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Proteins tightly associated with the termini of replicative form DNA of Kilham rat virus, an autonomous parvovirus

(terminal proteins/viral DNA replication)

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ABSTRACT **Revie *et al.* [Revie, D., Tseng, B. Y., Grafstrom, R. H. & Goulian, M. (1979) *Proc. Natl. Acad. Sci. USA* 76, 5539–5543] have proposed that the double-stranded replicative form (RF) DNA of the autonomous rodent parvovirus H-1 has protein of 60 kDa covalently bound at its 5′ termini. We present evidence that the RF DNA of a similar rodent parvovirus, Kilham rat virus (KRV), also has covalently bound protein. NaDodSO$_4$/polyacrylamide gel electrophoresis of purified, ^{125}I-labeled RF DNA shows that proteins of 68–72, 66, 64, and 55 kDa copurify with the DNA during velocity and equilibrium sedimentation in the presence of detergents and 4 M guanidine HCl. Phenol extraction in the presence of 2-mercaptoethanol removes the 68- to 72-kDa proteins, but the 66-, 64-, and 55-kDa proteins remain tightly, but noncovalently, bound. The latter polypeptides also appear to associate with protease-treated RF DNA when mixed with uninfected cell extract. Following removal of these proteins by electrophoresis in NaDodSO$_4$/agarose gels, two proteins (called RF TP-90 and RF TP-40), of about 90 and 40 kDa, become evident. These remain bound to the DNA and are released only after nuclease digestion of the DNA. These two proteins, apparently not of viral origin, are associated with terminal restriction fragments of the RF DNA and appear to be covalently bound to the 5′ termini of both strands.**

The commonly studied autonomous rodent parvoviruses [H-1, minute virus of mice (MVM), and Kilham rat virus (KRV)] have a number of properties in common. They have ≈5000 nucleotide-containing linear single-stranded (ss) DNA genomes with short terminal hairpins (1, 2). The DNA is packaged into an icosahedral capsid about 240 Å in diameter (3–5). The viruses replicate only in host cells undergoing DNA synthesis (6) and are presumed to rely largely, if not entirely, on host factors for DNA synthesis, although no reconstituted replication system using purified proteins has been developed to demonstrate this. Because of their dependence on host S phase, autonomous parvoviruses provide a model system for studying cellular DNA replication.

Following infection, the ss viral DNA is converted to a linear, double-stranded (ds) replicative form (RF) DNA molecule (7–10) by the host replicative machinery, presumably using the 3′ hairpin terminus as a primer-template. This duplex RF DNA is then further replicated and acts as template for the synthesis of the viral DNA strand from a proposed unique replication origin at the 3′ end of the complementary strand (11, 12). These observations have led to the proposal of a "rolling hairpin" model for parvovirus replication (13–16).

Based on its aberrant electrophoretic mobility in agarose and an increase in its buoyant density in CsCl following protease treatment, most of the intracellular RF DNAs of H-1 and MVM appear to have protein covalently bound at its termini (16, 17). To date, no protein has been isolated that can be identified as the parvovirus RF DNA terminal protein (RF TP) based on (*i*) covalent binding to RF DNA and (*ii*) location at the termini of RF DNA. In this paper, we have presented experiments to show that KRV RF DNA also has covalently bound proteins at its termini and we have discussed the origin of these proteins.

MATERIALS AND METHODS

Materials. Guanidine HCl (grade I), Tris (reagent grade), 2-mercaptoethanol (type I), and phenylmethylsulfonyl fluoride (PhMeSO$_2$F) were purchased from Sigma. Sucrose and CsCl were Schwarz/Mann "ultrapure." *Hpa* II and electrophoresis-grade agarose were from Bethesda Research Laboratories. Materials for polyacrylamide gels were Bio-Rad electrophoresis grade. Na^{125}I (Amersham) and Tagit (Calbiochem) were used for the preparation of Bolton–Hunter reagent by the procedure of Feitelson *et al.* (18).

Cells and Virus. Normal rat kidney (NRK) cells (19), strain N153, kindly provided by W. K. Yang of this Division, were maintained in Eagle's minimum essential medium (EMEM) with 10% fetal bovine serum (GIBCO). KRV strain 171 (20) was used for all infections.

Infection of Cells and Purification of RF DNA. Parasynchronous cultures of NRK cells, obtained after trypsinization of confluent cultures, were infected with KRV in serum-free medium 1 hr after seeding (multiplicity of infection = 10) at 20% confluence. One hour later, the serum was added back to the medium. In some cases L[^{35}S]methionine (New England Nuclear; 1244 Ci/mmol; 1 Ci = 37 GBq) was added (15 μCi/ml) at this time in methionine-free EMEM containing 10% dialyzed fetal bovine serum and 10 μM methionine, and the cells were incubated until 16 hr after infection. For the preparation of [^{3}H]thymidine-labeled RF DNA, infected cells were allowed to grow at 37°C until 16 hr after infection, at which time [*methyl*-^{3}H]thymidine (New England Nuclear, 200 Ci/mmol) was added to the cells (50 μCi/ml) for an additional 30 min.

Following the indicated labeling periods, RF DNA was purified from infected cells by a modification of the procedure of Lavelle and Li (21). Briefly, cells were lysed in buffer containing 20 mM Tris·HCl (pH 8.0), 10 mM EDTA, 0.5% Sarkosyl NL97 (CIBA–Geigy), 4 M guanidine HCl, 1 mM PhMeSO$_2$F, and, where indicated, 250 mM 2-mercaptoethanol at 37°C for 60 min. The lysate was layered onto a 10–30% sucrose gradient in buffer containing 20 mM Tris·HCl (pH 8.0), 2 mM EDTA, 0.1 mM PhMeSO$_2$F, 4 M guanidine HCl,

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Abbreviations: RF, replicative form; ds, double-stranded; ss, single-stranded; KRV, Kilham rat virus; MVM, minute virus of mice; NRK, normal rat kidney; kbp, kilobase pair(s); RF TP, RF DNA terminal protein; PhMeSO$_2$F, phenylmethylsulfonyl fluoride.

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and, in some cases, 15 mM 2-mercaptoethanol and centrifuged at 26,000 rpm in an SW 28 rotor (Beckman) for 38 hr at 10°C. Peak fractions containing the RF DNA (Fig. 1*A*) were dialyzed against buffer containing 20 mM Tris·HCl (pH 8.0), 2 mM EDTA, 0.1 M NaCl, 0.1% Sarkosyl, and 0.1 mM $PhMeSO_2F$. Following ethanol precipitation, RF was isopycnically banded in 5.0 ml of 53% (wt/wt) neutral CsCl [in 20 mM Tris·HCl (pH 8.0), 2 mM EDTA, 0.1 mM $PhMeSO_2F$, 0.1% Sarkosyl, with or without 15 mM 2-mercaptoethanol] at 40,000 rpm for 48 hr in a Beckman type 50 rotor at 10°C (Fig. 1*B*). These preparations of RF DNA were free of cellular DNA, as judged by $NaDodSO_4$/agarose gel electrophoresis (Fig. 1*D*).

Radioiodination of Proteins. Purified RF DNA was treated with 50–150 μCi of ^{125}I-labeled Bolton–Hunter reagent (^{125}I-Bolton–Hunter reagent) prepared as described (18). The DNA was separated from the unreacted reagent by gel filtration on Sephadex G-25 (Pharmacia) in 50 mM NH_4HCO_3 (pH 7.5) and 0.1% $NaDodSO_4$. Iodinated RF DNA was stored at −80°C in 70% ethanol and pelleted immediately prior to use. Labeled preparations were routinely analyzed within 2 days to minimize radiochemical damage to proteins.

Restriction Endonuclease Digestion. One to five micrograms of KRV RF DNA was digested with 4 units of *Hpa* II in 50 mM Tris·HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 μg of bovine serum albumin per ml, and 1 mM $PhMeSO_2F$ at 37°C for 2 hr. The restriction fragments were separated on neutral $NaDodSO_4$/agarose gels and electroeluted.

Gel Electrophoresis. RF DNA or its restriction fragments were electrophoresed in neutral agarose gels in Tris/acetate/EDTA buffer (22) containing 0.1% $NaDodSO_4$. Prior to loading, samples were heated to 37°C for 10 min in 10 mM EDTA/20% glycerol/2.0% $NaDodSO_4$/0.1% bromophenol blue.

Proteins were separated on $NaDodSO_4$/polyacrylamide gels by the procedure of Laemmli (23) using a 4.5% acrylamide stacking gel with a 12% acrylamide resolving gel (acrylamide:bisacrylamide = 37.5:1). Iodinated proteins were detected by autoradiography with XAR-5 film (Kodak).

RESULTS

Proteins Associated with CsCl-Purified RF. [^{35}S]Methionine, added to infected cells, was incorporated into RF DNA and copurified with RF DNA through guanidine HCl/sucrose (Fig. 1*A*), neutral and alkaline CsCl (Fig. 1 *B* and *C*), and $NaDodSO_4$/agarose (Fig. 1*D*). As seen in Fig. 1*D*, a small amount of dimer-length RF DNA (fraction 12) was also observed; it too was labeled with ^{35}S. Protease treatment resulted in a loss of ^{35}S label and a downward shift in the ^{3}H

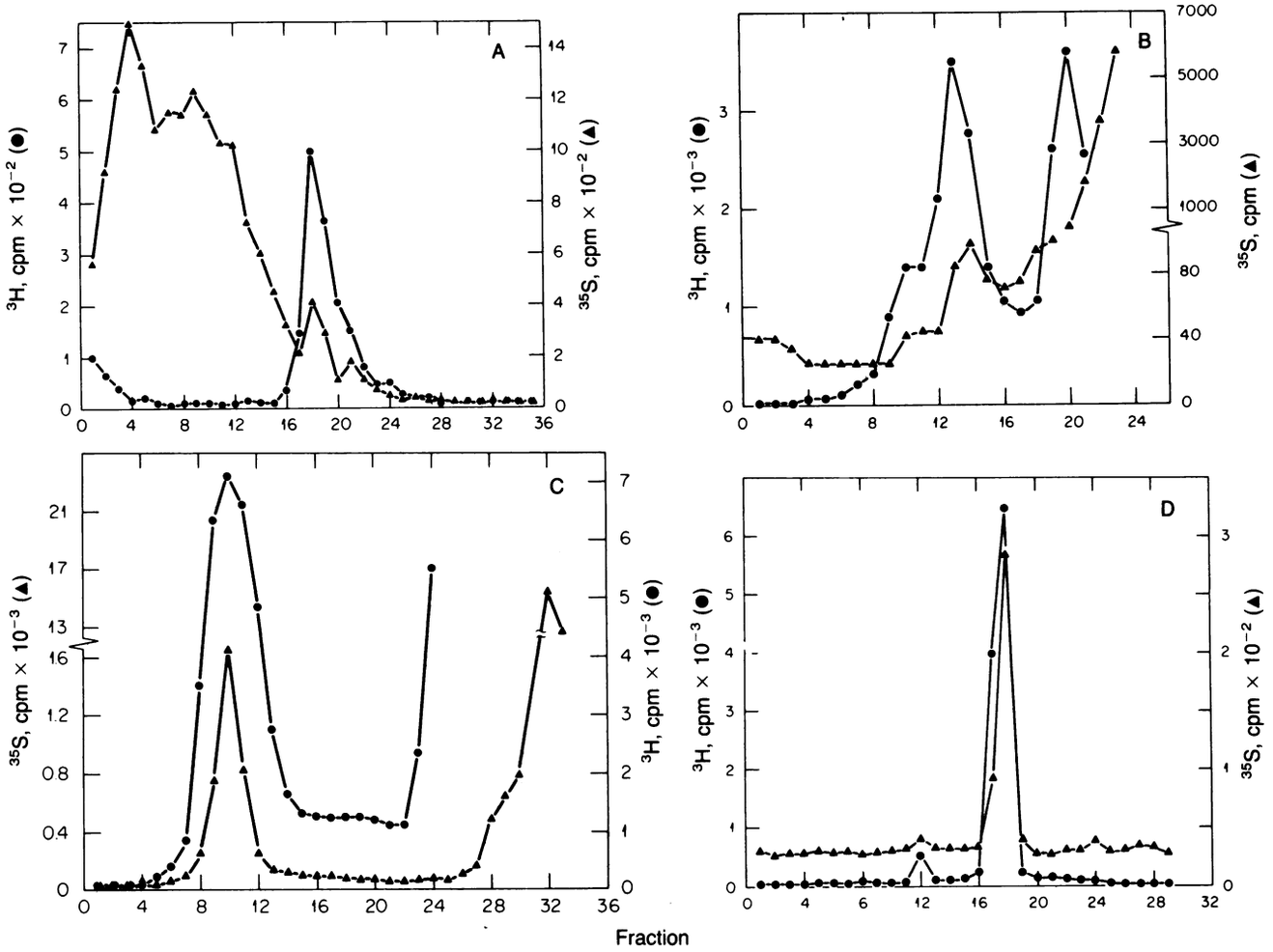

FIG. 1. Copurification of ^{35}S label with RF DNA. KRV-infected NRK cells were labeled, harvested, and lysed. (*A*) A 10–30% sucrose/4 M guanidine HCl gradient of cell lysate; 1-ml fractions were collected from the top, and 5 μl was assayed for ^{3}H or ^{35}S. (*B*) A 53% (wt/wt) neutral CsCl gradient of material peak (fractions 17–21) in *A*; 10-drop fractions were collected from the bottom, and 10 μl was assayed for radioactivity. (*C*) A 56% (wt/wt) alkaline CsCl gradient (17) of peak material from *B*; 10-drop fractions were collected from the bottom and assayed for radioactivity. (*D*) $NaDodSO_4$/agarose gel electrophoresis of peak material from *B*; 0.5-cm slices of the gel were melted and assayed for ^{35}S or ^{3}H.

peak of 8–10 fractions on alkaline or neutral CsCl sedimentation (data not shown).

Incorporation of ^{35}S label into RF DNA was very poor, and, as a result, we chose to label proteins with ^{125}I-Bolton–Hunter reagent. This reagent is highly specific for labeling primary amino groups of proteins (18); in our hands 500–1000 times more iodine was incorporated into bovine serum albumin than into an equal mass of *Escherichia coli* DNA (unpublished observation). Iodinated RF DNA gave a protease-sensitive labeled band on NaDodSO$_4$/agarose gels (Fig. 2) and a large amount of much faster migrating material; protease treatment resulted in only a slight increase in the mobility of RF DNA stained with ethidium bromide (data not shown).

When CsCl-purified RF DNA was iodinated, radioactive proteins of approximately 200, 68–72, and 55 kDa were seen in polyacrylamide gels (Fig. 3*A*, lane 1). The fact that these bands were observed without digestion of RF DNA and that micrococcal nuclease treatment did not alter their intensity (Fig. 3*A*, lane 2) suggests that they are not covalently bound to RF DNA. Nuclease digestion of the RF DNA resulted in the appearance of a new protein at about 40 kDa (Fig. 3*A*, lane 2), which may be covalently associated. All of these bands were eliminated by protease treatment prior to electrophoresis (data not shown).

Attempts to remove the noncovalently associated proteins by phenol extraction in the presence of detergent (Fig. 3*B*, lane 1) were unsuccessful. However, phenol extraction in 2% 2-mercaptoethanol removed the 68- to 72-kDa material, whereas proteins of 66, 64, and 55 kDa became more prominent (Fig. 3*B*, lane 3). As above, release of the material in these bands from non-nuclease-treated RF DNA and their protease sensitivity (Fig. 3*B*, lanes 2 and 4) indicated their noncovalent association and proteinaceous nature, respectively. These proteins probably correspond to the faster migrating material seen on NaDodSO$_4$/agarose gels (Fig. 2). The sensitivity of some of these proteins to 2-mercaptoethanol led us to include this reagent during subsequent purifications of the RF DNA (see *Materials and Methods*). Iodination by Bolton–Hunter reagent does result in small changes in the apparent molecular mass of some proteins (18) so the true molecular masses may be different from those observed here by a few percent.

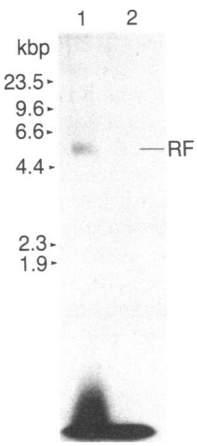

FIG. 2. NaDodSO$_4$/agarose gel electrophoresis of ^{125}I-Bolton–Hunter reagent-labeled RF DNA. Autoradiogram of 0.1% NaDodSO$_4$/agarose gel of labeled RF purified, radioiodinated, and electrophoresed. Lane 1, ^{125}I-labeled RF; lane 2, ^{125}I-labeled RF DNA digested with 200 μg of proteinase K per ml (37°C, 30 min). RF, position of ethidium bromide-stained RF DNA. kbp, Kilobase pairs of ethidium bromide-stained *Hin*dIII-digested λ phage DNA marker fragments.

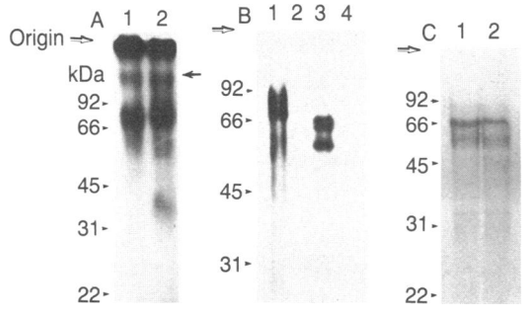

FIG. 3. NaDodSO$_4$/polyacrylamide gel electrophoresis of ^{125}I-labeled proteins tightly associated with KRV RF DNA. (*A*) Autoradiogram of ^{125}I-labeled proteins associated with neutral CsCl-purified RF DNA. Lane 1, undigested RF; lane 2, RF digested with 200 μg of micrococcal nuclease per ml, 200 μg of venom phosphodiesterase per ml, and 100 μg of bacterial alkaline phosphatase per ml at 37°C for 30 min. (*B*) ^{125}I-labeled proteins associated with RF DNA following phenol extraction in the presence of 1% Sarkosyl (lanes 1 and 2) and after phenol extraction in the presence of 1% Sarkosyl/2% 2-mercaptoethanol (lanes 3 and 4). Lanes 1 and 3, undigested RF DNA; lanes 2 and 4, RF digested with proteinase K. (*C*) Uninfected host cellular proteins reassociating with protease-treated RF DNA. Lane 1, undigested RF; lane 2, RF digested with nucleases as described above. The positions of protein standards in the gels indicated by their size (kDa) are given. The arrows on the left indicate the origin and the arrow on the right of *A* indicates the position of the 200-kDa band.

Binding of Uninfected Cellular Proteins to Deproteinized RF. Some of the proteins associated with RF DNA may have arisen from aggregation of cellular proteins onto the DNA during cell lysis or from virus capsid assembly intermediates. [Two of the capsid proteins have molecular masses of 64 and 59 kDa (24).] To test this possibility, RF DNA purified by centrifugation in guanidine HCl/sucrose was digested with proteinase K, extracted with phenol, and mixed with uninfected cells lysed by the procedure used for RF DNA preparation. After rebanding in guanidine HCl/sucrose and neutral CsCl, this DNA was radioiodinated and electrophoresed in NaDodSO$_4$/polyacrylamide gels with and without nuclease digestion. Major proteins of 64–66 and 55 kDa were found to be noncovalently associated with this RF (Fig. 3*C*), suggesting that the noncovalent proteins observed above (Fig. 3 *A* and *B*) are not of viral origin. Nuclease treatment resulted in no new proteins being released from the RF DNA (Fig. 3*C*, lane 2), indicating that no covalent DNA–protein linkages are induced by this procedure.

Proteins Associated with Electrophoretically Purified RF. Because only a small amount of ^{125}I label comigrated with CsCl-purified RF DNA in NaDodSO$_4$/agarose gels (Fig. 2), we chose to use electrophoresis as a purification step following CsCl banding. RF DNA purified in this manner appeared to be free of noncovalently associated proteins (Fig. 4, lane 1), whereas nuclease digestion resulted in the appearance of two protease-sensitive bands at 90 and 40 kDa (Fig. 4, lanes 2 and 3). These protein bands were absent in all of the RF DNA preparations tested in the absence of nuclease treatment.

Association of Protein with Terminal Restriction Fragments. If either the 40- or 90-kDa protein is covalently bound to the termini of RF DNA, it should be exclusively released from the terminal restriction fragments of the RF DNA by nuclease digestion. CsCl-purified, ^{125}I-labeled RF DNA was digested with *Hpa* II that cleaves KRV RF DNA at map positions 0.50 and 0.71 (from the 3′ end of the virus DNA strand) (25). The restriction fragments were separated by NaDodSO$_4$/agarose gel electrophoresis, electroeluted, and analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis before and after nuclease treatment. The results (Fig. 5) indicate that the 40-

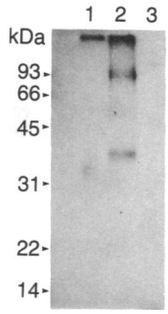

FIG. 4. Proteins associated with electrophoretically purified RF. ^{125}I-labeled RF DNA purified by NaDodSO$_4$/agarose gel electrophoresis was electroeluted and loaded onto a NaDodSO$_4$/polyacrylamide gel before (lane 1) or after (lane 2) digestion with nucleases, as described in the legend to Fig. 2, or after digestion with nucleases and proteinase K (lane 3).

and 90-kDa proteins are associated exclusively with the terminal restriction fragments (A and B) and not with the internal (C) fragment (24, 25). Similar results were obtained with *Pst* I, which generates four fragments of the RF (data not shown and ref. 26). Because of their association with RF DNA terminal restriction fragments, these proteins are designated RF TP-90 and RF TP-40, for RF terminal protein, 90 and 40 kDa, respectively. Because the extent of labeling of terminal proteins with ^{125}I might not be uniform, it is not possible to determine the stoichiometric relation of RF TP-90 and RF TP-40.

DISCUSSION

This paper describes isolation of two proteins, RF TP-40 and RF TP-90, which appear to be covalently bound to the termini of KRV RF DNA. Previous studies (17) provided evidence suggesting that a protein of 60 kDa was covalently linked to the RF DNA of parvovirus H-1. Subsequently, Astell *et al.* (16) claimed to have isolated a 60-kDa RF TP for MVM, consistent with the predicted result (17), but did not demonstrate an exclusive association with RF DNA termini. We have also observed polypeptides in the 60-kDa range; however, our results clearly indicate that they are noncovalently bound. This difference may also be due to the virus and cell systems used or to differences in sample preparation. The difference between the observed molecular masses of the

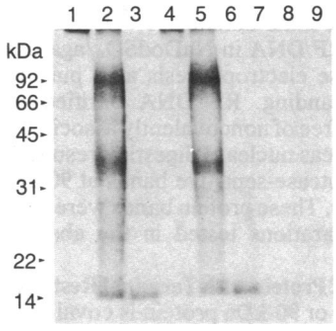

FIG. 5. Proteins associated with electrophoretically purified *Hpa* II restriction fragments of RF DNA (25). NaDodSO$_4$/polyacrylamide gel of *Hpa* II A (lanes 1–3), B (lanes 4–6), and C (lanes 7–9) fragments. Lanes 1, 4, and 7, undigested fragments; lanes 2, 5, and 8, fragment RF digested with nucleases (see Fig. 2); lanes 3, 6, and 9, fragment digested with nucleases and proteinase K. NaDodSO$_4$/agarose gel electrophoresis and electroelution of fragments were done as described in the text. The size and location of the protein markers are given.

KRV RF TPs and the predicted value of 60–70 kDa (17) can be explained by considering the "RF" to be a mixed population of molecules with either RF TP-40 or TP-90 at either end. Thus, the predicted molecular mass would be the average of the two, weighted by their relative abundance.

The copurification of RF TP-40 and TP-90 with RF DNA through a number of harsh treatments expected to eliminate noncovalent association and the fact that they are released only after digestion of the RF DNA (Figs. 4 and 5) strongly suggest that they are covalently bound to the DNA. The specific association of these proteins with terminal restriction fragments (Fig. 5) of the RF DNA leads us to propose that they are covalently bound to the termini of the DNA. The sensitivity of the KRV RF DNA to exonuclease III and resistance to λ exonuclease suggest that the covalent linkage is to the 5′ termini of the duplex DNA (27). Though our data indicate that both ends of the RF DNA have both proteins bound, it remains to be seen whether both proteins are simultaneously bound to the same RF DNA end or if there are two populations of ends, one with RF TP-40 and one with RF TP-90. Preliminary data from NaDodSO$_4$/alkaline agarose gel electrophoresis of *Pst* I-digested RF DNA show two populations of 5′ terminal fragments (26) and these results are consistent with the possibility that RF TP-40 and RF TP-90 are not both attached to the same terminus of a DNA molecule.

The origin of these proteins remains to be determined. They do not appear to be related to the virus capsid proteins (26, 27). Antisera to the virus-encoded nonstructural proteins of KRV, H-1, MVM, and porcine parvovirus (28, 29), which have molecular masses close to RF TP-90, do not cross-react with either terminal protein (26). A 90-kDa protein covalently bound to H-1 RF DNA has been isolated recently with the same antigenic behavior (P. Paradiso, personal communication) as KRV RF TP-90. These observations suggest that RF TP-90 is not encoded by any of the virus' open reading frames (26, 28) and may be of host origin.

As with RF TP-90, RF TP-40 appears to be immunologically unrelated to either the capsid proteins or the nonstructural proteins of KRV (26, 27). We have found evidence (26) consistent with the possibility that RF TP-40 is generated from RF TP-90 by site-specific proteolysis analogous to the processing of the adenovirus terminal protein (30).

Covalent nucleic acid–protein complexes have been observed in a number of viral systems (31–34) and have been suggested to occur in the chromosomal DNA of a number of higher organisms (35, 36). The viral covalently bound proteins have usually been implicated in the initiation of the replication of the DNA to which they are bound (34, 37–39). As yet, there has been no direct demonstration of such a role for the parvovirus terminal protein, although RF DNA synthesized *de novo* in the cell-free system of Muller and Siegl (40, 41) behaved as if it had covalently bound protein. Several authors have proposed that this protein is the site-specific endonuclease in the "hairpin transfer" replication model for parvoviruses (16, 17, 42). This role would be, at least superficially, analogous to that of the gene A protein in φX174 DNA replication (34)—namely, generation of a site-specific nick with a 3′ OH, which is elongated by host replicative enzymes, and a 5′ phosphate to which the protein is covalently bound. This similarity continues in that, in both systems, the covalent DNA–protein complex is not observed in the mature virion. It appears that the virion DNA of LuIII parvovirus has covalently bound RF TP that is later removed during encapsidation of the DNA (40, 41).

The origin of the noncovalently bound proteins is not entirely clear. Again, none appears to be immunologically related to capsid proteins (26, 27). Mixing experiments with protease-treated RF DNA and uninfected cell extracts (Fig. 3*C*) suggest that some of these proteins are of cellular origin,

and our preliminary data indicate that the proteins of 60-kDa range are identical to the well-characterized proteins of the nuclear lamina (26), which are known to be DNA binding proteins (43, 44). The proteins removed by 2-mercaptoethanol treatment may be related to proteins with similar properties observed by others (43–45) that have been implicated in maintenance of the stability of the nuclear matrix and the binding of cellular DNA to this structure (43, 44). The possible interaction of KRV RF DNA with the nuclear matrix is particularly interesting in view of proposals that this structure is a site for cellular DNA replication (46, 47) and transcription (48) and that the DNA of a number of viruses can be found tightly associated with it (49–51). Binding of KRV RF DNA to the nuclear matrix has not yet been demonstrated, although LuIII DNA has been found associated with non-histone "chromosomal" protein (52) and Bates *et al.* have found evidence for the binding of bovine parvovirus DNA to the nuclear matrix (R. C. Bates, personal communication).

It is not known at what point during synthesis of parvovirus RF the terminal proteins become attached or whether all RF DNA molecules have them throughout the viral life cycle. Although the amino acid and nucleotide involved in the presumed covalent bond are unknown, the alkali stability suggests a phosphotyrosine linkage is involved. Investigations in these areas should shed light on the function of the terminal proteins during parvovirus replication.

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