In Vitro Transcription of Herpes Simplex Virus Genes: Identification of a New Initiation Site and Second Intervening Sequence in the Immediate-Early RNA-5 Gene

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Received 18 April 1984/Accepted 9 October 1984

We have used partially purified preparations of RNA polymerase II from mock-infected and herpes simplex virus type 1-infected HEp-2 cells to transcribe the herpes simplex virus type 1 strain F *Bam*HI Z DNA fragment containing the promoter for immediate-early RNA-5 (α 47), a 1.8-kilobase, spliced RNA. In agreement with the *in vivo* transcriptional regulation of herpes simplex virus type 1 immediate-early genes, electrophoretic analyses of runoff and truncated transcripts from this template showed that RNA polymerase II from mock-infected cells initiates transcription more selectively than does that from the herpes virus-infected cells at the immediate-early RNA-5 promoter. S1 nuclease mapping of the 5' ends of *in vivo*- and *in vitro*-synthesized mRNA placed the initiation site ca. 110 base pairs upstream from the previously published site and also demonstrated the presence of a second, smaller intervening sequence between this new cap site and the previously characterized intervening sequence. S1 analyses also suggested that some splicing of the larger but not the smaller intervening sequence occurred *in vitro*.

It is well established that gene expression of herpes simplex virus type 1 (HSV-1) is transcriptionally regulated, with three major gene classes being sequentially expressed during the infectious cycle—immediate-early (IE), early (E), and late (L), or α , β , and γ (6, 16, 18, 38). To date, only certain aspects of this transcriptional control have been elucidated. Although RNA polymerase II appears to be involved in viral transcription throughout the infectious cycle (9), alterations in the host cell transcriptional system by virally encoded factors undoubtedly play a role in this regulation (20, 32, 39). There is good evidence that a virion component is required for optimal transcription of the IE genes (1, 8, 31), and the switch from IE to E and L transcription clearly involves at least one IE gene product, a 175-kilodalton phosphoprotein (ICP 4) specified by IE gene 3 (10, 20, 32, 39). The activation of L gene expression and turnoff of IE and E gene expression has been shown to require prior synthesis of both IE and E polypeptides and is strongly dependent upon viral DNA synthesis (6, 16, 18, 38). At least one early (β) polypeptide, ICP 8, appears to be intimately involved in negatively regulating transcription of viral genes at middle to late times after infection (15).

Studies on the regulation of HSV gene transcription have focused on the mechanisms by which RNA polymerase II is able to distinguish the different temporal classes of viral genes. One approach has been to analyze the transcription of cloned HSV-1 genes with an extract from HeLa cells described by Manley et al. (25). Only results from uninfected cell extracts have been reported, and the selectivity of transcription has varied. For example, Read and Summers transcribed the *Bam*HI Q DNA fragment containing the HSV-1 thymidine kinase (TK) gene and reported that the

HeLa cell extract efficiently recognized the early TK promoter as well as a L gene promoter present in the same fragment (33). Ikura et al. also found that the uninfected cell extract initiated transcription *in vitro* at both an E and L promoter mapping within the HSV-1 *Bam*HI J fragment (17). In contrast, Frink et al. compared *in vitro* transcription from an E and a L gene promoter and reported that the uninfected HeLa cell extract efficiently initiated transcription only from the E gene promoter (14).

Our laboratory recently described a partially purified RNA polymerase II preparation from HEp-2 cells that is about 10-fold more purified than the HeLa cell extract and essentially free of RNA polymerases I and III, nucleases, and nucleic acids. This preparation exhibits a selectivity of HSV-1 DNA transcription *in vitro* that is similar to that seen *in vivo* (2, 3). Transcription of whole HSV DNA showed that, even though the enzymes prepared from both mock-infected (Pol II-M) and HSV-1 infected cells (Pol II-H) transcribed essentially all regions of the genome, only Pol II-M preferentially transcribed the repeat regions of the genome which contain primarily the IE genes (3). We further showed, by *in vitro* transcription studies with the cloned *Bam*HI Q fragment of HSV-1, that Pol II-H initiates transcription *in vitro* much more efficiently than Pol II-M at the early TK promoter (2, 3).

To extend these studies, we analyzed transcription with both preparations with the cloned *Bam*HI Z fragment mapping between 0.955 and 0.966 map units on the HSV-1 genome. This fragment contains the promoters for IE RNA-3 (α 4) and IE RNA-5 (α 47). IE RNA-5 is a 1.8-kilobase (kb) spliced RNA that spans the U_g -TR_s junction and codes for a 12-kilodalton protein of unknown function (26, 35, 40). By using truncated template and S1 nuclease analyses, we showed that (i) Pol II-M initiates transcription *in vitro* more selectively than does Pol II-H at the IE RNA-5 promoter; (ii) initiation both *in vivo* and *in vitro* occurs preferentially at a site upstream from the previously published start site (29, 35, 41); (iii) a second, smaller intervening sequence (IVS) of 75 base pairs (bp) exists 5' to the previously characterized IVS

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(29, 35, 40, 41) and near the RNA start site; and (iv) some splicing of the larger, but not the smaller, IVS occurs in vitro with the Pol II-H preparation.

(This work partially fulfills master's thesis requirements for K.L.F. in the department of Immunology and Microbiology.)

(This work was presented, in part at the 1983 meeting of the American Society of Virology.)

MATERIALS AND METHODS

Cells and virus. HEP-2 cells and HSV-1 strain F, originally obtained from Bernard Roizman, University of Chicago, were propagated and harvested as previously described (28).

Purification and assay of RNA polymerase II. RNA polymerase II was prepared as previously described (2) from mock-infected and HSV-1-infected HEP-2 cells harvested at 8 h postinfection. Briefly, this procedure consisted of sonication in hypertonic medium, differential centrifugation, and subsequent fractionation on DEAE-cellulose to separate RNA polymerase II from RNA polymerase I and III activities. Polymerase activity was assayed by incorporation of labeled precursors into trichloroacetic acid-precipitable material as published (2).

Plasmid preparation. Plasmid pRB122, containing the HSV-1 strain F *Bam*HI Z fragment inserted into pBR322 (30), was kindly provided by Bernard Roizman. Plasmid DNA was prepared either by the method of Clewell and Helinski (7) or by a modification of the alkaline lysis procedure of Birnboim and Doly (5) as described by Maniatis et al. (24). Form I DNA was isolated from cesium chloride-ethidium bromide gradients.

Restriction and electrophoresis of DNA. Plasmid DNA was digested with various restriction enzymes (Bethesda Research Laboratories, Rockville, Md.) at 4 to 6 enzyme U/ μ g of DNA for 2 h under conditions recommended by the supplier. Reactions were terminated by adding EDTA to twice the molar concentration of Mg^{2+} and sodium dodecyl sulfate (SDS) to 0.2%, heating for 3 min at 65°C, and chilling on ice. After one extraction with phenol-chloroform-isoamyl alcohol, (24:24:1 [PCIA]), the DNA was precipitated with 2 volumes of ethanol for 10 min in a dry ice-ethanol bath and then centrifuged for 15 min in an Eppendorf Micro Centrifuge at $12,800 \times g$. DNA pellets were dried in vacuo, dissolved in TE buffer (10 mM Tris-hydrochloride [pH 8.0], 1 mM EDTA), and analyzed by electrophoresis through 1% agarose (Bethesda Research Laboratories) horizontal slab gels with a TBE buffer system (90 mM Tris, 90 mM boric acid, 4 mM EDTA [pH 8.3]). Gels were stained with ethidium bromide (1 μ g/ml), and DNA bands were visualized under UV light.

Preparation of DNA restriction fragments. For in vitro transcription or for S1 nuclease protection experiments, DNA fragments were purified by electrophoresis through a preparative 1% agarose gel followed by electrophoresis onto Whatman DEAE cellulose paper (11). The paper was washed with 10 mM Tris-hydrochloride (pH 8.0)–0.1 mM EDTA–0.1 M NaCl, and the fragments were eluted with 10 mM Tris-hydrochloride (pH 8.0)–0.1 mM EDTA–1 M NaCl, extracted once with PCIA, and precipitated with 2 volumes of ethanol. The DNA pellets were washed with 80% ethanol, dried in vacuo, and dissolved in TE buffer.

In vitro transcription and purification of RNA. Transcription was performed in 25- μ l reactions containing 50 mM Tris-hydrochloride (pH 8.0), 7 mM $MgCl_2$, 120 mM KCl, 0.6 mM ATP, 0.6 mM GTP, and 0.6 mM UTP, 50 μ M [α - 32 P]CTP at 7 to 20 μ Ci/nmol (Amersham Corp., Arlington

Heights, Ill., or New England Nuclear Corp., Boston, Mass.), 50 μ g of DNA per ml, and 10 μ l of RNA polymerase II (ca. 7 μ g of protein) (2). RNA was purified by extraction with PCIA, treatment for 15 to 30 min at 37°C with iodoacetate-treated (19) DNase I (100 μ g/ml; Worthington Biochemicals Corp., Freehold, N.J.), and precipitation with ethanol. For size analysis, the RNA was dried in vacuo, dissolved in a solution containing 50% dimethyl sulfoxide, 1 M glyoxal, and 10 mM sodium phosphate (pH 6.8), and glyoxalated for 1 h at 50°C (21). The glyoxal was previously deionized by stirring with Bio-Rad AG 501-X8D mixed-bed resin for 4 h at 4°C (34). Bromphenol blue was added to 0.01%, and the samples were analyzed by electrophoresis through a horizontal 1.6% agarose slab gel containing 10 mM sodium phosphate (pH 6.8), 0.1% SDS, and 5% glycerol (34). The gels were dried onto Whatman 3MM paper in vacuo and exposed to X-ray film (Kodak XR-P or XA-R) at -70°C .

Isolation of RNA from HEP-2 cells. HEP-2 cells in 850-cm² roller bottles were infected at 37°C with HSV-1 (F) at 50 PFU per cell in Dulbecco modified Eagle medium containing 1% heat-inactivated calf serum (DME-1% ICS). For the isolation of immediate early RNA, cells were infected with HSV-1 in the presence of cycloheximide (50 μ g/ml). After 1 h, virus was removed, and the cells were overlaid for 6 h with DME-1% ICS containing cycloheximide (50 μ g/ml). Late RNA was isolated from cells infected for 1 h in DME-1% ICS and further incubated for 10 h. Mock-infected RNA was prepared in the same manner as was late RNA, but without virus. Cells from two roller bottles were harvested by removing the medium and scraping them into 25 ml of RSB (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-hydrochloride [pH 7.5]). Following centrifugation for 10 min at 3,000 rpm (IEC PR-J centrifuge), the cells were suspended in 5 ml of RSB and lysed by adding an equal volume of 1% Nonidet P-40 and incubating for 10 min on ice with occasional mixing. Nuclei were pelleted by centrifugation for 5 min at 2,000 rpm (IEC PR-J centrifuge). The cytoplasmic fraction was removed, brought to 0.2% SDS–5 mM EDTA, and frozen at -70°C . After thawing, the cytosol was extracted three times with PCIA and precipitated with 2 volumes of ethanol.

Labeling of DNA fragments. DNA was end labeled by the method of Maxam and Gilbert (27). In preparation for labeling, DNA fragments were incubated for 1 h at 65°C in restriction buffer or TE buffer containing 20 U of bacterial alkaline phosphatase (Bethesda Research Laboratories) per pmol of DNA 5' ends, extracted twice with PCIA, and precipitated with 2 volumes of ethanol. For labeling, DNA was dissolved in 50 μ l of forward reaction buffer (50 mM Tris-hydrochloride [pH 7.5], 10 mM $MgCl_2$, 5 mM dithioerythritol, 0.1 mM spermidine, 0.1 mM EDTA) containing 20 U of polynucleotide kinase (Bethesda Research Laboratories) per 50 pmol of 5' ends and 50 pmol of [γ - 32 P]ATP (New England Nuclear Corp., Boston, Mass.); the mixture was incubated for 30 min at 37°C (27). After two extractions with PCIA, unincorporated nucleotides were removed by four or five precipitations with ethanol from 0.3 M sodium acetate (NaOAc).

DNA fragments were 3' end labeled by incubation for 30 min at room temperature in 20 μ l of nick translation buffer (50 mM Tris-hydrochloride [pH 7.2], 10 mM $MgSO_4$, 0.1 mM dithiothreitol, 50 μ g of bovine serum albumin per ml) containing 2 nmol of unlabeled dGTP and dTTP and 2 pmol of [α - 32 P]dCTP and [α - 32 P]dATP, and ca. 5 U of *E. coli* Klenow fragment (Bethesda Research Laboratories) (24). The reaction was stopped by addition of EDTA, the mixture was

FIG. 1. Map of HSV-1 *Bam*HI Z fragment and IE RNAs. The prototypic arrangement of the HSV-1 genome is shown at the top. Beneath it is an expanded map of the *Bam*HI Z fragment as published by Murchie and McGeoch for strain 17 (30), showing the positions and orientations of IE RNA-5 and IE RNA-3. Distance on *Bam*HI Z is in kb. The locations of relevant restriction sites are designated by X (*Xho*I), S (*Sph*I), B (*Bst*EII), and E (*Eco*RI).

extracted once with PCIA, and the RNA was precipitated from 0.3 M NaOAc several times to remove unincorporated nucleotides.

S1 nuclease mapping. End-labeled DNA probes were mixed with either in vitro synthesized RNA (from 100- μ l transcription reactions) or 10 μ g of whole cytoplasmic RNA in buffer containing 40 mM Pipes [piperazine-*N,N'*-bis(2-ethanesulfonic acid) (pH 6.6)], 0.4 M NaCl, 1 mM EDTA, 80% (vol/vol) formamide, denatured by heating at 90°C for 3 min, and hybridized overnight at 57°C (4, 12). The hybridization mixtures were then quickly added to cold S1 buffer (30 mM NaOAc [pH 4.5], 1 mM ZnCl₂, 250 mM NaCl, 5% glycerol) containing 6,000 U of nuclease S1 (Boehringer-Mannheim Chemicals, Indianapolis, Ind.) The samples were digested with S1 nuclease for 1 h at 37°C, after which the S1-resistant hybrids were extracted once with PCIA and precipitated with 2 volumes of ethanol. Products were analyzed under denaturing conditions by dissolving in 100% formamide followed by electrophoresis through a polyacrylamide-8 M urea sequencing gel, or under nondenaturing conditions by dissolving in TE buffer and electrophoresis through a neutral polyacrylamide gel in TBE buffer (24). All S1 analyses were performed in duplicate.

RESULTS

Truncated template mapping of RNA transcribed in vitro from the HSV-1 *Bam*HI Z fragment. Murchie and McGeoch, using HSV-1 strain 17 (29), and Watson and Vande Woude, using the Patton strain (41), have mapped IE RNA-5 (α 47) within the *Bam*HI Z fragment with the mRNA initiation site located 722 bases from the *Bam*HI site (Fig. 1). To analyze transcription from the IE RNA-5 promoter, we isolated the cloned *Bam*HI Z fragment of HSV-1 strain F and used this fragment and several of its subfragments as templates for in vitro transcription by Pol II-M and Pol II-H (Fig. 2). With *Bam*HI Z as a template, three distinct, major products were synthesized by Pol II-M (Fig. 2B, lane 2; Fig. 2C, lane 3). These consisted of a 1.85-kb RNA, corresponding in size to an end-to-end transcript of the entire fragment, a 1.28-kb transcript, presumably a runoff transcript from the IE RNA-5 promoter, and a 0.65-kb product. Minor quantities of a 0.34-kb transcript were also apparent in these analyses. With all the *Bam*HI-Z-derived templates, Pol II-H produced primarily end-to-end transcripts with only a small percentage of the specific transcription products (Fig. 2B, lane 3; Fig. 2C, lane 4).

By analyzing the transcription products from several *Bam*HI Z subfragments, we were able to confirm the position of the IE RNA-5 start site relative to the ends of the DNA templates. If transcription initiated at the previously described site 722 bases from the left end of *Bam*HI-Z (29) and proceeded to the right as shown in Fig. 2A, restricting the fragment with *Sph*I, *Xho*I, or *Bst*EII should have resulted in runoff transcripts of 0.94, 0.86, and 0.79 kb, respectively. Observed RNA products migrating with mobilities corresponding to 1.04 kb (Fig. 2C, lane 7), 0.90 kb (Fig. 2B, lanes 4 and 5), or 0.88 kb (Fig. 2B, lanes 6 and 7), for templates restricted by *Sph*I, *Xho*I, or *Bst*EII, respectively, are consistent with initiation at this site. Restriction with *Eco*RI, which cuts at the opposite end of the fragment upstream from the mRNA-5 start site, resulted in a shorter end-to-end transcript, but, as expected, it produced no reduction of the major 1.28-kb runoff product (Fig. 2C, lanes 5 and 6). The predominant bands visible in the lower portion of Fig. 2B, lanes 4 through 7, and Fig. 2C, lanes 7 and 8, represent end-to-end transcripts of the smaller truncated fragments. The 0.65-kb RNA present in large amounts in the Pol II-M products of *Bam*HI-Z was also apparent in variable amounts in the products of all truncations of this fragment, including the *Sph*I truncation (visible in darker exposures). Although some of this product might have originated by transcription from the RNA B promoter located roughly 600 bp downstream from the IE RNA-5 promoter (35, 36), at least some of this RNA could be the result of termination,

FIG. 2. Truncated template transcriptional analysis of RNA transcribed in vitro from *Bam*HI-Z. Isolated *Bam*HI-Z DNA, as well as several restrictions of it, were used as templates for in vitro transcription. RNA products were extracted, glyoxalated as described, and analyzed by electrophoresis through 1.6% agarose gels. (A) Relative position of IE RNA-5 on *Bam*HI-Z (inverted prototypic orientation). The size of the DNA is given in kb and the restriction sites used to generate subfragments are as shown in Fig. 1. (B) Lane 1, 5' ³²P-labeled *Alu*I-restricted pBR322; lanes 2 and 3, *Bam*HI-Z DNA transcribed in vitro by Pol II-M and Pol II-H, respectively; lanes 4 and 5, *Xho*I-restricted *Bam*HI-Z transcribed by Pol II-M and Pol II-H, respectively; lanes 6 and 7, *Bst*EII-restricted *Bam*HI-Z transcribed by Pol II-M and Pol II-H, respectively; lane 8, 5' ³²P-labeled *Hind*III-restricted lambda DNA. (C) Lanes 1 and 9, 5' ³²P-labeled *Hind*III-restricted lambda DNA; lane 2, 5' ³²P-labeled *Taq*I fragments of pBR322; lanes 3 and 4, *Bam*HI-Z transcribed by Pol II-M and Pol II-H, respectively; lanes 5 and 6, *Eco*RI-restricted *Bam*HI-Z transcribed by Pol II-M and Pol II-H, respectively; lanes 7 and 8, *Sph*I-restricted *Bam*HI-Z transcribed by Pol II-M and Pol II-H, respectively.

processing, or both. This possibility is supported by the sequence data of Murchie and McGeoch (29), which indicate that an internal AATAAAA sequence (13) exists 160 bases 3' to the characterized splice acceptor site and 618 bases from the IE RNA-5 start site for HSV-1 strain 17.

S1 nuclease mapping of the splice donor site. Because IE RNA-5 contains a well-characterized splice ca. 250 bases from the published initiation site (29, 35, 40, 41), and since we have evidence that our polymerase preparations produce spliced adenovirus type 2 and simian virus 40 transcripts in vitro, we were interested in determining whether splicing of IE RNA-5 occurs in vitro. A *HinfI* fragment which spans the

published intervening sequence was 3' end labeled with ^{32}P , recut with *BstEII*, and the 620-bp *HinfI*-*BstEII* probe labeled only at the *HinfI* site was isolated and hybridized to in vitro and in vivo-synthesized RNA (Fig. 3). The hybrids were treated with nuclease S1, and the resistant hybrids were glyoxalated and separated by electrophoresis through a 1.6% agarose gel (Fig. 3B). In vivo-synthesized IE (Fig. 3B, lane 5) and L (Fig. 3B, lane 6) RNA both protected a fragment of 175 bases which corresponds in size to the portion of the 5' exon homologous to the probe. RNA synthesized in vitro by Pol II-H (Fig. 3B, lane 3) and possibly by Pol II-M (Fig. 3B, lane 2) appeared to protect a fragment of approximately this

FIG. 3. Nuclease S1 mapping of the in vivo and in vitro splice donor site in IE RNA-5. RNA synthesized in vitro from *Bam*HI-Z (100- μl reactions), or whole cytoplasmic RNA isolated from mock-infected and HSV-1 infected HEP-2 cells, was hybridized with the 620-bp *HinfI*-*BstEII* probe, which was 3' ^{32}P labeled at the *HinfI* site. The hybrids were digested with nuclease S1 as described, and the hybrid-protected fragments were analyzed by gel electrophoresis. Sizes of protected fragments are indicated in bases. (A) Map of *Bam*HI-Z, showing the location of the 620-bp *HinfI* (H)-*BstEII* (B) probe, the IE RNA-5 runoff transcript, and the 175-base protected fragment corresponding to the 5' exon sequences extending to the labeled *HinfI* site. (B) Agarose gel electrophoretic analysis of glyoxalated DNA fragments protected from S1 digestion of hybrids. Lane 1, 5' ^{32}P -labeled *AluI* fragments of pBR322; lanes 2 and 3, hybridization with RNA synthesized in vitro by Pol II-M and Pol II-H, respectively; lane 4, hybridization with 10 μg of whole mock-infected cytoplasmic RNA; lanes 5 and 6, hybridization with 10 μg of whole immediate early cytoplasmic RNA (6 h after infection and treatment with cycloheximide) and late cytoplasmic RNA (10 h postinfection), respectively; lane 7, untreated probe; lane 8, 5' ^{32}P -labeled *Hae*III-restricted ϕX174 DNA. (C) Denaturing polyacrylamide gel electrophoretic analysis of S1 digestion products. Hybridization was carried out in 90% formamide buffer with the NaCl concentration of the S1 reaction buffer reduced to 50 mM to minimize DNA-DNA reassociation. Lane 1, 5' ^{32}P -labeled *AluI*-restricted pBR322; lane 2, hybridization with 10 μg of whole mock-infected cytoplasmic RNA; lane 3, hybridization with 10 μg of whole IE cytoplasmic RNA; lanes 4 and 5, hybridization with RNA synthesized in vitro by Pol II-H and Pol II-M, respectively; lane 6, 5' ^{32}P -labeled *Hpa*II-restricted pBR322.

same size. An additional fragment of 450 bases was protected by the Pol II-H product (Fig. 3B, lane 3) and, to a lesser extent, by the Pol II-M product (apparent in darker exposures of this gel). The mock-infected control (Fig. 3B, lane 4) and the control containing only tRNA (Fig. 3B, lane 7) protected mainly the whole probe.

To obtain better sizing, we repeated this experiment and ran the protected samples on an 8% polyacrylamide gel in 8 M urea (Fig. 3C). Because this area of the genome is high in G+C content and repeat sequences, the experiment was performed under more stringent conditions to minimize DNA-DNA reassociation. Hybridization was carried out in 90% formamide, and concentration of NaCl in the S1 reaction buffer was reduced from 250 to 50 mM. In vivo-synthesized IE RNA protected a major fragment of 170 bases (Fig. 3C, lane 3), whereas RNA synthesized in vitro by Pol II-H protected fragments of 160 and 450 bases as well as the total probe (Fig. 3C, lane 4). RNA synthesized by Pol II-M gave major protection of only the 450-base fragment and the total probe (Fig. 3C, lane 5); however, trace amounts of a 160-base fragment can be seen on the original autoradiograph. The latter fragment is presumably indicative of splicing at an alternate site near the major in vivo splice site. This is supported by the observation that, although it is not apparent in the exposure shown in Fig. 3C, RNA synthesized in vitro by Pol II-H also protected minor amounts of the 170-base fragment and, likewise, in vivo IE RNA protected minor amounts of the 160-base fragment. These results suggest that a portion of the Pol II-H products may be spliced at a site located near the in vivo site. The DNA band migrating with an apparent size of 450 bases protected by the in vitro-synthesized RNA (Fig. 3B, lane 3; Fig. 3C, lanes 4 and 5) could correspond to the 650-base transcript observed in the *Bam*HI-Z transcription products (Fig. 2) and represent a processed or terminated product. The mock-infected RNA control (Fig. 3C, lane 2) showed no protection of the labeled probe.

S1 nuclease mapping of the IE RNA-5 initiation site. To further define the 5' ends of the in vitro- and in vivo-synthesized RNAs, we isolated the 117-bp internal *Hinf*I-*Sma*I fragment, 5' end labeled at the *Hinf*I site, from *Bam*HI-Z (Fig. 4). This probe, which spans the published IE RNA-5 initiation site, was hybridized to in vitro- and in vivo-synthesized RNA. The hybridization mixture was treated with S1 nuclease and analyzed by electrophoresis through 8% polyacrylamide under denaturing conditions. If initiation occurred at the published site, (29, 35, 41), a fragment of 60 bases should have been protected. As shown in Fig. 4, lane 5, infected-cell IE RNA protected the expected fragment of 60 bases in addition to total probe. Control RNA from mock-infected cells gave no probe protection (Fig. 4, lane 6). In contrast, RNA synthesized in vitro by Pol II-M and Pol II-H (Fig. 4, lanes 3 and 4) protected mainly the total probe. These results indicate that the in vitro RNA and possibly some of the in vivo RNA initiated from a site located to the left of the probe.

To resolve this, we isolated a larger probe and used this in further S1 nuclease mapping experiments. A 0.54-kb *Sau*3A fragment of *Bam*HI-Z was 5' labeled with ³²P and recut with *Sph*I (Fig. 5A). The 508-bp *Sau*3A-*Sph*I fragment was electrophoretically isolated and used as probe in S1 mapping analysis of in vivo- and in vitro-synthesized RNA. The hybrid-protected DNA fragments were separated by electrophoresis through both denaturing (8 M urea) and nondenaturing 5% polyacrylamide gels. Under nondenaturing conditions (Fig. 5B), RNA synthesized in vitro by both Pol II-M

FIG. 4. (A) S1 nuclease mapping of the IE RNA-5 initiation site. A 5' end-labeled *Hinf*I (H) subfragment of *Bam*HI-Z was recut with *Sma*I (S) to yield the 117-bp probe uniquely labeled at the *Hinf*I site. (B) This probe was hybridized to in vitro- and in vivo-synthesized RNA, and the hybrids were treated with nuclease S1 for 1 h at 20°C. Protected DNA fragments were analyzed by electrophoresis through a denaturing 8% polyacrylamide gel. DNA fragment sizes are indicated in bases. Lanes 1 and 7, 5' ³²P-labeled, *Hpa*II-restricted pBR322; lanes 2 and 8, 5' ³²P-labeled, *Hae*III-restricted pBR322; lanes 3 and 4, hybridization with RNA synthesized in vitro by Pol II-M and Pol II-H, respectively; lane 5, hybridization with 10 µg of whole IE cytoplasmic RNA (6 h after infection and treatment with cycloheximide); lane 6, hybridization with 10 µg of whole mock-infected cytoplasmic RNA.

(lane 3) and Pol II-H (lane 4) protected one major fragment of ca. 264 bases, whereas both immediate early and late HSV-1-infected cell RNA (Fig. 5B, lanes 5 and 6) protected two fragments of 145 and 110 bases. These results indicate that either there are two initiation sites used in vivo which differ from the one used in vitro, or the in vivo-synthesized RNA is spliced at an IVS which lies within the region being probed (Fig. 5A).

Analysis of the same products under denaturing conditions (Fig. 5C) revealed only one band of 105 bases protected by both immediate early and late in vivo RNA (Fig. 5C, lanes 4 and 5, respectively) and one fragment of 215 bases protected by RNA synthesized in vitro by either Pol II-M (Fig. 5C, lane 2) or Pol II-H (Fig. 5C, lane 3). Repetition of this experiment resulted in essentially the same findings under denaturing conditions, but under nondenaturing conditions, immediate early and late in vivo-synthesized RNA sometimes protected only the larger of the two fragments (145 bases, data not shown). The larger apparent sizes of fragments in nondenaturing gels is presumably because of the somewhat lower mobility of RNA-DNA hybrids as

FIG. 5. S1 nuclease mapping of the IE RNA-5 initiation site. A 538-bp *Sau3A* (Sa) subfragment of *Bam*HI-Z was isolated, 5' end labeled with ^{32}P , and recut with *SphI* (Sp) to obtain the 508-bp *Sau3A-SphI* probe labeled at the *Sau3A* site. This probe was hybridized with RNA, and the hybrids were treated with nuclease S1 as described. The products were divided in half and analyzed under both denaturing and nondenaturing conditions. (A) Mapping of the *SphI-Sau3A* probe and the proposed 5' mapping of in vitro- and in vivo-synthesized IE RNA-5 based upon the data from this experiment and previously published data on the IE RNA-5 initiation site (∇) (29, 41). Fragment sizes are given in bases. (B) Nondenaturing 5% polyacrylamide gel electrophoretic analysis of S1-protected fragments. Lanes 1 and 9, 5' ^{32}P -labeled *Hpa*II-restricted pBR322; lane 2, untreated probe; lanes 3 and 4, hybridization with RNA synthesized in vitro by Pol II-M and Pol II-H, respectively; lanes 5 and 6, hybridization with 10 μg of whole IE cytoplasmic RNA (6 h after infection and treatment with cycloheximide) and L cytoplasmic RNA (10 h postinfection), respectively; lane 7, hybridization with 10 μg of whole mock-infected cytoplasmic RNA; lane 8, control hybridization with *E. coli* tRNA. (C) Denaturing 5% polyacrylamide gel electrophoretic analysis of S1 nuclease-protected fragments. Lanes 1 and 9, 5' ^{32}P -labeled *Hpa*II-restricted pBR322; lanes 2 and 3, hybridization with RNA synthesized in vitro by Pol II-M and Pol II-H, respectively; lanes 4 and 5, hybridization with 10 μg of whole IE cytoplasmic RNA and late cytoplasmic RNA, respectively; lanes 6 and 7, control hybridizations with 10 μg of mock-infected cytoplasmic RNA and *E. coli* tRNA, respectively; lane 8, untreated probe; lane 10, 5' ^{32}P -labeled *Hae*III-restricted pBR322.

compared to double-stranded DNA (35). We therefore consider the size values obtained under denaturing conditions as the more valid ones.

These results indicate that the major IE RNA-5 start site and a second IVS exist 5' to the previously published site. Under nondenaturing conditions, in vivo RNA protected a 145-base fragment equal in size to the sum of the 5' terminal exon and the portion of the second exon extending to the *Sau3A* site, as well as varying amounts of a smaller fragment (110 bases) derived from protection of only the 3' exon sequences. The smaller fragment was probably produced by S1 nuclease nicking the hybridized RNA at the splice junction; the efficiency of this is known to depend on the S1 digestion conditions (12). If this were the case, the same samples run under denaturing conditions would have shown protection of only the smaller, second exon fragment. As shown in Fig. 5C, this in fact was observed. In vitro-synthesized RNA, on the other hand, appeared not to be spliced at this site and protected a DNA fragment of 215 bases. These results place the initiation site for this RNA ca. 110 bases upstream from the published site. From the above S1 nuclease analyses, we present in Fig. 6 a revised transcriptional map for IE RNA-5.

DISCUSSION

In these studies, we used cloned HSV-1 DNA fragments containing the promoter and part of the coding sequence for IE RNA-5 to investigate the transcription of IE genes in vitro. Our results support the following conclusions: (i) Pol II-M initiates transcription from the IE RNA-5 promoter in vitro more selectively than the analogous enzyme from HSV-1 infected cells (Pol II-H); (ii) initiation both in vitro and in vivo occurs at a site upstream from the previously described site; (iii) a second IVS of ca. 75 bp is located 5' to the previously described IVS (29, 35, 40, 41) and about 35 bp from the mRNA start site; (iv) in vitro splicing of the larger IVS was detected in the Pol II-H transcripts.

Although both enzyme preparations were able to initiate at the IE RNA 5 promoter, as evidenced by runoff transcript and S1 analyses, Pol II-M yielded a greater percentage of the specific products and thus exhibited a greater degree of selectivity on this promoter. Since RNA from equal reaction volumes was analyzed, the larger amount of S1-protected fragments observed in the Pol II-H products is presumably because Pol II-H is a more active preparation than Pol II-M and thus synthesizes a greater overall amount of RNA in vitro.

The greater percentage of nonspecific transcription exhibited by Pol II-H is not a common property of this preparation. We have previously shown that Pol II-H, but not Pol II-M, efficiently initiates transcription from the early TK promoter (2, 3). Furthermore, in more recent studies, we have demonstrated that only Pol II-H is able to initiate with high selectivity from a promoter in the glycoprotein B gene region, as well as from two L promoters, one for the

FIG. 6. Proposed 5' mapping of IE RNA-5 within the *Bam*HI Z DNA fragment as derived from results presented in this paper. The previously published initiation site (29, 41) is represented by (∇). Map distances are given in kilobases (*Bam*HI-Z) or bases (IE RNA-5).

glycoprotein C gene and one for a 5.2-kb β, γ RNA located in the *EcoRI*-F region (Cleveland and Millette, manuscript in preparation). In addition, we have found that Pol II-H, but not Pol II-M, is able to yield discrete L transcripts from cloned simian virus 40 DNA fragments in vitro (Millette, unpublished data). The results presented here thus provide further evidence for specific in vitro transcriptional differences that parallel the transcriptional regulation pattern that is observed in vivo.

Mapping of the IE RNA-5 initiation site by S1 nuclease analysis showed that RNA synthesized in vitro initiated about 110 bp upstream from the site mapped previously and was unspliced. Results from these experiments also indicated that in vivo-synthesized RNA initiated at or near this new upstream site, but, in contrast to in vitro-synthesized RNA, contained a splice junction between a 35-nucleotide leader sequence and an acceptor site located at or near the originally described cap site. In fact, the DNA sequence at this site (29) contains a sequence, CCGCAG, which conforms to the splice acceptor consensus sequence (37).

We have recently performed preliminary exonuclease VII analyses that confirm our S1 mapping results. The results showed that initiation, both in vivo and in vitro, occurs primarily at the position defined by S1 nuclease analysis ca. 205 bp from the *Sau3A* site. From the size difference between the exonuclease VII- and S1-protected fragments, the intervening sequence appears to be ca. 85 bp in length. Most importantly, these experiments provided no evidence for initiation at the previously published site.

It may be useful, at this point, to consider the differences between our results and the earlier findings. First, the mapping results of Murchie and McGeoch (29) and Watson and Vande Woude (41) did not exclude the possibility of a second 5' exon and initiation further upstream, since their data were obtained with probes which would not have spanned the additional upstream exon reported here. Second, Rixon and Clements (35), using a larger probe which spanned both the previously described initiation site and the upstream site we have mapped, reported somewhat conflicting results of exonuclease VII and S1 experiments. Whereas their exonuclease VII analysis placed the RNA 5' terminus at approximately the same upstream location we have mapped, they believed this to be an artifact of the DNA sequence in the region and thus favored their S1 nuclease results, which agreed with the initiation site previously mapped (29, 41).

An examination of the DNA sequence of HSV-1 strain F (Edward Mocarski, personal communication), as well as that for the Patton (41) and Glasgow 17 (29) strains, reveals that the region surrounding the new start site possesses some features characteristic of eucaryotic promoter regions. The potential initiation site (CTGGCGC) shows six out of seven bases in agreement with the cap consensus sequence, CN^GANC^T, described by Temple Smith (personal communication). In addition, there is a sequence of 18 A's and T's, at a position -21 bp from this site (AATATATATATATTA-TTA), that might easily function as the TATA box homolog. This new start site should still allow functioning of the upstream control regions described by Mackem and Roizman (22, 23). In addition, it would place the IE RNA-5 cap site ca. 100 bp closer to the control sequences in a spacing that would be more consistent with that of the other IE regulatory regions.

Results from S1 nuclease experiments with a 3'-labeled probe spanning the second, larger intervening sequence (Fig. 3) suggested that some splicing of this sequence occurs

in vitro, at least with Pol II-H. This was not surprising, since we have previously found that the polymerase preparation efficiently splices both adenovirus IE RNAs (E1a and E4) and simian virus 40 L transcripts in vitro (unpublished data). Spliced transcripts were not apparent in the runoff products, possibly because of their lesser abundance and the relatively small size difference between spliced and unspliced products. However, in more recent analyses of transcripts from the isolated 1.27-kb *SphI* fragment containing the IE RNA-5 promoter, RNAs of the expected sizes for spliced products have been detected in the Pol II-M transcripts (unpublished data). The in vivo- and in vitro-synthesized RNA protected fragments were of different sizes (170 versus 160 nucleotides, respectively); this may reflect either sequence differences between the cloned HSV-1 strain F virus used to generate the in vivo RNAs and the *BamHI*-Z clone of HSV-1 strain F (30) or differences in splice donor site selection in vitro (there are three closely spaced, potential splice donor sites in this region [29]).

Based upon the size of the probe used to map the second IVS (Fig. 3) and assuming the exon sequences mapping within the probe are homologous between strains, the size of this IVS for HSV-1 strain F is ca. 110 bp. This is in contrast to intron sizes of 149 and 212 bp for strains Patton and 17, respectively. These differences are not unexpected, since it has been shown that although the exon sequences of IE RNA-5 are 98% homologous between strains, the length of the second intron varies because of differences in number and size of its tandem repeats (29, 35, 41).

ACKNOWLEDGMENTS

We thank Bernard Roizman for the generous gift of the pRB122 clone and Edward Mocarski and Temple Smith for providing unpublished data. We also thank Rose Callahan for excellent technical assistance, John Cleveland for critical reading of this manuscript, and Rosemarie Klaiber-Franco for technical assistance and help in preparation of figures.

This investigation was supported by Public Health Service grant CA-21065 awarded by the National Cancer Institute.

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