Herpes Simplex Virus Defective Genomes: Structure of HSV-1 ANG
Defective DNA of Class II and Encoded Polypeptides

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SUMMARY

Sequence organization and origin of HSV-1 strain Angelotti (ANG) class II defective DNA (HSV-1 ANG dDNA1) were examined in detail by establishing physical maps and by molecular cloning. dDNA1 consists of concatemers of tandem repeat units in which sequences from the UL region spanning map coordinates 0.37 to 0.415 of standard HSV ANG DNA are covalently linked to TRs/IRs sequences. The size of the repeat unit was determined to be about 8.9 kilobase pairs (kb), comprising sequences of 7.3 kb from UL and 1.6 kb from TRs/IRs regions. UL sequences were delineated by restriction enzyme sites KpnI N-P and EeoRI F-M, and were colinear with the corresponding sequences of the standard (wild-type) virus genome. Expression of dDNA1 was studied in African green monkey kidney cells and in Xenopus laevis oocytes. A major polypeptide of approx. mol. wt. 135000 (135K) was overproduced, suggesting that this protein was encoded by dDNA1. By several parameters, e.g. size, immune cross-reactivity, and affinity for native and denatured DNA, the 135K polypeptide was identified as the major HSV DNA-binding protein. It was further shown that the repeat unit contains part of the DNA polymerase gene as demonstrated by its ability to rescue some mutations in this gene.

INTRODUCTION

Defective virus particles arise during serial undiluted propagation of herpes simplex viruses (HSV) and contain only a subset of the genomic information of standard (wild-type) virus (for review, see Frenkel, 1981). We have previously shown that HSV-1 strain Angelotti (ANG) is capable of generating different classes of defective particles when propagated at high m.o.i. (Schröder et al., 1975/76; Stegmann et al., 1978). Two types of defective HSV-1 ANG DNA have so far been characterized (Kaerner et al., 1979, 1981). dDNA1, representing class II defective HSV-1 DNA (Frenkel et al., 1980), was found to be resistant to restriction endonucleases XbaI, EcoRI, HindIII, HpaI and BglII. dDNA2, representing class I defective HSV-1 DNA (Frenkel et al., 1980), was resistant to HindIII and HpaI but sensitive to EcoRI, and was found to contain sequences exclusively from the S-region of HSV-1 DNA (Schröder et al., 1975/76; Stegmann et al., 1978; Kaerner et al., 1979).

Examination of the genome structure of dDNA1 revealed that dDNA1 purified from enveloped virions is composed of tandem repeat units. Restriction enzyme maps were established, and it was shown that the total population of dDNA1 molecules consists of different homopolymers, each of which is made up of identical repeats with a constant copy number of a 500 base pair (bp) insertion sequence originating from the TRs or IRs sequences of the HSV ANG genome (Kaerner et al., 1981).

It was the aim of this study to correlate the sequences of the dDNA1 repeat unit with those of the standard virus genome. dDNA1 was analysed in more detail by molecular cloning of dDNA1 restriction enzyme fragments and blot hybridization. In addition, the polypeptide-coding potential was studied. It was demonstrated that dDNA1 encodes for at least one
functional gene product expressed both in African green monkey kidney cells and in *Xenopus laevis* oocytes. The results confirm recent mapping data of the major HSV-1 DNA-binding protein and the HSV-1 DNA polymerase on the HSV-1 genome (Weller et al., 1983; Chartrand et al., 1979, 1980; Coen & Schaffer, 1980; Knopf et al., 1981).

**METHODS**

**Virus and cells.** HSV-1 ANG was propagated in African green monkey kidney (AGMK) cells (Rita clone-37, Italidiagnostics, Rome, Italy), and virus pools enriched in defective particles of series I were obtained by undiluted passage as described previously (Schröder et al., 1975/76; Kaerner et al., 1979).

**Labelling of DNA.** Isolation of HSV-1 ANG standard and defective DNA has been described in detail previously (Stegmann et al., 1978). Nick-translation of DNA was carried out according to the method described by Rigby et al. (1977) employing $[\alpha^{32}P]dCTP$ (3000 Ci/mmole; Amersham Buchler) and 0.5 mM each of dATP, dGTP and dTTP; incubation was at 37 °C for 30 min. For 3' end-labelling of DNA, the Klenow fragment of *Escherichia coli* DNA polymerase I (Bethesda Research Laboratories) was used to fill in the first position with the appropriate $[\alpha^{32}P]dNTP$. After labelling, DNA was purified by gel filtration (Sephadex G75) and the DNA concentrated by extraction with butan-2-ol and by ethanol precipitation.

**Blot hybridization.** The method of Wahl et al. (1979) was employed using nick-translated HSV DNA or plasmid DNA as probe and omitting dextran sulphate.

**Recombinant DNA and bacteria.** Recombinant plasmids between pAT153 (Twigg & Sherratt, 1980; a gift of Dr R. Thompson, Institute of Virology, Glasgow) and HSV ANG dDNA1 were constructed by standard cloning procedures (Maniatis et al., 1982). For transformation, *E. coli* K12 HB101 cells and *E. coli* dam- NZ3050 cells (kindly provided by Dr K. Chowdhury, University of Heidelberg) were used, and growth was carried out in Luria Bertani broth at 37 °C. Isolation of recombinant plasmids both on small and large scale was performed according to the method described by Birnboim & Doly (1979). Cloning procedures were carried out under Category L2B2 containment conditions according to the guidelines ‘Bundesminister für Forschung und Technologie’, August 7, 1981, having obtained the approval of the ‘Bundesgesundheitsamt’, Berlin.

**DNA-cellulose chromatography.** Denatured and native DNA-cellulose was prepared as described previously (Knopf et al., 1976) using 20 mg salmon sperm DNA (Sigma) per g purified cellulose. Columns containing 1 ml bed volume of DNA-cellulose were washed extensively with column buffer as described by Purifoy & Powell (1976) and then loaded with cellular extracts. Elution was performed stepwise using different concentrations of NaCl in column buffer and collecting 10 fractions of 0.5 ml per salt step.

**Immediate-early polypeptides.** Immediate-early polypeptides were prepared as described by Preston (1979a). Confluent AGMK cells growing in 50 mm diam. Petri dishes were either mock-infected or infected with defective or standard virus at an m.o.i, of 20 in the presence of 200 μg/ml cycloheximide for 1 h at 37 °C. Infected cells were maintained in M199 supplemented with 5% foetal bovine serum and 200 μg/ml cycloheximide for 5 h at 37 °C. Monolayers were washed three times for about 2 min with prewarmed medium. Labelling was then carried out in the presence of 2.5 μg/ml actinomycin D in 1 ml of one-tenth the concentration of M199 and 100 μCi $^3$H-labelled amino acid mixture for 2 h at 37 °C. After removal of medium and washing, cells were lysed in 250 μl sample buffer for SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970).
micromanipulator. Injected oocytes were incubated at 18 °C in modified Barth medium (Gurdon, 1976) and [³⁵S]methionine (0.8 mCi/ml) was added at appropriate times to label polypeptides.

**Gel electrophoresis.** Agarose gel electrophoresis was carried out in horizontal and vertical slab gels at 4 °C and 3 V/cm using 40 mm-Tris–HCl pH 8.3, 20 mm-sodium acetate and 2 mm-EDTA as electrophoresis buffer in the absence or presence of 0.5 μg/ml ethidium bromide. Gels were either dried and exposed for autoradiography or stained with ethidium bromide and photographed under u.v. light. DNA fragments were isolated electrophoretically from agarose gels (Maniatis et al., 1982).

Protein samples were precipitated with 20% trichloroacetic acid and washed twice with ether prior to PAGE (Knopf & Kaerner, 1980). Two-dimensional gel electrophoresis was carried out as described by O'Farrell et al. (1977). In the first dimension separation was achieved by non-equilibrium pH gradient electrophoresis (NEPHGE) and in the second dimension by SDS–PAGE. Oocyte extracts were prepared in lysis buffer. Aliquots of supernatants cleared by centrifugation and free of lipids were treated with DNase I and RNase A and then analysed as described. Protein standards were added to all samples to monitor electrophoretic mobilities.

**Marker rescue.** The method of Stow et al. (1978) was used. Cotransfection of BHK-21 cells was performed with 1 μg each of intact infectious HSV-1 DNA from a temperature-sensitive (ts) mutant and plasmid DNA. Incubation was performed for 5 days at 34 °C in Dulbecco's minimal essential medium containing 5% foetal bovine serum. Cells were then harvested with the growth medium, disrupted by sonic treatment and the infectious progeny virus was titrated on BHK cell monolayers at 34 °C and 38.5 °C.

**RESULTS**

**Structure of the class II defective HSV ANG genome**

For a study of the expression of the class II defective HSV ANG genome (dDNA1), it was important to investigate the organization and coding capacity of its DNA sequences. In a previous report we have shown that the head-to-tail joined tandem repeats of dDNA1 contained sequences homologous to the UL as well as TRs/IRs regions. A variably amplified sequence of approximately 500 bp which shares homology with sequences from the TRs/IRs region of the standard virus genome is contained within the dDNA1 genome (Kaerner et al., 1981). As a consequence of this amplification, restriction fragments of dDNA1 comprising this sequence, for example all fragments A and right-hand terminal fragments of dDNA1 (Fig. 1 e and 2a to c), appear as step-ladders of bands migrating with an increment of 500 bp in agarose gels. To examine the organization of dDNA1 sequences, authentic as well as cloned dDNA1 fragments were compared with the corresponding sequences of the standard virus genome with respect to restriction enzyme analysis and sequence homology.

**Sequence homology of authentic dDNA1 and standard virus DNA restriction fragments**

**BamHI** and **KpnI** maps of HSV-1 ANG standard DNA were established using different cloned overlapping restriction fragments. The maps obtained are shown in Fig. 1 (b) and the corresponding BamHI and KpnI restriction patterns are shown in Fig. 1 (c and d, lane 1). Due to heterogeneity in the Tₜ and Tₛ regions of HSV-1 ANG standard virus genome, five DNA fragments both of BamHI- and KpnI-restricted DNA appear as sets of minor bands differing in size by constant increments as follows: the terminal (BamHI R, S and KpnI K, I, R) and L-S joint fragments (BamHI K and KpnI C, E), and BamHI N and Y.

Fig. 1 (d) summarizes the results of blot-hybridization of uncleaved dDNA1 and of dDNA1 fragments to BamHI- and KpnI-restricted HSV-1 ANG standard DNA as well as dDNA1. dDNA1 hybridized to BamHI standard DNA fragments G, U and Q which derive from Uₜ region, to the L-S joint fragments K, to the S-terminal fragment R, and to a lesser extent to the L-terminal fragment S. Isolated dDNA1 'step-ladder' fragment XhoI A (Fig. 1 a) exhibited the same hybridization to BamHI standard DNA fragments as the uncleaved dDNA1 (Fig. 1 d, lane 4). Furthermore, as similarly observed for uncleaved dDNA1, XhoI A hybridized to the following KpnI standard DNA fragments (Fig. 1 d, lane 10): to P, V, A' from Uₜ ; to the L-S joint fragments C, E; to the S-terminal fragments K, I; and weakly to the L-terminal fragment R. Isolated dDNA1 fragment XhoI D hybridized to standard BamHI G and KpnI P, and dDNA1 fragment XhoI E hybridized to standard BamHI G and U, and KpnI P. Since the analysed dDNA1 fragments XhoI A, D and E comprise the sequence information of one single repeat unit
The sequences, homologous to the TRs/IRs region and containing the variably amplified 500 bp element (subsequently termed 'ac' sequences), are located within dDNA1 step-ladder fragment XhoI A. Their location was further defined by analysing dDNA1 fragments overlapping with (BamHI D) or contained within (KpnI D) step-ladder fragment XhoI A (see Fig. 1a). Hybridization as well as comparison of the electrophoretic mobilities revealed that BamHI D and KpnI D were identical to standard fragments BamHI U and KpnI V respectively (Fig. 1d,e).

These results indicate that dDNA1 shared homology to the following UL sequences of the standard genome in the order of BamHI G, U, Q and KpnI P, V, A'. From the blot-hybridization data obtained with step-ladder fragment XhoI A as well as from size and heterogeneity of the right-hand terminal dDNA1 fragments, we concluded that the 'ac' sequences were joined to the UL sequences and formed the right-hand terminus of the defective genome as proposed from restriction mapping of dDNA1 (Fig. 1a, black boxes). The junction between UL and TRs/IRs sequences probably occurred at the right-hand end of the UL sequences within KpnI A sequences, as derived from the following. (i) dDNA1 is not cleaved by EcoRI, and the missing EcoRI F-M site of standard DNA is located within fragment KpnI A' at 0-415 fractional genome units (Chartrand et al., 1979, Fig. 1b). (ii) Hybridization of dDNA1 to KpnI X, the standard fragment adjacent to the right of KpnI A', was not observed (Fig. 1d, lanes 10 to 13), nor to standard fragment EcoRI M (data not shown).

For the sequences of the left-hand terminus of dDNA1 the following was deduced: dDNA1 fragment XhoI C, D and E comprise sequences of standard KpnI P but no sequences of KpnI N, the fragment adjacent to the left of KpnI P. Since the left-hand terminal dDNA1 fragment KpnI B was about 400 bp larger than expected for KpnI P, as demonstrated by comparing their electrophoretic mobilities as shown in Fig. 1(e), one could assume that in addition other sequences are linked to KpnI P sequences within the very left-hand terminal dDNA1 fragment XhoI C. The UL sequences could therefore maximally comprise sequences starting from KpnI N-P to EcoRI F-M site [7-3 kilobase pairs (kb)] mapped between 0-37 and 0-415 fractional genome units of the standard virus genome (Fig. 1b). The observation that ClaI is a single-cut enzyme for dDNA1, cleaving in the middle of BamHI fragment U (Fig. 2a), allowed the size of the tandem repeat unit to be estimated at 8-9 kb. The minimal size of the 'ac' sequences hence comprised 1-6 kb.

**Analysis of cloned dDNA1 restriction fragments**

To obtain additional confirmation of the proposed organization of the dDNA1 genome, we performed molecular cloning experiments. BamHI and ClaI dDNA1 fragments were inserted into pAT153, a plasmid derivative of pBR322 (Twigg & Sherratt, 1980).

Characterization of the recombinant clones by restriction enzyme analysis revealed that the majority of them displayed deletions of dDNA1 sequences. For example, 60% of the dDNA1

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Fig. 1. Organization and origin of HSV ANG dDNA1. (a) Model of dDNA1 genome according to Kaerner et al. (1981) illustrating the concatemeric arrangement of tandem repeat units composed of sequences of dU1 and of repetitive sequences ('ac') of the S-region of HSV-1 DNA (black boxes) containing a variably amplified 500 bp sequence; n equals the number of repeats per unit-length molecule of dU1 and varies from 9 to 17 depending on the copy number of inserts per repeat. The KpnI, XhoI and BamHI restriction enzyme sites and the notation of the resulting DNA fragments obtained by two neighbouring tandem repeats are indicated. All dDNA1 fragments A vary by amplification of a 500 bp sequence giving rise to a 'step-ladder pattern' of these fragments in agarose gels (see e). (b) BamHI and KpnI restriction enzyme maps of standard HSV-1 ANG DNA. (Mol. wt. shown x 10^-6). (c) The BamHI restriction pattern of standard wild-type (wt) HSV ANG DNA visualized by ethidium bromide staining. (d) Sequence homology of dDNA1 to standard HSV-1 ANG DNA. 32P-labelled standard (wt) and defective DNA (dDNA1), and isolated XhoI, BamHI and KpnI dDNA1 fragments designated according to the model presented in (a) were hybridized to nitrocellulose strips containing unlabelled BamHI and KpnI fragments of standard (wt) and defective DNA as specified under each individual autoradiogram. (e) KpnI DNA fragment pattern of standard (wt) HSV-1 ANG DNA and dDNA1 visualized by ethidium bromide staining in a 0.8% agarose gel. (Mol. wt. shown x 10^-6.)
**BamHI** recombinant clones exhibited sequence homology to **BamHI** U. However, none of these clones contained inserts of the size of standard fragment **BamHI** U (2.3 kb). Instead, inserts of 2 and 2.7 kb were observed, and fine mapping of restriction enzyme sites revealed that sequences were both deleted and apparently rearranged. Other investigators have reported similar observations and demonstrated that sequences located between the **KpnI** P-V site and the **BamHI** U-Q site of the HSV-1 KOS standard virus genome were deleted in cloned HSV DNA fragments (Weller et al., 1983; C. P. Gray, unpublished data). Of particular interest was the characterization of recombinant plasmids containing **ClaI** dDNA1 fragments. As mentioned above **ClaI** cleaves the dDNA1 repeat unit only once and generates three fragments (8-9, 5.1 and 4.25 kb): a major step-ladder fragment, containing the sequences of one repeat, and two terminal fragments with the larger one forming a minor step-ladder (Fig. 2a). In several independent cloning attempts, only the left-hand terminal sequences of the **ClaI** restricted dDNA1 could be cloned.

Although we do not have a simple explanation of how deletions in cloned dDNA1 sequences arise, three recombinant clones which were examined in detail with respect to deletions observed after propagation of the clones in **recA**- bacterial hosts are discussed.

1. Plasmid HD1003, containing an 8.3 kb **BamHI** dDNA1 insert, hybridized to standard HSV ANG and HSV KOS **BamHI** fragments Q (U_L region) and S, R, K (terminal and joint fragments), as well as to standard HSV ANG **KpnI** fragments V, A' (U_L region) and C, E, I, K, R (terminal and joint fragments) (Fig. 2b). Furthermore, it hybridized strongly to dDNA1 **BamHI** fragments A, B and to a lesser extent to C. Hybridization as well as restriction sites (Fig. 2d) indicated that HD1003 was probably derived from a **BamHI** dDNA1 step-ladder fragment. It contained part of the U_L sequences (1-4 kb) as shown for the **BamHI** step-ladder fragment (Fig. 1a) linked to an as yet unidentified portion (6.9 kb) of 'ac' sequences. The residual U_L sequences of the step-ladder (**KpnI** P sequences) were apparently deleted during propagation in the **recA**- bacterial host.

2. Plasmid HD1030, containing a 7.3 kb **BamHI** dDNA1 insert, hybridized to standard HSV ANG and KOS **BamHI** fragments G, Q, J' (U_L region) and R, K, weakly to S (terminal and joint fragments), as well as to standard HSV ANG **KpnI** fragments V, A', M (U_L region) and C, E, I, K, R (terminal and joint fragments) (Fig. 2b). The hybridization data and the restriction enzyme analysis as shown in Fig. 2(c, d) demonstrated that plasmid HD1030 could contain an undeleted **BamHI** dDNA1 step-ladder fragment A2 (Fig. 1a). Various restriction sites of the dDNA1 insert were arranged as seen for the U_L sequences of dDNA1 (Fig. 2d). In addition, this clone contained the **BamHI** J' (0.6 kb) fragment, probably as a result of contaminating standard DNA fragments present in the dDNA1 preparation used for molecular cloning.

3. Plasmid HD1344, containing a 4.3 kb **ClaI** dDNA1 insert, hybridized to standard HSV ANG **BamHI** fragments G, U (U_L region) and to S, R, K (terminal and joint fragments) (Fig. 2b). Restriction mapping of HD1344, cloned in dam- bacteria, indicated that all the restriction sites found on the left-hand terminal portion of the dDNA1 genome were present (Fig. 2d), and that only the **ClaI** site right next to the **HindIII** site of pAT153 was deleted.

Since both sequence homology and restriction enzyme analysis indicated that plasmid HD1344 contained the U_L sequences of the left-hand terminal **ClaI** dDNA1 fragment, we were interested in identifying the sequences of this dDNA fragment insert which hybridized to TR_5 and TR_6 regions of the standard virus genome. Assuming that these sequences are probably located near the deleted **ClaI** site, we have examined the sequence at the point of insertion.

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Fig. 2. Molecular cloning of dDNA1. (a) Digestion of 3'-32P-labelled dDNA1 with restriction enzymes **BamHI** and **ClaI**. Autoradiography of a 0.8% agarose gel. (b) Blot hybridization of 32P-labelled standard (wt) and recombinant plasmid DNA (HD1003, HD1030 and HD1344) to unlabelled **BamHI** and **KpnI** fragments of standard (wt) HSV-1 ANG and KOS DNA and dDNA1 as indicated below each individual autoradiogram. (c) Analyses of the **BamHI** restriction profiles of HD1030 DNA and dDNA1 by agarose gel electrophoresis. **EcoRI** λ DNA and **HindIII** SV40 DNA fragments served as size standards. (d) Scale maps of the cleavage sites of several restriction enzymes on dDNA1 and the recombinant plasmids HD1003, HD1030 and HD1344: **BamHI**, B; **ClaI**, C; **EcoRI**, E; **HindIII**, H; **KpnI**, K; **PstI**, P; **XhoI**, X.
starting from the HindIII site of pAT153 and leading into the dDNA1 insert (Fig. 2d). By 3'-end labelling of the HindIII site, we found that the base composition of the dDNA1 sequences directly adjacent to this site strikingly resembled the 'a' sequence portion located at the junction between a' and c' sequences (Fig. 3; Mocarski & Roizman, 1981, 1982; Davison & Wilkie, 1981). The orientation of the 'a' sequence determined from the presence of the direct repeat I (DRI) and from the unique Avall site, indicated that 'c' sequences were probably not present in the cloned Clal dDNA1 fragment. In support of this, the hybridization of plasmid HD1344 to BamHI-restricted dDNA1 could be interpreted. When autoradiograms were overexposed as shown in Fig. 2(b), weak hybridization to the right-hand terminal BamHI step-ladder fragments B containing 'ac' sequences was detected. Furthermore, as shown above (Fig. 2d), plasmid HD1003 containing 'ac' sequences and none of the U sequences of BamHI dDNA1 fragment C, hybridized weakly to these left-hand terminal fragments, again indicating that probably only 'a' sequences were present at the very left-hand terminus of the dDNA1 genome.

In conclusion, cloning of dDNA1, possessing both a U region (0.4 map units) known to be deleted during cloning, as well as highly reiterated sequences (500 bp amplification), lead readily to recombinant clones with partially deleted as well as rearranged sequences. However, identification of their U sequences further confirmed the colinearity of the U portion of the dDNA1 sequences with the U region of the standard genome mapping between 0.37 and 0.415 fractional genome units.

The coding potential of dDNA1 sequences

The following proteins have been mapped within the identified U sequences of dDNA1: the β-polypeptide ICP8 (Morse et al., 1978) and a 136' (130K) polypeptide (Marsden et al., 1978). These polypeptides are identical and represent the major HSV DNA-binding protein (Littler et al., 1981). In addition, parts of the sequences of the HSV DNA pol gene map within the dDNA1 U sequences. Physical mapping of ts and drug-resistant (phosphonoacetic acid-resistant, PAA R) mutants of HSV DNA polymerase by intertypic marker rescue (Chartrand et al., 1979, 1980) located at least part of the HSV pol gene to a 4-6 kb region spanning the Kpnl fragments V, A' and X with a coding capacity for a 150K polypeptide, the estimated size of the enzyme (Powell & Purifoy, 1977). dDNA1 contains the sequences of Kpnl V and about 60% of the sequences of Kpnl A'. The PAA R mutation of the majority of DNA pol mutants analysed to date have been mapped within the Kpn I A' sequences missing in dDNA1 and within Kpnl X (Chartrand et al., 1979, 1980; Knopf et al., 1981). This implies that the defective genome does not encode the complete HSV DNA polymerase. In support of this we found that the specific
Table 1. Marker rescue of tsC7 and tsD9

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<th>DNA</th>
<th>Marker rescue efficiencies with mutant DNAs*</th>
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<tr>
<td></td>
<td>tsC7</td>
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<tr>
<td>None</td>
<td>&lt;0.013</td>
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<tr>
<td>Wild-type ANG, sheared†</td>
<td>93.8</td>
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<tr>
<td>HD1003</td>
<td>0.7</td>
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<tr>
<td>HD1030</td>
<td>1.9</td>
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* Determined from plating efficiencies as (p.f.u. per ml at 39 °C/p.f.u. per ml at 34 °C) x 10³.
† Wild-type ANG DNA was sheared by forcing 0-6 ml of a solution containing 18 μg/ml DNA in HEPES-buffered saline three times through a 21 gauge needle. The plating efficiencies at 34 °C and 39 °C of the sheared DNA were less than 5 p.f.u./ml.

HSV pol activity of extracts of cells that were infected with virus stocks containing about 25% dDNA1 defective particles was about half that of wild-type-infected cell extracts, and did not correlate with the amount of dDNA1 sequences present in infected cells. However, marker rescue studies using the recombinant clones HD1003 and HD1030 to rescue the HSV-1 KOS mutants tsC7 and tsD9, both of which map within the HSV pol gene (Chartrand et al., 1980), revealed that dDNA1 sequences are capable of rescuing the tsC7 lesion mapped within KpnI V but not the tsD9 mutation mapped within sequences between KpnI A' to BglII I and KpnI X-C sites (Table 1). These results indicate that dDNA1 contains part but not all of the sequences of the HSV pol gene.

From DNA sequencing, polypeptide and transcript mapping data of the immediate-early polypeptides, one can further conclude that the dDNA1 sequences (1.6 kb) confined to BamHI R and K could not code for the immediate-early polypeptide ICP4, since only the 3'-end of the mRNA lies within the respective sequences at an approximate distance of 1.2 kb from the S-end of the standard wild-type virus genome (Watson et al., 1981; Anderson et al., 1980; Mackem & Roizman, 1980; Clements et al., 1979; Davison & Wilkie, 1981).

Synthesis of dDNA1-encoded viral gene products

Information about the polypeptides encoded by dDNA1 and their regulation were obtained by analysing the polypeptides induced in AGMK cells by a virus pool enriched in dDNA1 defective virus, and by microinjection of purified dDNA1 into the nuclei of X. laevis oocytes. Polypeptide synthesis was studied both with or without prior cycloheximide treatment. Since HSV-specific transcription as well as translation is regulated in a 'cascade-type fashion' (Honess & Roizman, 1974), cycloheximide prevents the translation of immediate-early mRNA, and proteins that would initiate early and late transcription are not formed. After the removal of cycloheximide the synthesis of immediate-early polypeptides can be followed by pulse-labelling in the presence of actinomycin D which prevents synthesis of new mRNA. Fig. 4 shows SDS-PAGE of polypeptides synthesized with or without prior cycloheximide treatment in cells infected with standard (wild-type) virus alone, stocks containing dDNA1 defectives and mock-infected controls. In the absence of cycloheximide, dDNA1 defective virus evidently induced the overproduction of a 135K polypeptide in AGMK cells. This polypeptide could be clearly distinguished at the earliest pulse-labelling time (2 to 4 h post-infection) both in defective and wild-type virus-infected cells, but was not detected in the profile of virus structural polypeptides (Fig. 4, lane 11). After cycloheximide treatment four major immediate-early polypeptides (175K, 120K, 74K and 63K) were detected in both wild-type and defective virus-infected cells. All of these were apparently largely overproduced in cells infected with defective virus. These polypeptides can be correlated fairly well with the immediate-early polypeptides described by Preston (1979b). In addition, a faint band identical to the 135K polypeptide could be detected in defective virus-infected cells (Fig. 4, lane 3). As well as a more pronounced shut-off of host protein synthesis, presumably mediated by the defective virus and a significant overproduction
of the 135K polypeptide as compared with wild-type infection, no further characteristic change in polypeptide synthesis could be recognized in cells infected with virus stock containing dDNA1 defectives.
The 135K polypeptide is expressed in Xenopus oocytes

Purified dDNA1 was microinjected into the nuclei of X. laevis oocytes in order to demonstrate that the overproduced 135K polypeptide is encoded by the defective genome, and the polypeptides synthesized between 18 and 31 h after injection were labelled. Immunoprecipitates of oocyte extracts were prepared using a general anti-HSV ANG rabbit serum. The precipitates were subjected to two-dimensional gel electrophoresis using the NEPHGE–PAGE technique of O'Farrell et al. (1977). The immunoprecipitates of polypeptides synthesized in control oocytes (Fig. 5a) and in dDNA1-injected oocytes (Fig. 5b) were examined. In the polypeptide profiles of immunoprecipitates of extracts of dDNA1-injected oocytes a 135K polypeptide could be detected as a broad band at pH 6 (Fig. 5b). Further studies by IEF–PAGE (O'Farrell, 1975) revealed that this polypeptide occurred in at least four differently charged forms with apparent pIs between 6-6 and 6-8. Another polypeptide spot not seen in the control pattern (at pH 6 and 68K in Fig. 5b) has not yet been identified as virus-specific.

The 135K polypeptide is identical with the HSV-1 major DNA-binding protein

From the molecular weight and the map coordinates of the UL sequences in dDNA1 we suspected that the 135K polypeptide might be identical to ICP8, which was previously characterized as the HSV-1 major DNA-binding protein and mapped within the corresponding region of HSV-1 standard DNA (Morse et al., 1978). In order to test this assumption we analysed the affinity of the 135K protein for native and denatured DNA. Cells were either mock-infected or infected with standard wild-type or dDNA1 defective virus in the presence of 3H-labelled amino acids, and cell extracts were prepared and subjected to DNA–cellulose chromatography. Denatured DNA–cellulose chromatography revealed that extracts of cells infected with defective virus stocks contained two- to threefold more acid-precipitable material eluting at 0-4 to 1 M-NaCl than extracts of standard virus-infected cells (Fig. 6). It was further demonstrated by PAGE analysis that the 135K polypeptide was a major constituent of defective virus-infected cells eluting with 0-4 M-NaCl from denatured DNA–cellulose columns (Fig. 6) and with 0-6 M-NaCl from native DNA–cellulose columns (Fig. 7a).

Additional studies using a monospecific antiserum to ICSP 11/12, the major binding protein of HSV-2 (Purifoy & Powell, 1976), showed that the 135K polypeptide could be precipitated by this antiserum, which is known to precipitate both the HSV-1 and HSV-2 major DNA-binding protein (Yeo et al., 1981) (Fig. 7b). This result confirmed that the HSV ANG 135K polypeptide encoded by dDNA1 is identical to the major HSV-1 DNA-binding protein exhibiting strong affinity for both native and denatured DNA.

DISCUSSION

In this paper we have identified the sequences of homology between a class II defective (dDNA1) and its parental HSV-1 ANG genome. As shown for other class II defective genomes (Locker et al., 1982), the dDNA1 molecule derives its sequences from separate regions of standard virus DNA. The tandem repeat unit of dDNA1 consists of a stretch of UL sequences, shown to be colinear with standard DNA sequences mapped between 0-37 and 0-415 fractional genome units. The hybridization studies reveal that the dDNA1 genome terminates at its right-hand end by 'ac' sequences containing a variable copy number of an amplified 500 bp sequence as previously proposed (Kaerner et al., 1981). There is some evidence that the left terminus of the dDNA1 genome contains an 'a' sequence and none of the sequence of the variably amplified 500 bp element.

Within the UL sequences of the dDNA1 repeat unit two proteins have so far been mapped on the HSV-1 standard genome: the major DNA-binding protein ICP8 [Morse et al., 1978, and in a different nomenclature 136' (130)K polypeptide, Marsden et al., 1978] and the HSV-1 DNA polymerase (Chartrand et al., 1979, 1980). The HSV pol gene has been previously fine-mapped between the restriction sites KpnI P-V and KpnI X-C by marker rescue studies using intertypic recombinants. The results of the present study show that dDNA1 does not comprise the complete HSV pol gene. Comparisons of the specific HSV pol activities of extracts of standard wild-type and defective virus-infected cells gave no indication that dDNA1 encodes an active
enzyme. The major polypeptide expressed in AGMK cells infected by dDNA1 defective virus was shown to be a 135K polypeptide (Fig. 4). This polypeptide is identical to the major HSV DNA-binding protein as judged by size, immunological parameters, and affinity for DNA.
Fig. 6. Denatured DNA-cellulose chromatography of proteins extracted from AGMK cells either mock-infected (a), or infected with standard wild-type (wt) virus (b) or defective (D) virus (dDNA1) (c). (d) The polypeptide profiles of the peak fractions (fraction 22) eluting at 0.4 M-NaCl of standard wild-type (b) and defective (c) virus extracts were made visible by fluorography of 7.5% SDS-polyacrylamide gels. 14C-amino acid-labelled proteins (Amersham Buchler) were used as mol. wt. standards (mol. wt. shown × 10⁻³).

Overproduction of ICP8 has also been reported for tsLB2 defectives of class II (Locker et al., 1982). By expression of ICP8 in X. laevis oocytes we could more directly demonstrate that dDNA1 encodes the complete major DNA-binding protein including the requisite expression signals (Fig. 5). Since the major DNA-binding protein belongs to the β-polypeptides, its expression and regulation should depend on α-protein expression. However, as similarly observed for HSV DNase (Preston & Cordingley, 1982), the expression of the major DNA-binding protein in X. laevis oocytes apparently does not absolutely require immediate-early gene expression. Thus, under immediate-early conditions a significant amount of 135K polypeptide is synthesized (Fig. 4). As a further indication that ICP8 expression is less stringently controlled by α-protein expression, one can interpret the data of Locker et al. (1982) which showed that ICP8 was overproduced even when cells infected with virus stocks containing tsLB2 defectives of class II were grown at the non-permissive temperature for the helper virus.

Recent studies have demonstrated that the major HSV DNA-binding protein has a highly
Fig. 7. Affinity of the dDNA1-specific 135K polypeptide to native DNA (a) and the cross-reaction of this protein by immunoprecipitation with an antiserum to the major HSV-2 DNA-binding protein (b).

(a) Native DNA-cellulose chromatography of proteins extracted from defective virus (dDNA1)-infected (D) AGMK cells performed as described in Methods. Aliquots of the peak fractions eluting at 0.05 M (wash), 0.15 M and 0.6 M NaCl were examined by SDS-PAGE in 8-75% gels. (b) SDS-PAGE of 3H-amino acid-labelled (5 x 10^6 cpm/min) proteins extracted from defective virus-infected AGMK cells (60% dDNA1) (D) and of material immunoprecipitated from this extract using the monospecific antiserum to ICSP 11/12 (IP). (Mol. wt. shown x 10^-3.)

conserved amino acid sequence and that a monospecific antiserum against this protein cross-reacts with the major DNA-binding proteins of several herpesviruses of the *Alphaherpesvirinae* subfamily (Littler *et al.*, 1981). Genetic analyses of ts mutants of the HSV-1 major DNA-binding protein have shown that the most likely coding region for this protein resides within *Kpn I P*
(Weller et al., 1983). Thus, the repeat unit of HSV-1 ANG class II defectives containing the complete major DNA-binding protein gene as well as the sequence information for replication and packaging might provide a useful system to study expression and role of the major DNA-binding protein as well as other viral genes in replication and recombination of the more complex standard wild-type virus genome.

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REFERENCES


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