Characterization of a herpes simplex virus type 1 deletion variant (1703) which under-produces Vmw63 during immediate early conditions of infection

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The herpes simplex virus type 1 deletion variant 1703 apparently fails to synthesize the essential IE2 gene product Vmw63 despite the deletion leaving the gene intact. Sequence analysis revealed that the deletion removes a region to the right of IE2 comprising the 3′ end of IE1, UL56 and the 3′ part of UL55, stopping 555 bp downstream of the IE2 polyadenylation signal. Further DNA sequencing has shown that there is no secondary mutation in the IE2 gene. Western blot analysis demonstrated that Vmw63 is made at reduced levels compared to that produced by the wild-type virus during immediate early conditions of infection. S1 nuclease protection mapping has revealed that this reduction is also apparent at the level of mRNA synthesis. A direct link between the deletion and the change in mRNA synthesis was provided by the insertion of a deletion-spanning fragment from 1703 into a 17+ genome, which resulted in the recombinant having a 1703-like phenotype. Evidence that down-regulation of IE2 mRNA during immediate early conditions of infection could be due to antisense RNA initiating from the IE1 promoter was obtained by the insertion of a novel transcriptional termination signal between IE1 and IE2 in the variant and the subsequent detection of wild-type levels of IE2 mRNA and protein.

Introduction

Herpes simplex virus type 1 (HSV-1) has five immediate early genes (IE1, 2, 3, 4 and 5) encoding the polypeptides Vmw110, 63, 175, 68 and 12 respectively. Immediate early genes are unique in that they are transcribed in the absence of prior viral protein synthesis and their products are required for the expression of early and late viral polypeptides. The immediate early genes and their encoded proteins have been the subject of close scrutiny and much information has been gained concerning their regulatory activities and functional domains. The isolation and characterization of temperature-sensitive (ts) IE2 mutants demonstrated that Vmw63 played an essential role in virus replication (Sacks et al., 1985) and the characterization of null mutants in this gene demonstrated the role of Vmw63 in the modulation of early and late gene expression at the transcriptional level (McCarthy et al., 1989). Further analysis demonstrated that Vmw63 performs its regulatory activities over time and mediates these activities indirectly via interactions with, and modification of Vmw175 and possibly other viral and cellular proteins (Rice et al., 1989; McMahon & Schaffer, 1990). Recent evidence indicates that Vmw63 may be involved at post-transcriptional levels to affect viral mRNA 3′ processing (Sandri-Goldin & Mendoza, 1992; McLauchlan et al., 1992).

In 1987 we reported the isolation of an HSV-1 strain 17+ variant (1703) with a 7.5 kb deletion encompassing part of the long internal repeat region (IRL) and the adjacent unique sequences, such that most of one copy of the IE1 gene, part of UL55 and all of UL56 were deleted (MacLean & Brown, 1987a). The deletion terminated approximately 500 bp downstream of the 3′ end of the IE2 gene. The mutant synthesized reduced levels of the IE1 gene product Vmw110 and synthesis of Vmw63 could not be detected at either protein or RNA levels under immediate early conditions despite there being no apparent deletion in the coding or controlling regions of the IE2 gene. Given that the isolation of ts and null mutants in IE2 demonstrated the essential nature of the gene, the isolation of a mutant which grew normally in vitro but failed to produce detectable levels of Vmw63 appeared to be inconsistent.

This paper discusses the characterization of the 1703 variant and demonstrates by DNA sequence analysis that there is no mutation in either the promoter or coding region of IE2 and that the deletion is distal from the 3′ end of the gene. We suggest that the down-

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regulation of IE2 under immediate early conditions may be due to antisense RNA initiating from the intact IE1 promoter. Preliminary data to support this proposition are presented.

Methods

Cells and viruses. BHK-21 C13 cells were maintained in 80 oz roller bottles in Glasgow-modified MEM supplemented with 10% newborn calf serum. HFL cells were maintained in the same medium supplemented with 10% fetal calf serum. Viruses used were HSV-1 17′ (Brown et al., 1973) and the HSV-1 17′ deletion variant 1703 (MacLean & Brown, 1987a).

DNA sequence determination. Two fragments of 1703, HpaI s and HpaI m′ were recovered by HpaI digestion of 1703 DNA and agarose gel electrophoresis. These fragments were cloned into the Smal site of pUC19, fragmented by digestion with Smal and cloned into the Smal site of M13 mp8 (Stratagene). Sequences of M13 clones were determined by the extended Sanger dideoxynucleotide method. The sequences obtained were then compared to those published for the wild-type virus (McGeoch et al., 1988).

Preparation of RNA. BHK-21 C13 cells were grown as monolayers in rotating 80 oz roller bottles. For the production of immediate early RNA, cell monolayers were infected with HSV-1 17′ or 1703 at a m.o.i. of 50 p.f.u./cell. The cell monolayers were pretreated and maintained in medium containing cycloheximide as described previously (Clements et al., 1977). Early and late RNA preparations were made by infecting monolayers with 20 p.f.u./cell and harvesting 7 and 16 h post-infection respectively. RNA was prepared by the method described by Kumar & Lindberg (1972).

S1 nuclease mapping of RNA. This method is a modification of that used by Whitton et al. (1983). Labelled probes were prepared by the incorporation of a 32P-labelled deoxyxynucleotide at the 3′ end of two digested plasmids. Less than 1 µg was precipitated with known amounts of RNA from infected or mock-infected cells. The probes used were pGX51 (SalI-digested) and pGX55 (BamHI-digested) and were supplied by Dr V. G. Preston. The DNA–RNA pellet was resuspended in 20 µl of 90% (v/v) formamide (deionized with Amberlite monobed resin MB-1), 0.4 M-NaCl, 49 mM-PIPES pH 6.8 and 1 mM-EDTA. The mixture was boiled for 2 min, incubated at 57 °C for 16 h and then quenched on ice prior to S1 nuclease digestion. This was performed at 30 °C for 1-5 h in 200 µl of 0.25 M-NaCl, 30 mM-sodium acetate pH 4.5 and 1 mM-ZnSO₄, with 150 units of S1 nuclease. The nucleic acids were extracted with phenol–chloroform and precipitated with ethanol. The products were analysed by electrophoresis through a 6% polyacrylamide sequencing gel at 70 W for 1 to 1.5 h. The gels were dried under vacuum and bands visualized by autoradiography using Kodak X5 film.

Preparation of immediate early polypeptides. The procedure was performed essentially as described by Preston et al. (1978). The infected cells were harvested into 300 µl of a 1:3 dilution of extraction buffer consisting of 1 ml of running gel buffer (0.11 M-Tris–HCl pH 6.7, 0.1% w/v SDS), 1 ml glycerol, 0.5 ml 2-mercaptoethanol, 20 µl of 1% bromophenol blue and 0.8 ml of 25% SDS.

Preparation of early and late polypeptides. These were made by infecting confluent BHK-21 C13 monolayers with 20 p.f.u./cell of virus and incubating at 37 °C for 7 and 16 h respectively. Infected cells were harvested into 300 µl of extraction buffer as described for immediate early polypeptides.

Western blot analysis. To generate a Vmw63-specific antisemur, sandy half-lop rabbits were immunized on day 0 with 100 µg of a peptide corresponding to the predicted first 16 amino acids of the protein, in Freund’s complete adjuvant (McGeoch et al., 1988). The immunogen was synthesized as described previously (McLean et al., 1990) in the form of a branched or multiple antigenic peptide (Tam, 1988) of sequence (MATDIDMLIDLGLDLS)₉-Kₐ. At days 10, 30 and 40 1000 µg of the peptide in Freund’s complete adjuvant was administered to the rabbits and sera were collected on day 50. Western blotting was carried out as described by Towbin et al. (1979).

Transfection of virus DNA. Intact HSV-1 DNA (0.2 to 2 µg/plate) was cotransfected with a 5:10 molar ratio of plasmid: virus DNA onto semi-confluent monolayers of BHK-21 C13 cells in 50 mm Petri dishes using the calcium phosphate precipitation–DMSO boost technique described by Stow & Wilkie (1976). Single plaques were obtained and stocks were grown in Linbro trays.

Restriction enzyme analysis of recombinant virus genomes. Analysis of single-plaque isolates was carried out by a modification of the technique of Lonsdale (1979).

Results

Sequence analysis of the gene IE2 defining the deletion in 1703

The initial restriction enzyme and Southern blot analysis of the variant demonstrated that the deletion was at the IR₈/U₁ junction and its size was estimated to be 7.5 kb. On HpaI digestion the v, r and one copy of the m fragment were deleted. A novel fragment, designated m′ and thought to be a composite of part of v and part of m, was apparent (MacLean & Brown, 1987a). The IE2 gene is contained entirely within HpaI s (Fig. 1b and c).

To determine whether there was a mutation in the coding region or upstream regulatory sequences of IE2, HpaI s from 1703 was cloned into pUC19 and, after Smal digestion, the subfragments were cloned into M13 mp8. Sequence analysis was carried out on nine subfragments, totalling 3192 bp, and no mutations were detected. The promoter and associated upstream and downstream regulatory sequences were found to have no mutations within 1681 bp. Part of the coding region of the gene (936 bp) was also free from alteration compared to the wild-type sequence. A downstream sequence of 575 bp including the known termination signals for IE2 was found to be mutation-free.

To determine the endpoints of the deletion in U₁/IR₈, the HpaI m′ fragment from 1703 was cloned into pUC19 and, after Smal digestion, the subfragments were subcloned into M13 mp8. The deletion was found to be 7784 bp in length, starting 555 bp downstream of the IE2 poly(A) signal at nucleotide position 115839 and terminating within the first intron of the IR₈ copy of IE1 at position 123623. As predicted the deletion totally removed the UL56 gene plus the 3′ part of UL55 (Fig. 1d and e). The sequencing substantiated our previous conclusion that the deletion stopped approximately 500 bp beyond the 3′ end of IE2 and therefore should not interfere with Vmw63 synthesis.
Western blot analysis of Vmw63 synthesis

Polypeptides were synthesized in BHK-21 C13 and HFL cells under immediate early, early and late conditions. Immediate early polypeptides were synthesized by accumulating mRNA in the presence of cycloheximide which was removed 5 h post-infection. Polypeptides were then made in the presence of actinomycin D and harvested 2 h later. Early and late polypeptides were harvested 7 and 17 h post-infection respectively. Results from Western blot experiments using the anti-peptide serum are shown in Fig. 2. Low amounts of Vmw63 were synthesized in BHK-21 C13 cells by the parental 17+ virus under immediate early conditions but none was detectable in cells infected with 1703 under these conditions (Fig. 2a). At early times the amount of Vmw63 made by 1703-infected cells was slightly less than that made by 17+-infected cells, and at late times 17+ and 1703 samples gave equivalent signals. Subsequent Western blot analysis experiments (Fig. 3) demonstrated that Vmw63 production by 1703-infected cells was equivalent to that of 17+ during early and late conditions of infection. This indicated that the reduction in Vmw63 synthesis by 1703 during early times of infection could have been due to experimental variation. Confirmation that the total proteins made by each virus at the three stages of infection were equivalent was achieved by autoradiography of the 35S-labelled proteins in SDS-polyacrylamide gels. Evidence that Vmw63 was produced by 1703 during immediate early times of infection was provided by repeating the immediate early polypeptide experiment in HFL cells. These cells have been shown to be more efficient producers of immediate early polypeptides than BHK-21 C13 cells (MacDonald, 1980).

Fig. 1. Map of the deletion in 1703 and the insertion in 1703PA. (a) Structure of the HSV-1 genome showing UL and US flanked by TRL/IRL and IRS/TRS respectively. (b) A portion of UL/IRL expanded to show the positions and the orientations of the genes. (c) BamHI and HpaI restriction enzyme sites (B and H respectively) and restriction enzyme fragments resulting from cleavage with these enzymes. (d) The region of DNA deleted in 1703 is between nucleotide positions 115 839 and 123 362, affecting genes UL55, UL56 and IE1. (e) The dotted line indicates the deletion. The restriction enzyme sites affected are shown. BamHI b becomes BamHI b’, the remaining DNA of HpaI v and HpaI m becomes HpaI m’. (f) The insertion of the poly(A) signal introduces two novel BamHI sites. BamHI b’ becomes BamHI b’ since the fragment now ends at the inserted poly(A) signal.

Fig. 2. Western blot analysis of the polypeptides of 17+ - (lanes 1), 1703- (lanes 2) and mock-infected (lanes 3) cells. (a) Immediate early (IE), early (E) and late (L) polypeptides synthesized in 17+, 1703- and mock-infected BHK-21 C13 cells. (b) Polypeptide extracts from HFL cells infected with 17+, 1703- and mock-infected. (c) Immediate early polypeptides synthesized in HFL cells. 17+ extract was diluted in a series with mock-infected cell extract; lane (i), 1:2; lane (ii), 1:4; lane (iii), 1:8; lane (iv), 1:16; lane (v), 1:32. Arrowheads indicate the presence of Vmw63. The antiserum used for this series of experiments was directed against the amino terminus of Vmw63.

Fig. 3. Western blot analysis of immediate early (a), early (b) and late (c) extracts of polypeptides synthesized in BHK-21 C13 cells. The cells were mock-infected (lanes 1), or infected with 1703PA (lanes 2), 1750 (lanes 3), 1703 (lanes 4) or 17+ (lanes 5). The position of Vmw63 is indicated by an arrowhead. The antisera used was the same as that described in Fig. 2.
S1 nuclease mapping of mRNA synthesized by 1703-infected cells as compared to wild-type virus

Fig. 2(b) shows that Vmw63 made in HFL cells by 1703 was again less than that synthesized by 17+ but this time could be visualized. Quantification of the amount of Vmw63 produced by 1703 during immediate early infection was achieved by blotting serial twofold dilutions of immediate early proteins extracted from 17+-infected cells, together with the extract from 1703-infected cells. Comparison of band intensity showed that the amount of Vmw63 produced by 1703 to be about an eighth of that synthesized by wild-type virus (Fig. 2c). These results indicate that there is interference with the pattern of Vmw63 synthesis in 1703-infected cells.

S1 nuclease mapping of mRNA synthesized by 1703

To determine whether the impairment of Vmw63 synthesis at immediate early times was the result of interference at the transcriptional level, the synthesis of mRNA of 1703 under immediate early, early and late conditions was analysed by S1 nuclease mapping. The probe chosen to detect the 3' end of IE2 was pGX51. Plasmid pGX55, which detects the 3' end of IE5, was used as a control. Both probes have been used for S1 nuclease mapping previously (Whitton et al., 1983; Rixon & Clements, 1982). The autoradiograph shown in Fig. 4(a) demonstrates that the amount of IE5 mRNA synthesized by 1703 was similar to that made by 17+ in cytoplasmic fractions of infected cells under immediate early conditions. IE5 mRNA in the nuclear fraction of 1703-infected cells was reduced in comparison with levels of the transcript in the same location in 17+-infected cells. When comparing the levels of IE5 mRNA detected in each cellular fraction, lower amounts of the transcript were observed in the nuclear fractions of cells infected with 1703 than in the cytoplasm of those infected with either 17+ or 1703. The amount of IE2 mRNA detectable at immediate early times in both the cytoplasmic and nuclear fractions of 1703-infected cells was significantly reduced when compared to wild-type levels. At early and late times, IE2 mRNA synthesis by 1703 returned to normal (only the late data are shown; Fig. 4b). It is evident from the results that temporal regulation of IE2 is disrupted at the transcriptional or post-transcriptional level.

Transfer of the 1703 UL/IR deletion into 17+ DNA and analysis of the resultant infection

The results of sequence analysis demonstrated that the alteration of temporal regulation of IE2 was not due to a secondary mutation within the gene or its regulatory sequences. It was therefore essential to confirm that the down-regulation of transcription was a direct consequence of the downstream deletion. To do this a 1703 restriction endonuclease fragment, BgIII f', containing the deletion, was cotransfected with strain 17+ DNA. Restriction enzyme analysis of resultant plaques revealed a recombinant, 1750, whose profile was the same as that of 1703. This virus was plaque-purified three times and its structure confirmed by restriction enzyme analysis (Fig. 5). The appearance of the BamHI k band was variable in all tracks but this was expected since the band corresponds to an end fragment and is therefore subject to variation.

Synthesis of Vmw63 by the recombinant 1750 was compared to 17+ and 1703 infections by Western blot analysis. The results are shown in Fig. 3. Under immediate early conditions, the amount of Vmw63 synthesized by 1750 was equivalent to that made by 1703 and was markedly less than that made by 17+ (Fig. 3a). At early and late times, Vmw63 synthesis in 1703-infected cells returned to normal (Fig. 3b and c).

Introduction of a poly(A) signal between the 5' end of IE1 and the 3' end of IE2 in 1703 and analysis of the recombinant

The down-regulation of transcription and translation from IE2 has been shown to be a direct consequence of the UL/IR deletion in 1703. One explanation for this phenomenon involves the deletion of the normal IE1 and UL56 poly(A) signals. This means that transcripts...
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initiating from the IE1 promoter can continue until a suitable poly(A) signal is reached, for example at UL51 and interfere with IE2 transcription. To test this theory, it was decided to insert a termination signal into the 1703 variant in the appropriate orientation between HpaI s and HpaI m' to prevent transcription into the IE2 open reading frame. An HSV-2 termination signal (from UL38) cloned into pGEM (Promega) was available and this was linked to the HpaI s fragment in pUC19 by a series of restriction endonuclease digestions and ligation. Similarly, HpaI m', in pUC19, was also attached to the construct. The orientation was confirmed by digestion and gel electrophoresis to determine the size of the resultant fragments.

The plasmid was cotransfected with 1703 DNA and the progeny analysed with restriction enzymes to identify a recombinant. This was plaque-purified three times and its structure confirmed. The BamHI profile of the recombinant, 1703PA, is shown in Fig. 5. A novel 1 M fragment of Mr 1.6 x 10^6 appears. This corresponds to BamHI b, containing a slightly larger deletion than the b' fragment of 1703, since there are now two BamHI sites flanking a poly(A) signal, in place of the original HpaI site. This is shown in Fig. 1 (f).

A Western blot of Vmw63 synthesized by 1703PA is shown in Fig. 3. At immediate early times the amount of Vmw63 was comparable to that made by 17+, and was markedly increased over the synthesis of Vmw63 by the parental 1703 virus strain (Fig. 3a). The amount of Vmw63 made by 1703PA at early and late times remained constant (Fig. 3b and c). Also, a protected fragment of 150 bp (Fig. 6) was detected during the S1 nuclease analysis of 1703PA transcripts when using the plasmid construct containing the termination signal as a probe. This band was absent from the 17+, 1703 and 1750 lanes. These results support the theory that the down-regulation of the IE2 gene is due to transcripts initiating from the IE1 promoter.

Discussion

Vmw63 is an essential immediate early polypeptide (Sacks et al., 1985; McCarthy et al., 1989) which trans-activates later genes, especially the true late genes, which are not synthesized in the absence of viral DNA replication. IE2 deletion variants are not viable in tissue culture, except in cell lines expressing Vmw63. These variants synthesize greatly reduced levels of viral DNA, early and late polypeptides and over-express early proteins.

Western blot analysis and S1 nuclease mapping established that Vmw63 production in 1703-infected cells was affected only during immediate early conditions of
infection and that the alteration in synthesis was at the level of transcription. This demonstrated that wild-type amounts of Vmw63 are not required for progression to further stages of virus expression during immediate early conditions of infection. The synthesis of IE2 mRNA and Vmw63 returned to levels equivalent to wild-type during early and late infection. The small amount of polyadenylated IE2 mRNA present during a 1703 immediate early infection was reflected in the small amount of Vmw63 synthesized, indicating that the block was at the transcriptional or post-transcriptional level. During immediate early conditions of infection, IE2 mRNA was present in both the nuclear and cytoplasmic RNA fractions indicating that a quantity of IE2 mRNA was polyadenylated and therefore transported to the cytoplasm. To explain this, several possibilities were considered. These included (i) a mutation in the IE2 immediate early-specific promoter TAATGARAT, (ii) the genes deleted in 1703 function to trans-activate IE2 or to enhance the stability of the transcript or (iii) antisense transcripts initiating from the IR, copy of the IE1 promoter controlled IE2 mRNA production. We shall discuss each of these explanations in turn.

The first possibility was studied by sequence analysis of the promoter region of IE2. A requirement for trans-activation of IE2 by the Vmw65–cellular factor complex was demonstrated by Ace et al. (1989). This group mutated the gene encoding Vmw65 in such a way as to abolish the trans-activating function of the protein. Cells infected with the resultant recombinant virus expressed reduced amounts of IE2 mRNA, demonstrating that to some extent the gene required functional Vmw65. Approximately an eighth of the wild-type amount of Vmw63 was expressed by 1703 during immediate early infection. This possibly indicates that the IE2 immediate early promoter was not recognized by the Vmw65–cellular factor complex through a mutation within the TAATGARAT immediate early consensus sequence. The product of IE5 was synthesized in amounts equivalent to the wild-type infection, indicating that Vmw65 was able to trans-activate other immediate early genes normally. The possibility of a mutation in the promoter was investigated by sequence analysis of IE2. This demonstrated that the immediate early promoter region was homologous to the published wild-type sequence (McGeoch et al., 1988). This conclusively shows that Vmw65 is capable of trans-activating 1703 immediate early genes and that a mutation in the promoter region of the 1703 IE2 gene is not responsible for the pattern of Vmw63 synthesis.

Sequencing of the 1703 DNA fragment containing the deletion has shown that there are 555 bp remaining between the 3' end of IE2 and the deletion endpoint. The deletion removes 343 bp of the 3' end of UL55, leaving its promoter elements and part of the open reading frame intact. All of UL56 and the 3' end of the IR, copy of IE1 are deleted as far as the first intron of IE1. Roles for UL55 and UL56 have been suggested. For example, Block et al. (1991) indicated that either gene product may act to control Vmw63 synthesis. However, it has already been shown that UL55 and UL56 are dispensable in tissue culture (Brown et al., 1984; Harland & Brown, 1988; MacLean & Brown, 1987a, b). Several variants have been isolated and characterized (MacLean & Brown, 1987b; Junejo et al., 1991; Rosen & Darai, 1985) whose deletions affect the UL55/UL56 region of the genome and do not appear to synthesize wild-type levels of Vmw63 during infection. There is no published evidence of an antisense RNA mechanism operating in cells infected with these viruses and the pattern of Vmw63 synthesis is attributed to the proximity of the deletion to the 3' end of the IE2 gene. In 1703-infected cells, Vmw63 synthesis returned to wild-type amounts after insertion of the poly(A) signal, indicating that transcripts initiating from the IE1 promoter are responsible for the pattern of Vmw63 synthesis and that UL55 and UL56 play no role in the regulation of IE2 mRNA or protein production. To ensure that the properties of 1703 could not be attributed to a mutation other than the deletion, a wild-type recombinant of 1703 was constructed by cotransflecting BgII f' from 1703 into 17' DNA. The previously cloned HpaI m' fragment was not used for this procedure because there is only about 55 bp between the deletion endpoint and one end of HpaI m', which may have resulted in inefficient recombination. The recombinant 1750, generated from the BgII f'–17' cotransfection experiment, produced IE2 gene products in amounts similar to those produced by 1703. This indicates that the deletion was responsible for the underproduction of Vmw63.

The preceding two hypotheses fail to explain adequately the pattern of Vmw63 synthesis by 1703. A third possibility is that Vmw63 synthesis in 1703-infected cells is controlled by the production of a transcript initiating from the intact promoter of the IR, copy of IE1. In 1703PA, an HSV-2 poly(A) signal was placed in the correct orientation to result in termination of a potential antisense transcript before reaching IE2 coding sequences. We successfully detected a small transcript which corresponded to the truncated mRNA between the IR, copy of the IE1 promoter and the inserted poly(A) signal. When 1703PA IE2 gene products were characterized, it was shown that the synthesis of Vmw63 returned to wild-type levels during all transcriptional conditions. This could be due to transcripts initiating from the IR, IE1 promoter.

Further work is required to provide definite proof that Vmw63 production by 1703 is temporally controlled by
an antisense transcript, including the detection of the transcript in 1703-infected cells. The data presented do not give conclusive proof of a link between Vmw63 transcript in 1703-infected cells. The data presented do suggest the possibility of controlling the production of defined viral proteins in a temporal manner using antisense technology, but without the requirement for the co-infection of virus and antisense genes.

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References


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