatory signals of the TK gene and the upstream sequences of the ICP8 gene. Analysis of promoters of other delayed-early (beta) genes showed that they all contained regions of significant homology with the distal signals of the upstream sequences of the TK or ICP8 gene.

The major DNA-binding protein of herpes simplex virus (HSV), ICP8, is essential for viral DNA replication (3, 36, 45). In infected cells before viral DNA replication, ICP8 is localized to specific sites in the nuclei named prereplicative sites. The protein migrates to large globular structures (replication compartments) as viral DNA replication occurs (39, 41), where it is bound to progeny or replicating viral DNA (22, 24). In vitro, ICP8 binds to DNA nonspecifically, with some preference for single-stranded DNA (1, 23, 38). It lowers the melting temperature of poly(dA-dT) helices (36). It has also been shown that ICP8 can regulate the level of viral RNA accumulation in infected cells by controlling the transcription rate from parental viral genomes (11-13).

As a delayed-early (or beta) gene of HSV, the ICP8 gene requires the products of HSV immediate-early (or alpha) genes for expression; of these, ICP4 has been demonstrated to activate ICP8 gene expression (17, 20, 37). Either ICP4 or ICP0 can stimulate ICP8 gene expression in a transient assay system, and their effects seem to be cooperative (40), as determined by measuring the levels of ICP8 protein in the transfected cells.

Transcripts from the ICP8 gene in cells infected with HSV-1. ICP8 is encoded by a gene which maps between coordinates 0.397 and 0.411 (3, 16, 25, 26, 43, 45; Fig. 1). Two major RNA species, of 4.2 to 4.6 and 10.0 kilobases, have been identified from this region (16, 43). Using strand-specific probes, Holland et al. (16) demonstrated that both of the RNA species from this region are transcribed leftward in the prototypic arrangement of the HSV type 1 (HSV-1) genome. The initiation sites of the transcripts were determined to be within the *Kpn*I-*Bam*HI fragment from 0.407 to 0.411 map units.

To map the initiation site for the transcription of the ICP8 gene, we performed S1 nuclease analysis of RNA-DNA hybrids by using RNA isolated from monkey cells infected with HSV-1. The *Kpn*I-*Bam*HI DNA fragment (0.407 to 0.411 map units), labeled at the 5' end of the *Kpn*I site (Fig. 1C), was hybridized with the RNA, and the hybrids were

digested with S1 nuclease. A major protected band was observed, corresponding to protection of about 477 bases of DNA to the right of the *Kpn*I site at map unit 0.407 (Fig. 2A, lane 4). Digestion of the RNA-DNA hybrids with exonuclease VII gave a major protected band of approximately the

FIG. 1. HSV DNA probes used for mapping the ICP8 gene transcripts. (A) Arrangement of HSV genomic sequences and physical map coordinates of HSV-1 DNA sequences used in this study. (B) *Eco*RI-F clone pSG18 (14), containing the ICP8 (DBP) gene and part of the DNA polymerase (POL) gene (10, 46). (C) *Bam*HI-V clone pSG18-B51 (16), containing the 5' ends of the ICP8 and polymerase genes. The probes were prepared as follows. Plasmid pSG18-B51 was labeled at the *Kpn*I or *Bst*EII restriction sites with polynucleotide kinase and [γ - 32 P]ATP (ICN Chemicals, Inc.; 27). The desired fragments were resolved in and isolated from an agarose gel. The arrowheads indicate the deletion in the clones (14, 16). B, *Bam*HI; Bt, *Bst*EII; E, *Eco*RI; K, *Kpn*I; *, 32 P-labeled 5' end.

* Corresponding author.

FIG. 2. 5' ends of ICP8 gene transcripts from infected cells. Total cellular RNA was isolated as described previously (12). The RNA sample (40 μ g) was dissolved in 20 μ l of hybridization buffer [80% formamide, 400 mM NaCl, 40 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid)] (PIPES), 150 ng of the labeled DNA probe. After being heated at 78°C for 15 min, the hybridization mixture was incubated at 62°C for 6 h. It was then diluted into 200 μ l of ice-cold S1 nuclease buffer (280 mM NaCl, 50 mM sodium acetate, 4.5 mM zinc acetate, 25 μ g of salmon sperm DNA per ml, 5% glycerol, 100 U of S1 nuclease [Boehringer Mannheim Biochemicals]). The reaction was performed at 37°C for 30 min and stopped by putting the mixture on ice and adding 33 μ l of stop buffer (4 M ammonium acetate, 0.1 M EDTA) and 10 μ g of tRNA. The mixture was then extracted with 0.2 ml of phenol-chloroform-isoamyl alcohol (25:24:1). The products of the reaction were resolved by electrophoresis in an 8% polyacrylamide gel containing 7 M urea (2, 27). (A) S1 nuclease mapping of the ICP8 gene transcripts with the *KpnI-BamHI* probe. Lanes: 1, intact probe; 2 and 6, DNA size markers; 4, hybrids formed with RNA from cells infected with HSV-1 KOS1.1 (wild type) virus (11); 3 and 5, hybrids formed with RNA from mock-infected cells. (B) S1 nuclease mapping of the ICP8 gene transcripts with the *BstEII-BamHI* probe. Lanes: 1 and 2, hybrids formed with RNA from HSV-1 KOS1.1-infected cells (20-fold less sample was loaded on lane 2); 3 through 6, the sequencing reactions of A, G, C, and T, respectively (27); 7, DNA size markers. (C) Mapping of the left end of the deletion in the probe. The *BstEII-BamHI* probe (approximately 150 ng) was hybridized with 120 μ g of HSV-1 KOS genomic DNA (purified from infected cell lysates by NaI density gradient centrifugation) in 20 μ l of buffer containing 10 mM EDTA, 5 $\times$ Denhardt solution, 0.5% sodium dodecyl sulfate, 6 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and 0.1 mg of salmon sperm DNA per ml. The S1 nuclease treatments were performed as described above. Lanes: 1, hybrids formed with total RNA from cells infected with HSV-1 KOS1.1 virus; 2, hybrids formed with RNA from mock-infected cells; 3, hybrids formed with HSV-1 KOS DNA; 4, DNA size markers; 5, intact probe.

same size as the major band from S1 nuclease treatment (data not shown), indicating that no RNA splicing was detected within the first 477 nucleotides of the transcript.

To map the major start site for ICP8 gene transcription more precisely, a smaller probe labeled at the 5' end of the *Bst*EII site, *Bst*EII-*Bam*HI (0.409 to 0.411 map units, Fig. 1C), was used to perform similar nuclease analysis experiments. The RNA-DNA hybrids digested with S1 nuclease were run on a DNA sequencing gel along with a sequence ladder generated from the probe. The major transcription start site was determined to be 89 base pairs (bp) to the right of the *Bst*EII site, although protected fragments of 88 and 90 bp were also observed (Fig. 2B, lane 1). This heterogeneity was detected by using various conditions of S1 nuclease reaction, suggesting that the transcripts may vary slightly in their 5'-end nucleotide. A similar pattern of protected bands was observed with RNAs isolated at either early or late

TABLE 1. Accumulation of ICP8 gene transcripts in transfected cells^a

Plasmids transfected (μg)	Relative stimulation
pSG18-Sac1 (5), pBR325 (5).....	1
pSG18-Sac1 (5), pSG1-ES1 (2.5), pBR325 (2.5).....	25
pSG18-Sac1 (5), pSG1-EK1 (2.5), pBR325 (2.5).....	89
pSG18-Sac1 (5), pSG1-ES1 (2.5), pSG1-EK1 (2.5).....	205

^a An autoradiogram (Fig. 3) was scanned, and the amounts of ICP8 gene transcripts initiated from the major start site were determined by planimetry (40).

FIG. 3. ICP8 gene transcripts from transfected cells. The cloned ICP8 gene (pSG18-Sac1) was transfected with or without the stimulating genes into Vero cells (39). Total cell RNA was isolated at 48 h posttransfection (Fig. 2). An RNA sample (80 μg) from each transfection was annealed to 150 ng of the *Bst*EII-*Bam*HI probe. The S1 nuclease reaction was performed as described in the legend to Fig. 2. (a) Lanes: 1, hybrids formed with RNA from HSV-1 KOS1.1-infected cells; 2 through 5, the sequencing reactions of A, G, C, and T, respectively; 6, hybrids formed with RNA from cells transfected with pSG18-Sac1 (ICP8); 7, hybrids formed with RNA from cells transfected with pSG18-Sac1 (ICP8) and pSG1-ES1 (ICP0); 8, hybrids formed with RNA from cells transfected with pSG18-Sac1 and pSG1-EK1 (ICP8 and ICP4); 9, hybrids formed with RNA from cells transfected with pSG18-Sac1, pSG1-ES1, and pSG1-EK1 (ICP8, ICP0, and ICP4); 10, hybrids formed with RNA from mock-infected cells. (b) A shorter exposure of panel a. (c) Plasmids containing the ICP8 gene. (d) Plasmids containing the ICP4 or ICP0 genes or both (40). E, *Eco*RI; K, *Kpn*I; S, *Sac*I.

times after HSV infection or with RNAs from cells infected in the presence or absence of phosphonoacetic acid, an HSV DNA replication inhibitor, at late times of infection (results not shown). This indicated that the major start site was used for initiating transcription in all of these situations.

All DNA clones of this region contain deletions (45, 46), which could interfere with the nuclease analysis results if the deletions were within or partly included in the ICP8 coding sequences. Therefore, we mapped the deletion by S1 nuclease analysis of the *Bst*EII-*Bam*HI DNA probe hybridized to HSV-1 genomic DNA. The left end of the deletion in pSG18-B51 was shown to be approximately 285 bp to the right of the *Bst*EII site (Fig. 2C, lane 4), a location similar to that of one of the deletions previously reported (46). Therefore, the deletion in this probe is well upstream from the 5'-end start site of the ICP8 transcripts and did not affect the results described above.

ICP8 transcripts in cells transfected with a cloned ICP8 gene. The cloned ICP8 gene can express functional ICP8 protein when introduced into monkey cells by transfection (41). It is also able to respond to positive regulation mediated by the immediate-early gene product ICP4 or ICP0 or both in transfected cells, as determined by immunoprecipitation of ICP8 protein from the transfected cells (40).

To characterize the transcripts expressed from the cloned ICP8 gene, RNA isolated from cells transfected with the ICP8 gene was hybridized with the *Bst*EII-*Bam*HI probe

FIG. 4. Comparison of the sequences of the promoter regions of the TK and ICP8 genes. A 120-bp segment upstream from the cap site of the TK gene was compared with the corresponding region upstream from the ICP8 gene (with the major initiation site as the mRNA cap site). The sequences shown indicate the positions of the ATA box (the proximal signal) and the two distal signals of the TK gene promoter along with the corresponding homologous regions of the ICP8 gene promoter. The proximal signal (ATA box) was located by visual inspection. The sequences underlined in the two distal signals are the DNA sequences which share significant homologies. The comparison was performed with the DECsystem 2060-IntelliGenetics program (Dana-Farber Cancer Institute) with the following parameters: (i) expect = 6 (the maximal number of random matches allowed in a random sequence of nucleotides of the same base composition and length as the sequences being examined); (ii) minimum match = 8 (the minimum number of exact matches in a homology required to report the match); (iii) percent match = 80 (the minimum ratio of matched nucleotides to total nucleotides in a homology match); (iv) after mismatch = 2 (the minimum number of the next three nucleotides that must be matched to continue a homology past a mismatch or loopout); (v) loopout = 3 (the length of insertion or deletion loops allowed in comparing two homologous sequences).

described above (Fig. 1C), and S1 nuclease analysis was performed. The same major transcription start site was detected, although at a very low level (Fig. 3a, lane 6). When a plasmid containing the HSV-1 immediate-early gene for either ICP4 or ICP0 was cotransfected with the ICP8 gene, the ICP8 gene transcripts initiated from this start site were increased (Fig. 3a and b, lanes 7 and 8). When both of the plasmids encoding ICP4 or ICP0 were cotransfected with the ICP8 gene, ICP4 and ICP0 appeared to act cooperatively to stimulate the accumulation of the ICP8 gene transcripts (Fig. 3b, lane 9, and Table 1). The increase in the amount of RNA with the authentic 5' end was consistent with the increase in ICP8 protein previously determined by immunoprecipitation with a monoclonal antibody against ICP8 (40). The synergistic effect of ICP4 and ICP0 has been reported for other systems (7, 9). It may be due to mutual regulation of their own expression (35) or to the possible interaction of the two proteins, because a mutant ICP4 protein has been reported to block the nuclear localization of the wild-type ICP0 (21). In studies of HSV TK gene expression (9), Gelman and Silverstein observed that either ICP4 or ICP0 could activate *in trans* the expression of the TK gene. They also detected TK gene transcripts that initiated at upstream sites when the cells were cotransfected with the plasmids encoding the ICP0 gene and the TK gene. We detected no ICP8 transcripts initiated at upstream sites when the ICP8 gene was cotransfected with the ICP0 gene. Thus, the upstream start sites observed with the TK gene plasmid may be unique to that region of the viral genome. Although we observed relative efficiencies of ICP4 and ICP0 stimulation of ICP8 expression different from those observed by others for other beta genes (7, 9, 34), the alpha-gene plasmids that were used contained different genomic inserts. It is difficult to determine at this point where the difference might be in the stimulatory or target genes.

Comparison of promoter regions of ICP8 and other beta genes. ElKareh et al. (6) compared the nucleotide sequences of a number of beta-gene promoters. A hyphenated palindrome with dyad symmetry was found to be a common feature of these promoters. Extensive studies have elucidated the structure-function relationship of the HSV-1 TK gene promoter (28–32). A 109-bp DNA segment upstream from the 5' end of the TK gene is sufficient and necessary for the regulated expression of this gene (30). Studies of a series of deletion and linker-scanning mutations in this region have

defined three essential elements within the 109-bp region, which have been named the proximal signal (ATA box), first distal signal, and second distal signal (29, 31, 32; Fig. 4A).

Using the major 5' end of the ICP8 gene transcript as the mRNA cap site, we compared the 120-bp nucleotide sequence upstream from the ICP8 gene (46) with that of the TK gene (Fig. 4). By visual inspection, a proximal signal (ATA box) was seen to be present at a similar position relative to the cap site (–20 to –25 from the putative cap site). Significant homology (greater than 80%) was detected in the other two distal signals by computer-aided search (Fig. 4). The core sequence (GGGCGG) for the binding of the SP1 cellular transcription factor, present in the distal signals of the TK promoter (19, 32), was also detected in the putative first distal signal of the ICP8 promoter (Fig. 4, –54 to –59). This suggested that these regulatory signals may also be essential for expression of the ICP8 gene during HSV infection. However, the CAT box (CCAAT), which is the binding site for another cellular transcription factor in the TK gene promoter and many other eucaryotic genes, is not present within this regulatory region of the ICP8 gene.

Similar analyses of promoters or upstream sequences of other beta genes were performed using the promoters of the TK and ICP8 genes as the standards. The promoters for the beta genes encoding the alkaline nuclease (4), DNA polymerase (10, 46), ribonucleotide reductase large and small subunits (8, 33), dUTPase (15), and glycoprotein D (18, 44) were included in the comparison. Only the two distal signals containing the SP1 transcription factor binding sequences were shared by all of these promoters. More than one copy of the distal signals were present in these promoter sequences, as copies of the first or second distal signal or both, as with TK and ICP8. Therefore, as McKnight et al. (32) showed for the TK gene promoter, the two distal signals may be at least partially equivalent, and two or more copies of the signals may be necessary for the regulated expression of HSV beta genes. Further work should define the function of these sequences in the beta-gene promoters.

While this manuscript was in preparation, Quinn and McGeoch (42) reported the DNA sequence of the ICP8 gene of HSV-1 strain 17. A single transcriptional start site was reported, but the data used for determining this site were not shown. This cap site is very close, but not identical, to the major initiation site reported here, being 3 bp further upstream from the ICP8 coding sequences.

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