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Localization of an origin of DNA replication within the TR_S/IR_S repeated region of the herpes simplex virus type 1 genome

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An assay has been developed and used to locate an origin of DNA replication on the herpes simplex virus type 1 (HSV-1) genome. Baby hamster kidney cells were transfected with circular plasmid molecules containing cloned copies of HSV-1 DNA fragments, and helper functions were provided by superinfection with wild-type HSV-1. The presence of an HSV-1 origin of replication within a plasmid enabled amplification of the vector DNA sequences, which was detected by the incorporation of [^{32}P]orthophosphate. By screening various HSV-1 DNA fragments it was possible to identify a 995-bp fragment that maps entirely within the reiterated sequences flanking the short unique region of the viral genome and contains all the *cis*-acting signals necessary to function as an origin of viral DNA replication. The products of plasmid replication were shown to be high mol. wt. DNA molecules consisting of tandem duplications of the complete plasmid, suggesting that replication was occurring by a rolling-circle mechanism.

Key words: herpes simplex virus DNA/origin of replication/defective viral genomes/DNA transfection

Introduction

The herpes simplex virus type 1 (HSV-1) genome is a linear DNA duplex of mol. wt. 98 x 10^6 (Becker *et al.*, 1968; Kieff *et al.*, 1971; Clements *et al.*, 1976), consisting of two components, L and S, each of which contains a region of unique sequences (U_L and U_S, respectively) flanked by inverted repeats (TR_L, IR_L, IR_S, and TR_S, Figure 1a) (Sheldrick and Berthelot, 1974; Hayward *et al.*, 1975; Delius and Clements, 1976; Roizman, 1979). The repeats flanking U_L and U_S differ but have in common a region of ~400 bp, the 'a' sequence, which is present as a direct repeat at the genomic termini and also in inverted orientation at the joint between the L and S segments (Grafstrom *et al.*, 1974, 1975; Wagner and Summers, 1978; Davison and Wilkie, 1981; Mocarski and Roizman, 1981). Four alternative structural arrangements for virion DNA are generated by the inversion of L and S relative to one another (Hayward *et al.*, 1975; Delius and Clements, 1976; Roizman, 1979).

Examination of replicating HSV-1 DNA by electron microscopy and analysis of the structures of defective genomes have been utilized in attempts to locate the origin(s) of viral DNA replication. Friedmann *et al.* (1977) suggested, from evidence obtained by electron microscopy, that the HSV-1 genome contained at least two origins of replication, one possibly within the TR_S/IR_S sequences and the other within U_L. This conclusion could not, however, be directly confirmed because it was not possible to orientate the observed molecules with respect to the physical map of the viral genomes.

When HSV-1 is passaged serially at a high multiplicity of infection, defective genomes consisting of tandem duplications of small subsets of the viral DNA sequences accumulate (Wagner *et al.*, 1974; Frenkel *et al.*, 1976, 1980; Schröder *et al.*, 1975/1976, Graham *et al.*, 1978; Locker and Frenkel, 1979, Kaerner *et al.*, 1979, 1981; Denniston *et al.*, 1981). The repeat unit from one type of defective DNA (class I) originates from either side of the S component (Frenkel *et al.*, 1980; Denniston *et al.*, 1981; Vlazny and Frenkel, 1981), whereas class II defective genomes contain sequences predominantly from within U_L (Schröder *et al.*, 1975/1976; Kaerner *et al.*, 1979, 1981; Frenkel *et al.*, 1980; Cuifo and Hayward, 1981). This observation again supports the presence of separate origins of replication located within these two regions. Direct evidence for the presence of an origin of replication within the repeat units of class I defective DNA was provided by Vlazny and Frenkel (1981). These workers co-transfected cells with restriction endonuclease-generated monomer units of class I DNA and non-defective HSV-1 DNA as helper, and demonstrated that the individual units were able to replicate and regenerate tandemly repeated genomic structures. This result therefore indicated the presence of an origin of replication within either the U_S or TR_S sequences which constituted the monomer units (0.94-1.00 map units).

Because the monomer units of this type of defective genome may contain deletions, insertions, or rearrangements (Denniston *et al.*, 1981) it is also important to be able to define the fragment(s) of non-defective HSV-1 DNA upon which replication may be initiated. This communication demonstrates that, in cells co-infected with wild-type HSV-1 as helper, certain defined fragments of non-defective HSV-1 DNA are able to replicate autonomously and also can potentiate the replication of foreign DNA sequences to which they are covalently linked. An assay based upon this property has been used to locate an origin of HSV-1 DNA replication within a 995-bp DNA fragment which contains only sequences from the repeats flanking U_S.

Results

Localization of a presumptive origin of replication

Figures 1a and b show the physical map locations of the DNA sequences which occur in class I defective genomes (Frenkel *et al.*, 1980; Denniston *et al.*, 1981) and the sites of cleavage by restriction endonuclease *Bam*HI within the S region of the HSV-1 genome (Wilkie *et al.*, 1978). The experiments of Vlazny and Frenkel (1981) indicated that the repeat units of class I defectives contain an origin of DNA replication. Initial attempts to identify an origin-containing fragment of non-defective HSV-1 DNA were therefore performed using plasmids pGX2, pGX33, pGX34, and pGX60 which respectively contain the *Bam*HI *k*, *n*, *x*, and *y* fragments of non-defective HSV-1 DNA inserted into the *Bam*HI site of the vector pAT153 (Figure 1d). As indicated, these four fragments represent the entire regions 0.81-0.90 and 0.95-1.00 map units (with the exception of the ~200-bp *m'* fragment) and therefore contain DNA sequences very similar

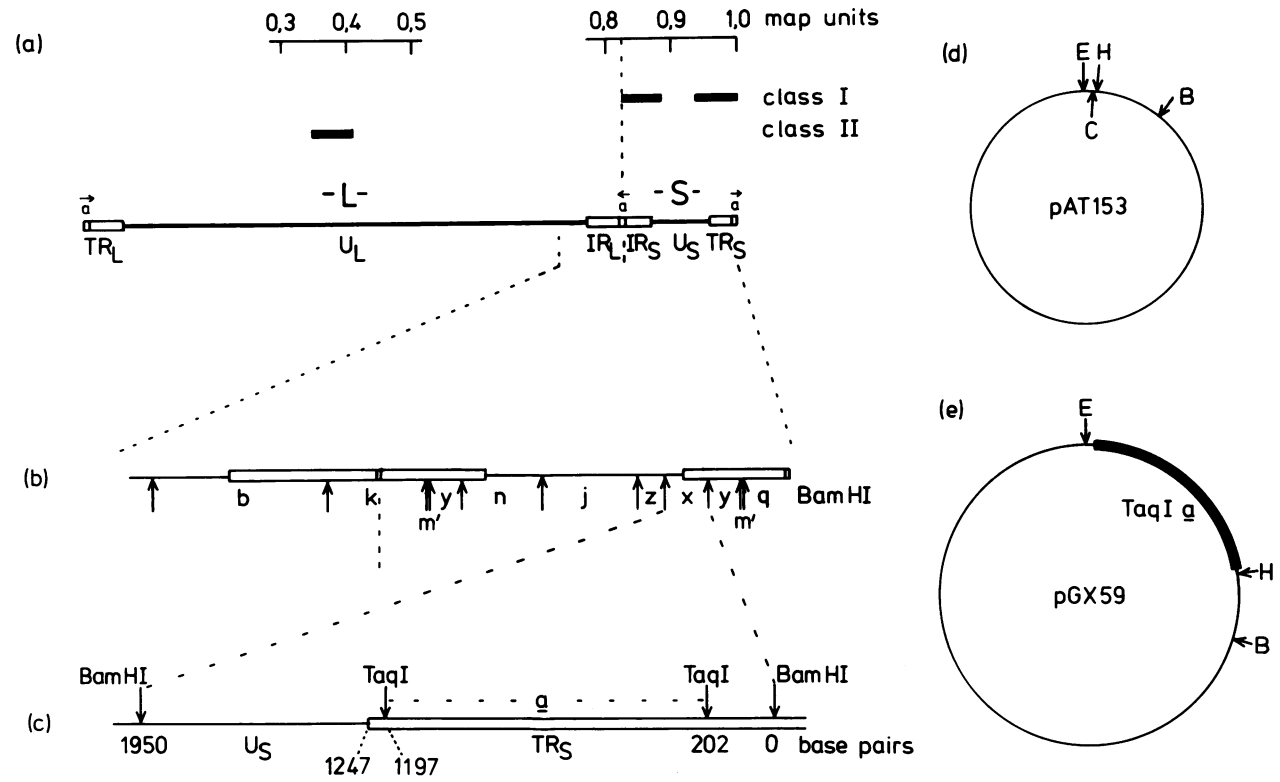

Fig. 1. (**a**) Structure of the HSV-1 genome showing the sizes and positions of the reiterated regions and the DNA sequences found in class I and class II defective genomes (for description and references see Introduction). (**b**) Position of *Bam*HI cleavage sites within the right-hand end of the HSV-1 genome (Wilkie *et al.*, 1978). It should be noted that the sequences of the terminal fragment, *Bam*HI *q*, are present within the "joint" fragment, *Bam*HI *k*. (**c**) Location of the *Taq*I *a* fragment within the reiterated region of *Bam*HI *x*. The position of this fragment is based upon the DNA nucleotide sequence data of Murchie and McGeoch (1982). (**d**) Location of cleavage sites for *Eco*RI (E), *Cla*I (C), *Hind*III (H), and *Bam*HI (B) within the plasmid vector pAT153 (Twigg and Sherratt, 1980). (**e**) Structure of pGX59, which contains the *Taq*I *a* fragment (**c**) inserted into the *Cla*I site of pAT153 (**d**).

to those found in class I defective genomes. Baby hamster kidney (BHK) cells were transfected with circular plasmid molecules, using a modification of the calcium phosphate technique (Graham and van der Eb, 1973; Stow and Wilkie, 1976), and non-defective HSV-1 was provided as helper by superinfection with wild-type (wt) virus particles at a multiplicity of infection of 5 plaque forming units (p.f.u.) per cell. It was reasoned that the presence of an HSV-1 origin of DNA replication within a plasmid might enable the linked vector sequences to be replicated in cells in which the necessary helper functions were supplied by the superinfecting virus. To monitor the replication of these sequences, the infected cells were labelled with [^{32}P]orthophosphate during a single infectious cycle and total cell DNA was purified and hybridised to nitrocellulose strips upon which separated fragments generated by restriction endonuclease digestion of HSV-1 or plasmid DNA had been immobilised. Each of the nitrocellulose strips shown in Figure 2 contains the HSV-1 *Bam*HI *b* fragment (12 kilobase pairs [kbp]) and linearized pAT153 (Twigg and Sherratt, 1980) and pMK16 (Kahn *et al.*, 1979) DNAs (3.6 and 4.4 kbp, respectively). The DNA sequences specifying tetracycline resistance are common to the two plasmids, and pAT153 DNA is therefore capable of hybridizing to both plasmid bands.

Figure 2 shows that hybridisation to the HSV-1 *Bam*HI *b* fragment (0.74–0.81 map units) occurred using ^{32}P-labelled DNA from each plate which received the superinfecting virus, indicating that the viral DNA had replicated, and demonstrating the efficacy of the labelling and hybridisation procedures. In contrast, hybridisation to the plasmid DNA bands occurred only when labelled DNA from cells which received plasmids containing either *Bam*HI *n* or *Bam*HI *x* (pGX33 and pGX34, respectively) in combination with superinfecting virus was used. This result demonstrates the presence within both *Bam*HI *n* and *Bam*HI *x* of sequences which, provided that the necessary virus helper functions are supplied, enable the replication of the covalently linked plasmid vector sequences, and therefore probably represent an origin for HSV-1 DNA synthesis. *Bam*HI *x* and *Bam*HI *n* originate from opposite sides of the S region suggesting that the presumptive origin of replication is located within the sequences they share, and therefore lies within the small region of IR_S and TR_S located adjacent to, and on either side of, U_S. To confirm this location, a 995-bp *Taq*I fragment (*Taq*I *a*; Figure 1c), mapping entirely within the reiterated region and containing the majority of these shared sequences, was subcloned from the *Bam*HI *x* fragment of pGX34 into pAT153. Figure 2 shows that in cells which received both the resulting plasmid (pGX59) and superinfecting helper virus replication of the linked pAT153 DNA occurred, and confirms that HSV-1 DNA sequences located within the reiterated IR_S and TR_S regions suffice for replication of the plasmid vector DNA.

Structure of the replicated plasmid DNA

To analyse the structure of the replicated plasmid DNA, restriction enzyme generated fragments of unlabelled whole cell DNA were transferred to nitrocellulose and subsequently hybridised to DNA of the vector, pAT153, which had been

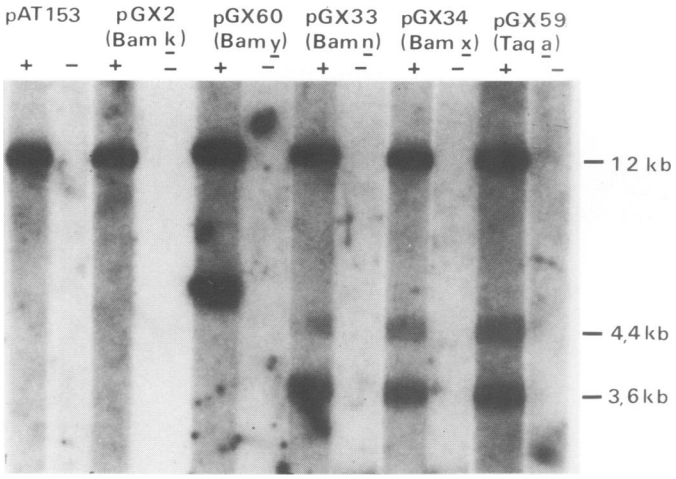

Fig. 2. Analysis of the replication of plasmid vector DNA sequences. Monolayers of BHK cells were transfected with 0.24 μg pAT153, 0.69 μg pGX2, 0.60 μg pGX33, 0.38 μg pGX34, 0.37 μg pGX60, or 0.30 μg pGX59 as indicated and described in Materials and methods. Six hours after transfection the monolayers were either mock-infected (−) or superinfected with wt HSV-1(+) as described. The cells were labelled with [^{32}P]orthophosphate from 9 to 24 h post-transfection and DNA purified and hybridized to nitrocellulose strips containing separated unlabelled DNA fragments. Each strip contains 0.04 μg of the 12-kbp HSV-1 *Bam*HI *b* fragment (Figure 1b) which had been generated from a recombinant plasmid, and linearized plasmid vector molecules generated by digestion of 0.2 μg pAT153 DNA (3.6 kbp) and 0.3 μg pMK16 DNA (4.4 kbp) with *Bam*HI. DNA recovered from a single cell monolayer was used in each hybridisation and the strips were finally washed and subjected to autoradiography. The heavy mark below the 12-kbp band in the pGX60(+) track is caused by non-specific binding of ^{32}P-labelled DNA to the nitrocellulose.

labelled *in vitro* with ^{32}P by nick translation. Figure 3A shows the results of an experiment in which the products of pGX59 replication were analysed in this way. DNA was isolated from cells which had been transfected with pGX59 and then either mock-infected or superinfected with wt HSV-1. A small amount of the input DNA was detected in the absence of its replication (i.e., DNA from mock-infected cells) upon a long exposure of the blot. However, replication of the vector sequences in the superinfected cells was clearly demonstrated by the massive increase in the signal obtained (compare tracks a and b).

Figure 3A shows that when the cellular DNA was either undigested or digested with an enzyme such as *Kpn*I, which does not cleave pGX59, the replicated vector sequences were detected in the form of high mol. wt. DNA. Rehybridisation of this region of the blot to an HSV-1 probe (Figure 3B) revealed that the *Kpn*I cleavage was complete and that the pAT153 DNA sequences were present within molecules significantly larger than the *Kpn*I *a* fragment (14 kbp), and possibly approaching the size of intact HSV-1 DNA. In contrast, cleavage of the infected cell DNA with *Eco*RI, *Hind*III, or a combination of these enzymes in each instance generated bands which hybridized to pAT153 DNA and co-migrated with the appropriate pGX59 markers (Figure 3A). *Eco*RI cleavage, as expected, yielded a fragment migrating slightly more slowly than linearized pAT153, whereas the fragment generated by *Eco*RI and *Hind*III cleavage co-migrated with the pAT153 marker (Figure 1d and e).

These results indicate that although the replicated vector DNA is found in high mol. wt. molecules, these molecules nevertheless contain apparently unaltered copies of the input plasmid, pGX59, which are therefore arranged as tandem head to tail repetitions.

Comparison of the intensity of the bands obtained from the cellular DNA (tracks b) with the marker DNA indicates that the final yield of plasmid DNA was ~0.2 μg/50 mm Petri dish. The amount of residual plasmid DNA detected in the mock-infected cell DNA was estimated to be at least 500-fold lower.

Discussion

The results presented here demonstrate that in cells in which the required helper functions are supplied by superinfecting wt HSV-1, a 995-bp DNA fragment originating from within the IR_S/TR_S region of the HSV-1 genome contains sequences sufficient to enable the replication of the pAT153 DNA to which it is covalently linked. This replication does not appear to occur as a result of recombination between the input plasmid and helper virus as suggested by the fact that only certain specific HSV-1 fragments enable plasmid DNA replication, and more strongly indicated by the analysis of the structure of the replication products.

The vector DNA sequences are found in high mol. wt. species which are resistant to digestion with *Kpn*I, which cleaves HSV-1 DNA but not DNA of the input plasmid, pGX59. In contrast, digestion of the replicated DNA with enzymes such as *Hind*III and *Eco*RI, which cleave pGX59, generates fragments which co-migrate with the corresponding fragments of the input plasmid (Figure 3). This establishes that the replicated vector DNA is found in structures consisting of tandem repeats of the entire input plasmid, and is inconsistent with either site-specific or random integration of the plasmid into the helper virus genome. The data therefore indicate that the *Taq*I *a* fragment of pGX59 contains sequences which enable the initiation of DNA synthesis and the subsequent amplification of the vector, pAT153 DNA, and therefore represent an origin for HSV-1 DNA replication.

From the studies of Vlazny and Frenkel (1981) it seems likely that it is these same sequences which function as origins for the replication of class I defective genomes. However, because studies of replicating HSV-1 DNA by electron microscopy (Friedmann *et al.*, 1977) have not allowed unambiguous localization of the origin(s) of replication, we do not yet know whether these sequences are essential for the replication of non-defective viral DNA. The concatameric arrangement of the products of plasmid DNA replication are indicative of a rolling-circle type mechanism, as previously suggested for the generation of tandemly reiterated genomes during passage of the virus at high multiplicities of infection (reviewed by Frenkel *et al.*, 1980), or following transfection with the individual monomer units of these defective genomes (Vlazny and Frenkel, 1981).

Studies based upon the sequence arrangement of class II defective genomes (Kaerner *et al.*, 1979, 1981; Frenkel *et al.*, 1980; Cuifo and Hayward, 1981) and upon electron microscopy of replicating viral DNA (Friedmann *et al.*, 1977) suggest the presence of another origin of replication within U_L. The mapping approach described here is therefore being extended to determine whether additional origins of replication can be identified elsewhere within the HSV-1 genome.

The nucleotide sequence of the repeat regions flanking U_S (including the complete *Taq*I *a* fragment) has been determined (Murchie and McGeoch, 1982) and reveals a number of in-

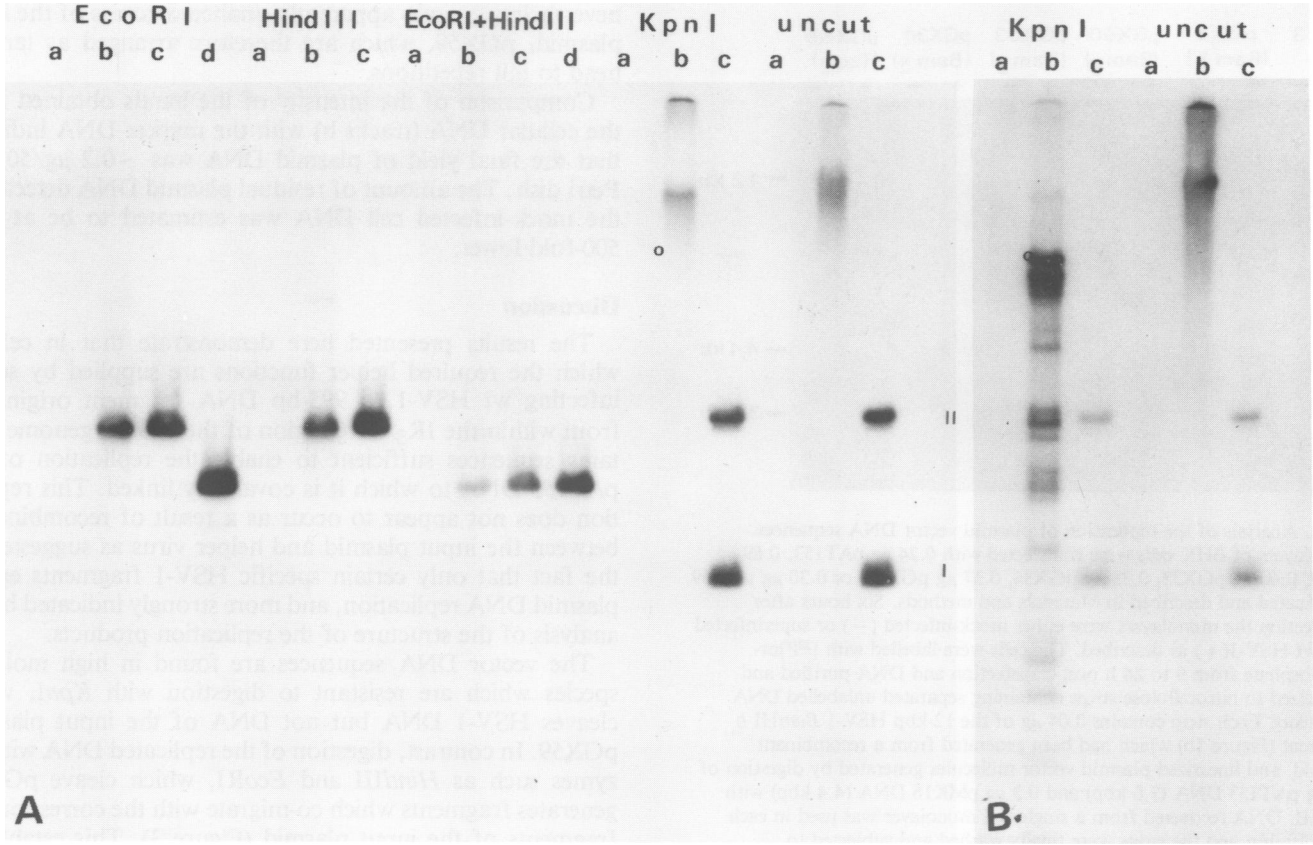

Fig. 3. Analysis of the products of pGX59 replication. Unlabelled DNA was purified from BHK monolayers which had been transfected with 0.30 μg pGX59 and either mock-infected (tracks, **a**) or superinfected with wt HSV-1 (tracks, **b**). The DNA was cleaved with the enzymes indicated and the resulting DNA fragments separated by electrophoresis through a 0.8% agarose gel. One tenth of the total DNA recovered from a 50-mm diameter Petri dish was loaded per slot (**a** and **b**) and the markers were either 20 ng pGX59 DNA (tracks, **c**) or 20 ng pAT153 DNA (tracks, **d**) digested with the enzymes indicated, and subjected to electrophoresis in the presence of 3 μg calf thymus DNA per track. The gel was soaked for 15 min in 0.25 M HCl prior to transfer of the DNA fragments to a nitrocellulose sheet by the technique of Southern (1975). The sheet was initially hybridised to *in vitro* labelled pAT153 DNA (**A**). After autoradiography the probe was removed by denaturation and the right-hand side rehybridised to labelled HSV-1 DNA (**B**). o, indicates the position of the HSV-1 *Kpn*I *a* fragment in **B** and the corresponding position in **A**. I and II, indicate the positions of supercoiled and open-circular pGX59 DNA molecules.

teresting features. The 5′ ends of divergently transcribed immediate early mRNAs have been located close to positions 30 and 724 in the *Bam*HI *x* sequence depicted in Figure 1c (Murchie and McGeoch, 1982). The intervening 700 bp, the majority of which lie within *Taq*I *a*, are not known to be transcribed and would represent an attractive site for an origin of replication. Furthermore, an almost perfect 45-bp palindrome, containing a stretch of 18 consecutive A or T residues at its centre is located between nucleotides 575 and 619 in Figure 1c (Murchie and McGeoch, 1982), and is a strong candidate for an important structural element of a DNA replication origin. By suitable manipulations of the *Taq*I *a*-containing plasmid, pGX59, it should be possible to define more precisely the role of these interesting DNA sequences in the initiation of viral DNA synthesis. Plasmids such as pGX59, which contain small DNA fragments in which origins of DNA replication are located, are also likely to prove valuable in studies on the mechanism of HSV DNA replication, and may additionally provide important components in the development of herpes viruses as cloning vectors (Stow and McMonagle, 1982).

Materials and methods

Cells and virus

BHK 21 C13 cells were grown in Eagle's medium supplemented with 10% tryptose phosphate broth and 10% calf serum (Macpherson and Stoker, 1962). Subconfluent cell monolayers (2 x 10^6 cells/50 mm plastic Petri dish) were used for DNA transfections, and the superinfecting virus was wt HSV-1 (Glasgow strain 17).

Plasmids

Plasmids pGX2, pGX33, pGX34, and pGX60 contain HSV-1 *Bam*HI fragments *k*, *n*, *x*, and *y*, respectively, inserted into the *Bam*HI site of plasmid vector pAT153 (Twigg and Sherratt, 1980). Plasmid pGX59 contains a 995-bp *Taq*I fragment from pGX34 inserted into the *Cla*I site of pAT153 (Figure 1d and e). Plasmid construction, propagation, and purification of supercoiled DNA by isopycnic banding on caesium chloride/ethidium bromide gradients were all essentially as described by Davison and Wilkie (1981).

Transfections with plasmid DNA and virus superinfection

Monolayers of BHK cells were each transfected with plasmid DNA in the presence of 5 μg calf thymus carrier DNA using the calcium phosphate technique (Graham and van der Eb, 1973) followed by a dimethyl sulphoxide boost (Stow and Wilkie, 1976) at 4 h post-transfection. Each monolayer received 0.24 μg pAT153 DNA or amounts of recombinant plasmids calculated to contain equal numbers of plasmid molecules. Incubation was at 37°C in Eagle's medium containing 5% calf serum (EC5). Six hours after transfection the cells were either mock-infected, or superinfected with wt HSV-1 at a multiplicity of infection of 5 p.f.u./cell, and incubation continued at 37°C in EC5.

Preparation of cell DNA

Replicating DNA was labelled *in vivo* by incubating cell monolayers in phosphate-free Eagle's medium containing 2% calf serum (E2C-Pi) and 0.3 mCi [^{32}P]orthophosphate from 9 to 24 h post-transfection. Total cell nucleic acids were isolated by proteinase K digestion and phenol extraction (Botchan *et al.*, 1973), digested with RNase A (10 μg/ml), re-extracted sequentially with phenol and chloroform, ethanol precipitated, and the resulting DNA redissol-

ved in H_2O. Unlabelled DNA was prepared similarly from nuclei obtained following Nonidet P40 lysis (Wilkie, 1973) of cells which had been incubated from 6 to 24 h post-transfection in EC5.

Hybridisation of ^{32}P-labelled cell DNA to unlabelled DNA fragments

DNA of plasmids pMK16 (Kahn *et al.*, 1979), pAT153 (Twigg and Sherratt, 1980), and pAT153 containing an insert of the HSV-1 *Bam*HI *b* fragment (a gift of V. Preston) were digested with *Bam*HI and the digestion products separated by electrophoresis through a 1% agarose gel. The DNA fragments were transferred to nitrocellulose sheets using the Southern (1975) blotting technique. ^{32}P-labelled cell DNA was denatured with alkali and hybridized to strips cut from the above blot. Hybridisation was for 16 h at 68°C using previously described conditions (Botchan *et al.*, 1976; Jeffreys and Flavell, 1977). After hybridisation, the strips were washed and autoradiographed using Kodak XR or XS film.

Analysis of unlabelled cell DNA

DNA was cleaved with restriction endonucleases using conditions recommended by the supplier. The products of digestion were separated by electrophoresis through a 0.8% agarose gel and transferred to nitrocellulose as described above. Hybridisation to pAT153 DNA, labelled *in vitro* with ^{32}P by nick translation (Rigby *et al.*, 1977), was also as described above. Prior to rehybridisation to nick-translated HSV-1 DNA, the labelled pAT153 DNA was first removed by denaturation in H_2O at 95°C. After hybridisation, the nitrocellulose sheets were washed and subjected to autoradiography.

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