Functional Mapping and DNA Sequence of an Equine Herpesvirus 1 Origin of Replication

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The genome of equine herpesvirus 1 (EHV-1) defective interfering (DI) particle DNA originates from discrete regions within the standard (STD) EHV-1 genome: the left terminus (0.0 to 0.04 map units) and the inverted repeats (0.78 to 0.79 and 0.83 to 0.87 map units of the internal inverted repeat; 0.91 to 0.95 and 0.99 to 1.00 map units of the terminal inverted repeat). Since DI DNA must contain *cis*-acting DNA sequences, such as replication origins, which cannot be supplied in *trans* by the STD EHV-1 virus, regions of the EHV-1 genome shown to be in DI DNA were assayed for the presence of a viral origin of DNA replication. Specifically, STD EHV-1 DNA fragments encompassing the genomic regions present in DI particle DNA were inserted into the vector pAT153, and individual clones were tested by transfection assays for the ability to support the amplification and replication of plasmid DNA in EHV-1-infected cells. The Sma-1 subfragment of the internal inverted repeat sequence (0.83 to 0.85 map units) was shown to contain origin of replication activity. Subcloning and BAL 31 deletion analysis of the 2.35-kilobase-pair (kbp) Sma-1 fragment delineated a 200-bp fragment that contained origin activity. The origin activities of all EHV-1 clones which were positive by the transfection assay were confirmed by methylation analysis by using the methylation-sensitive restriction enzymes *DpnI* and *MboI*. DNA sequencing of the 200-bp fragment which contained an EHV-1 origin of replication indicated that this region has significant homology to previously characterized origins of replication of human herpesviruses. Furthermore, comparison of known origin sequences demonstrated that a 9-bp sequence, CGTTCGCAC, which is conserved among all origins of replication of human lytic herpesviruses and which is contained within the 18-bp region in herpes simplex virus type 1 origins shown by others to be protected by an origin-binding protein (P. Elias, M. E. O'Donnell, E. S. Mocarski, and I. R. Lehman, *Proc. Natl. Acad. Sci. USA* 83:6322-6326) is also conserved across species in the EHV-1 origin of replication.

Equine herpesvirus 1 (EHV-1) is a natural infectious agent of horses and is an important cause of spontaneous abortions in pregnant mares (35-37, 39). Serial undiluted passage of standard (STD; infectious) EHV-1 results in the generation of defective interfering (DI) particles which display all of the properties which characterize classic DI particles (19, 20, 37, 38). EHV-1 DI particles have been generated both in tissue culture in L-M suspension cells (19) and in vivo in suckling Syrian hamsters (6). Infection of permissive hamster embryo cells with EHV-1 viral preparations enriched for DI particles results in the establishment of oncogenic transformation and persistent infection (10, 37, 45, 49). Cell lines established in this manner release both infectious EHV-1 and DI particles and have remained persistently infected after over 10 years in culture (10; R. Harty and D. J. O'Callaghan, unpublished observation). Our laboratory has continued to characterize the genetic structure and molecular organization of both STD and DI particle DNA with the aim of elucidating how DI particles participate in the establishment of persistent infection.

The EHV-1 genome is a double-stranded DNA molecule with a molecular size of 94 megadaltons, (MDa) which consists of two components: a long (L) region (73 MDa) covalently linked to a short (S) region (21 MDa). The S region contains two identical inverted repeats (IRs; 8 MDa) which bracket a short unique region (U_s ; 5 MDa) and which enable the entire S region to invert in orientation relative to the fixed L region (4, 21, 50, 64). Recent analysis of EHV-1 DI particle DNA has revealed that DI DNA originates from

the left terminus (0.0 to 0.04 map units) and the IR_s (0.78 to 0.79 and 0.83 to 0.87 map units; 0.91 to 0.95 and 0.99 to 1.00 map units) of the STD EHV-1 genome (1-3). Since DI DNA must contain *cis*-acting regions (e.g., replication origins) which cannot be supplied in *trans* by the helper virus, it is probable that an EHV-1 origin is contained within the STD genome left terminus and/or within the IR sequences known to be present in EHV-1 DI DNA.

The existence of replication origins in herpes simplex virus (HSV) DNA was initially suggested by electron microscope examination of replicating viral genomes (14, 22). The location of HSV origins was inferred from early studies on the DNA composition of HSV DI particles (for a review, see reference 14). Subsequent studies demonstrated conclusively that DI DNA contains a replication origin located within the c component of the IR_s (33, 54, 55, 62). Five distinct human herpesvirus lytic origins of replication have now been characterized and sequenced: two in HSV-1 (*ori_{S1}* [56, 57, 59] and *ori_{L2}* [17, 63]), two in HSV-2 (*ori_{S2}* [65] and *ori_{L2}* [28]), and one in varicella-zoster virus (VZV; *ori_S* [58]). In addition, analysis of cloned pseudorabies virus DI DNA has suggested that two origins of replication are present at different locations in the unique long section of the pseudorabies virus genome (66). In general the human herpesvirus origins of replication share extensive homology, and all contain nearly perfect palindromes which can be arranged in hypothetical stem-and-loop structures. In addition, all five origins contain a conserved 11-base-pair (bp) motif (CGT TCGCACTT [58]) which has been shown by nuclease protection studies of *ori_{S1}* to be bound by a protein present in HSV-1-infected cells (13). Recent mutagenesis studies have

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suggested that the left arm of the *ori_{SL}* palindrome containing the conserved sequence is essential for origin activity whereas the right arm can be deleted without significantly inhibiting replication (11). This study also suggests that the ability to form a cruciform structure is not essential for *ori_{SL}* activity. Lastly, transfection studies have indicated that HSV-1 origin-mediated plasmid amplification requires only seven viral gene products (9, 32, 67).

In this paper, we describe the mapping and DNA sequencing of an EHV-1 origin of replication within STD viral IR sequences previously shown to be present in EHV-1 DI particle DNA. The EHV-1 origin is shown to possess significant DNA homology across species with all five previously sequenced human herpesvirus origins, including a 9-bp motif noted by others (58) to be highly conserved in herpesvirus origins and which has recently been shown to be protected in the HSV *ori_{SL}* by an origin-binding protein (13). Unlike other herpesvirus origins, the EHV-1 origin does not contain a near perfect palindrome and is therefore limited in its ability to form hypothetical stem-and-loop structures.

MATERIALS AND METHODS

Cells and viruses. EHV-1 (Kentucky A strain) was grown in L-M suspension cell cultures as described elsewhere (36, 39, 40). Passage of EHV-1 was performed at low multiplicity of infection (0.005 PFU per cell). Virus was purified by polyethylene glycol 6000 precipitation of infected cell-free supernatants, followed by several cycles of rate-velocity centrifugation in Dextran 10 gradients (40). L-M TK⁻ cells were obtained from the American Type Culture Collection (ATCC CCL1.3) and were maintained in the absence of bromodeoxyuridine.

Isolation of viral DNA. Purified EHV-1 viral particles were suspended in TE buffer (0.01 M Tris hydrochloride, pH 7.4, 0.001 M EDTA) and treated with sodium dodecyl sulfate (SDS; final concentration, 1%, wt/vol and proteinase K (100 µg/ml) for 2 h at 37°C (19). Following incubation, DNA was extracted once with 80% phenol in TE buffer and twice with chloroform-isoamyl alcohol (24:1, vol/vol). DNA was precipitated by the addition of 2.5 volumes of 95% ethanol and a 1/20 volume of 4 M sodium acetate and was incubated at -70°C for 1 h. DNA was collected by centrifugation at 31,000 × g for 30 min and suspended in 0.1 × TE buffer.

Plasmid construction. The cloning of the STD EHV-1 genome and of DI DNA has been described previously (2-4, 46-48). All clones were inserted into the vector pAT153 (60), which lacks vector sequences known to poison the simian virus 40 origin of replication (30), or were inserted into our derivative, pAT12, which contains the multiple cloning site of pUC12 (61) inserted between the *EcoRI-HindIII* sites of pAT153 (pAT153 was a gift of Robert Perry). Cloning of the desired DNA fragments was performed by established procedures (31). When necessary, specific DNA fragments were sliced as individual bands from low-melting-point agarose (SeaPlaque; FMC Corp., Marine Colloids, Div., Rockland, Maine) and isolated by extraction. After transformation, bacteria (*Escherichia coli* HB101) were grown in the presence of the appropriate antibiotic, and plasmid DNAs were isolated from individual colonies by the minilysate procedure (23). DNAs were screened for inserts by minisubmersible gel electrophoresis through 0.8 to 2.0% agarose gels, following restriction endonuclease digestion. Individual clones were expanded into large bacterial growths, and the plasmid DNA was isolated by the lysis-by-boiling technique (23) and ethidium bromide-CsCl dibuoyant density gradient centrifugation.

Clones p1-121 *d11*, *d16*, *d17*, and *d18* were generated by BAL 31 digestion of clone p1-121. p1-121 was linearized by digestion with *XbaI* and treated for various times with 1.25 U of BAL 31 (New England BioLabs, Beverly, Mass.) at 30°C. Phosphorylated *XbaI* linkers (New England BioLabs) were ligated to the appropriate BAL 31-digested samples (T4 DNA ligase; Bethesda Research Laboratories, Gaithersburg, Md.), and samples were digested with *XbaI*, ethanol precipitated, and religated overnight at 4°C. Individual clones were screened for inserts as described above, and specific inserts were freed and ligated back into intact pAT12 to ensure that all clones contained identical vector sequences. The extent of deleted sequences was estimated by gel electrophoresis of restriction enzyme digests: *d16* (-200), *d17* (-400), *d18* (-600), and *d11* (-625).

Transfection assays. EHV-1 DNA fragments were tested for origin activity by using the transfection assay of Stow and McMonagle (59). L-M TK⁻ cells were selected for transfection analysis because they are permissive for EHV-1 replication, can readily be transfected, and will survive EHV-1 infection at a high multiplicity of infection (>5) for greater than 16 h (unpublished observation). Briefly, subconfluent monolayers of L-M cells in 25-cm² flasks were transfected with equimolar amounts of plasmid DNAs (approximately 200 ng) by the calcium phosphate technique (16). DNA precipitate (1 ml) was added to 10 ml of Eagle minimal essential medium EMEM (5% fetal bovine serum) in each 25-cm² flask and incubated at 37°C. Cells were shocked at 2 to 4 h posttransfection by the addition of 1 ml of 10% dimethyl sulfoxide-20% glucose for 5 min at room temperature (5), rinsed, and refed. At 6 h posttransfection, cells were infected with EHV-1 at a multiplicity of infection of 5 or greater, and at 20 to 24 h postinfection, cells were harvested by scraping into 2 ml of 1 × TE-0.6% SDS containing 10 to 20 µg/ml of proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Flasks were incubated overnight at 37°C. DNA was extracted twice with phenol saturated with 1 × TE buffer and once with chloroform-isoamyl alcohol (24:1, vol/vol). Following ethanol precipitation, DNA samples were resuspended in 0.5 ml of distilled water and quantitated spectrophotometrically and visually by ethidium bromide gel electrophoresis.

Restriction enzyme digestion and Southern blot hybridization. DNAs (10 to 15 µg or about 1/20 of the total DNA yield) were digested to completion with a three- to fivefold excess of the desired restriction endonuclease under conditions recommended by the supplier (Bethesda Research Laboratories). The resultant DNA digests were electrophoresed through 0.8% agarose, stained with ethidium bromide (0.5 µg/ml), and photographed in shortwave UV light (52). Gels containing viral DNA fragments were alkali denatured, neutralized, transferred to hybridization membranes (nitrocellulose; Schleicher & Schuell, Keene, N.H.) in 10 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) by the method of Southern (53), and immobilized by baking in vacuo at 80°C for 2 h. Filters were prehybridized in 6 × SSC-0.5% SDS-5 × Denhardt solution (1 × Denhardt solution is 0.02% each bovine serum albumin, polyvinylpyrrolidone, and Ficoll [Pharmacia Fine Chemicals, Piscataway, N.J.])-200 µg of salmon sperm DNA per ml for 4 h at 65°C. Probe DNA was ³²P labeled by nick translation (43), denatured, and hybridized (2.0 × 10⁶ cpm/ml) with filters in the same mixture overnight at 65°C.

Filters were rinsed once with 2 × SSC at room temperature and then washed for 1 h in 2 × SSC-0.5% SDS at 65°C, followed by a 1-h wash in 0.1 × SSC-0.1% SDS at 65°C.

FIG. 1. Restriction enzyme cleavage map and subcloning of the Sma-1 fragment. On the top lines are shown the DNA sequences present in EHV-1 DI DNA and the structure of the STD EHV-1 genome. IRs are shown as the boxed areas. The Sma-1 DNA fragment is shown in enlarged form below the STD EHV-1 genome. Subclones of the Sma-1 fragment are shown and are listed by number at the right. The construction of specific clones is described in Materials and Methods. The abbreviations for the restriction endonucleases are given at bottom left. The bottom line gives map units for the Sma-1 fragment.

Filters were air dried and allowed to expose Kodak X-Omat AR film at -70°C for 1 to 7 days in the presence of Cronex Lightning-Plus (Du Pont Co., Wilmington, Del.) intensifying screens.

DNA sequencing. The Sanger dideoxy technique was employed for DNA sequencing (51). A kit purchased from American Bionetics (Hayward, Calif.) containing deaza dGTP was routinely used for DNA sequencing. The 203-bp fragment between the *SpeI* and *ClaI* restriction sites (clone p1-133) was sequenced in M13 mp18 and mp19 as part of a general project to sequence the entire 2.35-kbp Sma-1 fragment (Yalamanchili et al., manuscript in preparation). The EHV-1 p1-133 DNA sequence was compared with the published sequences of the known human herpesvirus origins of replication (28, 34, 58, 63, 65) by using the BIONET national computer resource for molecular biology (26).

RESULTS

Selection and cloning of STD EHV-1 DNA fragments. Previous studies from this laboratory demonstrated that DI particle DNA originates from the left terminus (0.0 to 0.04 map units) and IRs (0.78 to 0.79 and 0.83 to 0.87 map units; 0.91 to 0.95 and 0.99 to 1.00 map units) of the EHV-1 STD genome. Analysis of DI particle DNA in other herpesvirus systems indicated that *cis*-acting sequences, such as cleavage encapsidation signals and replication origins, are located

in DI DNA (15). Therefore, we chose four STD EHV-1 DNA clones for replication origin analysis which together contained virtually all of the DNA sequences known to be present in EHV-1 DI DNA. These cloned were *Bg/II* N (0.00 to 0.03 map units), Sma-5 (0.78 to 0.79 map units), Sma-1 (0.83 to 0.85 map units), and Sma-2 (0.85 to 0.865 map units) (Table 1 and Fig. 1). As a precaution, each insert was recloned into the vector pAT153, which lacks DNA sequences shown to poison the replication of the simian virus 40 origin (60).

Individual EHV-1 clones in pAT153 were assayed for origin activity by the method of Stow and McMonagle (59). Plasmid DNAs in equimolar amounts (equivalent to 200 ng of pAT153) were transfected into duplicate flasks of L-M cells, and the transfected cells were either mock infected or infected (multiplicity of infection, 5 or greater) with EHV-1 STD virus. Cell DNAs were extracted, digested with *EcoRI*, and analyzed by Southern blot hybridization by using ^{32}P -labeled pAT153 DNA as a probe (Fig. 2). *EcoRI* cleaves only once in each plasmid construct and therefore should yield only a single detectable band in infected-cell lanes in which plasmid amplification has occurred in linkage to an EHV-1 origin of replication. Only the Sma-1 clone showed amplification of vector sequences in the lane with the infected cells (Fig. 2). The band seen in the Sma-5 mock-infected-cell lane represents weak homology between the probe and cell DNA

TABLE 1. Plasmid constructions used for origin analysis

Clone ^a	Size (bp) ^b	Map units	Origin activity ^c
BglII N	2,700	0.000–0.030	–
Sma-5	1,200	0.780–0.790	–
Sma-2	2,100	0.850–0.865	–
Sma-1	2,350	0.830–0.850	+
p1-115	450	0.830–0.834	–
p1-117b ^d	1,000	0.834–0.843	+
p1-118	700	0.834–0.848	–
p1-119	200	0.848–0.850	–
p1-120	2,150	0.830–0.848	+
p1-121	1,900	0.834–0.850	+
d16 ^e	1,700	0.836–0.850	+
d17	1,500	0.837–0.850	+
d18	1,300	0.839–0.850	+
d11	1,275	0.839–0.850	+
p1-127	700	0.834–0.840	–
p1-128	300	0.840–0.842	+
p1-131	370	0.840–0.843	+
p1-132	167	0.841–0.843	–
p1-133	203	0.840–0.841	+
p1-134	445	0.839–0.843	+
p1-135	470	0.839–0.843	+
p1-136	1,100	0.839–0.848	+

^a All clones were inserted into the vector pAT153 or its derivative pAT12 (see Materials and Methods).

^b Sizes were determined by DNA sequencing or estimated by comparison to electrophoretically separated restriction enzyme digests of pBR322 and lambda bacteriophage DNA.

^c Origin activity was determined by transfection assay and restriction enzyme methylation analysis (see Materials and Methods).

^d Clone p1-117 was found to contain two copies of the insert; a second clone containing only a single insert (p1-117b) was derived.

^e Clones p1-121 d16, d17, d18, and d11 were derived by BAL 31 deletion of clone p1-121.

sequences and was visible in all lanes upon prolonged exposure but was not observed reproducibly in different Southern blots. No amplification of vector sequences alone or of other STD EHV-1 clones (Fig. 2) was demonstrated. In fact, over the course of these experiments, amplification of vector sequences alone or EHV-1 DNA sequences outside of Sma-1 was never observed, even when conditions were intentionally varied and excessive amounts of DNA were used for transfection. These studies suggested that an EHV-1 origin of replication exists within STD EHV-1 IR sequences (Sma-1 fragment) known to be present in EHV-1 DNA particle DNA.

To confirm that the Sma-1 plasmid is actually replicating in EHV-1-infected cells, methylation analysis was conducted on cell DNA preparations. *E. coli* Dam⁺ strains methylate adenine residues within their DNA; in contrast eucaryotic cells neither methylate their DNA at adenine residues nor do they possess an adenine demethylase (27, 42). Thus, restriction enzymes which are sensitive to the methylation state of the DNA can be used to differentiate between DNA replicated in bacteria and DNA which has replicated in eucaryotic cells. Two such enzymes are *DpnI*, which cleaves the sequence GATC only when the A residue is methylated, and *MboI*, which cleaves at GATC only when the A residue is unmethylated. Plasmid DNA which has replicated in eucaryotic cells is *DpnI* resistant but *MboI* sensitive. *DpnI-MboI* analysis of amplified Sma-1 DNA (Fig. 3B) indicated that it was *DpnI* resistant (Fig. 3B, lane ED) but *MboI* sensitive (Fig. 3B, lane EM). When *DpnI*-sensitive plasmid DNA was mixed with cell DNA before digestion (Fig. 3B, lane ★ED), the exogenous plasmid was cleaved by *DpnI* (low-molecular-

weight smear). Hence, lack of *DpnI* cleavage in lane ED is not due to generalized inhibition of *DpnI* cleavage by the cell DNA sample.

Fine mapping of the EHV-1 origin. The EHV-1 *ori*_S was delineated further by subcloning of the Sma-1 fragment. A series of six different subclones of Sma-1 were derived in the vectors pAT153 and pAT12 and included p1-115, p1-117b, p1-118, p1-119, p1-120, and p1-121 (Table 1 and Fig. 1). Transfection analysis of the six clones indicated that clones p1-117b, p1-120, and p1-121 possessed origin activity but clones p1-115, p1-118, and p1-119 were origin negative (Fig. 3A and 4A). *DpnI-MboI* analysis of origin-positive clones indicated that amplified sequences were *DpnI* resistant and *MboI* sensitive, a pattern indicative of unmethylated DNA which has replicated in eucaryotic cells (Fig. 3B and 4B). Interestingly, our initial isolate of p1-117 contained two copies of the *HpaI-HindIII* fragment (Fig. 1) and demonstrated only minimal amplification in the origin assay. A single-copy clone (p1-117B [lanes with ★]) which was subsequently isolated displayed readily detectable origin activity (Fig. 3A). Perhaps tandem or multiply origin sequences in direct linkage are inhibitory to origin function.

The extent of amplification and the ability to detect input DNA varied among transfections, but the ability to be replicated was always consistent for individual viral DNA clones. For example, in Fig. 4A constructs p1-120 and p1-121 underwent marked amplification in infected cells, whereas the amplification seen for the parental Sma-1 clone was much more modest. *DpnI-MboI* analysis indicated that plasmid DNA in the mock-infected-cell lane (Fig. 4B, p1-117B, lane m) was unreplicated (i.e., *DpnI* sensitive and *MboI* resistant), even though input p1-117B DNA was detected in the mock-infected-cell lane (Fig. 4A). In contrast, plasmid DNA in the infected sample (*DpnI* resistant and *MboI* sensitive) had replicated [Fig. 4B, p1-117B (i)]. These studies indicate that EHV-1 replication origin sequences were contained in the 1-kbp fragment p1-117B which was bordered on the left by a *HpaI* site and on the right by a *HindIII* site (Fig. 1).

Clone p1-121 showed optimal origin activity in our transfection assay. Since p1-117B (the smallest origin-positive fragment) represents the left 1.0 kb of p1-121 (Fig. 1), the

FIG. 2. Origin analysis of STD EHV-1 clones homologous to sequences present in DI DNA. Individual fragments were inserted into the vector pAT153 (or pAT12) and analyzed for origin activity by transfection into L-M cells. All cell DNAs (10 to 15 µg) were digested with *EcoRI* to linearize transfected DNAs, electrophoresed through 0.8% agarose, and blotted to nitrocellulose. Nitrocellulose filters were probed with pAT153 DNA which had been ³²P labeled by nick translation. The individual clones used are shown at the top. Duplicate flasks of cells were used which were either mock infected (–) or virus infected (+). The map units of Sma-1 are 0.83 to 0.85.

FIG. 3. Origin analysis of subclones of the Sma-1 DNA fragment. (A) Transfected-cell DNAs (10 to 15 μ g) were digested with *Eco*RI, electrophoresed in 0.8% agarose, and transferred to nitrocellulose. The filter was probed with vector DNA which had been 32 P labeled by nick translation. The individual clones used for transfection are shown at the top. Lanes are designated m for mock infected or i for infected-cell monolayers. Clone p1-117B is designated by a star (*). p1-117 contains a two-copy insert. p1-117B contains a single-copy insert of the same fragment. v, EHV-1 genomic DNA digested with *Eco*RI; c, positive control from an infected-cell lysate which was transfected with Sma-1. (B) *Dpn*I-*Mbo*I analysis of Sma-1 subclones. Cell DNAs from infected-cell lanes (i) in panel A which were positive for origin activity were digested with *Eco*RI (E), *Eco*RI and *Dpn*I (ED), or *Eco*RI and *Mbo*I (EM). In lanes ED marked by stars (*) exogenous vector DNA was mixed with cell DNAs before digestion as a control to ensure that *Dpn*I was active. Total-cell DNAs were analyzed by Southern blot analysis as in panel A.

exonuclease BAL 31 was used to delete sequences from the *Hpa*I site located at the left end of p1-121. Four clones p1-121 d16, d17, d18, and d11 were characterized (Fig. 1 and Table 1). All four clones were origin positive (Fig. 5A), including p1-121 d11, which has approximately 625 bp deleted from the left end. *Dpn*I-*Mbo*I analysis (Fig. 5B) confirmed that all clones had replicated. These studies suggested that EHV-1 origin activity could be localized to the rightward 400 bp of clone p1-117B. Therefore, a new series of

FIG. 4. Origin analysis of subclones p1-117B, p1-120, and p1-121. (A) Total-cell DNAs were isolated after transfection, and 10 to 15 μ g was digested with *Eco*RI, electrophoresed through 0.8% agarose, and blotted to nitrocellulose. The filter was hybridized with pAT153 which had been 32 P labeled by nick translation. Clones used for transfection are listed at the top of each set of lanes. Sma-1 was cloned into either pAT153 (Sma-1 pAT) or pUC12 (Sma-1 pUC). V, EHV-1 genomic DNA digested with *Eco*RI; mock infected; i, infected. (B) Cell DNAs from panel A in amounts of 10 to 15 μ g were digested with *Eco*RI (E), *Eco*RI and *Dpn*I (ED), or *Eco*RI and *Mbo*I (EM). Cell DNAs were analyzed by Southern blot hybridization as described above.

FIG. 5. Origin analysis of BAL 31 deletion constructs of p1-121. Deletions were constructed in p1-121 by using BAL 31 as described in Materials and Methods. (A) After transfection, cell DNAs (10 to 15 μ g) were digested with *Eco*RI, electrophoresed through 0.8% agarose gels, and blotted to nitrocellulose. Filters were probed with pAT153 which had been 32 P labeled by nick translation. m, Mock infected; i, infected. (B) Cell DNAs from infected-cell (i) lanes were digested with *Eco*RI (E), *Eco*RI and *Dpn*I (ED), or *Eco*RI and *Mbo*I (EM) and then were subjected to Southern blot analysis as in panel A.

clones was developed which centered on this 400-bp region. These clones included p1-127, p1-128, p1-131, p1-133, p1-134, p1-135, and p1-136. As shown in Fig. 6 (also see Fig. 1 and Table 1), the smallest clone showing origin activity was the 203-bp clone p1-133 (0.840 to 0.841 map units) which is bordered on the left by an *Spe*I site and on the right by a *Cl*aI site. The origin-positive clones p1-128, p1-131, p1-134, p1-135, and p1-136 all contain clone p1-133 sequences as a subset of their total DNA sequences. In Fig. 6, cell DNAs in all lanes (including the mock-infected-cell lanes) were double digested with *Eco*RI and *Dpn*I. Only total-cell DNAs isolated from EHV-1-infected cells (Fig. 6, lanes i) contained *Dpn*I-resistant plasmid DNA (i.e., DNA which has replicated in eucaryotic cells).

DNA sequence analysis of the EHV-1 *orig*. The EHV-1 STD DNA fragment corresponding to the minimal origin of repli-

FIG. 6. Origin analysis of Sma-1 subclones. The 200-bp p1-133 clone has origin activity. After transfection, cell DNAs were isolated and 10 to 15 μ g were digested with *Eco*RI and *Dpn*I. Samples were electrophoresed in a 0.8% agarose gel and blotted to nitrocellulose. Filters were hybridized with pAT153 DNA which had been 32 P labeled by nick translation. m, Mock infected; i, infected.

FIG. 9. Hypothetical stem-and-loop structure for EHV-1 *ori_S*. Shown is the stem-and-loop structure that can be drawn in the area of maximal homology between the EHV-1 *ori_S* and the human herpesvirus lytic origins of replication: bp 71 to 129. Vertical lines designate areas of potential base pairing.

ing DNA fragments from regions shown to be present in EHV-1 DI DNA were assayed for origin activity in transfection assays. Of these clones, only a clone from the central portion of the IRs (Sma-1; 0.83 to 0.85 map units) was shown to have origin activity. A series of subclones of Sma-1 was generated and was used in transfection studies to demonstrate that origin activity is contained within a 200-bp fragment (0.840 to 0.841 map units). Comparison of the DNA sequence of this 200-bp fragment to those of human herpesvirus origins demonstrated that substantial homology exists among them, including a 9-bp sequence (CGTTCGCAC) previously noted to be conserved among the human herpesvirus origins of replication (58). To our knowledge, this work represents the first description of the location and DNA sequence of a herpesvirus replication origin other than those of a human herpesvirus. Thus, it appears that between herpesviruses of different species there is significant conservation of DNA sequences in viral origins of DNA replication. Structurally, the similarities between the EHV-1 origin and the human herpesvirus origins appear restricted, in that the EHV-1 origin does not contain a near-perfect palindrome. Although, a stem-and-loop structure can be proposed for the EHV-1 origin, the proposed structure has a 7-bp region in its center which does not have complementarity for the formation of a stem-loop structure; thus, one would presume that the formation of this hypothetical EHV-1 stem-and-loop structure would not be as favored as that of the near-perfect stem-and-loop structures proposed for the human herpesviruses. Interestingly, a recent study reports that an HSV-1 *ori_S* clone from which the right side of the palindrome was deleted retains origin activity (11). These results suggest that the ability to form a stem-and-loop structure may not be essential to origin activity.

Electron microscope visualization of replicating HSV genomes (14, 22) and molecular analysis of HSV DI particles (15) support the functional importance of the HSV *ori_S* and *ori_L* in productive infections. However, recent studies have indicated that the *ori_L* and at least one copy of the *ori_S* are dispensable for HSV-1 biological activity (29, 41). Furthermore, deletion of the *ori_L* was found not to affect the ability of HSV-1 to establish latency in the murine eye model. Interestingly, the HSV *ori_S* has been shown to be contained in a transcribed open reading frame (24), an observation which opens the possibility that *trans*-acting regulatory factors encoded by the origin region may act to modulate origin activity. The importance of interaction between replication origins and transcriptional elements in regulating viral growth, although postulated for HSV, has been clearly substantiated in other viral systems (7, 12). Indeed, others have noted some homology among the HSV origin and the adenovirus and papovavirus origins (25). Of course, the locations of the HSV *ori_L* and *ori_S* sequences are suggestive

of a role in transcriptional regulation. The HSV *ori_L* is flanked by the coding regions for the major DNA-binding protein (ICP8) and the DNA polymerase, while the *ori_S* is bracketed by the IE genes for ICP4 and ICP22/47. Similarly, the EHV-1 *ori_S* is located in the center of the IR_S where the single EHV-1 IE gene and the EHV-1 very early gene have been mapped (Fig. 7B) (8, 18, 44). Further investigation will be necessary to define the exact functional and regulatory properties of the herpesvirus origins.

The studies described here are part of our overall endeavor to characterize the genetic elements that constitute EHV-1 DI DNA. Ultimately, we hope that understanding STD and defective EHV-1 DNA at the molecular level will enable us to decipher how DI particles participate in the establishment of a persistent infection of permissive cells by STD EHV-1 (10, 37). Certainly, regulation of replication origin activity might be a major component in the establishment of a persistent infection. Currently, we are cloning and sequencing potential origins from cloned EHV-1 DI DNA to determine if they are complementary to the known sequence of the STD EHV-1 *ori_S* (Yalamanchili et al., in preparation).

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