Sequence of the Putative Origin of Replication in the UL Region of Herpes Simplex Virus Type 1 ANG DNA

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SUMMARY

Interest has been stimulated concerning the region mapping between 0.38 and 0.42 on the prototype configuration of the herpes simplex virus type 1 (HSV-1) genome due to the high probability of the presence there of a second origin of DNA replication. A 960 bp restriction fragment (HinfI E) of a class II defective HSV-1 ANG DNA has been sequenced using the viral DNA rather than molecularly cloned DNA. This fragment includes the BamHI U/R cleavage site, mapping at approximately 0.4. Part of the sequence derived in this study displays homology with the origins of DNA replication contained in TRs/IRs of HSV-1 and HSV-2 DNA. The homologous region comprising 76 bp occurs as two copies, each of which contains two palindromically arranged copies of an 8 bp sequence identical to the ‘consensus’ sequence reported to be part of the origin of DNA replication at the terminus of the mammalian adenoviruses. It can be deduced from a comparison of this structure to the TRs/IRs origin of HSV-1 and HSV-2 that there are two origins of replication in the UL region of HSV-1 ANG DNA. Assuming that the orientation of the consensus sequence is relevant to the direction of DNA replication, one can conclude that the UL origin(s) of HSV-1 ANG is (are) bidirectional. It has not yet been possible to clone DNA fragments molecularly which include the region spanning the UL origin(s) of HSV-1 DNA.

INTRODUCTION

The genome of herpes simplex virus (HSV) is a linear double-stranded DNA consisting of two covalently linked segments L (mol. wt. 82 × 10^6) and S (mol. wt. 18 × 10^6) (Fig. 1). Both segments contain unique sequences UL and US and are bracketed by two inverted repeats TRL/IRL and TRS/IRS, respectively. The repeats have in common a region of about 300 to 400 bp which is designated as the ‘a’ sequence. This sequence is located as a direct repeat at the termini of the HSV genome and in an inverted orientation at the L–S joint. The segments L and S can invert relative to each other, giving rise to four isomers of the HSV genome which exist in equimolar amounts (Sheldrick & Berthelot, 1974; Graffstrom et al., 1974; Hayward et al., 1975; Clements et al., 1976; Wilkie & Cortini, 1976; Delius & Clements, 1976; Skare & Summers, 1977; for review, see Roizman, 1979).

Investigation of replicating HSV-1 DNA by electron microscopy (Friedmann et al., 1977) suggested that the HSV-1 genome contains two origins of DNA replication. One is present in two copies, as it is located in the TRS and in the IRS repeat, whereas the other is located in the UL region between map positions approximately 0.385 and 0.435. It has also been shown that the HSV-1 TRS/IRS origin of replication is contained in the repeat units of repetitive class I defective HSV-1 genomes (Vlazny & Frenkel, 1981). Defective HSV genomes, which accumulate in the virus progeny during serial passages at high multiplicity of infection, have been shown to contain DNA consisting of tandem repetitions of short regions of the parental viral genome (for

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Two classes, I and II, of defective HSV-1 DNA have been identified: the repeat units of class I, originating from either TRs or IRs and the repeat units of class II defectives comprising the S terminus of the parental molecule linked to UL sequences mapping within the region 0.36 to 0.43 units (Frenkel et al., 1976; Kaerner et al., 1979). The class I defectives contain the TRs/IRs origin of replication (Stow, 1982; Murchie & McGeoch, 1982; Stow & McMonagle, 1983). From the sequence arrangement of class II defectives and from the findings of Spaete & Frenkel (1982) concerning the propagation of cloned class II defectives in mammalian cells, it must be concluded that the class II defectives contain an origin of DNA replication originating from the UL segment.

It has been shown that HSV-1 DNA fragments comprising sequences between coordinates 0.4 and 0.415 display deletions when they are cloned in Escherichia coli (Spaete & Frenkel, 1982; Weller et al., 1983). It has been suggested that these deletions might comprise in part the putative UL origin. This assumption was based upon the finding of Spaete & Frenkel (1982) that, following propagation of the cloned repeat unit of a class II defective HSV-1 Patton DNA in tissue culture in the presence of helper virus, the deleted sequences were always repaired in the resulting chimeric genomes. This apparent obligatory requirement for repair strongly argues that at least part of the deleted sequence is involved in replication and presumably contains an origin.

Recently, the origins of replication in TRs/IRs of HSV-1 DNA (Murchie & McGeoch, 1982; Stow, 1982; Stow & McMonagle, 1983) and HSV-2 DNA (Whitton & Clements, 1984) have been sequenced and, in the case of HSV-1, functionally proven (Stow, 1982; Stow & McMonagle, 1983). Here we describe the presumptive UL origin of replication in HSV-1 Angello (HSV-1 ANG) DNA, which we identified by nucleotide sequencing of the repeat unit of a class II defective DNA derived from this virus.

As with HSV-1 standard DNA, it was not possible to clone fragments of the repeat unit of the defective DNA corresponding to those mapping between 0-40 and 0-415 units on the parental viral genome, into E. coli without incurring a deletion. Thus, it was necessary to use native defective DNA for fine mapping and nucleotide sequencing.

METHODS

Virus and cells. HSV-1 ANG was propagated in African green monkey kidney cells RC-37 Rita as described previously (Schröder et al., 1975/76). Virus pools containing high percentages of class II defective DNA were obtained by serial virus passages at high m.o.i. as reported previously (Schröder et al., 1975/76; Stegmann et al., 1978).

HSV-1 ANG standard and class II defective DNA. HSV-1 ANG standard and class II defective DNA were prepared as described by Kaerner et al. (1979).

Preparation of recombinant plasmid DNA. E. coli strain HB101 containing the recombinant plasmids was grown in L-broth supplemented with 100 μg/ml ampicillin overnight in a bench-top shaker (Infors, Basel, Switzerland). The isolation of the plasmid DNA from the bacteria was essentially according to Birnboim & Doly (1979).

Labelling of DNA 3' and 5' ends with 32P. Labelling of the 3' and 5' termini of restriction fragments was essentially as described by Maniatis et al. (1982). In the case where the fragments were the product of restriction endonuclease BstNI, reverse transcriptase from avian myeloblastosis virus (AMV) was used for the 'fill-in' reaction.

Determination of nucleotide sequences. The chemical degradation method of Maxam & Gilbert (1980) was used. Uniquely labelled fragments isolated either from low-melting agarose gels as described below or from polyacylamide gels using the 'crush and soak' method of Maxam & Gilbert (1980) were subjected to the chemical degradation reactions specific for G, G+A, C+T and C residues.

Isolation of DNA fragments from low-melting agarose. The relevant fragment bands were cut out of the gels, and 0.5 ml 0.01 M-Tris–HCl pH 7.5, 0.001 M-EDTA and 0.01 M-NaCl (TEN buffer) was added to the gel slice. The mixture was heated to 65 °C for 5 to 10 min until the agarose was melted. After allowing the melted agarose to equilibrate at 37 °C it was extracted three times with 80% phenol equilibrated with TEN. The final aqueous phase was concentrated to about 50 μl with butan-2-ol (Stafford & Bieber, 1975) and the DNA was precipitated with ethanol.

Enzymes and 32P label. All the restriction enzymes and DNA modifying enzymes used in this study were purchased from either Bethesda Research Laboratories, New England Biolabs, or Boehringer Mannheim, except for the AMV reverse transcriptase which was purchased from Life Sciences, St Petersburg, Fla., U.S.A. 32P-labelled nucleoside triphosphates were purchased from Amersham.
**RESULTS**

Localization of a deleted sequence in a fragment of a class II HSV-1 ANG DNA cloned in E. coli

The origin of the defective HSV-1 ANG DNA on the parental viral genome (Kaerner et al., 1979; Schröder et al., 1975/76; Knopf et al., 1983) is schematically illustrated in Fig. 1 and cleavage site maps for several restriction endonucleases in its 8.55 kb repeat unit elucidated in our laboratory are shown in Fig. 2.

Apart from the routinely applied methods of mapping, use was made of several restriction endonucleases whose recognition sites include non-specific bases. For example, the enzyme *Hind*Ⅲ recognizes the sequence 5'-GNANTC-3', where N and N' can be any pair of complementary bases. After cleavage it was possible to label a subset of the termini of the resulting fragments by filling in the 5' overhang as described below using one α-32P-labelled deoxynucleoside triphosphate and one to three unlabelled nucleotides. For example, termini where N is C would be exclusively labelled by [α-32P]dCTP whereas the corresponding neighbouring fragments would be consequently labelled with [α-32P]dGTP, as N' is G. This selectivity successfully helped in the ordering of the fragments.

A peculiarity of the HSV-1 strain ANG, namely a 478 bp sequence (c amp) mapping in the 'c' region of the S repeat (TRs/IRs), is variably amplified in the standard viral genome as well as in the repeat unit of the defective DNA (Kaerner et al., 1981; C. P. Gray & H. C. Kaerner, unpublished results). The variably amplified sequence is indicated as a cross-hatched box in Fig. 2.

The *BamHI* restriction fragment U of HSV-1 ANG DNA corresponds to *BamHI* V of the HSV-1 strains KOS and F (Knopf et al., 1983; Kaerner et al., 1983; Smiley et al., 1981; Locker & Frenkel, 1979) and is contained within the repeat unit of a class II defective derived from HSV-1 ANG DNA. Post et al. (1980) have shown that it is not possible to clone the corresponding fragment from HSV-1 F into *E. coli*. The fragment *BamHI* U was isolated from a digest of defective HSV-1 ANG DNA and cloned into pBR322. A comparison of the restriction enzyme analyses of a recombinant plasmid, pHD1001, with corresponding analyses of the purified native de-
Fig. 2. Scale maps of the cleavage sites of various restriction enzymes in the repeat unit of defective HSV-1 ANG DNA. Numbers represent kb. B, BamHI; B", BstEII; C, Clal; E, EcoRV; K, KpnI; P, PstI; Pv, PvuI; S, SalI; X, XhoI. A to L in the outer circle indicate the map positions of the HindIII restriction fragments. A to G in the inner circle indicate the map positions of DdeI restriction fragments. The omission of fragments DdeI E and F results from the two cleavage sites in the amplified sequence amp, one copy of which is shown in the map (cross-hatched box). The amplification of part of the sequences in TRs/IRs is a peculiarity of the strain HSV-1 ANG. The black box between 0 and 0.44 kb marks the 'a' sequence. The black box between 5.1 and 5.4 kb designates the putative origin of replication in UL which corresponds to the deleted sequence in the cloned fragments HindIII E and BamHI U. S, Sequences stemming from the TRs/IRs repeat; L, sequences from the UL region of the standard HSV-1 ANG DNA. The Angelotti BamHI fragment U corresponds to the BamHI fragment V of HSV-1 strains KOS and F and is completely contained in the repeat unit of the defective ANG DNA.

The deleted sequence in the fragment HindIII E of defective HSV-1 ANG DNA contains the putative UL origin of replication

The HindIII fragment E of native defective HSV-1 ANG DNA was purified from agarose gels of a corresponding restriction enzyme digest of the DNA and sequenced as described in Meth-
Fig. 3. When HSV-1 ANG DNA fragments comprising sequences between 0-40 and 0.415 map units are cloned into E. coli a sequence of about 300 bp is deleted in the HSV DNA insertion. A comparison of the fragments produced by the restriction endonuclease *HinfI* and a combination of *HinfI* and *BamHI*, between the recombinant plasmid pHDI01 containing the restriction fragment *BamHI* U of HSV-1 ANG DNA isolated from defective class II ANG DNA, the vector plasmid pBR322 and the native defective DNA (Def). The DNA samples were cleaved with *HinfI*, 3'-labelled with [α-32P]dATP, and for the right-hand part of the figure recleaved with *BamHI*. The samples were then electrophoresed into a 6% (30:1) polyacrylamide gel and the gel was autoradiographed. The 300 bp deletion became apparent in one of the *HinfI*/*BamHI* fragments: the undeleted and the deleted fragments are indicated by triangles. The numbers to the left of the figure indicate the size in base pairs of the fragments produced by the *HinfI* cleavage of pBR322. The bars and the corresponding letters to the right designate the *HinfI* restriction fragments of the native defective DNA shown in the third lane.

ods. Fig. 5 (a) illustrates the nucleotide sequencing strategy for the 960 bp fragment in which *HinfI*, *BamHI*, *BstNI* and *AvalI* sites were used.

Nucleotide sequencing of native DNA is complicated by the problems of preparing sufficient quantities of highly purified material and in that native HSV DNA usually displays nicks and gaps (Wilkie, 1973). These give rise to internal radioactive labelling and hence a high back-
Fig. 4. Comparison of part of the nucleotide sequences of the deleted cloned BamHI/HindI fragment ΔE" and of the corresponding native fragment E". Both fragments, the native and the cloned BamHI product were 3'-labelled as described and cleaved with HindI. The relevant bands were isolated from an agarose gel and sequenced according to the protocols of Maxam & Gilbert (1980). The beginning of the deleted sequence is marked by the horizontal arrow in the enlarged section of the sequence. The vertical arrows designate an 8 bp direct repeat (DR) involved in the deletion as described in the text.

ground in sequencing gels. In the present study a number of special labelling techniques were used to overcome these difficulties. For example, 3' labelling of restriction fragments in some cases was performed using reverse transcriptase and only one α-32P-nucleotide without adding unlabelled nucleotides. By this means the background of radioactive labelling in the nicks and gaps was considerably reduced as compared to the usual labelling with the Klenow fragment of DNA polymerase I. Furthermore, it was possible to label uniquely a number of the fragments produced by the restriction endonucleases BstNI or AvaII by using either [α-32P]dTPP or [α-32P]dATP as the label. Fragments were thus sequenced without further cleavage and loss of material during re-extraction from gels. The sequence obtained of the HindI E fragment is shown in Fig. 5(b). It displays a sophisticated system of palindromic sequences which is schematically depicted in Fig. 6(a).

The 296 bp deletion in the molecularly cloned BamHI U fragment was identified as the sequence between, and including one of, an 8 bp direct repeat. This 8 bp was alternately directly and invertedly repeated within the deleted region (Fig. 6a). The most significant feature of the deleted region was the presence of two perfect palindromes (positions 204 to 334 and 345 to 489 in Fig. 5b and 6a). The smaller palindrome (positions 204 to 334) is repeated within the larger (345 to 489). A 76 bp stretch within these palindromes between the positions 222 to 297 and 370
Fig. 5. (a) Nucleotide sequencing strategy of the fragment HindIII E of class II defective HSV-1 ANG DNA. Scale numbers: base pairs. The arrows indicate the sequencing directions and the sequenced stretches of the individual strands. The numbers on the right designate the restriction endonuclease with which the fragment was first cleaved before labelling of either the 5' or 3' terminus, or both 5' and 3': 1, HindIII sites, 5'-labelled; 2, BamHI sites, 3'- and 5'-labelled; 3, BstNI sites, 3' - and 5'-labelled; 4, AvaII sites, 3'-labelled. The deleted sequence in the cloned fragment BamHI U of HSV-1 ANG DNA is shown as a black box. (b) Nucleotide sequence of the fragment HindIII E of class II defective HSV-1 ANG DNA. The section of the sequence deleted in corresponding cloned material is marked by arrows.
Fig. 6. (a) Scheme of the arrangement of the various repeats, palindromes and the 8 bp consensus sequences in the fragment Hinf I of class 11 defective HSV-1 ANG DNA. The numbers below the black bar indicate positions within the sequence shown in Fig. 5(b). All other numbers represent base pairs. The thin arrows indicate direct and inverted repeats. The thick arrows denote the two alternative localizations of the ends of the deletion. ( ), Palindromes; AATAAA, possible polyadenylation site. (b) Sequence homologies between the TRs/IRs replication origins of HSV-1 and HSV-2 and the UL origin of HSV-1 ANG. Numbers at the left refer to the following. 1, Sequence of the first of the two large palindromes in the UL origin region as determined in the present study (see Fig. 6a). The palindrome is marked by the brackets at positions 204 and 334. 2 and 3, Homologous 76 bp sequences in the TRs/IRs origins of HSV-1 (Murchie & McGeoch, 1982; Stow, 1982; Stow & McMonagle, 1983) and of HSV-2, respectively (Whitton & Clements, 1984). With the exception of the 8 bp consensus sequence, which is underlined and/or overlined, only non-identical nucleotides are indicated. The numbers designate the beginning and the end of the homologous sequence and refer to our sequence (1), the sequence of Stow & McMonagle (2) and to the sequence of Whitton & Clements (3).

to 445, respectively, can be aligned with a highly homologous 76 bp stretch being part of the TRs/IRs origin regions of HSV-1 (Murchie & McGeoch, 1982; Stow, 1982; Stow & McMonagle, 1983) with only eight mismatches, as well as with a directly repeated 76 bp stretch within the TRs/IRs replication origin of HSV-2 (Whitton & Clements, 1984) with as few as six mismatches. These findings are illustrated in Fig. 6(b). The 76 bp sequence in the HSV-1 origin maps from positions 550 to 624 of the sequence of Stow & McMonagle (1983) and from 758 to 833 and from 898 to 973, respectively in the HSV-2 origin sequence of Whitton & Clements (1984).

An A-T-rich sequence occurring as two copies at the points of symmetry of the two large palindromes (Fig. 6a) contains the 8 bp 'consensus' sequence which is part of an important structural element of the replication origin of the adenoviruses (Aleström et al., 1982; Rijnders et
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al., 1983). As the A–T-rich sequence is palindromic, the consensus sequence also occurs in both DNA strands. This octamer is also found in the nearly perfect palindromic 76 bp sequence in the TR<sub>S</sub>/IR<sub>S</sub> origin of HSV-1 DNA as shown by Stow (1982) and Stow & McMonagle (1983) (Fig. 6b). However, only one copy is present in this type of origin whereas the U<sub>L</sub> origin region presented here contains altogether four copies, two in one sense and the other two in the opposite orientation.

The HSV-2 TR<sub>S</sub>/IR<sub>S</sub> origin (Whitton & Clements, 1984), in contrast to the HSV-1 TR<sub>S</sub>/IR<sub>S</sub> origin, occurs within two 137 bp direct repeats. However, each repeat contains only one copy of the consensus sequence (positions 806 to 813 and 847 to 853). Thus, in HSV-2 DNA this sequence occurs twice, but in the same orientation.

Close to the deletion (Fig. 5b) there is a 45 bp structure consisting of two 9 bp inverted repeats separated by 27 bp. Within this 27 bp an AATAAA sequence was identified which possibly could represent a polyadenylation site.

**DISCUSSION**

The work of Spaete & Frenkel (1982) and others (Weller et al., 1983) has strongly suggested that class II defective HSV-1 DNA must contain an origin of replication which does not map within the S repeats but within the U<sub>L</sub> region of HSV-1 DNA between 0.40 and 0.415 units. Recent work performed in this laboratory has shown the requirement for this particular region to be present in conjunction with the ‘a’ sequence in order to maintain the interfering capacity of the class II defective DNA (Schröder et al., 1984). The identification of this origin on the viral genome has proved difficult because the DNA region in which it was assumed to map could not be cloned in E. coli without deletions occurring. We thus purified the BamHI fragment U from native HSV-1 ANG class II defective DNA (Kaerner et al., 1979). The same fragment was cloned into pBR322 and propagated in E. coli. A comparison of restriction enzyme patterns of the native and the cloned material revealed a 296 bp deletion in the latter. This deletion maps close to the BamHI U/R and to the KpnI P/V cleavage sites on the HSV-1 ANG standard DNA which are contained in the repeat unit of the class II defective genome (Kaerner et al., 1979, 1981; Knopf et al., 1983). Similar deletions although of different sizes were reported in corresponding cloned DNA fragments of the HSV-1 strains Patton (Spaete & Frenkel, 1982) and KOS (Weller et al., 1983).

The deletion in the cloned BamHI U fragment was found to map within the HinfI E fragment of defective HSV-1 ANG DNA and its nucleotide sequence had to be elucidated using native material. The results appear to confirm the suggestion of Spaete & Frenkel (1982) and of Weller et al. (1983) that the putative U<sub>L</sub> origin of DNA replication of HSV-1 maps within the deleted region. Additional strong evidence for this conclusion apart from the mapping data is based on the homology of 76 bp sequences contained within the two large palindromic regions (Fig. 6a) with those of the TR<sub>S</sub>/IR<sub>S</sub> origins of replication determined for HSV-1 (Murchie & McGeoch, 1982; Stow, 1982; Stow & McMonagle, 1983) and HSV-2 (Whitton & Clements, 1984) (Fig. 6b).

Considering the presence and the palindromic arrangement of four copies of the consensus sequence which has been shown to be involved in the binding of the terminal protein and the adenovirus DNA polymerase to the origin of adenovirus DNA (Rijnders et al., 1983) our results suggest that there are two functional U<sub>L</sub> origins located closely together in the BamHI fragment U of HSV-1 ANG DNA. The second origin probably accounts for the approximately 150 bp difference in size between the BamHI fragment U of HSV-1 ANG DNA and the corresponding fragments BamHI V of the HSV-1 strains KOS and Patton. This could possibly indicate that the latter strains contain only one copy of the palindromic sequence, i.e. one origin in the BamHI V fragment. If one assumes that the orientation of the consensus determines the direction of DNA replication, as is assumed in the case of adenovirus DNA, it would suggest that the U<sub>L</sub> origin in contrast to the TR<sub>S</sub>/IR<sub>S</sub> origins of HSV-1 and HSV-2 is bidirectional.

The significance of the AATAAA sequence between nucleotide numbers 546 and 552 of the fragment HinfI E and whether or not it functions as a polyadenylation site are at present unknown.


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