JOURNAL OF VIROLOGY, Feb. 1991, p. 957–960
0022-538X/91/020957-04$02.00/0

Vol. 65, No. 2

Isolation and Characterization of a Functional cDNA Encoding ICP0 from Herpes Simplex Virus Type 1

XIUXUAN ZHU, JIANXING CHEN, AND SAUL SILVERSTEIN*

Department of Microbiology, Columbia University, 701 West 168th Street, New York, New York 10032

Received 17 July 1990/Accepted 16 October 1990

The IE-0 gene of herpes simplex virus type 1 (HSV-1) contains two introns and encodes ICP0, a powerful transcriptional activator. We have isolated a cDNA clone that encodes ICP0 from a λgt10 cDNA library constructed from RNAs made from HSV-1-infected HeLa cells. DNA sequence analysis of this clone confirmed the predicted intron/exon boundaries (L. J. Perry, F. J. Rixon, R. D. Everett, M. C. Frame, and D. J. McGeoch, J. Gen. Virol. 67:2365–2380, 1986). Following transfection, a plasmid containing the cDNA copy of IE-0 directed the synthesis of ICP0, which was appropriately compartmentalized and distributed in the nucleus, as revealed by immunofluorescence. A transient expression assay was used to demonstrate that this cDNA copy retained the ability to transactivate the HSV-1 promoters for the IE-0 gene (an immediate-early gene), the thymidine kinase gene (an early gene), and the glycoprotein C gene (a late gene). The product of this cDNA clone cooperated with ICP4 to activate expression from the thymidine kinase gene promoter in a synergistic manner. The availability of a functional cDNA copy encoding ICP0 provides the opportunity to express this protein in vector systems that do not recognize eucaryotic donor and acceptor splicing signals to overexpress ICP0.

Herpes simplex virus type 1 (HSV-1) is a large double-stranded DNA virus whose genome of about 150 kb has the capacity to encode at least 72 unique proteins (14–16). During the course of productive infection, virus gene expression is coordinately regulated in a cascade fashion (11, 12). This temporal program of gene regulation consists of at least three groups of virus proteins: immediate-early (IE), early, and late. The expression of IE genes is required for activation of the early and late classes of virus genes and autoregulation of IE genes (3, 4, 12, 13, 17, 22, 24, 27, 30).

ICP0 is one of the five IE gene products. The gene encoding ICP0 is present in the repeated sequences bounding the long unique region of the HSV-1 genome and therefore is diploid (Fig. 1). Sequence analysis coupled with S1 nuclease mapping has revealed that the gene is 3,587 bp long, and it contains two introns in the coding region with predicted lengths of 767 and 136 nucleotides, respectively (21). IE-0 encodes a 775-amino-acid protein that is phosphorylated and found in the nucleus of infected cells (20). We and others have used a transient expression assay to show that ICP0 is a potent transcriptional activator and that it can activate the transcription of some herpesvirus genes in a synergistic manner when present together with ICP4 (8, 9, 18, 19, 23). Detailed insertion and deletion mutagenesis of the gene encoding ICP0 identified regions responsible for transactivation (1, 2, 5, 6). Deletion mutants of HSV-1 in IE-0 yield fewer infectious particles and demonstrate altered and delayed patterns of virus polypeptide synthesis (1, 7, 25, 29). Using a model in vitro latency system, we and others have shown that ICP0, alone of the HSV gene products, is sufficient to reactivate latent HSV-2 in an in vitro system and that the transactivation domains are required in this reactivation assay (10, 34).

The mechanism by which ICP0 transcriptionally regulates gene expression is still not known. Unfortunately, it has not been possible to overexpress ICP0 by using the available procaryotic and eucaryotic vector systems because of the lack of a cDNA copy encoding functional ICP0. In this study, a λgt10 cDNA library was constructed from RNAs made from HSV-1-infected HeLa cells and a cDNA clone that encodes ICP0 was isolated. Sequence analysis of this cDNA clone confirmed the predicted intron/exon boundary. This cDNA copy was able to direct the synthesis of ICP0 and retained its ability to transactive HSV-1 promoters from each of the three temporally regulated gene families.

Isolation of a cDNA clone encoding ICP0. An oligo(dT)-primed cDNA library cloned into the *Eco*RI site in λgt10 was generated by using poly(A)-containing RNAs from HSV-1-infected HeLa cells. This library was screened with a ^{32}P-labeled 290-bp *Msp*I fragment which included nucleotides −129 to +161 from the IE-0 gene (9). Sixteen positive plaques were picked and further analyzed by restriction endonuclease mapping and Southern blot hybridization. Seven clones contained *Eco*RI inserts of >1.8 kb, and the insert from one of these was subcloned into a pUC vector. Detailed restriction mapping revealed that the insert contained IE-0 sequences from the *Nco*I site (+147) to beyond the *Nru*I site (+1983) at the 3′ end (Fig. 1). The two introns in the genomic copy of IE-0 appeared to be deleted in this clone. A portion of the 0.8-kb sequence 5′ of the *Nco*I site was not derived from IE-0 and was a cloning artifact. To verify the sequence at the intron/exon junctions, a *Hin*fI fragment spanning the first intron/exon junction (−69 to +1025) and a *Kpn*I-*Nru*I fragment spanning the second one (+1545 to +1983) were each subcloned into an M13 vector and sequenced by the dideoxy-chain termination procedure (26). The consensus splice donor and acceptor sites were used at each junction (Fig. 1C), and the sequence is consistent with the predicted intron/exon boundaries based on sequence analysis of the genomic copy of IE-0 and S1 nuclease mapping (21). The *Nco*I-*Nru*I fragment from a genomic clone of IE-0 was replaced with the *Nco*I-*Nru*I fragment from the cDNA clone to generate a full-length cDNA copy (pDS-16) encoding ICP0 (Fig. 1).

* Corresponding author.

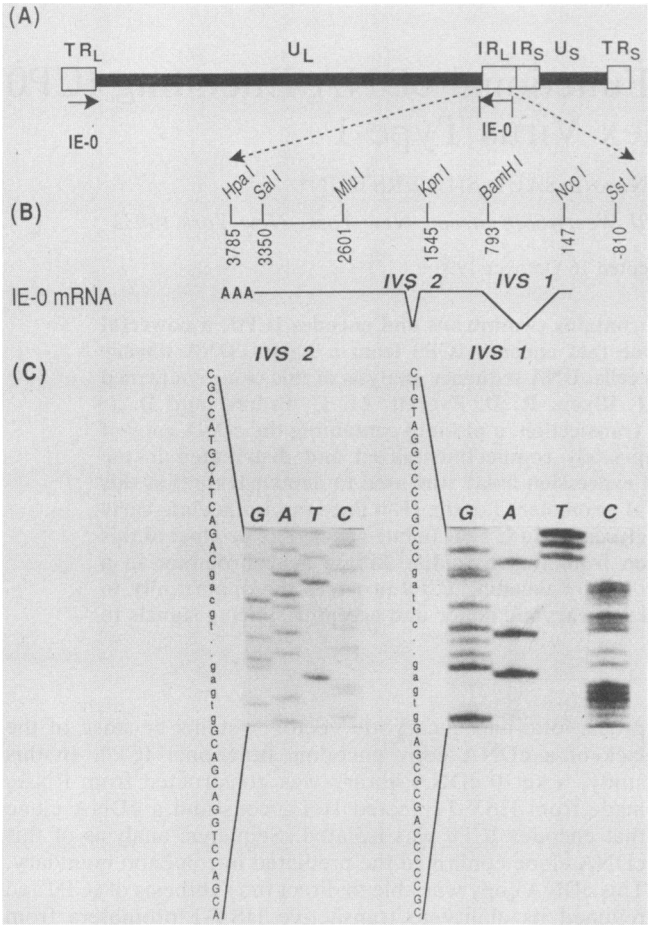

FIG. 1. Structure and location of the IE-0 gene from HSV-1. (A) Structure of the HSV-1 genome showing the prototype arrangement. The long unique region (U_L) and short unique region (U_S) are flanked by internal and terminal repeats (IR and TR). The sequences encoding ICP0 are contained entirely within TR_L and IR_L. (B) Restriction endonuclease map of the IE-0 gene and structure of the mRNA encoding ICP0. The structure of the mRNA encoding ICP0 is shown beneath the map. The numbering is with respect to the transcription initiation site at +1 (21). (C) The nucleotide sequence of a *Hin*fI fragment (spanning IVS1) and a *Kpn*I-*Nru*I fragment (spanning IVS2) derived from the cDNA copy of IE-0. The sequences are read from bottom to top in the 5′-to-3′ orientation. Capital letters represent nucleotides present in the cDNA, and lowercase letters refer to nucleotides present in the introns. For sequencing of the first intron/exon boundary, a *Hin*fI fragment from the cDNA clone was end filled with Klenow fragment of DNA polymerase I and subcloned into the *Sma*I site of M13mp19. Likewise, a *Kpn*I-*Nru*I fragment from the cDNA clone was subcloned into the *Kpn*I and *Sma*I sites of M13mp19 for determining the sequence surrounding the second intron/exon boundary. DNA sequence analysis was done by the dideoxy-chain termination method, using T7 DNA polymerase.

The IE-0 cDNA copy directs the synthesis of ICP0. To determine whether the cDNA clone was able to direct the synthesis of ICP0, Vero cells were transfected with plasmid containing either the IE-0 cDNA copy (pDS-16) or the IE-0 genomic copy (pXQ-1) and analyzed for the presence of immunoreactive protein by immunofluorescence analysis. Forty-eight hours after transfection, cells were fixed and reacted with a mouse monoclonal antibody specific for ICP0 (H1083). ICP0 was present in the nucleus of cells transfected with pDS-16 (Fig. 2B) as well as with pXQ-1 (Fig. 2C) but not with pUC19 (Fig. 2A). The ICP0 which is synthesized in these transfected cells is seen as punctate granules in the nucleus of cells transfected with either pDS-16 or pXQ-1.

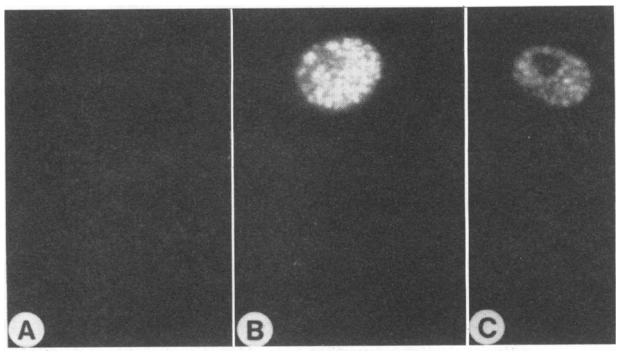

FIG. 2. Synthesis and localization of ICP0 after transient expression of a cDNA. Vero cells (2×10^5) were transfected with 2 μg of plasmid DNA; after 48 h, cells were washed three times with phosphate-buffered saline, air dried for 10 min, fixed in acetone for 10 min at room temperature, and then washed three times with phosphate-buffered saline. Mouse monoclonal antibody specific for ICP0 (H1083) was provided by Lenore Pereira. The antibody was diluted 1:100 in phosphate-buffered saline and incubated with the cells at room temperature for 1 h. Excess antibody was removed by three washes in phosphate-buffered saline, and the cells were then incubated with fluorescein-conjugated goat anti-mouse immunoglobulins (Organon Teknika-Cappel, West Chester, Pa.), washed three times in phosphate-buffered saline, and overlaid with a coverslip. Preparations were viewed at a magnification of × 600, using a Leitz Dialux Microscope with vertical illumination and optical systems for the selective visualization of fluorescein. Appropriate fields were photographed with Kodak TMAX film (ASA 3200) and a Wild automated photographic system.

The IE-0 cDNA copy retains transactivating activity. To determine whether the cDNA copy of the IE-0 gene retained biologic activity, plasmid pDS-16 was examined in a transient expression assay to test its ability to activate expression from HSV-1 promoters representing each of the three major transcriptionally regulated classes of genes. β-Galactosidase reporter cassettes driven by the promoters for the IE-0, thymidine kinase (TK), and glycoprotein C (gC) genes were cotransfected along with either clones containing the cDNA (pDS-16) or genomic (pXQ-1) copies of IE-0 into Vero cells. After 48 h, cells were harvested and assayed for β-galactosidase activity. pDS-16 was as competent as pXQ-1 at activating expression from each of the promoters tested; moreover, when present together with a plasmid containing the gene encoding ICP4 (JC-16), pDS-16 was able to cooperatively activate expression from the TK promoter (Table 1). From this analysis, we conclude that the cDNA clone encoding ICP0 retains its transactivating property.

The gene encoding ICP0 differs from the majority of HSV-1 genes (31) in having two introns in its coding sequences. This study reports the construction and characterization of a cDNA clone encoding ICP0. A transient expression assay demonstrated that this cDNA copy was able to direct the synthesis of ICP0 and that the expressed protein retained its transcriptional activating properties.

On the basis of sequence analysis and S1 nuclease mapping, Perry et al. (21) predicted that the splice donor for intervening sequence 1 (IVS1) was at +205 and that the

TABLE 1. Transactivation of promoters from three kinetic classes of HSV-1 genes

Effector	β-Galactosidase activity[a]		
	IE-0–β-gal	TK–β-gal	gC–β-gal
pXQ1	6.5	18.2	6.2
pDS16	9.7	23.5	8.5
pJC16	ND	13.5	ND
pXQ1 + pJC16	ND	116.2	ND
pDS16 + pJC16	ND	72.3	ND

[a] Fold induction above the level obtained with the reporter in the absence of any effector (assigned a value of 1). The β-galactosidase assay was performed as described by Spaete and Mocarski (28), with the following modifications. Vero cells transfected with reporter and effector plasmid DNAs (1:1 molar ratio) were washed twice with phosphate-buffered saline and scraped from the dishes into Tris-buffered saline containing 1 mM EDTA, pelleted, and resuspended in 100 μl of 0.25 M Tris hydrochloride (pH 7.8). The cells were disrupted by three cycles of freezing and thawing, and debris was removed by a 3-min centrifugation in a microfuge. Protein concentration was determined, and 100 μg of total protein from transfected cells was incubated with 0.77 mM 4-methylumbelliferyl-β-D-galactoside in a final volume of 230 μl of buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM 2-mercaptoethanol [pH 7.5]) at 37°C for 25 min in a Titertek Fluoroskan II. The fluorescence intensity of the released 4-methylumbelliferone is normalized to the value obtained with a cell extract prepared from cells transfected with pIC20R. Activity was determined for chimeric genes containing promoters from each of the three major kinetic classes of HSV-1 genes fused to a β-galactosidase (β-gal) reporter cassette. The promoter regions used for the fusions were −585 to +150 for IE-0, −775 to +56 for TK, and −1339 to +34 for gC. ND, Not determined.

acceptor was at +971; the donor and acceptor for IVS2 were calculated to be at +1637 and +1774, respectively. However, because of the high G+C composition and a nonuniform G+C distribution within this gene, they considered the information generated from these analyses to be inconclusive. Here, we have sequenced fragments from a cDNA clone that spanned both of the putative intron/exon boundaries in the IE-0 gene and demonstrated that the predicted consensus splice donor and acceptor sequences are used to generate a functional mRNA.

The first intron of IE-0 contains three copies of a 54-bp repeat, and the second intron is small, containing only 136 bp. It is not clear whether any function can be attributed to these introns; however, they do overlap with and comprise a portion of the latency-associated transcript RNA (32, 33). Although it is possible that the intervening sequences serve a function during the course of a productive infection with HSV-1 or in any of the steps involved in establishment, maintenance, or reactivation from the latent state, it appears that they are not required for expression of functional mRNA encoding ICP0 when tested in a transient expression assay.

The mechanism by which ICP0 transcriptionally regulates gene expression is still not known. The availability of the cDNA clone encoding functional ICP0 will permit the introduction of this sequence into vector systems in which a cDNA clone is required for expression. This should help to provide an enriched source of ICP0 for further biochemical characterization. Moreover, it is more convenient to introduce precise mutations into a cDNA clone of IE-0 than in clones containing the genomic sequence.

This study was supported by Public Health Service grant GM-38125 from the National Institutes of Health to S.S.

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