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Replication Process of the Parvovirus H-1.
VI. Characterization of a Replication Terminus of H-1 Replicative-Form DNA

SOLON L. RHODE III

Putnam Memorial Hospital Institute for Medical Research, Bennington, Vermont 05201

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The linear duplex replicative form (RF) DNA of the parvovirus H-1 has been characterized with respect to cleavage by the bacterial restriction endonuclease of *Escherichia coli*, *Eco*RI. RF DNA has a single cleavage site 0.22 genome length from the left end of the molecule. The molecular weight of H-1 RF DNA determined by gel electrophoresis is 3.26×10^6. H-1 RF DNA has been found to dimerize by hydrogen-bonded linkage at the molecular left end, and in some molecules the viral strand is covalently linked to the complementary strand. Some 10% of monomeric RF DNA also has a covalent linkage between the viral and complementary strands at the left end. The *Eco*RI-B fragment, containing the left end of the RF molecule, appears to be a replication terminus by its labeling characteristics for both RF and progeny DNA synthesis. These findings suggest that the left end of H-1 RF DNA has some type of "turn-around" structure and that this end is not an origin for DNA synthesis.

The parvovirus H-1 contains a genome of single-stranded DNA with a molecular weight of approximately 1.7×10^6 (15). We have previously characterized a double-stranded DNA replicative form (RF) synthesized during the replication of H-1-infected cells (17, 18). H-1 RF DNA has been found to be linear by isopycnic centrifugation in ethidium bromide-CsCl gradients, velocity sedimentation (17, 18), gel electrophoresis (in this paper), and by electron microscopy (23).

In this study, H-1 RF DNA and its dimer have been characterized further. An improved estimate of H-1 RF DNA molecular weight, 3.26×10^6, has been obtained by gel electrophoresis. Based on a DNA size of about 4,920 base pairs (bp), the H-1 genome has a total capacity to code for proteins with a cumulative molecular weight of about 172,000. To date, only the two polypeptides of the H-1 capsid, VP1 and VP2, have been demonstrated as viral-induced proteins in infected cells, and their total molecular weight of 164,000 is close to the coding capacity of the genome (14). It has not been determined whether the H-1 genome is transcribed to the extent of the other parvoviruses (50 to 100% of the rat virus viral strand is transcribed [20], and 75% of the adenovirus-associated virus [AAV] genome is transcribed [3-5]), or whether VP1 and VP2 have a common structural gene. Nevertheless, it is clear that the genetic capacity of H-1 is very limited, and thus its replication must be executed largely by cellular enzymes. A number of conditional lethal mutants of H-1 have been isolated and none of these has shown a viral protein requirement for RF DNA replication (19, and unpublished data). It is possible, but not established, that RF replication is totally under the control of cellular proteins. In any case, analysis of RF replication is a powerful probe of cellular DNA replication and could be useful in the study of the mechanisms of replication of linear molecules.

I have examined the physical map of H-1 RF DNA with respect to the bacterial restriction endonuclease of *Escherichia coli*, endo R·*Eco*RI (*Eco*RI). This enzyme has allowed a more detailed characterization of one end of the RF DNA molecule, and evidence for several structural alternatives for this end are presented. Experiments described here indicate that this end is a terminus for the semiconservative replication of RF DNA and for progeny DNA synthesis. This terminus is in some cases self-cohesive, as manifested by the formation of dimer molecules linked in an inverted manner rather than in tandem. The viral (V) and complementary (C) strands in some monomer or dimer RF molecules are covalently linked at this end. These findings suggest a structure for

the RF DNA replication terminus similar to one recently proposed for the AAV DNA termini (8, 24).

MATERIALS AND METHODS

Virus and cell strains. Parasynchronous cultures of secondary hamster embryo or NB cells were prepared and infected as previously described (14, 16). The H-1 strains used here were wild type (wt) and *ts* 1 (19). An *E. coli* (W1100) lysogen of λcI_{857} *sus*S was obtained from Herbert Boyer, San Francisco.

Infection, labeling, and viral DNA extraction. Parasynchronous hamster embryo cultures were infected at 12 h poststimulation, and NB cell cultures were infected at the time of reversal of the methotrexate block with medium containing thymidine (TdR; 10^{-5} M), adenosine (13 μg/ml), and minimal essential medium nonessential amino acids as a source of glycine (19). Virus adsorptions were performed for 30 min at 33°C. After virus adsorption, the cultures were incubated at the temperatures indicated. Viral DNA was labeled by changing the medium after two washes with Hanks balanced salts (5 ml per petri dish) to a prewarmed medium containing the appropriate labeled precursor. In most cases, labeled TdR was used in the presence of 5'-fluorodeoxyuridine (FUdR) at 0.5 μg/ml after endogenous pools of TdR had been depleted by a preincubation in medium with FUdR alone. Labeling with $^{32}PO_4$ was done in medium buffered by 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and with the appropriate Cl salts substituted for PO_4 in the medium.

Viral DNA was extracted by a modification of the Hirt procedure (12). The monolayer cultures were washed once with 5 ml of Tris-buffered saline per 100-mm dish and lysed with 0.5 ml of 0.6% sodium dodecyl sulfate in 50 mM Tris (pH 7.5)-10 mM EDTA per dish. After 15 to 20 min at room temperature, 0.5 ml of a solution containing 5 mg of Pronase per ml was added to each dish, and the incubation was continued for 30 min at 37°C. A solution of 5 M NaCl was then added dropwise to each dish to a final concentration of 1 M and gently mixed with the lysate. The lysates were collected into centrifuge tubes and incubated overnight at 0°C. After centrifugation at 25,000 × *g* for 50 min, the supernatant, containing viral DNA, was precipitated with 2.5 volumes of ethanol for 6 to 16 h at −20°C. The precipitates were redissolved in 50 mM Tris (pH 7.5)-1 mM EDTA and digested with pancreatic RNase (heated to 80°C for 20 min) at 50 μg/ml. They were then either extracted with phenol or directly fractionated by centrifugation in 5 to 20% neutral sucrose gradients as detailed previously (17). Generally, about two-thirds of the total isotopic incorporation was recovered as viral DNA in the Hirt supernatant. [^{3}H]TdR-labeled λ DNA was prepared as described (11).

Digestion with restriction endonuclease. Viral DNA dissolved in 50 to 100 μl of 100 mM Tris (pH 7.5)-50 mM NaCl-10 mM $MgCl_2$ was incubated with 10 to 100 U of *Eco*RI for 2 or 3 h at 37°C. Endonuclease R·*Eco*RI was purchased from Miles Laboratories, Inc. Digestions with endonuclease R·*Hae*III (*Haemophilus aegyptius*, kindly provided by David T. Denhardt) were carried out for 16 h at 37°C in 50 ml of 10 mM Tris (pH 7.5)-10 mM NaCl-10 mM $MgCl_2$-1 mM dithiothreitol and with 5 μl of enzyme. Reactions were stopped by addition of EDTA to a final concentration of 50 mM.

Analytical gel electrophoresis. Electrophoresis was conducted in cylindrical gels (0.6 by 15 cm) with various concentrations of agarose or 3% acrylamide-0.5% agarose in E buffer (40 mM Tris-20 mM Na acetate-1 mM EDTA) (9) at 23°C under constant voltage as specified in the text. DNA samples were mixed with 0.5 volume of a solution of E buffer, 50% glycerol, and 0.01% bromophenol blue for layering on the gel. Gels were sliced into 1.0-mm or 1.17-mm slices, and two slices per vial were incubated 2 to 16 h in 0.5 ml of NCS solubilizer (Amersham/Searle) and assayed for radioactivity by liquid scintillation spectrometry in 10 ml of a toluene-based scintillation fluid. Ninety percent or more of the activity is eluted from the gel slices by this method.

Preparative gel electrophoresis. Viral DNA was fractionated in cylindrical gels (1.2 by 6 cm) of 1% agarose in E buffer using a constant voltage of 150 V. DNA in fractions of 2 ml was collected every 10 min by continuous electroelution into a chamber of our design. Aliquots were taken for determination of radioactivity, and appropriate fractions were collected and precipitated with 0.1 volume of 3 M Na acetate (pH 5.0) and 2.5 volumes of ethanol for 16 or more h at −20°C.

Slab-gel electrophoresis. Slab-gel electrophoresis was carried out in gels (0.3 by 12 by 16 cm) of 1.8% agarose in an EC470 apparatus (E-C Apparatus Corp., Philadelphia, Pa.). The gel and electrode buffers were buffer E, and electrophoresis was for 18 h at 16°C with a constant voltage of 50 V. The gel was dried in vacuo and exposed to Kodak no-screen X-ray film NS2T for 4 days before development.

Velocity centrifugation. Neutral sucrose gradients were 5 to 20% sucrose in 50 mM Tris (pH 8.0)-1 M NaCl-1 mM EDTA-0.2% Sarkosyl centrifuged in an SW25 rotor for 18 to 22 h at 24,000 rpm. Alkaline sucrose gradients were 5 to 20% sucrose in 0.2 N NaOH-0.8 M NaCl-1 mM EDTA centrifuged in an SW50 rotor for 16 h at 30,000 rpm. Fractions were collected through the bottoms of the tubes. Aliquots were taken for determination of radioactivity either by solubilization directly in 10 volumes of NCS or after precipitation on cellulose acetate filters with 10% trichloroacetic acid and 100 μg of bovine serum albumin, followed by liquid scintillation spectrometry. Alkaline gradients were fractionated on 25-mm Whatman no. 3 filter paper disks, which were washed twice in cold 5% trichloroacetic acid and once in acetone, dried, and counted with 0.2 ml of NCS solubilizer and toluene scintillation fluid.

Isopycnic centrifugation. DNA was banded to equilibrium in Cs_2SO_4 as previously described (17, 19), and aliquots were measured for radioactivity as for alkaline sucrose gradients.

Benzoylated DEAE-cellulose (BDC) chromatography. BDC was purchased from Schwarz/Mann (Orangeburg, N.Y.). Columns (1 by 5 cm) were constructed and washed with 100 to 200 volumes of 0.3

M NaCl-50 mM Tris (pH 7.5)-1 mM EDTA (0.3 M TBS). DNA in 2 to 5 ml of 0.3 M TBS was applied to the columns with a flow rate of 1 ml/min, and the column was washed with 15-, 30-, and 30-ml volumes of 0.3 M TBS. Double-stranded DNA was eluted by two 10-ml applications of 1 M TBS, and DNA with single-stranded regions was similarly eluted with two 10-ml applications of 2% caffeine in 1 M TBS warmed to 40°C. In some cases, the caffeine elution was carried out with 20 ml of a linear gradient of 0 to 2% caffeine in 1 M TBS. The washes and aliquots of the 1 M TBS and 2% caffeine fractions were assayed for radioactivity by trichloroacetic acid precipitation as above. DNA in the eluates was precipitated for further studies by 2.5 volumes of ethanol overnight at −20°C.

Alkaline denaturation-BDC chromatography was done by incubating the DNA in a final concentration of 0.2 N NaOH in a volume of 200 μl for 20 min at ambient temperature; the solution was cooled to 0°C, neutralized, and diluted 25-fold with cold 0.2 M TBS containing 0.2 ml of 0.2 N HCl. To minimize intermolecular annealing, the DNA solution was immediately applied to the column of BDC within 1 to 2 min. The elutions were carried out as described above. Final recoveries were 80 to 100% of the DNA applied to the columns.

RESULTS

Endo R·*Eco*RI digestion of H-1 RF DNA. H-1 RF DNA labeled with [^{3}H]TdR was prepared from *ts*1 H-1-infected parasynchronous NB cells incubated at the restrictive temperature of 39.5°C (17). A portion of the major peak at 16*S* in the preparative neutral sucrose gradient was digested with *Eco*RI for 2 h at 37°C and a control incubated in the absence of the enzyme, and the digests were subjected to electrophoresis in replicate agarose gels; the results are illustrated in Fig. 1. *Eco*RI-cleaved H-1 RF DNA into a large fragment, RI-A, and one or more smaller fragments. This digestion did not go to completion, but identical results were obtained with complete digests using RF DNA prepared with either *ts*1 or wt H-1 and with electrophoresis in 3% acrylamide-0.5% agarose gels. The *Eco*RI-A fragment contained an aver-

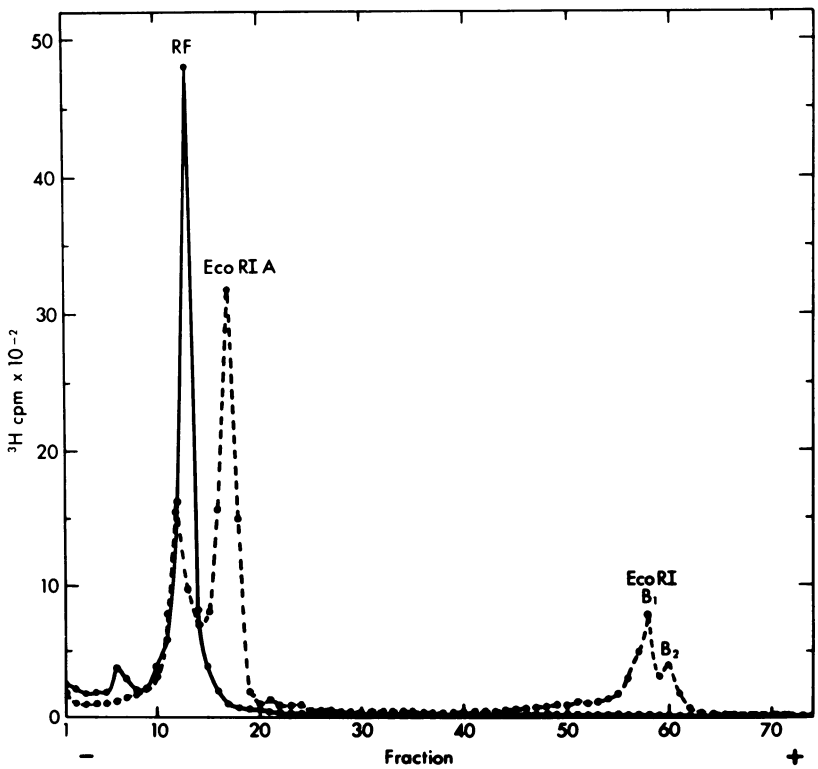

FIG. 1. *Analytical gel electrophoresis of H-1 RF DNA and EcoRI-digested H-1 RF DNA. H-1 RF DNA labeled with [^{3}H]TdR was isolated from hamster embryo cells infected with wt H-1 at 37°C. A portion of RF DNA that sedimented at 16.5S (cf. Fig. 3, fraction A) was digested in 50 μl of digestion buffer for 20 min at 37°C with 5 μl of EcoRI, and a control was incubated similarly without enzyme. The reaction was stopped with 20 μl of 2.5% sodium dodecyl sulfate-50% glycerol-0.01% bromophenol blue-10 mM EDTA, and the samples were subjected to electrophoresis in cylindrical gels of 1.4% agarose. In this case the electrophoresis buffer was 90 mM Tris-borate (pH 8.3)-2.5 mM disodium EDTA, and electrophoresis was for 20 h at 35 V at room temperature. In the illustration, the results of the separate gels are combined to simplify comparison.*

age of 78.4% of the [^{3}H]TdR or ^{32}P when $^{32}PO_4$ was used as a label, and the smaller fragments contained the remaining 21.6%. The dispersed electrophoretic pattern of the smaller fragments suggested several possible interpretations: (i) three *Eco*RI cleavage sites, producing one large fragment migrating as RI-A, one as RI-B_2, and two as RI-B_1 as labeled in Fig. 1; (ii) two *Eco*RI cleavage sites, one within *Eco*RI-B_1, yielding a small fragment lost from the gel and converting RI-B_1 to RI-B_2; and (iii) one *Eco*RI cleavage site, with structural heterogeneity of the *Eco*RI-B fragment giving heterogeneous migration in the gel electrophoresis.

The first alternative was ruled out by determining the size of the *Eco*RI-B fragments. Treatment of H-1 RF DNA with the restriction endonuclease endo R·*Hae*III of *H. aegyptius* produces 12 unique fragments, designated A to L, ranging in size from about 1,550 to 30 bp as determined by their contents of ^{32}P as shown in Fig. 2A. In a replicate gel (Fig. 2B), the *Eco*RI-B fragments were not resolved and migrated as one broad peak at a size position of about 1,050 bp, or 21% of the genome, in agreement with the proportion of counts recovered in all of the B fragments (Fig. 2C). Thus, the B fragments represent about 22% of the genome by size and composition and must be a heterogeneous collection of the same basic fragment.

The observed 8% difference in relative mobility of *Eco*RI-B_1 and *Eco*RI-B_2 with respect to *Eco*RI-A in 3% acrylamide gels suggests that a difference in size of about 50 to 70 bp would be expected. Although the proportions of *Eco*RI-B_1 to *Eco*RI-B_2 were constant at 2:1 independent of whether the digestion was complete or not as in Fig. 1, a further experiment to detect a possible second cleavage site near the first was conducted. A relatively large amount of [^{3}H]TdR-labeled RF DNA was digested to completion with *Eco*RI. This digest contained 40,000 cpm of *Eco*RI-B and was analyzed by electrophoresis for 3 h at 50 V in a 3% acrylamide gel (data not shown). Using the mobility of the *Eco*RI-B fragment and a linear extrapolation of mobility versus logarithm of molecular size as in Fig. 2C, the 3-h gel would have contained fragments with a relative mobility of 9 with respect to *Eco*RI-B and an estimated size of 2 to 4 bp. A putative fragment of 50 to 70 bp would have contained approximately 1% of the total counts per minute or about 2,000 cpm. No evidence for such a fragment was obtained. Evidence in support of the third alternative, namely, that there is one *Eco*RI cleavage site and that the *Eco*RI-B fragment is heterogenous, is presented below.

H-1 dimer RF DNA. It has been observed routinely in neutral sucrose gradients of Hirt extracts of H-1-infected cultures that the RF DNA peak has a fast-sedimenting shoulder (e.g., Fig. 3). Similarly, gel electrophoresis has revealed a species of DNA migrating more slowly than RF DNA, e.g., fraction 6 in Fig. 1. To determine the molecular weight of this DNA, the [^{14}C]TdR-labeled viral DNA illustrated in Fig. 3 was pooled as illustrated, and

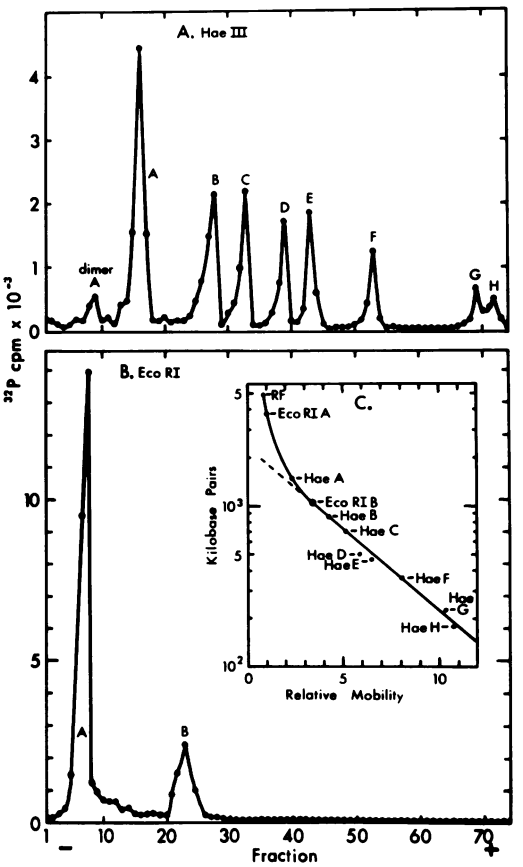

FIG. 2. *Analytical gel electrophoresis of ^{32}P-labeled H-1 RF DNA digested by HaeIII or EcoRI. H-1 RF DNA labeled with $^{32}PO_4$ was prepared in the usual manner through the sucrose gradient step. RF DNA in final volumes of 50 μl was digested for 16 h at 37°C with 5 μl of HaeIII (A) or for 2 h at 37°C with 5 μl of EcoRI (B). The DNA was analyzed by electrophoresis in cylindrical gels of 3% acrylamide-0.5% agarose at a constant voltage of 30 V for 16 h at room temperature. The counts recovered in each fraction were converted to percent total counts, and the number of base pairs for each fraction was calculated using a total of 4,920 (cf. Fig. 4). The electrophoretic mobility of each fragment expressed as a relative mobility in comparison to EcoRI-A fragment was plotted against the fragment size on a log scale in (C). The mobility of EcoRI-B indicates a fragment size of about 1,050 bp or 21.3% of the genome. In Fig. 2B, EcoRI-B contained 20% of the total ^{32}P recovered. The HaeIII fragments I-L migrated off this gel.*

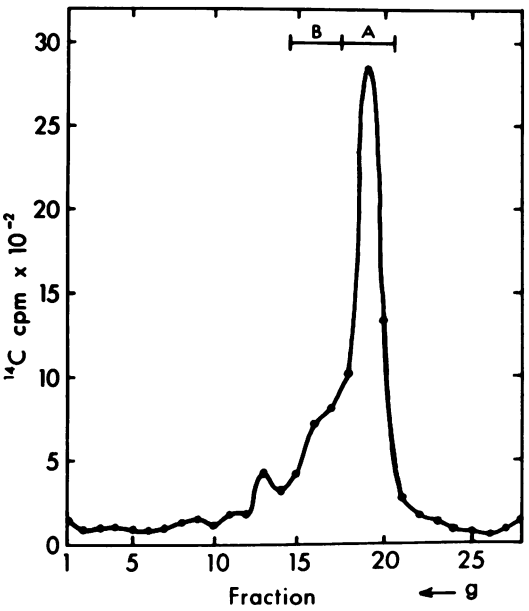

FIG. 3. *Preparative sucrose gradient centrifugation of H-1 RF DNA. H-1 RF DNA was labeled with [^{14}C]TdR by incubating parasynchronous hamster cultures infected with ts1 H-1 at 39.5°C 12 to 16 h p.i. in medium containing FUdR (0.5 μg/ml) and [^{14}C]TdR (1 μCi/ml, 57.1 mCi/mmol). Viral DNA was extracted by the Hirt method and sedimented in a gradient of 5 to 20% sucrose in 50 mM Tris (pH 8.0)-1 M NaCl-1 mM EDTA-0.2% Sarkosyl in the SW25 rotor for 18 h at 24,000 rpm. Fractions of 1 ml were collected through the bottom of the tube, 10-μl aliquots were assayed for radioactivity, and the fractions were pooled as in the figure. The DNA was precipitated with 2.5 volumes of ethanol at −20°C for 16 or more h. The precipitates were collected by centrifugation and washed once with 70% ethanol-50 mM Tris (pH 7.5)-1.5 M NaCl-1 mM EDTA, and the DNA was stored in 10 mM Tris (pH 7.5)-10 mM NaCl-0.1 mM EDTA at −20°C.*

pool B, previously determined to contain some of the RF DNA of pool A, was subjected to coelectrophoresis with an *Eco*RI digest of [^{3}H]TdR-labeled bacteriophage λ DNA in 0.7% agarose gels. The results are presented as the log molecular weight versus distance of migration in Fig. 4, and the molecular weights derived for the two peaks of H-1 DNA were 3.26×10^6 for H-1 RF DNA and 6.5×10^6 for the faster-sedimenting species. These values represent the average of two separate determinations, which differed by less than 1%. It should be noted that the curve is nonlinear in the regions where the faster-sedimenting H-1 DNA is represented. This DNA has the molecular weight expected of a dimer of H-1 RF DNA.

To clarify further the identity and structure of this putative dimer RF DNA, [^{3}H]TdR-labeled dimer was prepared as above and purified by preparative gel electrophoresis. A portion of this preparation was digested by *Eco*RI and analyzed by electrophoresis in a 1.4% agarose gel (Fig. 5). Three fragments were obtained; the largest had the electrophoretic mobility of *Eco*RI-A, the second migrated with a mobility equivalent to 2,100 bp or twice the size of *Eco*RI-B, and a smaller portion was at the position of *Eco*RI-B. This is the expected result for a dimer RF DNA linked through the *Eco*RI-B fragments, rather than in tandem. A similar result was obtained after a digestion of a mixture of equal parts of monomer and dimer RF DNA. The small amount of ^{3}H recovered at the position of *Eco*RI-B probably arose from con-

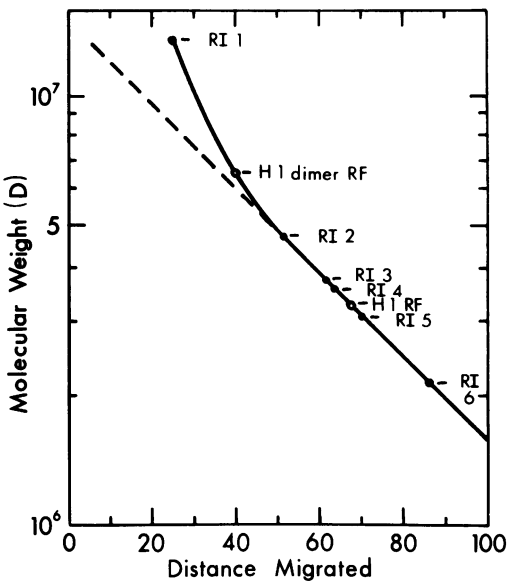

FIG. 4. *Determination of the molecular weights of H-1 monomer and dimer RF DNA. The molecular weights of H-1 monomer and dimer RF DNA were measured by coelectrophoresis of [^{14}C]TdR-labeled H-1 DNA prepared as in Fig. 3 with the [^{3}H]TdR-labeled EcoRI fragments of λ phage DNA. [^{3}H]TdR-labeled λ DNA was prepared as described (11) and digested to completion with 100 U of EcoRI in a volume of 100 μl. The reaction was stopped by the addition of EDTA to 50 mM, and 20 μl of the λ digest and a 5-μl aliquot of the [^{14}C]TdR H-1 DNA were analyzed by electrophoresis in duplicate in 1% agarose cylindrical gels as in Materials and Methods. The figure illustrates the distance migrated and the log molecular weight of EcoRI λ fragments (●) and the electrophoretic mobility of H-1 RF DNA monomer and dimer (○). The molecular weight of the smaller H-1 DNA was calculated using a linear regression constructed by the method of least squares for log molecular weight as a function of distance migrated for the λ EcoRI fragments 2–6 (11). The molecular weight of the larger H-1 DNA was estimated graphically, since it lay on the nonlinear part of the curve.*

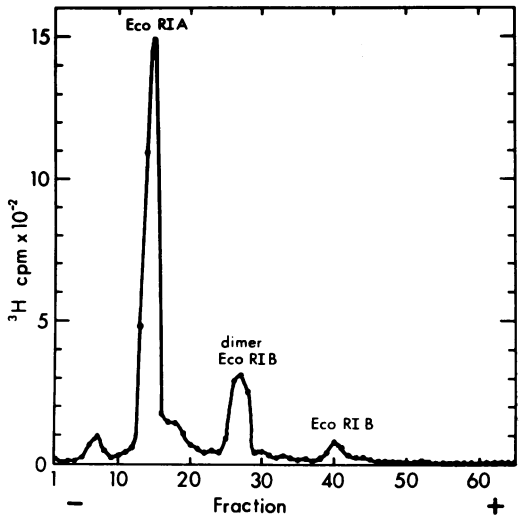

FIG. 5. *Analytical gel electrophoresis of an EcoRI digest of H-1 dimer RF DNA. Electrophoresis was in a 1.4% agarose gel as in Fig. 1 except buffer E was used for 16 h at 25 V at room temperature. DNA peaks were identified from the position of monomer fragments on a replicate gel and their mobilities relative to the marker EcoRI-A.*

tamination of the dimer preparation with monomer RF DNA.

To determine whether the dimer DNA contained covalently linked dimer length strands, [^{3}H]TdR-labeled dimer or monomer and ^{32}P-labeled monomer RF DNA were compared in alkaline sucrose gradients (Fig. 6). Virion DNA was sedimented in a replicate gradient (not shown), the position of which is indicated by the arrow (V), and the arrow (D) indicates the position of dimer length DNA expected using the equations of Studier (25). It can be seen that the majority of both monomer and dimer DNAs are genome length in the alkaline gradient and thus not covalently linked. Both preparations show fast-sedimenting shoulders not seen with virion DNA, which we will consider in detail below.

The relative proportions of dimer and monomer RF DNA synthesized at different times postinfection (p.i.) were examined in wt H-1-infected hamster embryo cells labeled with [^{3}H]TdR 11 to 12 h p.i., 11 to 12 h p.i. plus a 5-h incubation in the presence of unlabeled TdR, and 16 and 17 h p.i. The viral DNA was analyzed by electrophoresis in 0.7% agarose gels. The proportion of viral DNA that was dimer RF DNA varied little: 17% for the 11- to 12-h p.i. labeling period, 26% for 11 to 12 h plus 5-h chase, and 22% for the 16- to 17-h label. Since the majority of the dimers are not covalently linked, they may be formed by annealing of cohesive termini of the monomer RF DNA during extraction and subsequent handling. To determine whether all RF DNA could anneal, a portion of the 11- to 12-h RF DNA preparation above was incubated in 50% formamide for 4 h at 40°C before electrophoresis. No increase in dimer RF DNA occurred.

A more direct test for the possibility of random annealing of cohesive RF monomers was made using the density label bromodeoxyuridine (BUdR). In this experiment, wt RF DNA was prelabeled with [^{14}C]TdR and incubated 30 min with FUdR, followed by 30 min of labeling with [^{3}H]BUdR in the presence of FUdR. This

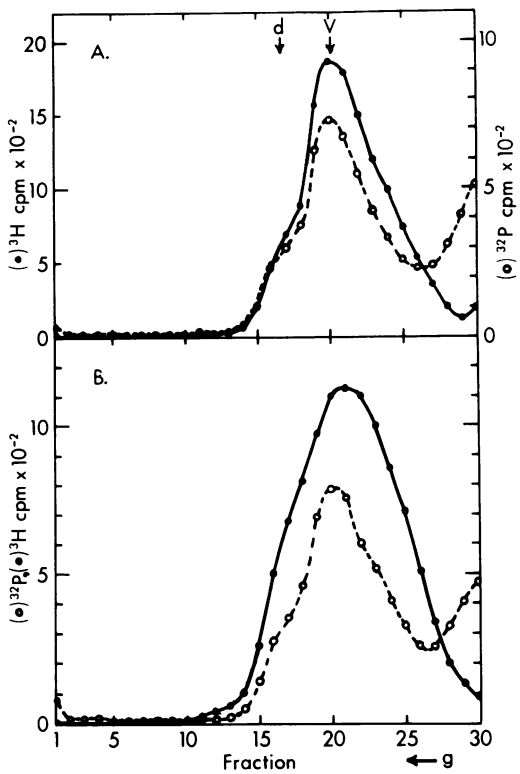

FIG. 6. *Centrifugation of dimer and monomer H-1 RF DNA in alkaline sucrose gradients. Labeled H-1 RF DNA was prepared as in Materials and Methods. The B fraction of the ^{3}H-labeled H-1 DNA shown in Fig. 3 was fractionated into monomer and dimer fractions by preparative gel electrophoresis in 1% agarose. ^{32}P-labeled monomer H-1 RF DNA was added to the ^{3}H monomer (A) or dimer (B) fractions, and after the mixtures were adjusted to 0.2 N NaOH, they were sedimented in a 5 to 20% alkaline sucrose gradient (cf. A in Fig. 3) for 6 h at 42,000 rpm at 4°C in the SW50 rotor. A replicate tube contained ^{3}H-labeled virion H-1 DNA, and its sedimentation position is indicated by the arrow V. The arrow D denotes the expected sedimentation position of dimer DNA relative to monomer by the equation of Studier (25).*

period of density labeling was chosen to produce a labeling of only a small portion of the total RF pool. Therefore, if dimers formed by random association of monomers, the majority of the dimers labeled with ^3H would contain only one [^3H]BUdR-substituted monomer RF DNA and be less than hybrid density. On the other hand, if dimers replicate semiconservatively during the density labeling or if they are generated by a failure of daughter RF monomers to segregate after replication, ^3H-labeled dimers would have hybrid density. Dimer RF DNA was prepared by sucrose gradient centrifugation and by preparative gel electrophoresis, and the dimer and monomer (sucrose gradient only) RF DNAs were analyzed by isopycnic centrifugation in Cs_2SO_4 (Fig. 7A and B). About 60% of the ^3H-labeled monomer is of hybrid density, whereas the density of the ^3H dimer is intermediate between light and hybrid, and virtually no ^3H dimer is of hybrid density. This is compatible with a random annealing of monomer RF DNA as the mechanism of dimer formation and rules out a failure of daughter RF monomer segregation as a means of dimer generation. However, this result would also be obtained if dimers replicated more slowly than monomers, such that a 30-min density label is less than or equal to the replication time for dimer RF under these conditions.

To examine this possibility, the experiment was repeated in a similar manner, except the period of density label was extended to 60 min (not shown). After the 1 h of density labeling, most of the ^3H-labeled dimer DNA was of hybrid density; however, appreciable amounts of the total monomer RF DNA were calculated to have become heavy (13%) and hybrid (38%) densities. Thus, the distribution of density label in the dimer RF DNA was compatible with a slower dimer replication process, random annealing of RF DNA monomers, or some more complex situation.

Covalent linkage of the V to C strand in the *Eco*RI-B fragment of monomer RF DNA. It was previously observed that the RF monomer and dimer DNA peaks had a faster-sedimenting shoulder in alkaline gradients, suggestive of the presence of single-stranded DNA of greater than genome length. Tattersall et al. have presented evidence for the existence of some RF DNA of the parvovirus MVM (minute virus of mice) that renatures unimolecularly after heat denaturation, and suggested that a covalently closed turnaround may exist in RF DNA (27). We have obtained evidence for a region at one end of H-1 RF DNA with a high melting temperature implicating a very high

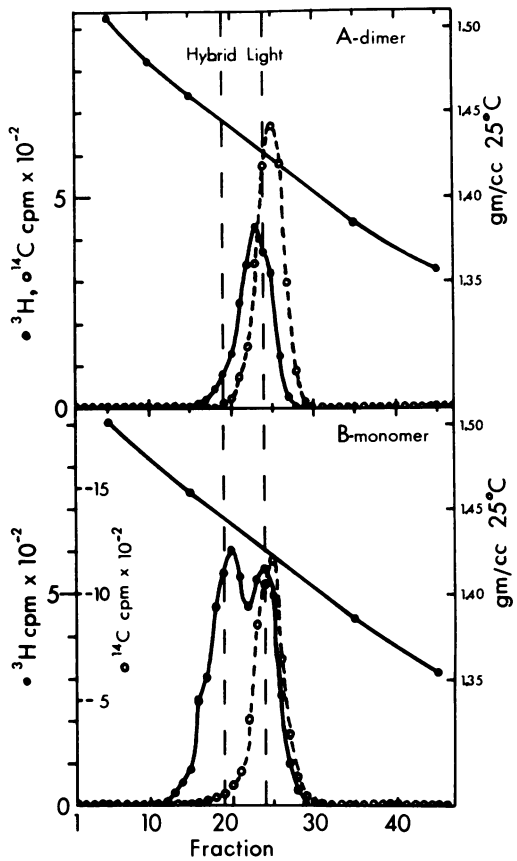

FIG. 7. *Isopycnic centrifugation of density-labeled H-1 RF monomer and dimer DNA. H-1 DNA was labeled in parasynchronous NB cultures infected with ts1 at 39.5°C first with [^{14}C]TdR from 12 to 15.5 h p.i. (FUdR, 0.5 μg/ml; [^{14}C]TdR, 1 μCi/ml, 57.1 mCi/mmol), followed by a 0.5-h treatment with FUdR alone, and then labeled with [^3H]BUdR from 16 to 16.5 h p.i. (FUdR, 0.5 μg/ml; [^3H]BUdR, 2 μCi/ml, 10^{-6} M). Viral DNA was extracted by the Hirt method and fractionated into monomer and dimer by centrifugation in a neutral sucrose gradient and by preparative gel electrophoresis in 1% agarose. Monomer and dimer DNA were banded to equilibrium in Cs_2SO_4: (A) dimer RF DNA, (B) monomer RF DNA. Centrifugation was for 48 h at 35,000 rpm at 4°C in the type 40 rotor.*

guanine plus cytosine content that might fail to denature with heat under the conditions used for MVM RF DNA (23). Nevertheless, if such a covalent cross-linkage is present in a portion of the H-1 RF monomer and dimer DNAs, then it could be isolated by using its rapid renaturation rate. This was tested by alkaline denaturation of wt monomer H-1 RF DNA followed by rapid neutralization and immediate application to a BDC column. Double-stranded DNA was

TABLE 1. *Akaline denaturation–BDC chromatography of H-1 RF DNA*[a]

Procedure	Cycle 1		Cycle 2	
	cpm	% Total	cpm	% Total
Washes	1,295		3,108	1.7
1 M TBS–total	261,560	18.5	128,560	73.1
Caffeine–total	1,151,340	81.4	44,300	25.2

[a] [^{3}H]TdR-labeled wt H-1 RF DNA was denatured by 0.2 N NaOH, neutralized at 0°C, and chromatographed on a column of BDC as in Materials and Methods. The DNA eluted with 1 M TBS was collected by ethanol precipitation, and the process was repeated in cycle 2. Values shown are total counts per minute recovered and their respective percentages.

selectively eluted with 1 M TBS and DNA containing single-stranded regions recovered by elution with 2% caffeine. The results are summarized in Table 1. When the double-stranded DNA was put through a second cycle of alkaline denaturation-BDC chromatography, the percentage recovered as double stranded increased from 18.5 to 73%. A portion of this DNA was then analyzed by alkaline sucrose gradient centrifugation (Fig. 8A). A majority of the DNA (68%) sedimented at the expected position of dimer length single strands, in constrast to the [^{14}C]TdR-labeled *ts*1 RF DNA monomer marker. The final recovery indicated that at least 9% of the RF monomer contained a covalent linkage between the V and C strands.

The localization of the V-C linkage was determined by digesting RF DNA with *Eco*RI before alkaline denaturation and BDC chromatography. The double-stranded DNA so obtained was analyzed by electrophoresis in a 1.4% agarose gel and compared with the total digest (without selection for the rapidly renaturing DNA) in a replicate gel. The results are illustrated together for convenience in Fig. 9A. The *Eco*RI-B fragment was clearly selected for by the procedure isolating rapidly annealing DNA, indicating that the covalent linkage is in the *Eco*RI-B fragment. A control experiment showed that BDC chromatography of an *Eco*RI digest of RF DNA without alkaline denaturation resulted in the retention of about 30% of the DNA after 1 M TBS elution, suggesting the presence of some single-stranded regions, but it was not selective for either fragment. This subject will be considered in more detail in a subsequent section of this study.

If the covalent linkage of V to C strand in monomer RF DNA was involved in the initiation of replication of RF DNA or progeny DNA synthesis, then rapidly labeled molecules would be enriched in this property compared with the total collection of RF DNA molecules. The [^{14}C]TdR-prelabeled RF monomer DNA used for the [^{3}H]BUdR density label in Fig. 7B was a ready source of DNA for comparison of old and new molecules. This DNA was fractionated by alkaline denaturation-BDC chromatography, followed by an alkaline sucrose gradient as above. The results in Fig. 8B show that the ^{3}H/^{14}C ratios were lower for the dimer length DNA than for the unfractionated monomer RF DNA, 0.29 versus 1.1. Thus, newly replicated monomer RF molecules (within 30 min) contain less of the covalent linkage than the general pool, as represented by the [^{14}C]TdR-labeled RF DNA.

Covalent linkage of V to C strand in dimer RF DNA. Experiments similar to those used to show linkage of V to C strands in monomer RF DNA were carried out with [^{3}H]TdR-labeled wt dimer RF DNA purified by gel electrophoresis. After one cycle of alkaline denaturation, 24% of the ^{3}H was recovered as double stranded in BDC chromatography, and 87% of this DNA sedimented as dimer length in an alkaline sucrose gradient (not shown). This represented about 21% of the original dimer preparation. The gel electrophoresis of this dimer preparation after *Eco*RI digestion is shown in Fig. 5. This DNA contained about 5% *Eco*RI-B monomer would be dimer length single-stranded 20% contamination with monomer RF DNA. Since we have seen that about 10% of the monomer would be dimer-length single-stranded DNA, this would constitute only 2% of the total dimer preparation, so that it can be deduced that some of the dimers contained covalently linked V and C strands.

To test whether the covalent linkage in the dimers is at the internal left end of the monomer, and probably at the site of linkage between the monomers, dimer DNA was digested with *Eco*RI and then subjected to the alkaline denaturation-BDC chromatography procedure. The rapidly renaturing DNA was analyzed by gel electrophoresis (Fig. 9B). It was found that the B fragment was isolated in a 40-fold molar excess compared with the A fragment and thus contained the V-C strand linkage. It is unlikely that a covalent linkage occurred during the Hirt extraction and subsequent handling, so

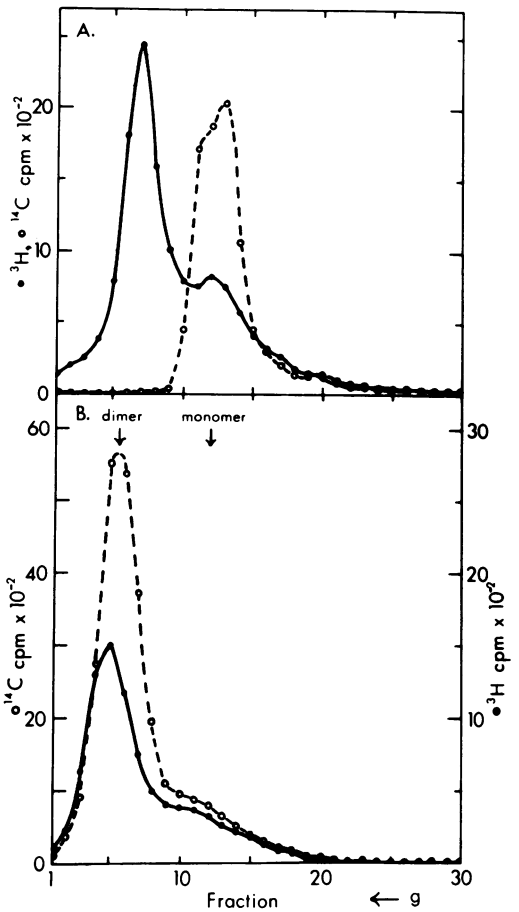

FIG. 8. *Alkaline sucrose gradient centrifugation of rapidly renaturing RF DNA. (A) H-1 RF DNA monomer was selected for its ability to reanneal rapidly by two cycles of alkaline denaturation-BDC chromatography (Table 1). The DNA in the 1 M TBS eluate of the second chromatography was adjusted to 0.2 N NaOH and sedimented in a gradient of 5 to 20% sucrose in 0.2 N NaOH-0.8 M NaCl-1 mM EDTA for 16 h at 32,000 rpm at 4°C. A marker of [^{14}C]TdR monomer RF DNA obtained from pool A of Fig. 3 was included. (B) H-1 RF DNA monomer prelabeled with [^{14}C]TdR (12 to 15.5 h p.i.) and pulse-labeled with [^{3}H]BUdR (16 to 16.5 h p.i.), as described in Fig. 7, was subjected to one cycle of alkaline denaturation-BDC chromatography. The rapidly annealing DNA in the 1 M TBS eluate was centrifuged in an alkaline sucrose gradient as in (A).*

this result strongly suggests that the dimer RF DNA exists in vivo.

Evidence that the B fragment is a terminus in RF DNA replication. By analyzing the distribution of [^{3}H]TdR in the *Eco*RI-A and -B fragments of H-1 RF DNA that have just completed replication during a short incubation in the presence of [^{3}H]TdR, it is possible to make a crude localization of the origin of replication. In these experiments, *ts*1 H-1 was used at the restrictive temperature to inhibit progeny DNA synthesis. This method has been successfully used by Danna and Nathans for simian virus 40 (7) and by Godson for ϕX174 (10). The first problem to be overcome with the linear H-1 RF

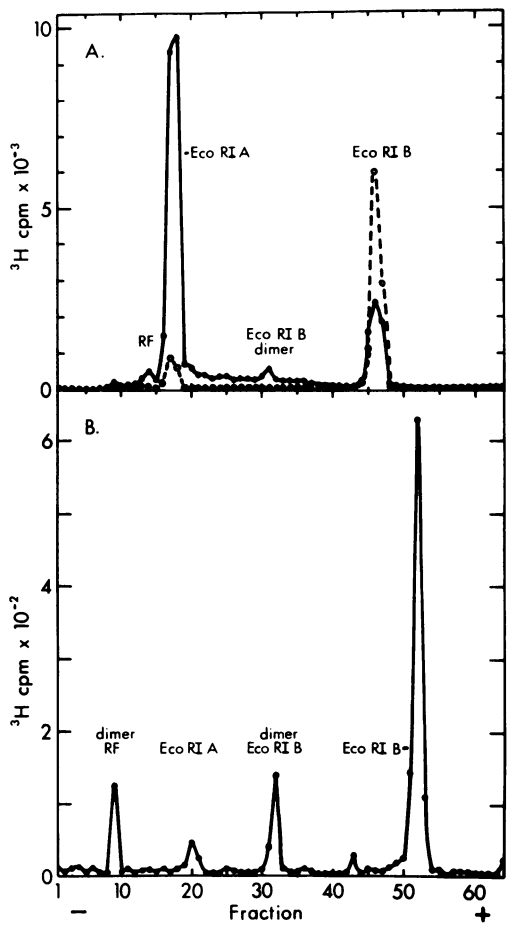

FIG. 9. *Analytical gel electrophoresis of EcoRI digests of RF DNA before and after alkaline denaturation-BDC chromatography. (A) RF DNA was digested to completion with EcoRI and then fractionated by alkaline denaturation-BDC chromatography. A sample of the rapidly annealing DNA in the 1 M TBS eluate and an aliquot removed before alkaline denaturation-BDC chromatography were analyzed separately in replicate gels of 1.4% agarose. Electrophoretic conditions were 17 h at 25 V at room temperature. Results of the replicate gels were plotted together for convenience: before alkaline denaturation-BDC (●), and after alkaline denaturation-BDC (○). (B) Dimer RF DNA was digested with EcoRI and fractionated by alkaline denaturation-BDC chromatography. The 1 M TBS eluate was analyzed in a gel of 1.4% agarose as in (A), except electrophoresis was for 20 h at 25 V.*

DNA is to devise a method of isolating the molecules that have just completed replication during the short pulse of [^{3}H]TdR. In these molecules, the specific activity of TdR at each position in the molecule will increase as the distance of that position from the origin increases. In comparison, RI molecules will have a declining specific activity in TdR as the TdR position from the origin increases. For this purpose, I have used chromatography on columns of BDC, which separates double-stranded DNA from the more tightly bound DNA that contains single-stranded regions (2, 27). This method or a similar one using benzoylated napthoylated DEAE-cellulose has been used for the separation of viral RI molecules from mature DNA for lambda (13), adenovirus (26), ϕX174 (21), MVM (27) and, most recently, herpes simplex virus (22).

Rapidly labeled H-1 RF DNA was found to bind preferentially to BDC and to require caffeine for elution, as has been shown for other examples of replicative intermediates cited above. Rapidly labeled viral DNA was prepared by labeling *ts*1-infected NB cultures, after incubation at 39.5°C, for 5 min at 22°C, or for 5 min or 15 min at 39.5°C, with [^{3}H]TdR before extraction of viral DNA. A uniformly labeled RF DNA was similarly prepared by incubating the cultures from 12 to 16 h p.i. in FUdR and [^{14}C]TdR (cf. fraction A in Fig. 3). After a preliminary purification in neutral sucrose gradients, the [^{3}H]DNA that sedimented as monomer RF DNA (but which may also include early RI molecules), was combined with a portion of [^{14}C]TdR-labeled RF DNA and was then chromatographed on BDC columns (Fig. 10). The percentage of the labeled DNA that eluted with caffeine was 95% for the 22°C, 5-min label (not shown), 80% for the 5-min ^{3}H label at 39.5°C, 61% for the 15-min ^{3}H label, and 38% for the 4-h [^{14}C]TdR-labeled DNA. Thus, the rapidly labeled RF DNA was preferentially retained after the 1 M TBS elution, even though the more rapidly sedimenting RI molecules were already partially excluded by the zonal centrifugation in neutral sucrose.

The rather high fraction of the [^{14}C]TdR-labeled DNA that remained bound after salt elution deserves further study. Electron microscope visualization of this fraction showed that only about 25% of the molecules were RI molecules (23), so that one explanation would be that these RF DNA molecules have large enough single-stranded DNA regions to bind BDC in 1 M TBS, as recently reported for herpes simplex virus (22). If this were true, then these regions may be uniquely located, so that after *Eco*RI cleavage, one of the fragments

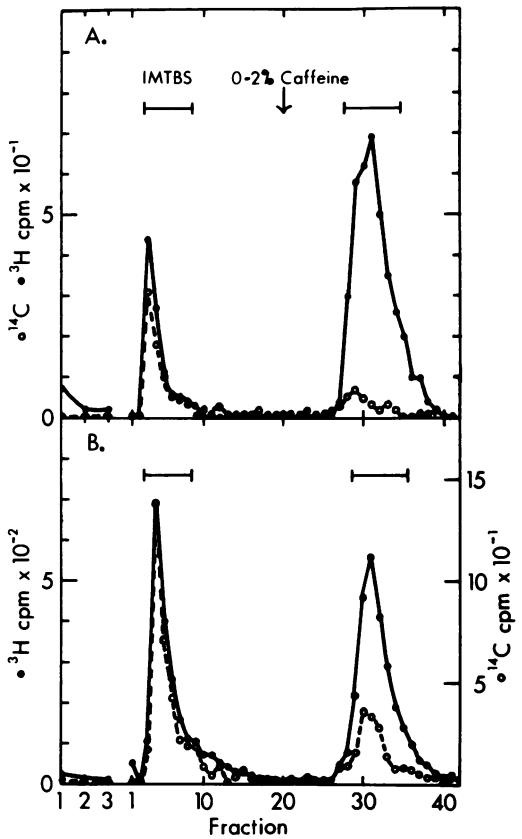

FIG. 10. *BDC chromatography of rapidly labeled H-1 RF DNA. Parasynchronous cultures of NB cells were infected with ts1 H-1 at 39.5°C and treated with FUdR for 30 min, 14.5 to 15 h p.i. At 15 h p.i. the cultures were labeled for 5 or 15 min with [^{3}H]TdR (20 μCi/ml, 50 Ci/mmol) in the presence of FUdR. Viral DNA sedimenting as monomer RF DNA was prepared in the usual manner and was chromatographed with a [^{14}C]TdR marker (cf. Fig. 3) on columns of BDC-cellulose using elutions of 20 ml of 1 M TBS and a 20-ml linear gradient of 0 to 2% caffeine in 1 M TBS. One-milliliter fractions were collected and 50-μl aliquots were assayed for radioactivity; (A) 5-min label, (B) 15-min label. The 1 M TBS and 2% caffeine eluates were pooled as illustrated and the DNA was precipitated with 2.5 volumes of ethanol at −20°C overnight.*

would be retained preferentially after the 1 M TBS elution. This was tested by digesting ^{3}H-labeled RF DNA (4-h labeling period) with *Eco*RI. The digest was extracted with phenol, precipitated with ethanol, redissolved in 0.3 M TBS, and chromatographed on BDC. Thirty-seven percent of the total ^{3}H eluted with caffeine. Portions of the 1 M TBS and 2% caffeine eluate were analyzed by gel electrophoresis, and there was no preferential binding of *Eco*RI-A compared with -B by the BDC (Table 2).

TABLE 2. *Composition of BDC chromatographic fractions of EcoRI-digested H-1 RF DNA*[a]

Elution with:	% Composition		
	RF	*Eco*RI-A	*Eco*RI-B
1 M TBS	11	67	22
2% Caffeine	23	60	17

[a] H-1 RF DNA labeled with ^3H was digested to near completion with *Eco*RI. The digest was extracted with phenol and precipitated with ethanol. The precipitate was redissolved in 0.3 M TBS and fractionated on a column of BDC. The 1 M TBS and 2% caffeine eluates were precipitated with ethanol, and the DNA was analyzed by electrophoresis in 1.4% agarose gels. The values shown represent the percent composition of RF, *Eco*RI-A, and *Eco*RI-B in each eluate.

The 2% caffeine eluate was enriched for the undigested RF DNA.

If the postulated single-stranded regions were distributed within both *Eco*RI-A and *Eco*RI-B, then RF DNA in the caffeine eluate should show a decrease in sedimentation velocity in alkaline sucrose gradients in comparison to the 1 M TBS eluate. In this experiment, uniformly labeled monomer RF DNA was fractionated on a BDC column, and the DNA in the 1 M TBS eluate and the 2% caffeine was precipitated by ethanol and then subjected to sedimentation in alkaline sucrose gradients. A marker of [^{14}C]TdR-labeled RF DNA (Fig. 3) was included, and its modal sedimentation is indicated by the arrow (Fig. 11). Although the caffeine eluate was enriched slightly for DNA sedimenting slower than the genome length DNA, both preparations were largely intact dimer length or monomer length single strands. This indicates that internal single-stranded gaps are not common in the RF DNA of the 2% caffeine eluate.

Another possibility is that the DNA is retained nonspecifically and not because internal single-stranded regions exist. If this is the case, rechromatography of the 1 M TBS eluate should again produce a sizable fraction retained after 1 M TBS elution. This experiment was done using a portion of the first 1 M TBS eluate of Fig. 11, which represented one-tenth of the total DNA used for the first BDC column. The result obtained was that 24% of the 1 M TBS fraction remained tightly bound and eluted in the 2% caffeine eluate. This is a smaller percentage than during the first chromatography (37%), but it does indicate a relatively high level of nonspecific retention after 1 M TBS elution. In conclusion, RI molecules are retained on BDC columns after salt elution on the basis of the kinetics of labeling and direct visualization by electron microscopy. Therefore, the salt fraction contains the nonreplicating molecules, even though some are retained by the column for an unknown reason.

Returning now to the *ts*1 RF DNA labeled for 15 min at 39.5°C and fractionated by BDC chromatography (cf. Fig. 10), the 1 M TBS fraction and 2% caffeine fraction were separately precipitated with ethanol and digested with *Eco*RI, and the digests were analyzed by gel electrophoresis (Fig. 12). Additional [^{14}C]TdR-labeled marker RF DNA, not fractionated by BDC, was added before the digest to improve the $^3H/^{14}C$ ratios (the absolute values for the $^3H/^{14}C$ ratios of the two column fractions were not adjusted to be equal). The $^3H/^{14}C$ ratios for the 1 M TBS eluate *Eco*RI-A and -B fragments prepared from this mixture were 3.32 and 4.42, respec-

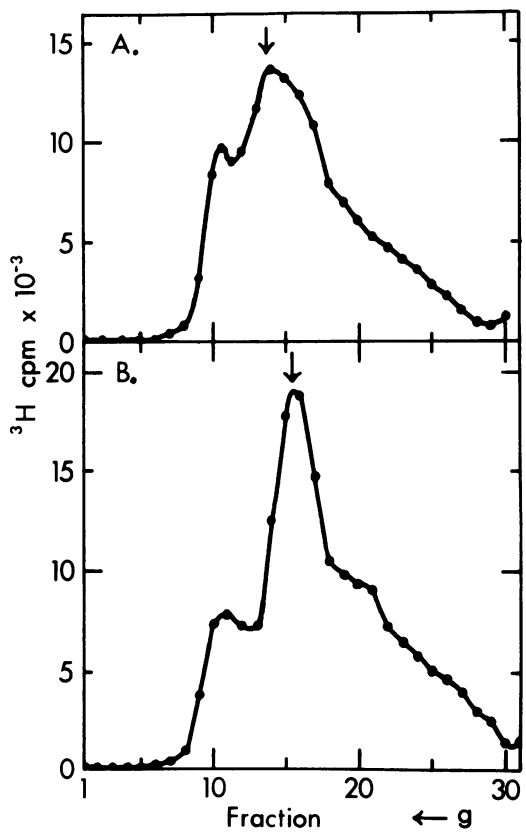

FIG. 11. *Alkaline sucrose gradient centrifugation of BDC chromatographic fractions of H-1 RF DNA. wt RF DNA labeled 4 h with [^3H]TdR was fractionated by BDC chromatography using batch elutions of 1 M TBS and 2% caffeine. The DNA fractions were collected by ethanol precipitation and sedimented as alkaline sucrose gradients as in Fig. 8. The marker DNA of [^{14}C]TdR-labeled RF sedimented at the arrow; (A) 1 M TBS eluate, (B) 2% caffeine eluate.*

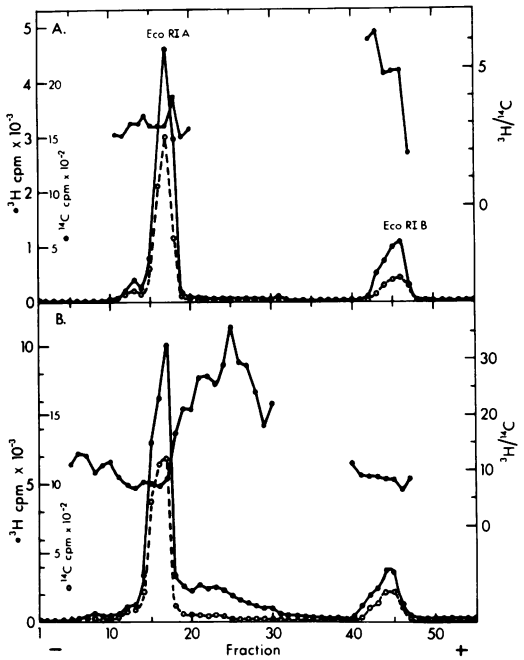

FIG. 12. *Analytical gel electrophoresis of EcoRI digests of RF and RI DNA labeled 15 min. H-1 ts1 RF DNA labeled 15 min with [^{3}H]TdR was fractionated by BDC chromatography as in Fig. 10. The DNA in the 1 M TBS and 2% caffeine eluates were separately digested with EcoRI after the addition of an aliquot of uniformly labeled [^{14}C]TdR-labeled ts1 RF DNA and analyzed by electrophoresis in 1.4% agarose gels as in Fig. 9. Electrophoresis was for 17 h at 25 V at room temperature; (A) 1 M TBS eluate, (B) 2% caffeine eluate.*

tively. Thus, if the ^{3}H/^{14}C ratio of *Eco*RI-A is normalized to 1, the ratio for *Eco*RI-B was 1.33. This increase is expected if *Eco*RI-B does not contain the origin of replication. The caffeine eluate shows ^{3}H/^{14}C ratios of 7.94 and 8.27 for *Eco*RI-A and -B, respectively. The normalized value for the *Eco*RI-B/*Eco*RI-A was 1.04. In the caffeine eluate the *Eco*RI-A fragment did not show the expected higher ^{3}H/^{14}C ratio compared with *Eco*RI-B, but it can be seen in Fig. 12B that a small portion of the rapidly labeled [^{3}H]-DNA with high ^{3}H/^{14}C ratios (as high as 40) migrated ahead of *Eco*RI-A. This DNA was not included in the average ratio of 7.94 cited for *Eco*RI-A above.

It would be expected that RI molecules would migrate more slowly than linear RF DNA due to their larger size, nonlinear configuration, and by analogy with the results reported for RI molecules of simian virus 40 (28). On the other hand, single-stranded regions and greater flexibility might accelerate their electrophoretic mobility. To determine whether this rapidly labeled DNA, migrating faster than *Eco*RI-A, arose as a result of the BDC chromatography or *Eco*RI digestion, the behavior of rapidly labeled RF DNA and RI DNA in gel electrophoresis was examined. ^{3}H-labeled *ts*1 RF DNA was prepared by a 5-min incubation with [^{3}H]TdR at 39.5°C and fractionated in a neutral sucrose gradient (Fig. 13). Tubes from the gradient were pooled to make three fractions, A, B, and C, as illustrated. After precipitation with ethanol, each fraction was subjected to agarose gel electrophoresis in the presence of a [^{14}C]TdR-labeled RF preparation containing monomer and dimer RF DNA (Fig. 14). Fraction A shows [^{3}H]DNA exhibiting a high ^{3}H/^{14}C ratio migrating in advance of monomer RF DNA (Fig. 14A). Therefore, the rapidly labeled DNA migrated ahead of RF DNA before BDC chromatography and *Eco*RI digestion, with a mobility equivalent to a duplex DNA 10 to 15% smaller than RF DNA. After *Eco*RI digestion this DNA migrated ahead of *Eco*RI-A, and its mobility increased about the amount expected if *Eco*RI has cleaved the *Eco*RI-B region from

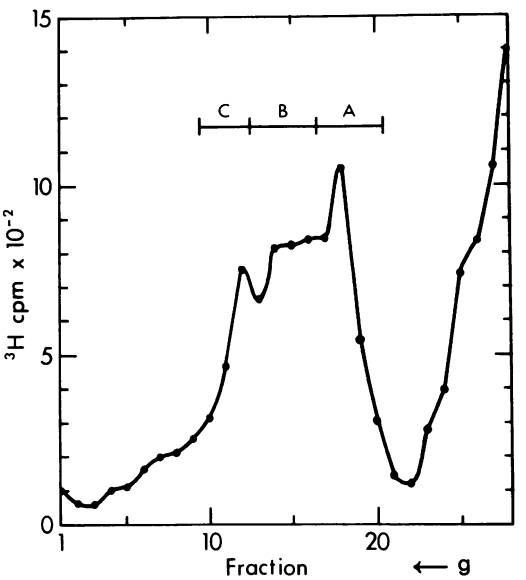

FIG. 13. *Preparative sucrose gradient centrifugation of rapidly labeled H-1 RF DNA. Parasynchronous NB cultures infected with ts1 H-1 at 39.5°C were treated 14 to 15 h p.i. with FUdR (0.5 μg/ml) and labeled at 39.5°C with [^{3}H]TdR (50 μCi/ml, 20 Ci/mmol, + FUdR 0.5 μg/ml) for 5 min. Viral DNA was extracted as in Materials and Methods and sedimented in a gradient of 5 to 20% sucrose as in Fig. 3. Fractions of 1 ml were collected, and 20 μl was assayed for radioactivity directly, accounting for the acid-soluble [^{3}H]TdR seen in fractions 23 to 28. Selected fractions were pooled as illustrated, and the DNA was collected by ethanol precipitation.*

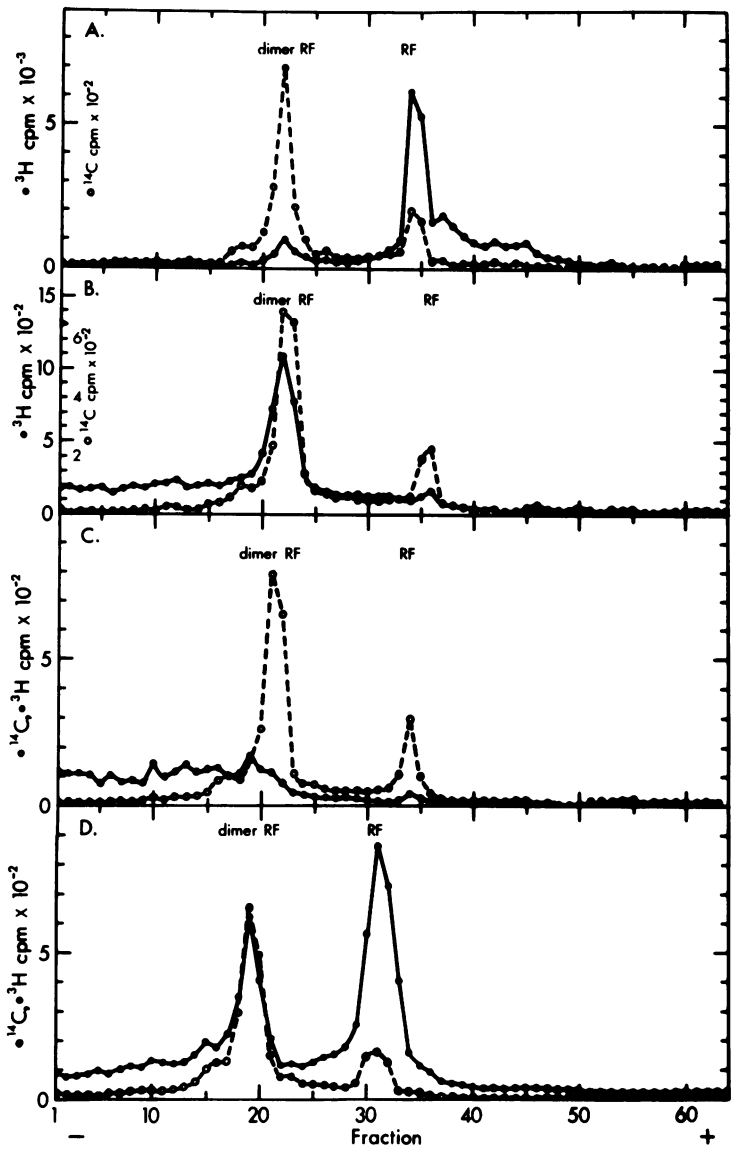

FIG. 14. *Analytical gel electrophoresis of rapidly labeled H-1 RF DNA. H-1 RF DNA was labeled for 5 min at 39.5°C as in Fig. 13. The DNA fractions pooled as A, B, and C from the preparative sucrose gradient of Fig. 13 were analyzed in 0.7% agarose gels. A marker DNA of [¹⁴C]TdR-labeled monomer and dimer RF DNA (pool B of Fig. 3) was added before electrophoresis. Pools A, B, and C are illustrated in (A), (B), and (C), respectively. It should be noted that the aliquot used for A was 10% of the total, and B and C were 25% of the total. Gel electrophoresis of the total DNA of a preparative sucrose gradient equal to a composite of fractions A + B + C of a ts1 RF DNA labeled similarly for 5 min at 22°C with [³H]TdR is shown in (D).*

these molecules. This suggests that this DNA contains a high specific activity of ^3H in the *Eco*RI-A portion of the molecule, which presumably contains the origin of replication. Experiments to demonstrate this more conclusively using other restriction endonucleases are in progress, and a similar conclusion has been reached by electron microscopy, as described in the following papers of this series.

Gel electrophoresis of fractions B and C of Fig. 13 is shown in Fig. 14B and C. The patterns revealed predominantly dimer RF DNA and some [^3H]DNA exhibiting high $^3H/^{14}C$ ratios migrating more slowly than dimer RF.

A DNA preparation from *ts*1 H-1-infected cells that had been labeled for 5 min at 22°C

was fractionated on a BDC column as described above, and the caffeine-eluted fraction was banded by isopycnic centrifugation in a CsCl gradient, precipitated with ethanol, and then subjected to electrophoresis in a 0.7% agarose gel (Fig. 14D). This electrophoretic pattern represents an approximate composite of Fig. 14A, B, and C. Thus, even with relatively short labeling times the majority of the label has the mobility of either the RF dimer or monomer.

Origin for progeny DNA synthesis is in the *Eco*RI-A fragment. In the preceding section, evidence was presented which implied that the origin for semiconservative replication of *ts*1 H-1 RF DNA is in the *Eco*RI-A fragment. Since it was found earlier (17) that about 80% of the labeled BUdR incorporated by wt RF DNA late in infection at 37°C was in the V strand, it can be estimated that approximately 60% of the total incorporation is asymmetric V-strand synthesis. This probably represents progeny DNA synthesis by displacement of the preexisting V strand from RF DNA by the nascent V strand. Using the experimental design of the preceding section, the distribution of [^{3}H]TdR in newly completed wt RF DNA was used to determine the polarity of synthesis and thus a crude estimate of the location of the origin for progeny DNA synthesis.

Parasynchronous NB cultures infected with wt H-1 at 37°C were labeled for 5 min at 37°C with [^{3}H]TdR in the presence of FUdR after a 15-min preincubation with FUdR. The monomer RF region of the neutral sucrose gradient of the Hirt-extracted viral DNA was fractionated by BDC chromatography and 10% of the applied DNA was eluted with 1 M TBS. This DNA and the 2% caffeine eluates were collected as before and digested with *Eco*RI in the presence of uniformly labeled ^{32}P *ts*1 RF DNA, and the digest was analyzed by gel electrophoresis as in Fig. 11 (not shown). The ^{3}H/^{32}P ratios were as listed in Table 3. In the 1 M TBS eluate, the *Eco*RI-B ^{3}H/^{32}P ratio was 7.4-fold higher than the ratio for *Eco*RI-A. There were only small differences (no more than 12%) in [^{3}H]TdR/^{32}P ratios for *Eco*RI or *Hae*III fragments when the [^{3}H]TdR-labeled RF was labeled for a 4-h period. Therefore, the distribution of TdR in RF DNA is not sufficiently asymmetric to affect these results. This suggests that *Eco*RI-B is a terminus for wt DNA synthesis.

The digest of the 2% caffeine eluate was complete, and the ^{3}H/^{32}P ratio was again higher for *Eco*RI-B (0.512) than for *Eco*RI-A (0.200), but the difference was not as great as for the salt-elutable RF DNA, 2.5 versus 7.4 (normalized values). The ratio for *Eco*RI-B would be elevated slightly by the 30 to 40% contamination of the 2% caffeine eluate with the 1 M TBS DNA as previously discussed. The expected high ^{3}H/^{32}P ratio for *Eco*RI-A may be absent because the RI molecules are selectively lost by this procedure. The sucrose gradient fraction used here excluded DNA migrating faster than monomer RF DNA. Also, examination of the 2% caffeine eluate by electron microscopy revealed that RI molecules were not the major form present (23). Another factor complicating the analysis of the 2% caffeine eluate is that [^{3}H]DNA representing one-third of the total of the fraction and with the highest ^{3}H/^{32}P ratio (38.25, normalized to *Eco*RI-A) did not migrate as *Eco*RI-A or *Eco*RI-B, but was in the first fraction of the gel. Why this DNA did not migrate into the gel is unknown. In summary, the distribution of label in newly completed RF DNA, as represented by the ^{3}H label in the 1 M TBS fraction, is not uniform and has a higher specific activity in the *Eco*RI-B fragment than in the *Eco*RI-A fragment. Thus, this fragment appears to include the terminus for both RF replication and progeny DNA synthesis. The complementary analysis of RI molecules as represented by the 2% caffeine eluate is complicated by several factors and is not conclusive for the localization of the origin.

***Eco*RI-B$_2$ fragment contains the turnaround.** The left end of H-1 RF DNA has been shown to be heterogenous in its structure, as

TABLE 3. *Distribution of nascent DNA in wt RF DNA*[a]

Prepn	1 M TBS		2% Caffeine	
	^{3}H/^{32}P	Normalized	^{3}H/^{32}P	Normalized
Gel top			7.65	38.25
RF	0.457	3.07		
*Eco*RI-A	0.149	1.0	0.200	1.0
*Eco*RI-B	1.107	7.43	0.512	2.56

[a] wt RF DNA was labeled with [^{3}H]TdR for 5 min at 16 h p.i. at 37°C (50 μCi/ml; specific activity, 20 Ci/mmol). The DNA sedimenting in a neutral sucrose gradient as monomer RF DNA (e.g., fraction A, Fig. 3) was fractionated by BDC chromatography. The 1 M TBS and 2% caffeine fractions were precipitated with 70% ethanol at −20°C, collected by centrifugation, and dried in vacuo. The DNA was redissolved in 50 μl of *Eco*RI digestion buffer combined with a portion of ^{32}P-labeled RF DNA and digested for 2 h at 37°C with 100 U of *Eco*RI. The digest was fractionated by gel electrophoresis as in Fig. 12. The ^{3}H and ^{32}P for each DNA species were summed, and the ^{3}H/^{32}P ratios were calculated. Values shown are ^{3}H/^{32}P ratios and the ratios normalized to the ratio for *Eco*RI-A.

manifested by two forms of the *Eco*RI-B fragment, i.e., B_1 and B_2. We have also presented other evidence for structural diversity in this region: a covalent linkage of V strand to C strand in 10% of the RF forms and self-cohesiveness of the left end as manifested by dimerization. When the distribution of [^{3}H]TdR in newly replicated RF DNA was analyzed, it was observed in both experiments that the ratio of ^{3}H to the uniform label of ^{14}C or ^{32}P was higher for the slower-migrating portion of the *Eco*RI-B fragment peak corresponding to B_1- than for *Eco*Ri-B_2 (cf. Fig. 12). The slightly faster migration of the "snap-back" *Eco*RI-B versus the total *Eco*RI-B in Fig. 9 suggests that it migrates as *Eco*RI-B_2. A more definitive analysis of these findings was carried out by a direct comparison of an *Eco*RI digest of ^{32}P-labeled H-1 RF DNA before and after alkaline denaturation-BDC chromatography by slab-gel electrophoresis (Fig. 15). It is clear that the rapidly renaturing *Eco*RI-B fragment migrates as *Eco*RI-B_2. In addition, digestion of dimer RF DNA produced the expected dimer *Eco*RI-B fragment, which shows a single electrophoretic mobility. Fractionation of the *Eco*RI-B digest on a BDC column was also done to determine whether either fragment had a significant single-stranded region, and electrophoresis of the 1 M TBS eluate shows that both *Eco*RI-B_1 and -B_2 are present. Therefore, it can be concluded that newly replicated RF DNA molecules contain the configuration characterized by an *Eco*RI-B_1 fragment and that these molecules are probably converted to the foldback configuration with or without the covalent linkage of the V to C strand afterward. The differences in electrophoretic mobilites of *Eco*RI-B_1 and -B_2 suggest that the foldback region is about 50 to 70 bp in length, assuming that they are both linear duplexes.

DISCUSSION

The linear duplex RF DNA of the parvovirus H-1 replicates semiconservatively and provides the template for the asymmetric progeny DNA synthesis (17, 18). The latter process was found to require at least one of the capsid proteins by analysis of temperature-sensitive capsid protein mutants of H-1 (19). In this study, the cleavage of H-1 RF DNA by the bacterial restriction endonuclease *Eco*RI is shown to occur at a unique cleavage site 0.22 genome length from the empirically designated left end of the molecule. The smaller *Eco*RI fragment B was heterogeneous in electrophoretic mobility, and a number of alternative structures were shown to exist in this fragment. The differences between the two electrophoretic species for *Eco*RI-B were due to differences in size and not strandedness, since neither B_1 nor B_2 was removed by BDC chromatography. The *Eco*RI-B fragment with the covalent V to C linkage had the electrophoretic mobility of the smaller *Eco*RI-B_2 fragment. The two types of *Eco*RI-B fragments were produced by a single cleavage by *Eco*RI, and a similar result has been obtained with the *H. aegyptius* restriction endonuclease endo R·*Hae*II. This enzyme also cleaves the molecule once near the *Eco*RI site (0.20 genome length from the left end), producing two *Hae*II-B fragments (unpublished data). Since both *Eco*RI-B_1 and -B_2 were found with *ts*1 RF as well as wt RF, both fragments were preserved during the cloning of *ts*1. It is probable that the *Eco*RI fragments differ by about 50 to 70 bp in length, as the difference in their electrophoretic mobilities suggests, or they may have some more complicated structural heterogeneity, such as proposed for AAV DNA (8). Probably the simplest explanation is that there is a turnaround or hairpin at the left end that is about 50 bp in length in the B_2 fragment, as illustrated in Fig. 16 and, in the B_1 fragment, it is extended by a full-length complementary strand, but this remains to be confirmed.

Analysis of H-1 intracellular viral DNA by agarose-gel electrophoresis has shown that H-1 RF DNA exists in two forms, a monomer and a dimer. The identity of the larger species as a dimer RF was made by establishing its molecular weight as twice that of monomer RF DNA and by the specificity of cleavage of the molecule by *Eco*RI endonuclease. H-1 monomer and dimer RF DNA molecular weights were measured by agarose-gel electrophoresis using the *Eco*RI fragments 2 through 6 of bacteriophage λ as markers of known molecular weights (11). Monomer RF DNA had an estimated molecular weight of 3.26×10^6, and the dimer was found to be a "tail-to-tail" linkage of monomer RF DNA at their left ends rather than a tandem union. This was revealed by digestion of the dimer RF DNA with *Eco*RI, and the *Eco*RI fragments obtained included two *Eco*RI-A molecules and a new fragment equal to twice the molecular weight of *Eco*RI-B. Sedimentation of dimer RF DNA in alkaline sucrose gradients showed that most of the DNA sedimented as monomer length single strands. Thus, the dimers were inferred to be linked by hydrogen bonds in the self-cohesive termini at the left end of monomer RF DNA. Incubation of RF DNA in 50% formamide for 4 h at 37°C resulted in a slight decrease in the percentage of the preparation that was dimer length, revealing that the majority

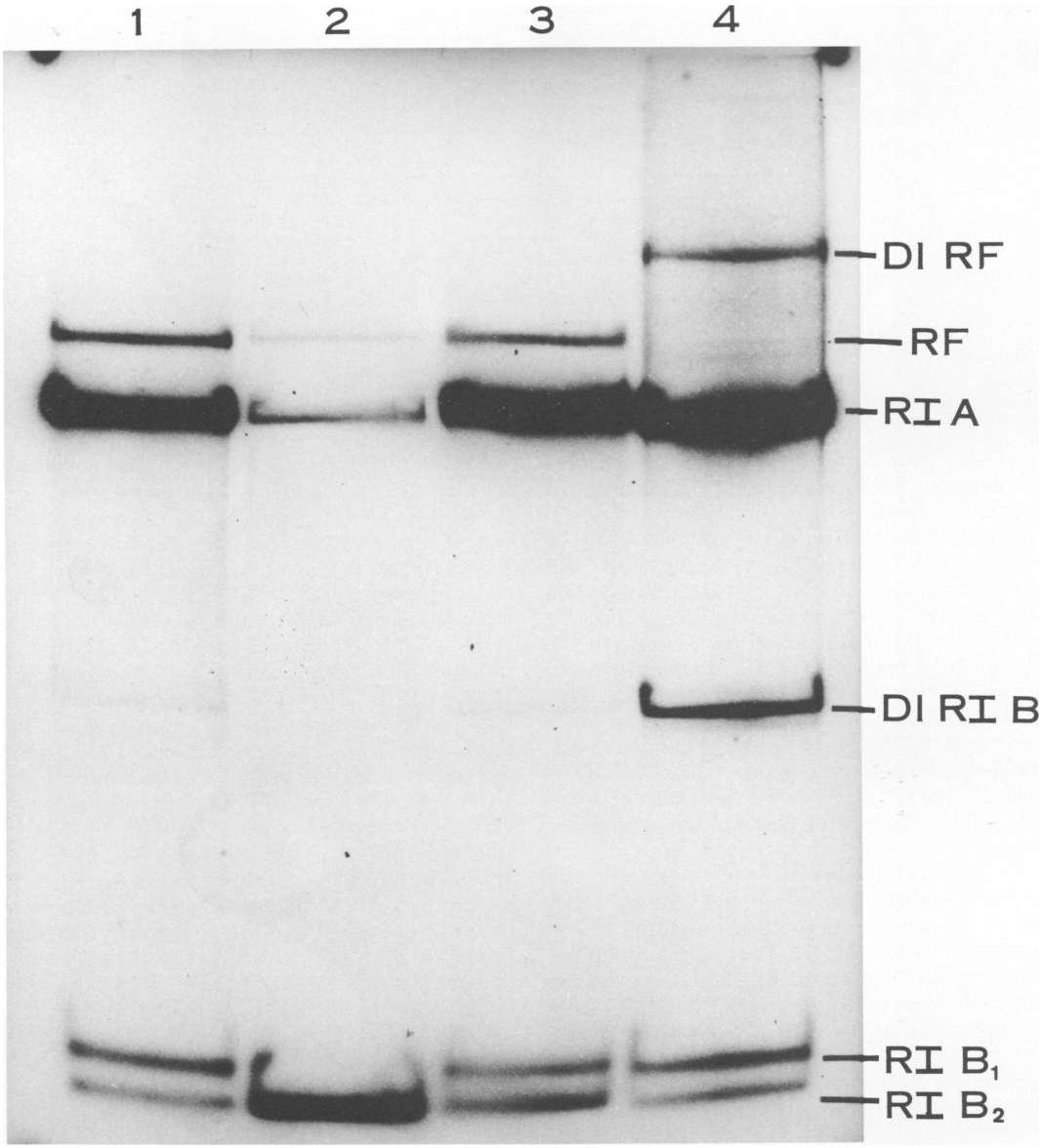

FIG. 15. *Slab-gel electrophoresis of EcoRI digests of RF DNA. RF DNA labeled with ^{32}P was digested for 3 h at 37°C with 100 U of EcoRI. The digest was extracted with phenol, precipitated with ethanol at −20°C, and redissolved in 20 M Tris (pH 7.5)-1 mM EDTA. Aliquots were separately fractionated by alkaline denaturation-BDC or neutral BDC chromatography. A portion of ^{32}P-labeled dimer RF DNA (cf. pool B, Fig. 3) was also digested with EcoRI. Electrophoresis was for 18 h at 16°C at a constant voltage of 50 V. Samples were: (1) EcoRI digest of RF DNA; (2) 1 M TBS eluate of alkaline denaturation-BDC chromatography of (1); (3) 1 M TBS eluate of neutral BDC chromatography of (1); (4) EcoRI digest of dimer RF DNA. Viral DNA and fragments are labeled as follows: dimer RF DNA, DIRF; RF DNA, RF; EcoRI-A, RIA; dimer EcoRI-B, DIRIB; and EcoRI-B_1 and -B_2, RIB_1 and RIB_2, respectively.*

of the monomer RF DNA molecules do not dimerize in vitro.

Dimer RF DNA was found to be relatively constant in proportion to monomer RF DNA at 15 to 25% of the total at several times p.i. and also with pulse-chase labeling. Experiments using the density label BUdR were done to determine whether dimer molecules may form by: (i)

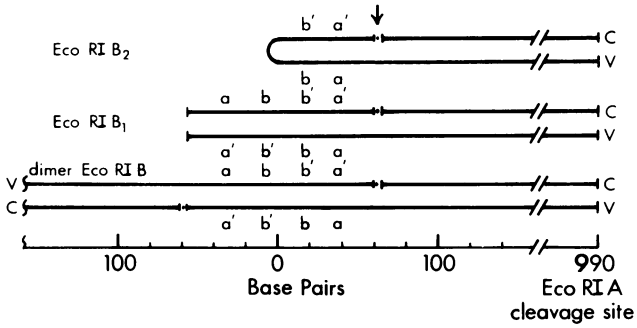

FIG. 16. *Model for the structure proposed for the left end of H-1 RF DNA. Letters represent terminal sequences of 50 to 70 nucleotides, and sequence complementarity is indicated by a primed letter. The structures shown are illustrated with a gap (arrow) between adjacent termini of the V and C strands, which in some cases is covalently closed as indicated by a dot.*

random annealing of monomers with cohesive left termini, perhaps in vitro; (ii) replication of previously formed dimer DNA; or (iii) failure of replicature intermediates of monomer RF DNA to segregate. The results showed that dimer RF DNA incorporated the density label more slowly than monomer RF DNA (i.e., became hybrid density) and ruled out nonsegregation of monomer RI molecules as the immediate mechanism of formation of dimer DNA. The complexity of density labeling with labeling periods greater than 30 min precluded a definitive distinction between (i) possible formation of labeled dimers by random association of monomer RF DNA, or (ii) by replication of parental dimer DNA.

Tattersall et al. reported what appeared to be intramolecular reannealing of MVM RF DNA after heat denaturation at 100°C in a solution of moderate ionic strength (27). In a subsequent report in this series, we will describe evidence for a region at the right end of RF DNA with a very high melting temperature, which, if present in MVM DNA, could have accounted for incomplete denaturation at 100°C. In fact, the alkaline sucrose gradient analysis of MVM RF DNA did not show the expected proportion of single-stranded DNA greater than monomer length (26). Nevertheless, a more rigorous test for covalent linkage of V to C strands in H-1 RF DNA was carried out using alkaline denaturation followed by rapid neutralization and BDC chromatography to separate double-stranded DNA that had reannealed rapidly from DNA with single-stranded regions. After two cycles of this procedure, a fraction was isolated that represented 19% of the total RF DNA, and this fraction sedimented as a dimer length single-stranded DNA in alkaline sucrose gradients. During the preparation of this manuscript a similar structure was reported for intracellular AAV DNA (23). It was suggested that covalent linkage between plus and minus strands of AAV may be the result of the 3′ end of one strand acting as the primer for the initiation of the C strand synthesis, as had also been proposed previously for MVM DNA (26). If this were the case for H-1, then newly labeled RF DNA would be expected to contain relatively more of the covalent linkage than the total RF DNA pool. In the present study the contrary result was obtained; RF DNA labeled for 3.5 h followed by a 1-h chase had a higher proportion of the covalently linked V to C strands than DNA labeled for the 0.5 h preceding the extraction.

The location of the covalent linkage was shown to be in the *Eco*RI-B fragment, and it seems most likely that it is located at the left end in an inverted self-complementary "turnaround" suggested by the dimerization observed occurring at this site. Dimer RF DNA was analyzed in a similar manner, and covalent linkage of V to C strands was found for at least 19% of the population. This linkage was again demonstrated to occur in the *Eco*RI-B fragment and probably at the molecular left end of RF DNA. The presence of dimer length V-C single strands in some of the dimer RF DNA molecules strongly implies that at least this type of dimer existed in vivo, whatever the mechanism of their formation.

Finally, the distribution of isotope in the two *Eco*RI fragments, after short labeling times, was examined to gain insight as to the location of the origin for replication of RF DNA and progeny DNA synthesis. When isotope is first introduced into the precursor pools, it is incorporated into RI molecules. After a labeling period that does not greatly exceed the time required for a round of replication, RI molecules that have completed replication during the la-

beling period will exhibit a declining specific activity of the thymidine at each location within the molecule as the origin of replication is approached. This type of analysis has been used successfully in mapping the origin for a number of viral systems (7, 10). In this study, BDC chromatography was used to isolate RF DNA from RI DNA so that newly completed RF DNA would be separated from RI molecules; the latter showed a preferential increase in incorporation of isotope as the distance to the origin decreased. The results of analysis of replicating H-1 DNA by BDC chromatography agreed with the experience of others; namely, that rapidly labeled DNA was preferentially retained after 1 M TBS elution (13, 25, 21, 26, 22). Confirmation of the presence of RI molecules in the 2% caffeine eluate was made by electron microscopy (23). Thus, the 1 M TBS eluate was suitable for isolation of newly completed RF DNA, and the *Eco*RI-B fragment of this DNA was shown to be enriched for ^{3}H after a short label with [^{3}H]TdR in comparison with [^{14}C]TdR, which was used as a uniform label. This implies that the left end of the molecule is a terminus during *ts*1 RF DNA replication at the restrictive temperature. This is in agreement with the previous finding that the left end, with the covalent linkage, was not implicated as an origin of replication. Also, electron microscopic analysis of the replication of *ts*1 RF DNA, to be described elsewhere, has confirmed that the left end is a terminus for replication (23).

A similar experiment was carried out with wt H-1 RF DNA incubated for 5 min at 37°C. Previous analysis of the incorporation of the density label BUdR into RF DNA indicated that about 75% or more of the wt RF DNA synthetic events at 37°C consisted of asymmetric synthesis of the V strand (17). This has been interpreted as representing progeny DNA synthesis by a displacement mechanism. The distribution of isotope from brief incorporation in the RF DNA in the 1 M TBS eluate was enriched in the *Eco*RI-B fragment, and this again implied that the left end is also a terminus for progeny DNA synthesis. The complementary analysis of the 2% caffeine eluate was complicated by several factors. First, the 2% caffeine eluate was contaminated with about 30% of the 1 M TBS RF DNA, which is retained nonspecifically, perhaps due to localized denaturation occurring at the ends of the molecule. Second, the most rapidly labeled DNA, which exhibited the highest $^{3}H/^{14}C$ ratios for the *ts*1 experiment or $^{3}H/^{32}P$ ratios for the wt experiment, did not migrate as either fragment. In the case of *ts*1 RF DNA, this DNA migrated just ahead of RF DNA or, after digestion with *Eco*RI, just ahead of the *Eco*RI-A fragment. With wt RF DNA, the DNA with the highest $^{3}H/^{32}P$ ratios was trapped at the top of the gel. The reason for these anomalous electrophoretic mobilities is unknown. The wt RF DNA trapped at the gel surface may have occurred due to differential difficulty in solubilizing the DNA after ethanol precipitation, or due to an association with protein.

It was apparent in both electropherograms that the [^{3}H]TdR in RF DNA that had recently completed RF replication or progeny DNA synthesis was associated with the *Eco*RI-B_1 fragment and not *Eco*RI-B_2. This is in agreement with the data indicating that the covalently closed turnaround that is present only in *Eco*RI-B_2 fragments was not associated with replication. These data suggest that, after replication or progeny DNA synthesis, the left end of RF DNA is in the extended configuration (*Eco*RI-B_1) and that it converts to the foldback or turnaround structure (*Eco*RI-B_2) afterward.

In conclusion, these studies imply that the origin for replication for H-1 RF DNA synthesis and progeny synthesis is in the *Eco*RI-A fragment. The left terminus of the molecule is characterized by self-cohesiveness and, in some molecules, this may result in tail-to-tail dimerization with (and by) a covalent linkage between the V and C strands.

It is thus likely that the left end of H-1 RF DNA has an inverted self-complementary sequence as proposed for AAV (1, 8). More detailed analysis of the physical map of H-1 RF DNA is in progress. It is possible that the 3'-hydroxyl at the turnaround at the left end of H-1 RF DNA is serving a primer function for a specialized system of end replication, but not for initiation of replication for the whole molecule.

No direct evidence for intracellular cleavage of the covalent linkage of V to C strands in H-1 RF DNA has been described in this report. However, the presence of this structure is suggestive of the existence of a site-specific nuclease capable of endonucleolytic cleavage of one strand in H-1 RF DNA, indicated by the arrow in Fig. 16, as has also been suggested for adenovirus-AAV-infected cells (24).

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