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Covalent association of protein with replicative form DNA of parvovirus H-1

(covalent protein–DNA complex/DNA synthesis/parvoviral replicative intermediates/viral nucleocapsid complex)

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ABSTRACT **The double-stranded replicative form (RF) DNA of the autonomous parvovirus H-1 can be isolated from infected cells in a covalent complex with protein. The protein is present on most or all of the RF DNA, including actively replicating molecules, and is associated with the 5′-terminal endonuclease *Hae* III fragments of both the viral and complementary strands of RF. The size of the protein is estimated to be 60,000–70,000 daltons from its effect on buoyant density of DNA. DNA with covalently bound protein has not been found in H-1 virions.**

Since the description of plasmid relaxation complexes (1, 2) there have been reports of covalent complexes between protein and nucleic acid in several RNA and DNA viruses. The protein has been found at the 5′ end of the nucleic acid molecules in virions (3–13) and, in a few cases, also associated with the intracellular replicative forms of the nucleic acids (3, 9, 11).

The studies described here were carried out with the autonomous parvovirus H-1 (14), which has a linear single-stranded DNA genome of 1.6×10^6 daltons with hairpin duplexes at both the 5′ and 3′ ends (14–16). Replication of viral DNA in the infected cell proceeds through a double-stranded intermediate, replicative form (RF), only one strand of which is packaged into the virion. We report here the presence of protein covalently associated with intracellular H-1 RF DNA. The RF was found in the form of a complex of nucleic acid with a capsid-like structure which, after treatment with strong protein-denaturing conditions, left protein covalently bound to RF DNA. The covalently bound protein was not found in virions, in contrast to the results with other viral DNA–protein complexes. Some of the features of the covalent protein–DNA complexes are described here.

MATERIALS AND METHODS

Preparation of Labeled H-1 Viral Intermediates. Crude stocks of unlabeled H-1 virus were prepared from infected newborn hamsters by a procedure suggested by Solon Rhode (personal communication), which included homogenization in sodium dodecyl sulfate ($NaDodSO_4$) and centrifugation through a 30% sucrose pad. NB cells (human embryonic kidney cells transformed by simian virus 40), grown as monolayers in modified Eagle's medium with 10% calf serum, were infected with H-1 virus (15 infectious units per cell) at a cell density of 4×10^6/10-cm plate (14, 17). The cells were labeled 18–20 hr after infection with [^{3}H]dThd at 10 μCi/ml or [^{14}C]dThd at 0.5 μCi/ml (1 Ci = 3.7×10^{10} becquerels), and intracellular viral DNA intermediates were separated from host DNA by $NaDodSO_4$ lysis/NaCl precipitation (18).

Samples requiring Pronase treatment were kept at 60°C for 3 hr in 1% $NaDodSO_4$/30 mM Tris·HCl/20 mM EDTA, pH 8, with three additions of Pronase (CB grade, Calbiochem) at 1 mg/ml followed by incubation for 16 hr at 37°C with additional Pronase at 2 mg/ml. Where indicated, this was followed by extraction with phenol and precipitation with ethanol.

Isopycnic Centrifugation. Neutral CsCl solutions for equilibrium density centrifugation contained 50 mM Tris·HCl, pH 8.1, and 25 mM EDTA. Alkaline CsCl solutions contained 50 mM Na_3PO_4 and 25 mM EDTA, and the final pH was adjusted to 12.8 (corrected for Na^+). The CsCl solutions also contained 0.2% Sarkosyl, calf thymus DNA at 25 μg/ml, and bovine plasma albumin at 25 μg/ml; densities are specified in figure legends. Centrifugations were for 42 hr, at 20°C (neutral) or 4°C (alkaline), in the Beckman 65 rotor (6-ml volume) at 40,000 rpm or SW 60 rotor (1.5-ml volume) at 35,000 rpm. Density was measured by pycnometry, and acid-precipitable radioactivity was determined.

RESULTS

Labeling of intracellular DNA with [^{3}H]dThd was carried out on cells infected with parvovirus H-1 during the period of most rapid synthesis of viral RF DNA, and a low molecular weight fraction of DNA was extracted by the Hirt procedure (18). When the Hirt supernatant, containing the RF, was banded in CsCl most of the DNA label was found in fractions much lower in density than DNA, suggesting association of the RF with large amounts of protein, similar to that of intact virus (density $\rho \approx 1.4$ g/ml). The properties of this material—including buoyant density in CsCl, sedimentation velocity in neutral sucrose gradients, sensitivity to S1 nuclease, and sedimentation velocity of the DNA (after treatment with $NaDodSO_4$/Pronase) in neutral and alkaline sucrose gradients—indicated that these protein-containing components, which make up the bulk of intracellular viral DNA intermediates, resemble the nucleocapsid complexes in cells infected by Kilham rat parvovirus described by Gunther and Revet (19).

Although stable to treatment with detergent at 25°C in the Hirt extraction and during the CsCl banding, the nucleocapsid complexes were disrupted by heating to 60°C in detergent or treatment with 8 M urea at 25°C. With removal of protein the DNA label shifted to a higher density in CsCl, closer to that of DNA (≈1.7 g/ml); however, it remained less dense than a protease-treated ^{14}C-labeled sample present in the same CsCl density gradient (Fig. 1). The density anomaly is not an artefact of labeling with ^{3}H because the result was the same when the labels were reversed—i.e., when the ^{3}H-labeled extract was treated with protease, it was the ^{14}C-labeled sample not treated with protease that had the lower density (not shown).

Because treatment with protease restored the density to that of DNA, the results suggest that the lower density of viral RF results from a small amount of protein that remains associated with the DNA in spite of the treatment with detergent at 60°C or with urea. Reassociation between DNA and protein cannot

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Abbreviations: RF, replicative form; $NaDodSO_4$, sodium dodecyl sulfate.

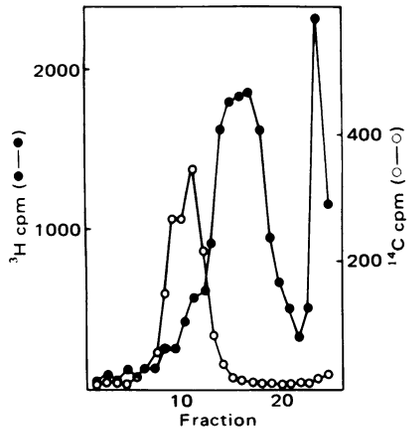

FIG. 1. Neutral CsCl centrifugation of native H-1 DNA–protein complex. Hirt extract from ^{3}H-labeled H-1-infected cells was mixed with a ^{14}C-labeled sample prepared as for the ^{3}H-labeled sample but, in addition, deproteinized with NaDodSO$_4$/Pronase/phenol. The mixture was brought to 2% Sarkosyl and 8 M urea (added as solid) and after 30 min at 25°C was centrifuged to equilibrium in 1.5 ml neutral CsCl ($\bar{\rho}$ = 1.68 g/ml). The ^{14}C sample contained a mixture of single- and double-stranded DNA.

explain the results because the protease-treated [^{14}C]DNA was present with the non-protease-treated [^{3}H]DNA during the disruption by hot detergent/urea. No counterpart to this type of tight protein–DNA association was detected in chromatin from uninfected cells. DNA from uninfected cells lysed with detergent at room temperature banded in CsCl at the density of purified DNA without further treatment (not shown).

The protein–DNA linkage also survived treatment with 8 M urea at 100°C (Fig. 2), or 0.1 M NaOH at 25°C (20), or 1 M NaOH at 37°C for 30 min (see below). In each case a protease-treated ^{14}C-labeled sample was present during the treatment and analysis.

Tests using S1 nuclease indicated that the density difference could not be accounted for by preferential renaturation of the non-protease-treated samples (20). In addition, the equilibrium density centrifugation analyses were carried out in alkali (Fig. 3). As in the neutral CsCl analyses, samples not treated with protease consistently banded in alkaline CsCl at a distinctly lower density compared to a protease-treated sample (^{14}C-labeled) present in the same analysis. We infer from these results that the association between protein and DNA is covalent.

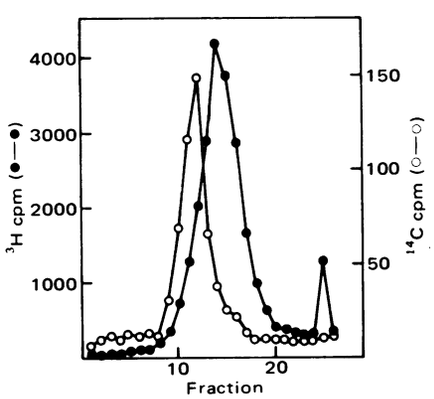

FIG. 2. Neutral CsCl centrifugation of denatured H-1 DNA–protein complex. Hirt extract from ^{3}H-labeled H-1-infected cells together with a ^{14}C-labeled deproteinized sample (Fig. 1) was heated to 100°C for 2 min in 0.1 ml of 50 mM Tris·HCl, pH 8.1/5 mM EDTA/3% Sarkosyl/8 M urea and then centrifuged to equilibrium in a 1.5-ml neutral CsCl gradient ($\bar{\rho}$ = 1.67 g/ml).

The samples described thus far were Hirt extracts of cells infected with 15 infectious units per cell and labeled for 120 min; however, the results were similar if cells were infected with 150 infectious units per cell, or labeled for 2 min, or lysed with 1% Sarkosyl (and sheared) prior to equilibrium centrifugation analysis in alkaline CsCl (not shown).

A variable proportion of viral RF (0–30%) remained very low in density (<1.58 g/ml), on the top of the CsCl gradients, at either neutral or alkaline pH (Figs. 1, 2, and 3). The amounts of this very low-density component, apparently containing a larger proportion of protein, appeared unrelated to the particular procedure used to dissociate the nucleocapsid complex and was not affected by addition of reducing agent (dithiothreitol). This heterogeneity was partially accounted for by the size of DNA contained in the protein–DNA complex. Fractions from the top of an alkaline CsCl gradient (Fig. 3) after deproteinization contained a 12S component by sedimentation in alkaline sucrose gradients. However, at least a third of the same fraction was 16S in alkali, as was the main fraction from the alkaline CsCl gradient ($\rho \approx 1.72$ g/ml).

In alkaline CsCl analyses of fresh unfractionated Hirt extracts of infected cells, most of the labeled DNA was found to be covalently bound to protein (Fig. 3), indicating that both viral and complementary strands of the RF are complexed. This was confirmed by hybridization analysis of fractions from preparative alkaline CsCl gradients (Fig. 3, Table 1). The hybridization studies also uncovered further heterogeneity. The principal component (fraction II, Fig. 3) contained predominantly viral strands, whereas the very light component (fraction IV, Fig. 3) consisted predominantly of complementary strands (Table 1); the significance of this remains unclear.

An estimate of protein size can be made from the density shift in CsCl isopycnic centrifugation. Taking the molecular weight of 16S RF, purified by velocity sedimentation (Fig. 4*A*), to be 3.3×10^6 (22), and the density of pure protein as 1.29 g/ml, the difference in density between protease-treated RF and RF not treated with protease by equilibrium centrifugation in neutral CsCl (Fig. 4*B*) gives a figure of 119,000 daltons of protein per RF molecule (23). If we assume that protein is present on both strands of RF, which appears to be the case for most molecules both by analysis in alkaline CsCl (Fig. 3) and by gel electrophoresis, and if the protein on each end (see below) is the same size, then each protein molecule is approximately 60,000 dal-

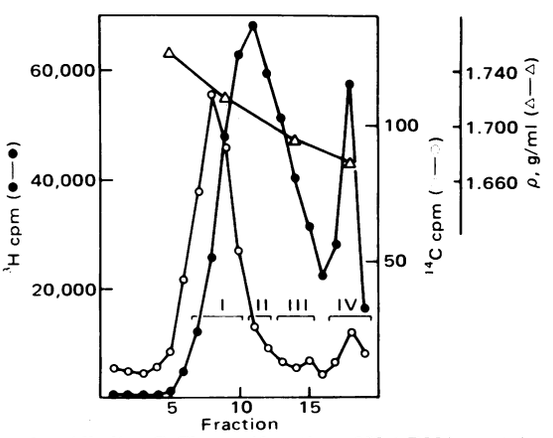

FIG. 3. Alkaline CsCl centrifugation of H-1 DNA–protein complex. Hirt extract from ^{3}H-labeled H-1-infected cells together with ^{14}C-labeled deproteinized sample was held at 37°C for 30 min in 1 ml of 1 M NaOH/20 mM EDTA and then centrifuged to equilibrium in a 6-ml alkaline CsCl gradient ($\bar{\rho}$ = 1.71 g/ml). After the radioactivities of 10% aliquots had been measured, fractions were pooled as shown (I–IV) for further analysis.

Table 1. Hybridization analysis of alkaline CsCl fractions

Sample	Strand content, %	
	Viral	Complementary
I	44	17
II	53	17
III	26	29
IV	6	43

^{3}H-Labeled samples (I–IV) are the pooled fractions from the alkaline CsCl equilibrium centrifugation described in Fig. 3. All samples were deproteinized by treatment with Pronase/NaDodSO$_4$/phenol and sonified. ^{3}H-Labeled samples were annealed with unlabeled DNA (1000-fold excess of virion DNA or 2000-fold excess of RF DNA) to $30 \times C_0t_{1/2}$ ($C_0t_{1/2}$ is the product of DNA concentration and incubation time at 50% hybridization). The figures for content of "viral" strand are based on % hybridization with unlabeled H-1 RF DNA minus % hybridization with unlabeled virion DNA; for "complementary" they are based on % hybridization with unlabeled virion DNA, alone. Extent of self-hybridization of each ^{3}H-labeled sample (8–11%), which was largely accounted for by "snap back" [extent hybridized at 0 time, 0°C, presumably due primarily to the terminal "foldbacks" (14)], was subtracted from each, and additional correction was made for difference in ^{3}H specific activity of viral and complementary strands resulting from the difference in % thymidine (21).

tons. A fraction of RF DNA consists of hinged molecules (terminally crosslinked) (14) that may bear covalent protein on one end only; however, the error from this must be small because at most 5–10% of the molecules in this preparation were double length in alkali (not shown). An experiment was carried out similar to the one described in Fig. 4, but in which the 16S monomer single strands were isolated in alkaline sucrose and then directly banded in alkaline CsCl (not shown). From the density shift in alkali, an estimate was made for a protein size of 71,000 daltons per 1.6×10^6 dalton single strands.

Mature virions were also examined for protein–DNA covalent linkage. When purification of the virus included either treatments with DNase and trypsin (which do not reduce infectivity) (24, 25) or selection of 110S particles by sucrose gradient velocity sedimentation, the density of DNA released from virions by alkali could not be distinguished from that of protease-treated viral DNA. Virus prepared by simply banding a lysate of infected cells in CsCl did contain protein covalently associated with DNA as judged by analysis in alkaline CsCl, which may be due to contamination with nucleocapsid material.

RF purified without protease digestion did not enter agarose gels (Fig. 5, lane 1) or, in some cases, entered the gel but smeared out widely. After protease digestion, RF moved into the gel to the correct position for its size (Fig. 5, lane 2). This is consistent with other observations that DNA bound to certain proteins does not enter agarose or acrylamide gels (27–29). It should be pointed out that even with salt (0.2 M NaCl) or detergent (0.2% NaDodSO$_4$ or Sarkosyl) present in samples of RF not treated with protease, the migration in gels remained anomalous—i.e., smearing or not entering. H-1 viral DNA purified without protease treatment entered agarose gels (Fig. 5, lane 3), confirming results of equilibrium centrifugation in alkaline CsCl, described above, which also did not detect covalently bound protein.

The location of the protein on RF DNA was analyzed by restriction enzyme cleavage of the DNA. Digestion of purified H-1 RF DNA (protease treated) with restriction endonuclease *Hae* III results in 12 fragments (22), only the largest 8 of which can be seen in Fig. 6 (lane 3). When protease treatment is omitted, the two largest fragments, A and B, are reduced or absent, and label is found at the origin of the gel, presumably representing protein-associated A and B fragments unable to enter the gel (Fig. 6, lane 2). The remaining bands are present in equal amounts in both samples. The positions on the H-1 genome of the fragments produced by *Hae* III digestion determined by Rhode (22) are shown in Fig. 7. The two fragments bound to protein, A and B, are located on the two ends of the RF DNA molecule.

The protein-bearing fragments were further analyzed to determine which strand is attached to protein. *Hae* III-digested RF was purified on alkaline CsCl gradients, yielding the protein-associated single-strand fragment in low density fractions at the top of the gradient (Fig. 8, fractions 15 and 16). Gel analysis of this fraction resulted in a wide smear, consistent with

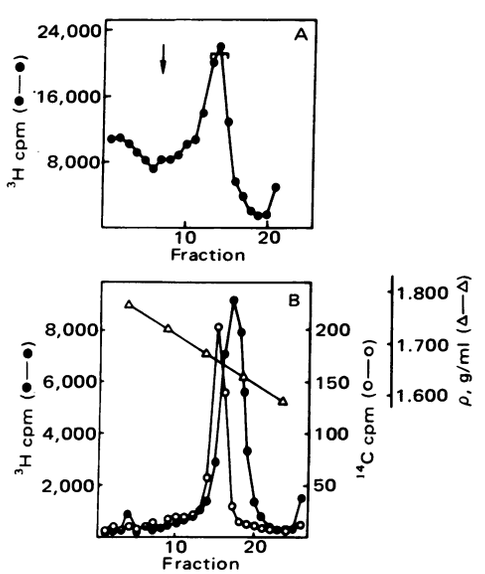

FIG. 4. Preparation of H-1 [^{3}H]RF and equilibrium centrifugation in neutral CsCl. (*A*) Hirt extract from ^{3}H-labeled H-1-infected cells was heated at 60°C for 30 min in 1% NaDodSO$_4$/1% Sarkosyl/50 mM Tris·HCl, pH 8.1/10 mM EDTA and then kept at 25°C in 8 M urea for 30 min to disrupt the nucleocapsids (see text). The mixture was then centrifuged in a 4-ml neutral sucrose gradient (5–20% sucrose in 1 M NaCl/0.05 M Tris·HCl, pH 8.1/5 mM EDTA/0.2% Sarkosyl) in the Beckman SW 60 rotor at 20°C for 2.5 hr at 50,000 rpm. A portion (1%) of each fraction was assayed for radioactivity. The arrow indicates the position of an external 30S marker of coliphage fd DNA. (*B*) Fractions 13 and 14 from the neutral sucrose gradient (*A*), containing double-strand monomer (RF), were pooled and 10% was mixed with a ^{14}C-labeled fraction prepared similarly but also deproteinized (Pronase/NaDodSO$_4$/phenol) and centrifuged to equilibrium in a 1.5-ml neutral CsCl gradient ($\bar{\rho}$ = 1.67 g/ml).

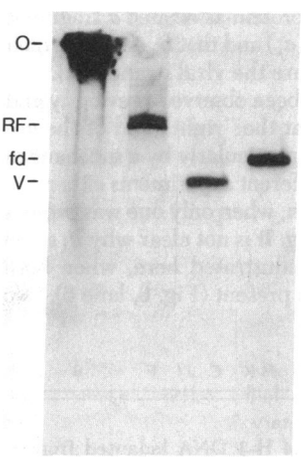

FIG. 5. Effect of protease treatment on behavior of H-1 RF in agarose gel (1%) electrophoresis. ^{3}H-Labeled double-strand monomer (RF) was prepared as described in Fig. 4*A*. Agarose gel was run in 40 mM Tris acetate, pH 8.1/2 mM EDTA, at 30 V for 16 hr, followed by fluorography (26). Lane 1, RF; lane 2, RF treated with Pronase; lane 3, H-1 viral DNA (V) (released from virions by 1% NaDodSO$_4$ at 60°C for 30 min); lane 4, coliphage fd DNA (released by 1% NaDodSO$_4$ at 60°C for 30 min), included for comparison. O, origin.

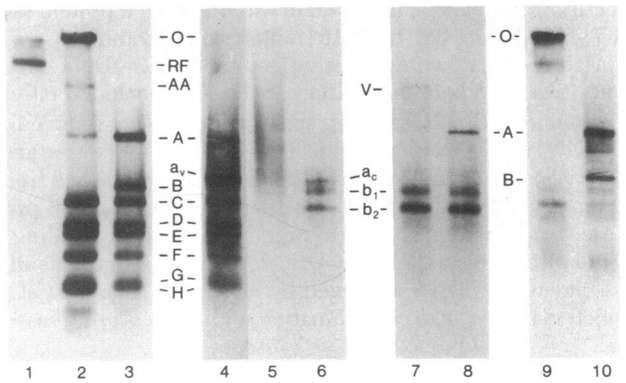

FIG. 6. Agarose gel (1.7%) analysis of isolated protein-linked strands of *Hae* III fragments. O, origin; capital letters A–H refer to *Hae* III restriction fragments (Fig. 7); AA, dimer A; V, viral DNA; a_v and a_c, A single strands, viral and complementary, respectively; b_1 and b_2, B single strands (see text). "RF" refers to the double-strand monomer (16S) purified from Hirt extract of H-1-infected cells by neutral sucrose gradient centrifugation after treatment with Na-DodSO$_4$ at 60°C (Fig. 4). Lane 1, RF, Pronase-treated; lane 2, RF, *Hae* III-digested; lane 3, RF, *Hae* III-digested and Pronase-treated; lane 4, non-protein-associated *Hae* III fragments of RF (DNA density pool from alkaline CsCl centrifugation, Fig. 8); lane 5, protein-associated *Hae* III fragments of RF (light density pool from alkaline CsCl centrifugation, Fig. 8); lane 6, sample 5, treated with Pronase; lane 7, sample 6, hybridized to unlabeled viral DNA; lane 8, sample 6, hybridized to *Hae* III-digested unlabeled viral DNA; lane 9, sample 6, hybridized to unlabeled RF; lane 10, sample 6, hybridized to *Hae* III-digested unlabeled RF. Hybridizations were carried out with 40-fold excess of unlabeled DNA to $>100 \times C_0t_{1/2}$. (Samples 9 and 10 were run in a different gel under the same conditions.)

its being associated with protein (Fig. 6, lane 5). After protease treatment, the fraction showed three bands which, by comparison with alkali-denatured *Hae* III-digested RF DNA (not shown), were tentatively identified as single strands of A and B (*a*, *b*) (Fig. 6, lane 6).

To determine whether the protein was associated with the viral or complementary single strands of the fragments, they were annealed (after deproteinization) with an excess of unlabeled viral strand DNA and then reanalyzed by agarose gel electrophoresis. Only fragment *a* had an altered mobility in the agarose gel due to hybridization with viral strand, resulting in its disappearance from the normal position and the appearance of a new band, the hybrid, at or near the position of intact viral strand (Fig. 6, lane 7). When *Hae* III-digested viral single strand was annealed with the fragments, only fragment *a* diminished in amount and a new band appeared at the A (double strand) position, confirming the strand and fragment identification (Fig. 6, lane 8). We infer that the protein-associated *a* fragment is the complementary strand of A (a_c) and that b_1 and b_2, which do not hybridize to viral strand, are the viral strand of B.

Multiplicity of B fragments has been observed previously and accounted for by heterogeneity at the "right" end of the genome (5′ end of the viral strand), particularly by a mechanism of "hairpin unfolding" (22). In different experiments either one or both B or *b* fragments were seen; when only one was present it was usually the faster-migrating. It is not clear why in some experiments, including the one illustrated here, when both single-strand (*b*) fragments were present (Fig. 6, lane 6), two

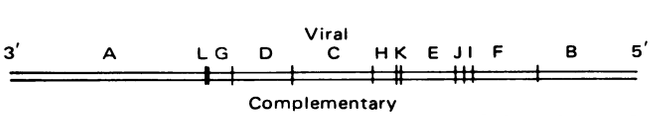

FIG. 7. *Hae* III cleavage map of H-1 DNA [adapted from S. Rhode (22)].

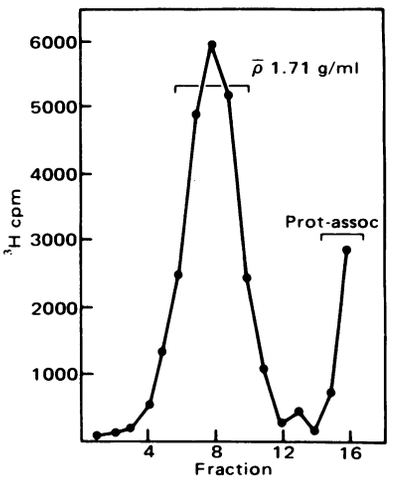

FIG. 8. Isolation of protein-linked strands of *Hae* III fragments by alkaline CsCl centrifugation. ^{3}H-Labeled RF purified from Hirt extract by sucrose gradient velocity sedimentation (Fig. 4) was digested with *Hae* III (2 units/μg of DNA) at 37°C for 16 hr. The sample was denatured by addition of NaOH to 0.2 M (25°C, 10 min), and centrifuged to equilibrium in a 1.5-ml alkaline CsCl gradient ($\bar{\rho}$ = 1.71 g/ml). The radioactivities of 1% aliquots were determined and fractions of DNA density (fractions 5–9) and the light density (protein-associated) fractions (fractions 15 and 16) were pooled.

double-strand (B) fragments were not always observed (Fig. 6, lane 3); the inverse discrepancy (one *b* and two B fragments) was also seen in some experiments.

Controls showed that all three fragments, a_c, b_1, and b_2, hybridized to RF DNA (Fig. 6, lane 9), and when annealed with *Hae* III fragments of RF migrated at their expected double-strand positions (Fig. 6, lane 10). There was no evidence of hybridization to DNA from uninfected cells (not shown). From these results, which show that the protein is associated with the complementary strand of the *Hae* III A fragment and the viral strand of the B fragment, and the location of these fragments on the H-1 genome (Fig. 7), it is evident that protein is bound only to the terminal fragments, on the strands with 5′ ends.

Hae III fragments that banded at DNA density in alkaline CsCl (Fig. 8, fractions 5–9), after being allowed to renature, showed all of the normal fragments present, except for A and B (Fig. 6, lane 4), consistent with the above results indicating that protein is associated with the *Hae* III A and B fragments. A new band that appeared in this fraction was tentatively identified as the viral strand of A (Fig. 6, lane 4a_v), which migrated at a rate different from that of the complementary strand. The absence of *b* bands may have been due to selective loss or obscuring by other bands. In other experiments *b* bands were present in this fraction.

DISCUSSION

Most of the intracellular viral DNA intermediates of H-1-infected cells are associated with a capsid-like structure, here referred to as nucleocapsid, reported previously for Kilham rat parvovirus by Gunther and Revet (19). In studies not described here, in which the nucleocapsid was purified by its binding to nitrocellulose filters, it was found that even short pulses with [^{3}H]dThd (e.g., 0.5 min) appeared in nucleocapsid complexes. Taken together with the effect on viral DNA synthesis of mutations in the virion structural proteins (17, 30), this suggests that DNA replication is closely coupled to virion assembly.

We have found that the DNA in the nucleocapsid complex is covalently bound to one or more proteins. The presence of the protein was determined by anomalous behavior of RF DNA with isopycnic banding in CsCl and electrophoresis in agarose

gels, both corrected by treatment with protease. The covalent nature of the association is indicated by the stability of the complex to conditions that include strong alkali.

It should be pointed out that, although protein and DNA are found covalently bound under the conditions described here, this does not necessarily imply that the association is covalent in the cell. There are examples in which a covalent bond can be caused to form in protein–DNA complexes (1, 31, 32) and a similar possibility exists here, for example, with the initial exposure to detergent in lysing the cells, or with the alkali.

It is shown here that protein is associated with the terminal *Hae* III fragments of H-1 RF. In both of these fragments it is the 5′ terminal strand that is attached to protein, and from this we make the provisional conclusion that the protein is attached at the 5′ ends of both the viral and complementary strands. The results from both alkaline CsCl and gel electrophoresis indicate that most of the component single strands of the RF duplex are protein-bound, suggesting that most of the RF molecules bear protein at both ends. Because the virion-like protein aggregate was found at only one end of RF DNA molecules (19), it appears that the covalent protein is not present only as a component of the capsid-like structure of the nucleocapsid complex. Similarly, in the case of poliovirus RF, in addition to a protein covalently bound to each end, there is a larger aggregate of noncovalently bound protein at one end (33).

The DNA that was not protein-bound consisted predominantly of viral strands. This could represent DNA being processed for inclusion in virions, or already packaged, and would be consistent with our inability to demonstrate the covalent protein–DNA complex in virions. Because no effort was made to isolate virus prior to the proteolytic processing step that results in a fall in density and enhanced infectivity (34, 35), the presence of the protein–DNA link in that particular form of virion has not been excluded. However, the results do show that protein capable of forming a covalent bond with viral DNA is not essential for infectivity of the virus.

Salzman and Koczot (36) have described an 8–10S complex from Kilham rat virus consisting of protein associated with different lengths of DNA. The complex resulted from treatment of the virus with alkali, but could be dissociated with detergent. A similar structure was not found in H-1 virions and the relationship, if any, between the protein–DNA complex formed from virions of Kilham rat virus and the protein–DNA covalent complex of H-1 RF is not clear.

It is not known whether the covalent protein is of viral or host origin. Similar to the results with parvovirus H-1, characterization of the site of attachment between protein and nucleic acid has shown this to be at the 5′ terminus for ColE1 plasmid (2), phage ϕ29 (9, 10), adenovirus (12), poliovirus (4–6), encephalomyocarditis virus (6), and cowpea mosaic virus (8).

It has been speculated that the proteins associated with adenovirus and poliovirus serve a priming function in new strand initiation (4, 5, 11, 12). However, if priming is accomplished in H-1 by the 3′ hairpin termini, as has been proposed (37, 38), it would suggest that the covalent protein does not function as primer. A covalent protein–DNA link may serve as intermediate in enzyme–nucleic acid interactions in which a phosphodiester bond is broken and rejoined without input of energy. This has been demonstrated for topoisomerases and gyrase (23, 31, 32), and such a function could play a role in some of the mechanisms proposed for the replication of parvoviruses (22). However, the replication mechanism for H-1 DNA and the role of the covalently bound protein await clarification.

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