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The DNA replication origins of herpes simplex virus type 1 strain Angelotti

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ABSTRACT

The nucleotide sequences of the origins of DNA replication (ori) of the S- and L-component (ori_S, ori_L) of the herpes simplex virus type 1 (HSV-1) standard genome were determined from HSV-1 strain Angelotti (ANG). In contrast to other HSV-1 strains, the ANG ori_S sequence revealed an insertion of an TA-dinucleotide in an otherwise very conserved but imperfect palindromic sequence of 47 bp. The ori_L sequence of the standard ANG genome was found to be identical to that of an ANG class II defective genome which exhibits a duplication of a 144 bp palindrome. A model is presented to explain the origination of the amplified ANG ori_L sequences from the parental genome with a single copy of ori_L via illegitimate recombination. Alignment of the ori sequences of HSV, adeno- and papovaviruses unveiled that the HSV ori region can be subdivided into two distinct sites of homology to the DNA initiation signals of papova- and adenoviruses, suggesting that the HSV origins of replication comprise elements for DNA replication by both, cellular and virus-encoded DNA polymerases.

INTRODUCTION

Studies of defective HSV-1 DNA led to the identification of three origins of DNA replication on the HSV-1 genome, one in the unique sequences of the L-component (ori_L) and two in the c inverted repeat sequences of the S-component (ori_S) (1-4). Until recently (5, 6), molecular cloning of the ori_L sequences has been unseccessful. Transfection experiments ("replicon assays") with ori_S and ori_L cloned into plasmids have directly demonstrated the functionality of the cis-acting ori signals through amplification of the input DNA by super-infecting HSV as helper (2, 5, 6, 7). By this kind of assays, a 90 bp segment of ori_S was shown to be sufficient for origin function (7).

Sequence analysis revealed a palindromic organization of ori_S and ori_L (5-10). Ori_S of HSV-1 strain KOS and strain 17 is composed of an almost perfect 45 bp palindrome. In HSV-2 strain HG52 two 137 bp direct repeats containing each a 45 bp ori_S palindrome are observed (11). The ori_L sequences of HSV-1 strains KOS and 17, and of HSV-2 strain 333 exhibit a single perfect palindrome of 144 bp (5, 10) and of 136 bp (6), respectively. The ori_L sequences determined from class II defective DNA of HSV-1 ANG (9) differ from all wild-type strains examined so far by an apparent duplication of the palindromic sequences: beside the 144 bp palindrome identical to that of strain KOS

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and 17, a further 130 bp palindrome is present. The smaller palindrome is repeated within the larger one. Since these results were obtained from defective DNA, we were interested to prove whether the standard genome of HSV-1 ANG embodied a similarly organized ori_L region. Previous studies have revealed that standard ANG Bam HI fragments N and Y, both comprising the ori_S sequences, appear on agarose gels as a heterogeneous family of bands differing in size by an amplified, not yet identified, sequence of approximately 450 bp (12). It appeared of interest to know whether the ori_S sequences were also effected by amplification events, which seem to occur frequently in this strain (13). A further goal was to compare the individual HSV origin sequences in order to find a possible mechanism for the origination of the ori_L palindrome duplication of HSV-1 ANG.

Considering our present knowledge, both about the structural organization of the origin region and about the interaction of viral and cellular proteins in the initiation of replication of adeno- and papovavirus DNA, we have searched for a relationship to the HSV origin regions. With the recently gained sequence information of the surroundings of ori_S and ori_L (8, 10, 14, 15), it was possible to perceive a structural similarity to the papovavirus origin region. In accordance with the organization of the simian virus 40 (SV40) ori (16), the HSV ori sequences are flanked by the 5' terminal regions including promotor recognition signals and regulatory elements of such important genes as the immediate early proteins at the ori_S, and the major DNA-binding protein and the DNA polymerase at the ori_L. Beyond the structural similarities in the positioning of ori sequences between important genes, this report will demonstrate that the ori sequences of HSV and of papovaviruses (16, 17) as well as of adenoviruses (18) share significant homology.

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney cells (RC-37, Italdiagnostic products) were routinely passaged in Dulbecco's MEM (Seromed), supplemented with 10 % fetal bovine serum (Seromed), 0.2 % glutamine, 100 µg/ml streptomycin, 100 units/ml penicillin and appropriate concentrations of $NaHCO_3$. Stocks of standard HSV-1 strain ANG were prepared as described (19).

HSV-1 ANG standard DNA. For routine DNA preparation, confluent monolayers of RC-37 cells (20 Roux bottles) were infected with HSV-1 ANG standard virus at a MOI of 0.05, and further cultivated in the above described culture medium except with 5 % fetal bovine serum. The supernatant medium was saved 2 to 3 days p.I., and the cells were harvested in 20 mM EDTA (pH 7.5) using a rubber policeman. Cells were disrupted by homogenization and the nuclei as well as the debris removed by low speed centrifugation (2000 rpm, 10 min, 4° C). The supernatants of the cell homogenates and the

supernatant medium were combined, and the virions were pelleted by centrifugation (Sorvall GSA-rotor, 11000 rpm, 2 h, 4° C). The pellets were resuspended in TNE-buffer (10 mM Tris.Cl (pH 7.5), 100 mM NaCl, 10 mM EDTA), 1 % sarkosyl and 0.5 mg/ml proteinase K was added, and the lysate was incubated at 37° C overnight with gentle agitation. To 5 ml of a lysate, 6.6 g CsCl was added and the final density adjusted to 1.71 g/cm^3. The viral DNA was separated from cellular DNA by equilibrium density gradient centrifugation in polyallomer tubes (Beckman rotor 50Ti, 42000 rpm, 48 h, 20° C). HSV DNA fractions were collected and stored at 4° C before use. Aliquots were precipitated with 3 volumes of 80 % ethanol at room temperature, washed with 80 % ethanol and the DNA was redissolved into TE-buffer (10 mM Tris.Cl (pH 7.5), 1 mM EDTA).

Cloning procedures. Standard cloning procedures were employed (20). HSV-1 ANG Bam HI fragment n was inserted into plasmid vector pAT153 (21). For transformation E. coli strain OM214 (RRIΔM15, leu, pro, thi, strA, hsd, r$^-$, m$^-$, lacZ,Δmis F' lacI^{Q}z,mis, pro$^+$) was kindly provided by Dr. W. W. Franke (Heidelberg). Deletion clones were prepared using Bal31 nuclease treatment followed by linker addition as described (22). Plasmid DNA was prepared employing the clear-lysate procedure (23) by proteinase K treatment and phenol extraction. If necessary, plasmid DNA was further purified by NACS-column (BRL) chromatography, and DNA fragments separated on horizontal agarose gels were isolated using DEAE-Nylon membranes (NA-45, Schleicher & Schüll) according to the protocols of the manufacturer.

Sequencing. The nucleotide sequence of DNA inserts of recombinant plasmids was determined using the Maxam & Gilbert technique (24) after restriction and 3' end-labeling. Routinely the following five degradation reactions were used: G, A+G, A>C, C+T, and C. Sequencing gels (5, 6, and 10 %) were run at 55° C and 50 V/cm using the LKB Macrophor sequencing unit equipped with a thermostatic circulator. Analysis of the sequence data was performed using the BSA program devised by Dr. S. Suhai at the German Cancer Research Center, Heidelberg.

RESULTS AND DISCUSSION

The ori$_S$ of HSV-1 ANG

In previous studies, the site of the ori$_S$ sequences in HSV-1 DNA of strain 17 was localized within the restriction enzyme fragments Bam HI n and x (2, 8). For the determination of the ori$_S$ sequences of HSV-1 strain ANG (ANG ori$_S$) the corresponding Bam HI n fragment (12) was cloned into plasmid pAT153. The ori$_S$ region was then approached by deleting the downstream sequences essentially as described by Guo et al. (22). The recombinant plasmid was cleaved at the unique Pvu II site within Bam HI fragment n (at pos. 637 as published (14)). The flanking sequences of this site were

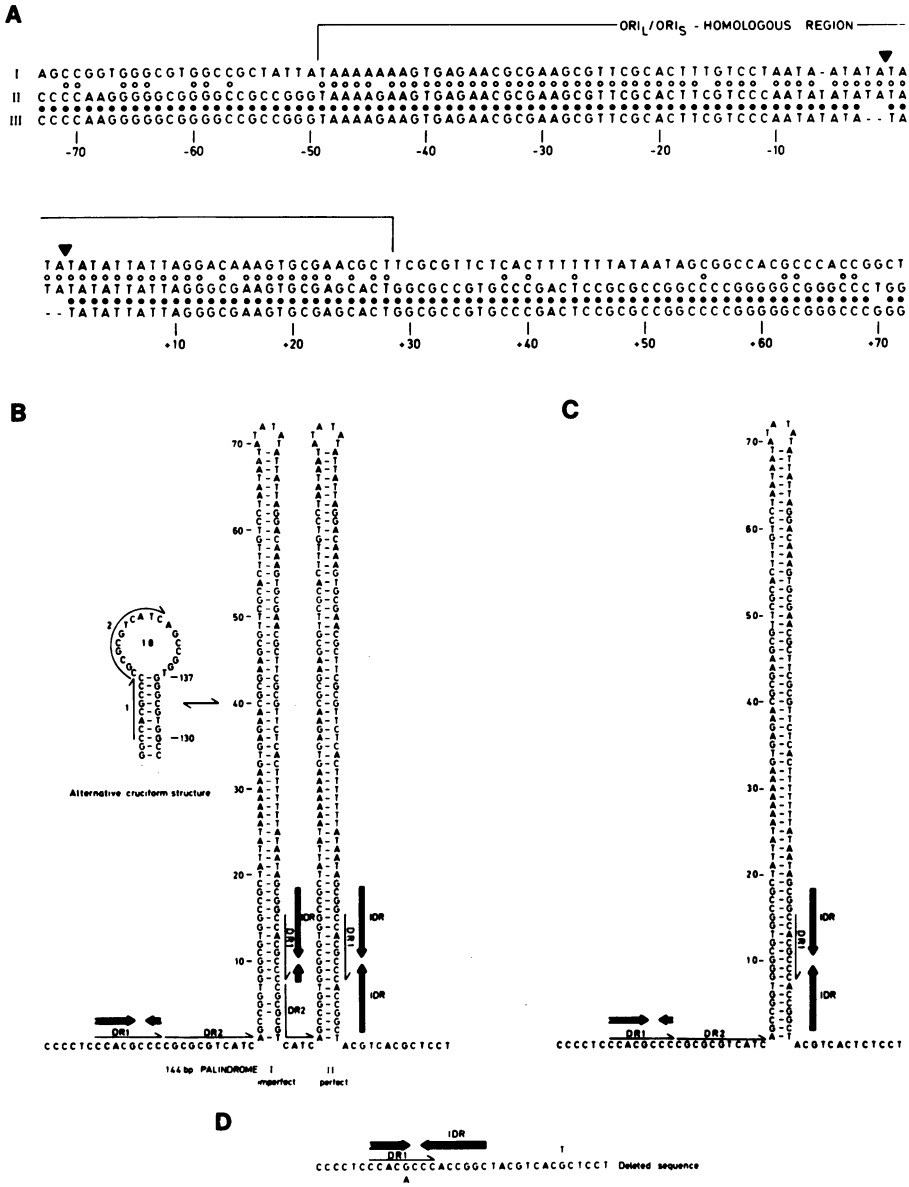

Figure 1. The HSV-1 ori$_S$- and ori$_L$ -sequences. A. Comparison of the 144 bp palindrome in ori$_L$ with ori$_S$ sequences from different HSV-1 strains. I, ori$_L$ -palindrome of HSV-1 strains KOS, 17 and ANG (= palindrome II), II, ori$_S$ of strain ANG, III ori$_S$ of strains KOS and 17. Identical nucleotides of sequences I and II, and II and III are marked by open and closed circles, respectively. The ori$_L$/ori$_S$-homology region is bracketed. Nucleotide numbering begins at the center of the ori$_L$ palindrome assigned by a triangle. B. HSV-1 ANG ori$_L$. The wild-type sequence is identical to the published sequence from

defectives of class II between pos. 173 to pos. 502 (9) and is schematically represented as a hairpin doublet or alternatively as extended cruciform structure. C. HSV-1 strain 17 ori_L. The sequence spans from pos. 4118 to pos. 4299 of the previously published sequences (10). D. Deleted HSV-1 ANG ori_L sequence. Cloning of HSV-1 ANG wild-type Bam HI fragment u into pAT153 yielded a 296 bp deletion as compared to B. Above and below the deleted sequence the differing nucleotides of the ori_L sequences of strain 17 and KOS, respectively, are indicated. Hooked bars refer to direct repeats, DR1 and DR2, and arrows designate indirect repeats, IDR.

deleted by Bal31 digestion, and blunt-ended molecules were joined with Hind III linkers. After Hind III cleavage, ligation and transformation, a bank of deletion clones was established. Suitable recombinant plasmids were 3' end-labeled at the unique Hind III site, recleaved by Bam HI, and the isolated fragments used for sequence determination according to the isolated fragments used for sequence determination according to the chemical degradation method (24). In Fig. 1A, 145 nucleotides of the determined ANG ori_S sequence are presented, and aligned with corresponding sequences of ori_S of HSV-1 strain 17 and KOS, respectively. In contrast to the latter strains, ANG ori_S exhibits an imperfect 47 bp palindromic sequence resulting from an insertion of a TA- dinucleotide at the AT-rich central region of the palindrome. The residual sequences except for a substitution at pos. +70 in Fig. 1A, are identical to the reported ori_S sequences of other HSV-1 strains (5, 7). Interestingly, a G-insertion was found in the center of the 45 bp palindrome of HSV-2 ori_S (11). Although it has been argued that cruciform structures are less likely in eukaryotic DNA because of the lacking superhelical strain (25), the insertions in the ANG and HSV-2 ori sequences may provide arguments for a possible existence of such hairpin structures in vivo: the base-pairing sequences of the palindrome arms are higher conserved than the out-looping AT-rich sequences of the palindrome center. Furthermore, shortly after infection HSV DNA is found in a circularized form (26). Circularization may supply the necessary torsional strain for initiating the transition to intra-strand base pairing that leads to a cruciform structure. At least it is a conceivable hypothesis that circularization is necessary for the initiation of DNA replication.

The ori_L of HSV-1 ANG

The location of the ori_L sequences on the HSV-1 ANG genome was deduced from studies on class II defective DNA of this strain (9, 12). Nucleotide sequencing of a Bam HI fragment of defective DNA which correlated in size to Bam HI fragment u of standard HSV-1 ANG DNA demonstrated that the ori_L sequences are located about 120 bp upstream from the Bam HI u/q site at pos. 594 of the reported sequence (9). Since the standard Bam HI fragment u in strain ANG can be separated from Bam HI fragment v in contrast to strains KOS and 17, and further, because cloning of this fragment was unsuccessful in the past, the Bam HI fragment u was isolated from native HSV-1 ANG DNA extracted from virion particles as described in Materials and

Methods. After 3' end-labeling and recleaving by the restriction enzymes Xho I or Bst E II (for orientation see ref. 9), the fragments were chemically degraded (24) and the sequencing reactions analyzed as described on 55 cm and 1 m long sequencing gels. Additionally, Bam HI u of standard DNA was cloned into pAT153 and the deleted sequences of the ori_L region were analyzed for comparison. The results of the sequence analysis of the standard ori_L confirm an identical organization as previously reported for the ori of HSV-1 ANG class II defective genomes: Two palindromes of 144 bp, an imperfect one (I), and a perfect one (II) could be distinguished and are shown in Fig. 1B as putative hairpin-like structures. The palindrome II exhibits a nucleotide composition identical to that of ori_L of strain 17, illustrated in Fig. 1C. The imperfect palindrome I is organized as follows: it contains the 72 bp direct repeat of the left arm but only 65 bp of the inverted repeat of the right arm of palindrome II, and in addition 7 bp of a sequence found as direct repeat in the upstream sequences directly flanking palindrome I. Both palindromic structures are linked by the sequence CATC found also upstream of palindrome I. Outside of the palindromic sequences minor differences between the ori_L sequences of HSV-1 ANG, KOS and 17 were observed and are indicated in the deleted sequence shown in Fig. 1D, which was obtained from cloned material. As shown previouly for the deleted sequences of the equivalent Bam HI fragment of class II defective DNAs (9) the standard Bam HI fragment u revealed an identical deletion of 296 bp comprising both palindromic sequences. As illustrated in Fig. 1B, the two palindromes can be arranged as a hypothetical cruciform structure with a 137 bp stem and a 18 b loop. Evidence for ori_L cruciform structures playing eventually a role in vivo derives from the analysis of the nucleotide changes between the ori_L sequences of HSV-1 and HSV-2 (6). Most of the differences occurring in pairs within the palindrome stem sequences serve to maintain the potential of intrastrand base-pairing, a prerequisite for the cruciform arrangement.

A model for the generation of the HSV-1 ANG ori_L.

A direct comparison of the ori_L sequences of HSV-1 strains ANG and 17 (Fig. 1B and C) suggest that the ANG ori_L duplication could have originated by asymmetrical recombination from parental genomes with a single ori_L copy. The possible crossover point can be assumed to lie within the direct repeat DR2 (Fig. 1B) located between palindrome I and II of strain ANG DNA, immediately adjacent to the 5' end of the palindromes I of strain ANG, and of strain 17 (Fig. 1C). In the hypothetical cruciform structure of the ANG palindromes, the DR2 sequences are contained within the 18 base loop in the center of the stem-loop structure (Fig. 1B). A closer search for the possible crossover site revealed another 8 bp direct repeat, designated DR1, which flanks DR2 and is also present in a region of dyad symmetry at the 3' end of the ori_L palindromes. It comprises a stretch of 17 bp with two indirect repeats, "IDR" (marked by arrows in

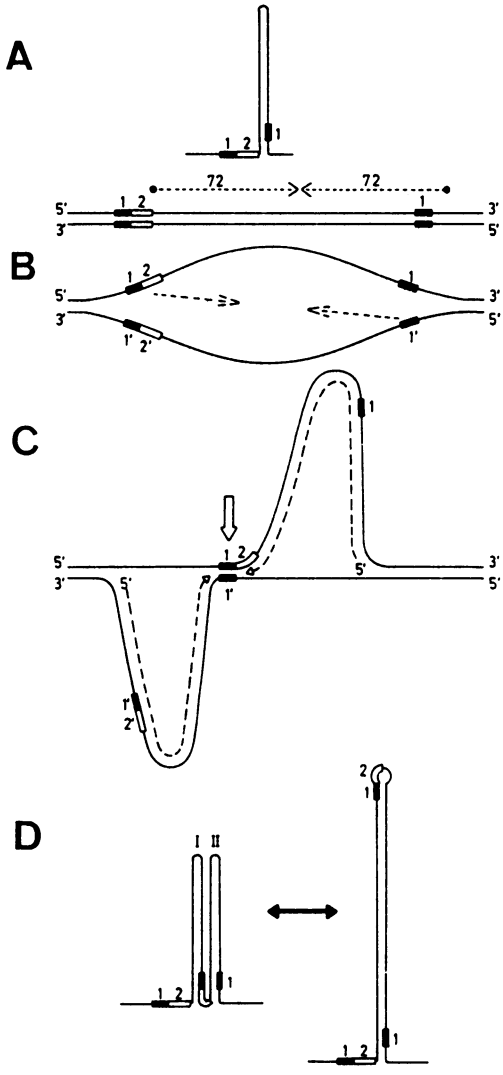

Figure 2. Hypothetical model for the origination of the ANG ori$_L$ tandem duplication from a genome with a single ori$_L$ copy. A. Hairpin-structure of the ori$_L$ sequences of the parental genome e.g. strain 17 or KOS with a single ori$_L$ copy and below the double-stranded DNA region with the 144 bp ori$_L$ palindrome, the sequences of DR1 are marked by black boxes and those of DR2 as white boxes. B. Initiation of replication results in "bubble formation". C. Illegitimate recombination via regions of homology "protein-mediated DNA-bridging". Nicking-closing reaction within DR1 by topoisomerase activity combines parent with daughter strands. D. Result of this asymmetrical intramolecular recombination is the duplication of the ori$_L$ palindrome seen in HSV-1 strain ANG. Two alternative structures are presented as either 2 perfect palindromes à 130 bp (I) and à 144 bp (II) or a stem-and-loop structure with a 137 bp stem and a 18 b loop.

Fig. 2B and C) of 7 bp (CGGCCAC/CACCGGC). The DR1 sequence is in consensus with sequences being part of DR4 which is considered to be the cis-acting site within the 'a' sequence responsible for the inversions of the L- and S-component of the HSV-1 genome (27).

Tandem duplications are generally thought to be produced by illegitimate recombination involving short tracts of homology on either side of the DNA to be duplicated (11, 28, 29, 30). Fig. 2 shows a possible model for the origination of the ori_L duplication by this mechanism. The formation of a bubble structure at the time of initiation of DNA replication (Fig. 2B) would allow mispairing between complementary DR1 sequences (black boxes). Protein-DNA interaction or conformational changes within the DNA structure could accomplish the stabilization of the recombinant structure (Fig. 2C). Joining of the parental strands with the newly synthesized daughter strands could then occur at the site of mispairing (DR1) by the nicking-closing reaction of a topoisomerase of type II. This enzyme activity has been demonstrated to play a role in illegitimate recombination events and in the organization of the chromatin structure (31, 33-36), and topoisomerase II activity was recently found to be associated also with the HSV-1 DNA polymerase (37). The described crossover would render two types of recombinant DNA molecules: i) molecules containing the ori_L duplication as observed in the HSV-1 ANG genome (Fig. 2D), and ii) molecules in which the ori_L palindromic sequences are deleted. HSV genomes of the latter type have not been described so far. It should be mentioned that cloning of ori_L sequences in prokaryotic recA and $recA^+$ E. coli hosts routinely failed in that these sequences are deleted (Fig. 1D (6, 9, 10)). This fact could possibly be a consequence of a similar illegitimate recombination event. Varicella-zoster virus (VZV) which also belongs to the subfamily Alphaherpesvirinae, does not contain an origin of replication in a position equivalent to that of HSV-1 ori_L (54), i.e. at the 5' terminal regions of the two indispensable genes, the DNA polymerase and the major DNA-binding protein (10). Furthermore, it has been recently shown that one copy of the HSV-1 ori_S sequences may be removed from the genome without a deleterious effect on DNA replication (32). It appears of interest to further investigate (i) whether both, ori_S and ori_L are required for HSV DNA replication, (ii) whether the ori_L duplication which so far has been described only for the strain ANG genome, would provide a selective advantage for the virus as similarly known in the case of papovaviruses (38-40), and (iii) whether the ori_L duplication can naturally occur in HSV strains with a single ori_L copy.

Similarities in the organization of the origin of replication of HSV, adenovirus and papovaviruses.

As noted previously (5, 6) and as demonstrated in Fig. 1A, the palindromic sequences of ori_S and ori_L of the HSV genome share extensive sequence homologies.

The region of highest homology between ori_S and ori_L is asymmetrically distributed with regard to the center of the ori_L palindrome. A stretch of 45 nucleotides of the ANG ori_S comprising the palindromic sequences and 30 bp upstream flanking sequences match with sequences of the left arm and 24 nucleotides with the right arm of ori_L, respectively. Replicon assays with ori_S deletion plasmids (7) revealed that those ori_S sequences spanning the homologous region of ori_L and ori_S are essential for the origin-function. Considering the present knowledge of the initiation of DNA replication of the papovaviruses and adenoviruses , we have searched for relationships to the HSV origin organization by aligning the available ori sequences of simian virus 40 (SV 40) (41), polyoma virus (PV) (42), human papovavirus (BKV) (43), and adenovirus (Ad5) (18, 44) with those of HSV-1.

To understand a possible relationship between the different origin sequences, the basic features of their organization are briefly described in the following. With the exception of initiation of DNA replication, papovavirus replication and assembly to minichromosomes depend on host-cell components, exclusively (for review see ref. 16). Initiation of SV40 DNA replication requires the cis-acting 63 bp ori-core sequence, SV40 large tumor antigen (T-Ag), and permissive cell factors from monkey or human cells. Initiation of bidirectional DNA synthesis begins at any one of several possible sites within the ori-core using the same components (DNA primase-DNA polymerase alpha) necessary to synthesize Okazaki fragments at the replication forks (45, 46). Of the three known binding sites of T-Ag, site II located within the ori-core has been involved in initiation of DNA replication. Replicon assays (17) similar to the system developed by Li & Kelly (47) have been used to identify the DNA-binding site for a limiting factor(s) required to initiate SV40 DNA replication. This site includes the three 21 bp repeats and a 17 bp A+T-rich sequence residing between pos. 5243 to pos. 72 on the SV40 genome (41) and only partially overlapping with the ori-core sequences.

The mechanism of adenovirus DNA replication has been studied extensively since the development of an in vitro assay for initiation and elongation (48). DNA replication initiates at either molecular end by the formation of a covalent complex between the precursor of the 55 kd terminal protein and dCMP, the 5'-terminal nucleotide of all human adenovirus DNA sequences. A virus-encoded DNA polymerase catalyzes the initiation reaction and elongates the 3'-OH group of the protein-bound dCMP by a displacement mechanism (for review see ref. 18, 49). In addition to the two viral proteins, initiation appears to require a 47 kd protein, encoded by the host cell, i.e. the nuclear factor I (NFI), that enhances the initiation reaction significantly (50). Nuclear factor I is a sequence-specific DNA-binding protein that has high affinity for a region of the Ad2 inverted repeat (ITR) (51). Footprint analysis has shown that a region between nucleotides 17 and 48 (51) or 19 and 43, respectively (18) of the ITR is protected against

DNase I attack by NFI. Elongation of full-length Ad DNA chains requires the viral DNA polymerase, a 72 kd virus-encoded DNA-binding protein (DBP) and another cellular protein of 30 kd, nuclear factor II (INFII), which is a type I DNA topoisomerase (52).

The results of the alignment of ori sequences of HSV, adenovirus and papovaviruses are presented in Fig. 3. Homologies to the papovavirus ori-core sequences have been detected in sequences both, contained in the HSV-1 ori_L palindrome as well as in sequences found outside the ori_S palindrome of the HSV-1 genome. The homologous region displays a significant division into G+C-rich and A+T-rich sequences and spans from pos. 5236 to pos. 35 in SV40. This DNA stretch, in addition to the SV40 ori-core, contains the T-Ag binding site II and part of the sequences required for DNA-binding by the SV40 initiation factor(s). The G+C-rich sequences of SV40 contain the pentanucleotide sequence element 5'-GAGGC-3'/5'-GCCTC-3' that can be found in one or more copies in all high affinity binding sites for T-Ag (53). The A+T-rich sequences of HSV ori_S have been demonstrated to be required for ori-function (7). This complies well with the observed homology to the corresponding papovavirus ori-core sequences. The G+C-rich HSV sequences homologous to the papovavirus ori are flanking the region of highest homology between HSV ori_L/ori_S as shown in Fig. 1A suggesting that these sequences are required for a function other than origin activation.

Comparison of the 5'-terminal sequences of Ad5, which comprise both the signals for DNA initiation and for DNA-binding by NFI with the HSV ori sequences revealed extensive homologies to the center of the ori_S as well as to the ori_L palindrome, exclusively. An A+T-rich sequence of 9 identical nucleotides in the region required for initiation of Ad DNA replication is found in both HSV ori sequences. In addition, the downstream flanking sequences of the NFI binding site exhibit a remarkable homology (Fig. 3). The comparative study on VZV and HSV origins of DNA replication and on activation of the VZV ori_S by HSV-1-encoded products (54) has revealed two sequences common to both viruses: (i) a tract of alternating A and T residues and (ii) a 11 bp sequence (CGTTCGACTT). Both sequences - the 11 bp sequence as inverted repeat - are found in the center of the HSV ori_S and ori_L palindromes within the limits of the AD5/HSV homologous region as shown in Fig. 3.

From that we infer that the center of the HSV ori_S and ori_L palindromes spanning from position -27 to +26 (Fig. 1A) contains the signals for DNA initiation by virus-encoded DNA polymerases, and that the domain between position -72 to -37 (Fig. 1A) by virtue of homology to the ori sequences of papovaviruses contains 'rudimentary' signals for DNA initiation by cellular DNA polymerases (papovavirus-like organization). An in vitro system to study initiation and elongation of HSV DNA replication combined with site-directed mutagenesis of the ori sequences should allow to identify the proposed host- and virus-specific DNA initiation signals.

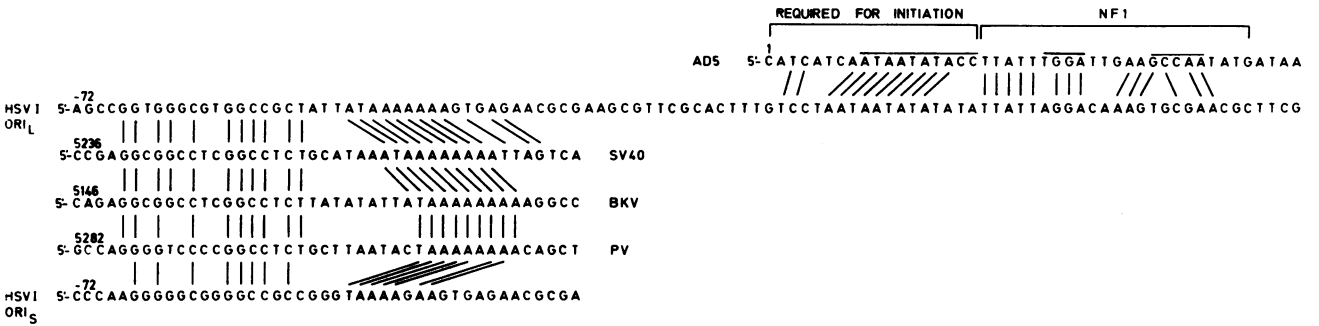

Figure 3. Alignment of the ori-sequences of HSV-1, adenovirus and papovaviruses. Ori$_L$ and ori$_S$ sequences are numbered according to Fig. 1A. Numbering of the other sequences is as published (41, 42, 43, 44). The Ad5 sequences required for initiation of DNA replication and for binding nuclear factor I are indicated.

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