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Ribonucleotides in Unintegrated Linear Spleen Necrosis Virus DNA

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The structure of unintegrated linear spleen necrosis virus DNA was characterized by using various chemical and enzymatic treatments in conjunction with denaturing gels and nucleic acid hybridization probes. Throughout the course of the viral infection, the predominant species of viral DNA was that of a linear double-stranded molecule containing ribonucleotides covalently joined to the DNA. The majority of both − and + strands were continuous. The ribonucleotide linkages appeared to be relativly short, and the base composition and distribution of the ribonucleotide linkages were heterogeneous. On the average, the − strand had fewer of the ribonucleotide linkages than did the + strand. Viral DNA containing ribonucleotide linkages was infectious in DNA transfection assays. The structure of spleen necrosis virus DNA was different from that of Schmidt-Ruppin Rous sarcoma virus-D, and mixed infections demonstrated that the observed differences are a result of *cis*-acting functions.

Retroviruses are + strand RNA viruses that replicate via a DNA intermediate. Various forms of the viral DNA, including chromosomally integrated, unintegrated linear, and unintegrated circular, have been described previously (4, 7, 8, 13, 17, 20, 21, 27, 29, 30, 40).

The structure and kinetics of synthesis of the − and + strands of the unintegrated linear viral DNA have been characterized extensively (4, 9, 27, 29). For the avian sarcoma and murine leukemia viruses, these experiments demonstrated that the − strand of the linear viral DNA is continuous and of genome size, whereas the + strand is segmented and composed of fragments smaller than genome size.

We have characterized the structure of the unintegrated linear viral DNA of another retrovirus species, the avian reticuloendotheliosis viruses. Our results demonstrate a structure for the − and + strands of unintegrated linear spleen necrosis virus (SNV) DNA that is different from that previously reported for other retroviruses. Here we present evidence that both − and + strands are of genome size and contain ribonucleotides that are covalently linked. We have characterized the distribution, size, and base composition of the ribonucleotide linkages and the synthesis and infectivity of the DNA molecules containing these ribonucleotides.

MATERIALS AND METHODS

Cells and viruses. The general sources and procedures for obtaining and propagating avian cells have been described previously (7).

SNV, a member of the avian reticuloendotheliosis virus species of retroviruses, has been described previously (36, 37).

Schmidt-Ruppin Rous sarcoma virus-D (SR-RSV-D) was a generous gift of S. Weller (McArdle Laboratory for Cancer Research).

SNV titers were assayed as described previously (36) by endpoint dilution of cytopathic effects or DNA polymerase activity. SR-RSV-D titers were determined by focus formation.

DNA extraction and purification. Chicken embryo fibroblasts were infected at a multiplicity of 1 PFU/cell, unless otherwise indicated. Unintegrated viral DNA was prepared from these infected cells 3 days after infection by the Hirt fractionation procedure (16). The Hirt supernatant fraction was then treated with 250 μg of predigested pronase per ml at 37°C for 30 min. The digested lysate was extracted twice with redistilled phenol (saturated with 0.01 M Tris-hydrochloride, pH 7.4, and with 0.16% 8-hydroxyquinoline [wt/vol]) and twice with chloroform-isoamyl alcohol (24:1, vol/vol), followed by ethanol precipitation.

Assay of infectious viral DNA. Chicken embryo fibroblast cells were prepared for assay of infectious viral DNA as described previously (7). Infectious viral DNA was assayed by the calcium phosphate precipitation method of Graham and van der Eb (7, 10). Media were changed every 3 days, and the plates were scored by the appearance of cytopathic effects (36) or DNA polymerase activity (36) or both at 8 to 9 days after DNA infection. Quantification of the infectivity of DNA samples was determined by endpoint dilution, as described previously (5).

All DNA samples in any one experiment were assayed at the same time, since considerable variation in titers was observed between infectious DNA assays performed at different times.

Gel electrophoresis and DNA transfer to nitro-

cellulose filter paper. DNAs were subjected to electrophoresis through 6- to 9-mm-thick horizontal 0.7% agarose slab gels containing 1 μg ethidium bromide per ml. For all glyoxal-treated DNA samples, the electrophoresis buffer consisted of 50 mM sodium phosphate buffer (pH 7.0) and 2 mM EDTA. Otherwise, the electrophoresis buffer consisted of 40 mM Tris-acetate (pH 7.9), 50 mM sodium acetate, and 1.0 mM EDTA.

Fragments of lambda DNA generated by digestion with *Eco*RI or *Hin*dIII restriction enzymes served as molecular mass markers and were visualized by ethidium bromide-UV light fluorescence. For glyoxal-treated DNA samples, lambda DNA molecular mass markers (0.5 to 1 μg/slot) were visualized by ethidium bromide-UV light fluorescence after in situ alkali treatment and subsequent neutralization of the agarose gel. This procedure hydrolyzes the glyoxal adducts and allows detection of the marker DNAs.

Transfer of DNA onto nitrocellulose filter paper was performed as described by Southern (31).

Molecular hybridization. (i) Hybridization probes. Viral RNA was extracted from virions that had been purified by banding in sucrose (19), and 60 to 70S RNA was isolated by centrifugation through 15 to 30% glycerol gradients (42).

SNV [^{125}I]RNA (specific activity, approximately 5×10^7 cpm/μg), prepared as previously described (21), was a generous gift of S. Mizutani (McArdle Laboratory for Cancer Research).

SNV and Rous-associated virus type 61 (RAV-61) ^{32}P-labeled complementary DNA ([^{32}P]cDNA; calculated specific activity, approximately 4×10^8 dpm/μg) was synthesized by avian myeloblastosis virus reverse transcriptase with oligomers of calf thymus DNA as primers (35) and 70S viral RNA as template as previously described (20). Actinomycin D (25 μg/ml) was included to restrict synthesis to − strand cDNA. Nick-translated lambda [^{32}P]DNA (specific activity, 5×10^7 cpm/μg) was a generous gift of Tim Schedl (McArdle Laboratory for Cancer Research).

(ii) Hybridization conditions. Presoaking and hybridization of nitrocellulose filters were carried out as previously described (20).

Filters were washed by using a modification of the procedure described by Shank and Varmus (29). Filters were incubated in 2× SSC, (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 63°C for 1 h and 0.1× SSC–0.1% sodium dodecyl sulfate at 50°C for 1.5 h and finally rinsed with 0.1× SSC. Some filters hybridized with viral [^{125}I]RNA were treated with 20 μg of RNase A per ml in 2× SSC at 37°C for 1 h in place of the first wash.

Rehybridization of filters was accomplished by eluting the former probe from the filter-immobilized DNA with a solution consisting of 50% formamide and 3× SSC at 63°C for 20 min. The filter was washed in 2× SSC at 50°C for 15 min and rinsed briefly with 0.1× SSC. The filter was then presoaked and hybridized as above.

Autoradiography and densitometry tracings. Kodak X-Omat X-ray film was exposed at −70°C with the use of Ilford fast tungstate intensifying screens (22). Exposure times varied from 0.5 h to 10 days for different experiments.

Autoradiographs were scanned with a Joyce-Loebl microdensitometer.

Treatment of DNA with glyoxal. DNA samples were treated with glyoxal by a modification of the procedure described by McMaster and Carmichael (23). Samples were incubated at 50°C for 1 h in a solution of 1 M glyoxal, 50% dimethyl sulfoxide, and 20 mM sodium phosphate (pH 7.0).

Treatment of DNA with RNase. Heat-denatured DNA samples were treated with either 40 μg of RNase A (Worthington Biochemicals Corp.) per ml in 5 mM EDTA (pH 7.0) at 37°C for 12 h or 1 U of RNase T_2 (Calbiochem) in 10 mM sodium acetate buffer (pH 4.5)–5 mM EDTA.

Chicken embryo RNase H was a generous gift of S. Mizutani. The preparation was shown to be free of DNA endonuclease activity by incubation with lambda DNA followed by agarose gel electrophoresis under nondenaturing and denaturing conditions. Viral DNA was treated with RNase H in 20 mM Tris (pH 8.0)–25 mM NaCl–5 mM $MgCl_2$ at 37°C for 1 h.

Mock-treated samples were treated as above except enzymes were omitted from the reaction.

Treatment of DNA with S1 nuclease. S1 nuclease from *Aspergillus oryzae* was a generous gift of S. Mizutani. Reaction conditions were 30 mM sodium acetate buffer (pH 4.5)–30 mM NaCl–0.1 mM $ZnSO_4$–7 μg of restriction enzyme *Eco*RI-digested lambda DNA per ml added as carrier and to monitor the extent of nonspecific digestion. Reactions were performed at 37°C for 1 h. One unit of S1 nuclease is defined as that amount of enzyme required to convert 1 μg of denatured DNA to trichloroacetic acid-soluble material in 1 h.

NaI density gradient centrifugation. Saturated NaI solution in 0.1 M Tris-hydrochloride (pH 8.0)–0.01 M EDTA–0.8 mM Na_2SO_3 was passed through a column of chelating resin (Bio-Rad Laboratories) and then filtered through a membrane filter (0.45 μm; Millipore Corp.) (3). The total volume of gradients was adjusted to 5 ml by mixing saturated NaI solution, sample, and the buffer above.

Hirt supernatant DNA was prepared as described above. After precipitation by ethanol, the precipitate was dissolved in RNase-free 0.01 M Tris-hydrochloride (pH 7.5)–0.001 M EDTA–0.1 M NaCl (TEN). The solution was mixed with NaI solution, and the initial refractive index was adjusted to 1.4375. Centrifugation was performed in a fixed-angle 50 Ti rotor at 40,000 rpm for 72 h at 20°C. Gradients were fractionated from the bottom, and 200-μl fractions were collected.

Each fraction was diluted 1:5, and DNA was precipitated with the addition of 2 volumes of ethanol and carrier tRNA. Each precipitate was resuspended in TEN. A sample of each fraction was subjected to agarose gel electrophoresis in parallel lanes. The DNA was transferred to nitrocellulose filters. SNV-specific DNA or lambda DNA was detected quantitatively by hybridization under conditions of probe excess with either SNV [^{32}P]cDNA or lambda viral [^{32}P]DNA. Relative concentrations of virus-specific DNA in each fraction were determined by referring to the linear range of densitometry of the autoradiogram (20).

RESULTS

Structure of SNV − and + strand DNAs. The general design of the experiments to study the structure of the unintegrated linear viral DNA of SNV was as follows. Unintegrated SNV DNA was prepared from infected chicken embryo fibroblast cells as described above. After treatment with different reagents, the SNV DNA was reacted with glyoxal and subjected to electrophoresis in neutral agarose gels. Under these conditions, viral − and + strands migrate independently as a function of molecular weight. Glyoxal adducts were hydrolyzed in situ by alkali. DNA fragments were then transferred to nitrocellulose filters by the Southern technique (31). Virus-specific − or + strand DNA was detected by hybridization with viral [^{125}I]RNA or viral [^{32}P]cDNA, respectively.

The structure of unintegrated linear SNV DNA is shown in Fig. 1. As expected, native viral DNA was detected as duplex molecules of 6.0 megadaltons (Md) (lane A). After denaturation with glyoxal, the majority of both − and + strand viral DNAs were detected as single-stranded molecules of genome size, 3.0 Md (lanes B and C). These results demonstrate that the predominant, unintegrated linear form of SNV DNA consists of continuous − and + strands of genome size.

Preliminary experiments using alkaline gel electrophoresis indicated the presence of alkali-labile bonds in the viral DNA. The nature of these bonds was analyzed further with the techniques of glyoxal denaturation and agarose gel electrophoresis.

Figure 2 shows the presence of alkali-labile bonds in both − and + strands of unintegrated linear SNV DNA. Portions of viral DNA were treated with alkali before treatment with glyoxal. If alkali-labile bonds are present in viral DNA, the alkali treatment would hydrolyze these bonds and reduce the size of the viral DNA. Relative to those glyoxal-treated samples that were not treated with alkali, the alkali-treated DNAs were reduced in size (Fig. 2a, compare lanes B and C and lanes E and F). This alkali-induced reduction in size was observed for both the − and + strands of viral DNA (Fig. 2a, lanes C and F, respectively). The degradation of the viral DNA was not due to nonspecific breakage of DNA under the conditions of alkali hydrolysis, since restriction enzyme fragments of lambda DNA included in the alkali-treated reactions were not degraded relative to the non-alkali-treated reaction (data not shown).

Although the majority of the SNV DNA fragments observed after alkali hydrolysis were considerably smaller than genome size (Fig. 2), a small fraction of the viral DNA molecules(<5%) remained genome size (3.0 Md) after alkali hydrolysis (Fig. 2b). These genome size molecules which do not contain alkali-labile linkages were observed among − and + strands of the viral DNA from each of several different preparations of DNA (data not shown).

Except for the genome size molecules which are resistant to alkali hydrolysis, no discrete size classes of molecules were observed after hydrolysis of the alkali-labile bonds (Fig. 2a, lanes C and F; Fig. 2c). The broad distribution of molecules of different molecular weights appears to result from a heterogeneous distribution of the

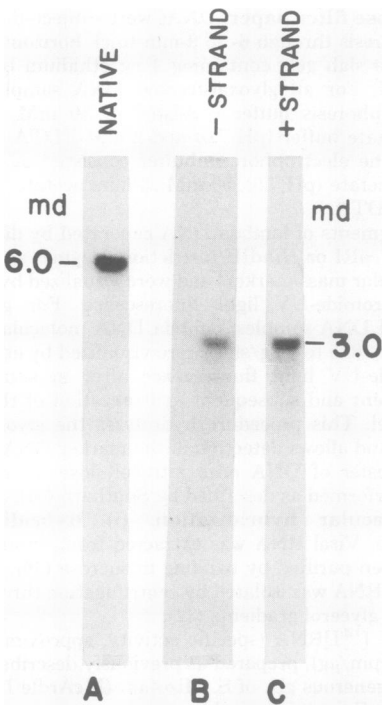


FIG. 1. *− and + strands of freshly prepared samples of unintegrated SNV DNA. Unintegrated viral DNA was prepared 3 days after infection as described in the text. Immediately after preparation, the DNA samples were treated as follows. Portions of the DNAs were either untreated or treated with glyoxal. A portion of each sample was electrophoresed in parallel in 0.7% agarose gels. The DNAs were transferred onto nitrocellulose filters, and virus-specific − or + strand DNAs were detected by hybridization with viral [^{125}I]RNA (lane B) and viral [^{32}P]cDNA (lanes A and C), respectively, followed by autoradiography. Fragments of lambda DNA generated by restriction enzyme digestion were included in each sample as internal molecular mass standards. The indicated molecular masses for lane A and for lanes B and C are double-stranded DNA and single-stranded DNA molecular masses, respectively.*

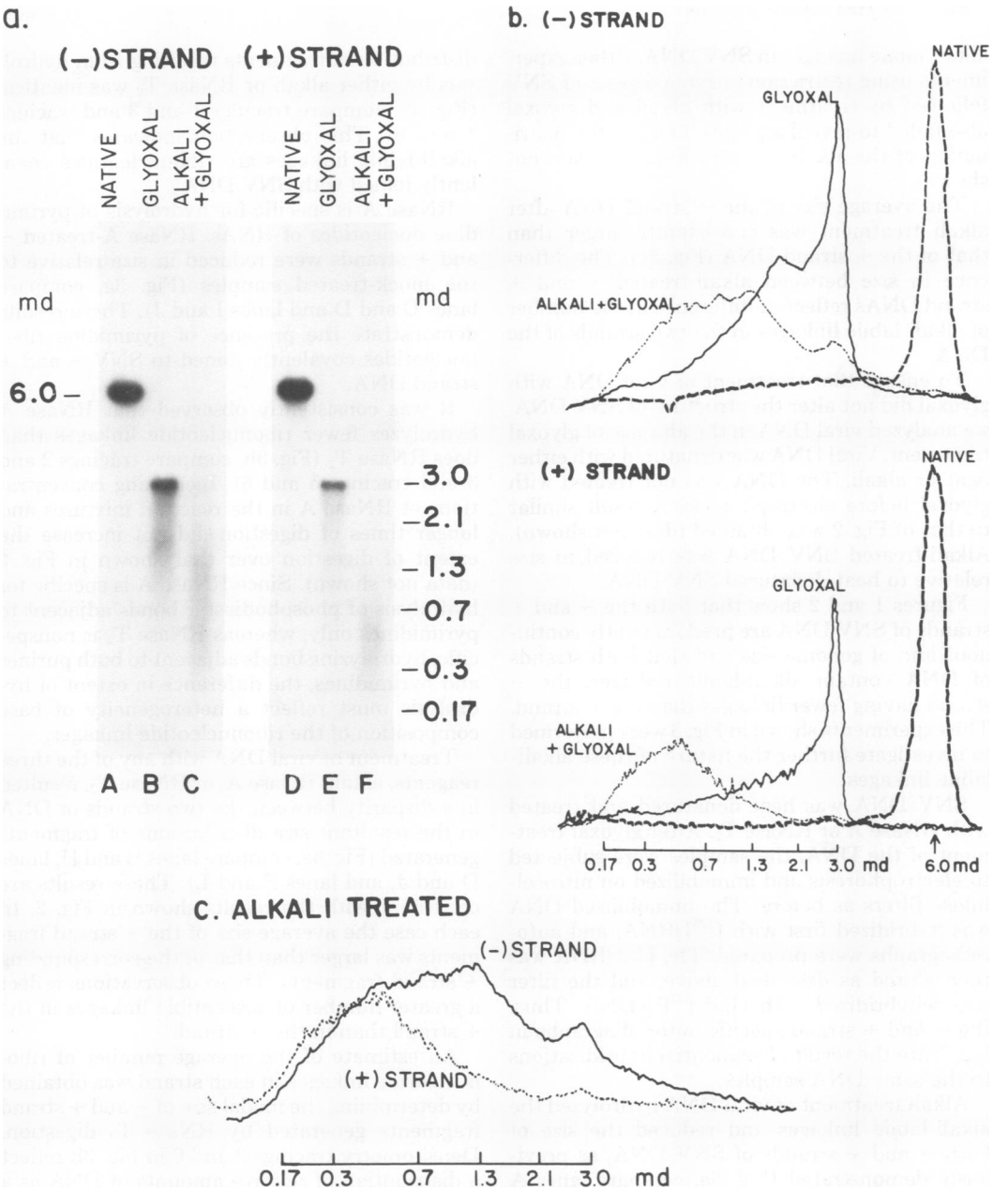


FIG. 2. *Alkali-labile bonds in the − and + strands of unintegrated linear SNV DNA. Unintegrated viral DNA was prepared 3 days after infection as described in the text. Portions of this DNA were untreated (a, lanes A and D); treated with glyoxal (a, lanes B and E); or treated with 0.1 N NaOH for 10 min at 80°C, neutralized, and treated with glyoxal (a, lanes C and F). A portion of each sample was electrophoresed in parallel in a 0.7% agarose gel. Equal amounts of viral DNA were present in lanes A, B, and C and in lanes D, E, F. The virus-specific DNA was detected as in Fig. 1. Fragments of lambda DNA generated by restriction enzyme digestion were included in each sample as internal molecular mass standards. The lambda DNA was not affected by alkali treatment (data not shown). (a) Autoradiographs of viral DNA hybridized with viral [^{125}I]RNA (lanes A, B, and C) and viral [^{32}P]cDNA (lanes D, E, and F). Molecular masses of double-stranded DNAs and single-stranded DNAs are to the left and right of the autoradiographs, respectively. The large amount of − and + strand DNA molecules detected in lanes A and D that are smaller than genome size are a result of hydrolysis of the alkali-labile linkages during preparation of this DNA. − and + strand DNA molecules that are smaller than genome size comprise less than 10% of the total in freshly prepared samples of viral DNA (Fig. 1). (b, top) Densitometry tracing of (a), lanes A to C; (b, bottom) densitometry tracing of (a), lanes D to F; (c) densitometry tracing of (a), lanes C and F.*

alkali-labile linkages in SNV DNA. Other experiments using restriction enzyme digests of SNV followed by treatment with alkali and glyoxal also failed to reveal any specificity in the distribution of the alkali-sensitive linkages (data not shown).

The average size of the − strand DNA after alkali treatment was consistently larger than that of the + strand DNA (Fig. 2c). The difference in size between alkali-treated − and + strand DNAs reflects a difference in the number of alkali-labile linkages in the two strands of the DNA.

To ensure that treatment of viral DNA with glyoxal did not alter the structure of SNV DNA, we analyzed viral DNA in the absence of glyoxal treatment. Viral DNA was denatured with either heat or alkali. The DNA was not treated with glyoxal before electrophoresis. A result similar to that of Fig. 2 was obtained (data not shown). Alkali-treated SNV DNA was reduced in size relative to heat-denatured SNV DNA.

Figures 1 and 2 show that both the − and + strands of SNV DNA are predominantly continuous and of genome size and that both strands of DNA contain alkali-labile linkages, the − strand having fewer linkages than the + strand. The experiments shown in Fig. 3 were performed to investigate further the nature of these alkali-labile linkages.

SNV DNA was heat denatured and treated with RNase A or RNase T_2. After glyoxal treatment of the DNA, the samples were subjected to electrophoresis and immobilized on nitrocellulose filters as before. The immobilized DNA was hybridized first with [^{125}I]RNA, and autoradiographs were prepared. The [^{125}I]RNA was then eluted as described above, and the filter was rehybridized with viral [^{32}P]cDNA. Thus, the − and + strand-specific autoradiographs in Fig. 3 are the result of sequential hybridizations to the same DNA samples.

Alkali treatment of viral DNA hydrolyzed the alkali-labile linkages and reduced the size of both − and + strands of SNV DNA, as previously demonstrated (Fig. 3a, compare lanes A and B and lanes G and H).

RNase T_2 specifically hydrolyzes phosphodiester bonds at ribonucleotides, irrespective of base composition. After digestion of heat-denatured SNV DNA with RNase T_2, it was observed that both the − and + strands of SNV DNA were reduced in size relative to mock-treated samples (Fig. 3a, compare lanes E and F and lanes K and L). These results demonstrate the presence of ribonucleotides that are covalently linked to the − and + strands of SNV DNA.

For both − and + strand DNAs, the size distribution of fragments resulting from hydrolysis by either alkali or RNase T_2 was identical (Fig. 3b, compare tracings 1 and 3 and tracings 4 and 6). This observation indicates that the alkali-labile linkages are ribonucleotides covalently joined with SNV DNA.

RNase A is specific for hydrolysis of pyrimidine nucleotides of RNAs. RNase A-treated − and + strands were reduced in size relative to the mock-treated samples (Fig. 3a, compare lanes C and D and lanes I and J). These results demonstrate the presence of pyrimidine ribonucleotides covalently joined to SNV − and + strand DNA.

It was consistently observed that RNase A hydrolyzes fewer ribonucleotide linkages than does RNase T_2 (Fig. 3b, compare tracings 2 and 3 and tracings 5 and 6). Increasing concentrations of RNase A in the reaction mixtures and longer times of digestion did not increase the extent of digestion over that shown in Fig. 3 (data not shown). Since RNase A is specific for hydrolysis of phosphodiester bonds adjacent to pyrimidines only, whereas RNase T_2 is nonspecific, hydrolyzing bonds adjacent to both purines and pyrimidines, the difference in extent of hydrolysis must reflect a heterogeneity of base composition of the ribonucleotide linkages.

Treatment of viral DNA with any of the three reagents, alkali, RNase A, or RNase T_2, resulted in a disparity between the two strands of DNA in the resultant size distributions of fragments generated (Fig. 3a, compare lanes B and H, lanes D and J, and lanes F and L). These results are consistent with the results shown in Fig. 2. In each case the average size of the − strand fragments was larger than that of the corresponding + strand fragments. These observations reflect a greater number of susceptible linkages in the + strand than in the − strand.

An estimate of the average number of ribonucleotide linkages in each strand was obtained by determining the modal size of − and + strand fragments generated by RNase T_2 digestion. Densitometry tracings 3 and 6 in Fig. 3b reflect a distribution of relative amounts of DNA as a function of molecular mass. A distribution of the relative number of molecules as a function of molecular mass was determined from these densitometry tracings (Fig. 3c). The modal size of − strand fragments was about 0.5 Md, or one-sixth genome size, and the modal size of + strand fragments was about 0.4 Md, or one-eighth genome size. Based upon these numbers, the average number of linkages in the − strand was approximately five, and the average number in the + strand was approximately seven.

NaI density gradients have been used to frac-

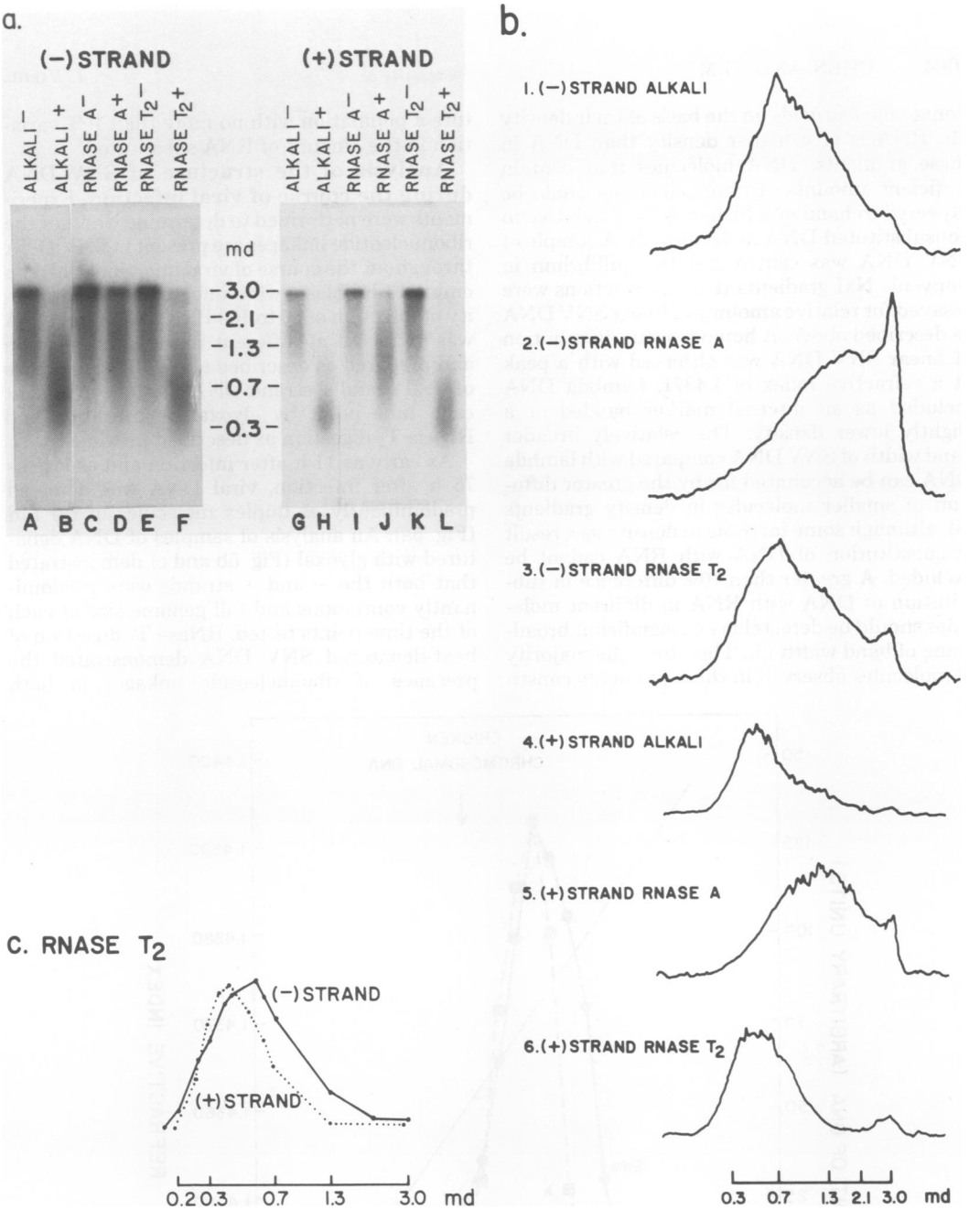


FIG. 3. *RNase-sensitive bonds in the − and + strands of unintegrated linear SNV DNA. Unintegrated viral DNA was prepared 3 days after infection as described in the text. This viral DNA was denatured at 100°C for 5 min. Portions of this denatured DNA were treated in 0.1 M NaCl at 80°C for 10 min (a, lanes A and G), treated with 0.1 N NaOH at 80°C for 10 min followed by neutralization with HCl (a, lanes B and H), mock treated with RNase A (a, lanes C and I), treated with RNase A as described in the text (a, lanes D and J), mock treated with RNase T_2 (a, lanes E and K), and treated with RNase T_2 as described in the text (a, lanes F and L). All samples were treated with glyoxal as described in the text and subjected to electrophoresis in parallel in a 0.7% agarose gel. The DNA was transferred onto nitrocellulose filters, and virus-specific − strand DNA was detected by hybridization with viral [^{125}I]RNA followed by autoradiography. The viral [^{125}I]-RNA was eluted from the filter as described in the text. Virus-specific + strand DNA was then detected by hybridization with viral [^{32}P]cDNA followed by autoradiography. Fragments of lambda DNA generated by restriction enzyme digestion were included in each sample as internal molecular mass standards. The lambda DNA was not affected by the various enzymatic treatments (data not shown). (a) Autoradiographs of filter-immobilized viral DNAs hybridized first with viral [^{125}I]RNA (lanes A to F) and then with viral [^{32}P]cDNA (lanes G to L). (b, tracings 1, 2, 3, 4, 5, and 6) Densitometry tracings of (a), lanes B, D, F, H, J, and L, respectively. (c) Distribution of the relative number of molecules as a function of molecular mass. This distribution was determined by dividing the relative amount of DNA (b, densitometry tracings 3 and 6) by its respective molecular mass.*

tionate nucleic acids on the basis of their density (3). RNA is of a higher density than DNA in these gradients. DNA molecules that contain sufficient amounts of ribonucleotides would be expected to band at a higher density, relative to nonsubstituted DNA molecules (3). A sample of SNV DNA was centrifuged to equilibrium in isopycnic NaI gradients (Fig. 4). Fractions were assayed for relative amounts of linear SNV DNA as described above. A homogeneous distribution of linear SNV DNA was observed with a peak at a refractive index of 1.4371. Lambda DNA included as an internal marker banded at a slightly lower density. The relatively broader band width of SNV DNA compared with lambda DNA can be accounted for by the greater diffusion of smaller molecules in density gradients (6), although some increase in density as a result of substitution of DNA with RNA cannot be excluded. A greater than 10% difference in substitution of DNA with RNA in different molecules should be detectable as a significant broadening of band width (3). Therefore, the majority of molecules observed in these gradients constitute a population with no more than 10% variation in the amount of RNA substituted.

Analysis of the structure of SNV DNA during the course of viral infection. Experiments were performed to determine whether the ribonucleotide linkages are present in SNV DNA throughout the course of viral infection. Chicken embryo fibroblasts were infected at a multiplicity of infection of 50 to 100 PFU/cell. Viral DNA was extracted at different times after infection and prepared as described above. The structure of viral − and + strand DNA was determined at each time point by glyoxal denaturation and RNase T_2 digestion as described previously.

As early as 11 h after infection and as late as 75 h after infection, viral DNA was detected predominantly as duplex molecules of 6.0 Md (Fig. 5a). An analysis of samples of DNA denatured with glyoxal (Fig. 5b and c) demonstrated that both the − and + strands were predominantly continuous and full genome size at each of the time points tested. RNase T_2 digestion of heat-denatured SNV DNA demonstrated the presence of ribonucleotide linkages in both

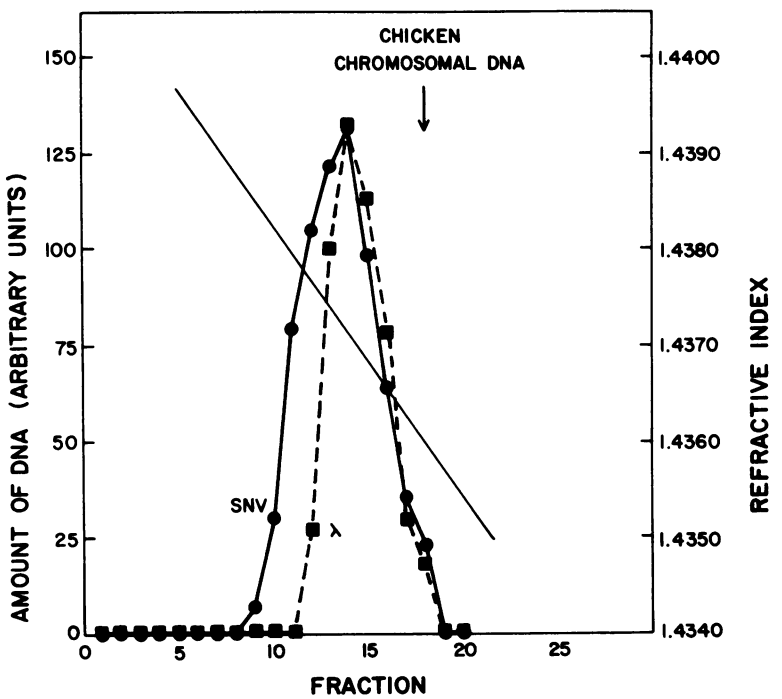


FIG. 4. *Buoyant density of SNV DNA in NaI isopycnic gradients. Unintegrated viral DNA was prepared 3 days after infection as described in the text. About 10^7 cell equivalents of viral DNA and 1 μg of lambda DNA were mixed with a saturated NaI solution. The initial refractive index and the final volume were adjusted to 1.4375 and 5 ml, respectively, by addition of the buffer in which NaI was dissolved. Conditions of centrifugation are as described in the text. Fractions were collected and treated as described in the text. Relative amounts of SNV-specific DNA and lambda DNA in each fraction were determined by hybridization to SNV [^{32}P]cDNA and lambda [^{32}P]DNA, respectively, as described in the text. For comparison, SNV and lambda DNAs were normalized to the same arbitrary amount of DNA in the respective peak fractions.*

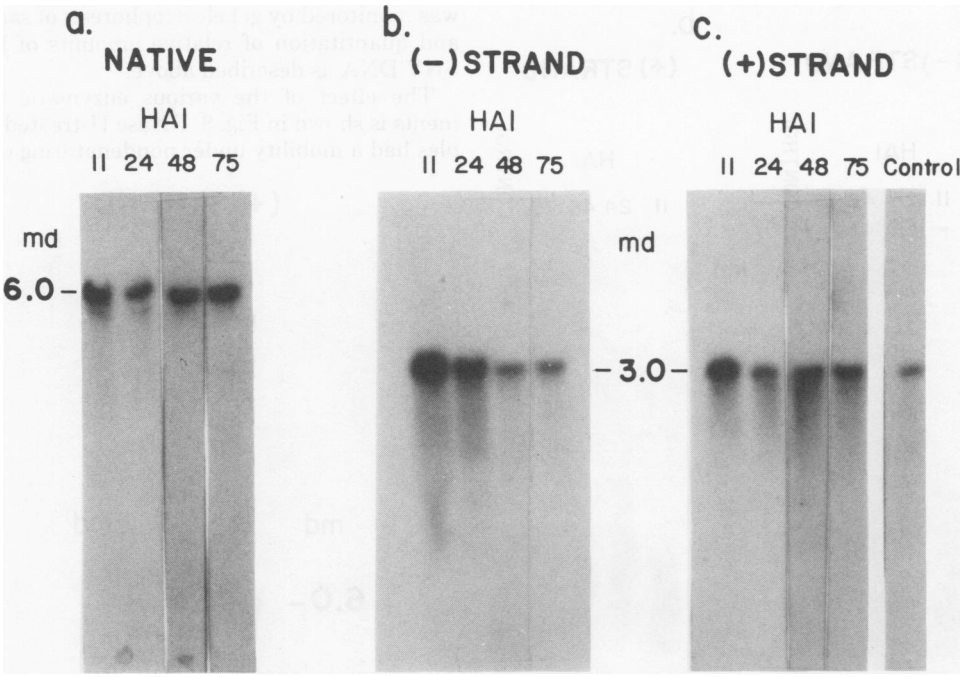


FIG. 5. *Presence of continuous genome size − and + strands of SNV DNA during the course of infection. Chicken embryo fibroblasts were infected with SNV at a multiplicity of infection of 50 to 100 PFU/cell. Unintegrated viral DNA was isolated at the times indicated, as described in the text. The control lane contains a sample of DNA prepared as in Fig. 1 to 4. Portions of DNA from each time point were either untreated or treated with glyoxal and electrophoresed in parallel in a 0.7% agarose gel. The virus-specific DNA was detected as described in the legend to Fig. 1. The amounts of DNA and autoradiograph exposures were adjusted to demonstrate the structure of individual DNA samples. Thus, the autoradiograph intensities do not reflect relative amounts of DNA. Fragments of lambda DNA generated by restriction enzyme digestion were included in each sample as internal molecular mass standards. (a) Autoradiograph of untreated viral DNA hybridized with viral [^{32}P]cDNA. Double-stranded molecular mass is indicated. (b) Autoradiograph of glyoxal-treated DNA hybridized with viral [^{125}I]RNA. Single-stranded molecular mass is indicated. (c) Autoradiograph of glyoxal-treated DNA hybridized with viral [^{32}P]cDNA. Single-stranded molecular mass is indicated. HAI, Hours after infection.*

strands of viral DNA at each time point (Fig. 6).

Small amounts of unintegrated linear viral DNA are present in chicken embryo fibroblasts chronically infected with SNV (20). The structure of this DNA was studied in the same manner as described in the legend to Fig. 1. Figure 7 shows that viral DNA present 30 days after infection contains alkali-labile linkages in the + strand. The large amount of subgenomic sized + strand fragments in the glyoxal-treated, non-alkali-treated sample may have been an artifact arising during preparation of this particular DNA sample. Other preparations of SNV DNA extracted from chronically infected chicken cells contained predominantly continuous genome size + strands (data not shown). The − strand also contained alkali-labile linkages (data not shown).

These results demonstrate that the basic structure of unintegrated SNV DNA as early as 11 h after infection and as late as 30 days after infection is the same.

Infectivity of DNA molecules containing ribonucleotide linkages. Infectious DNA assays have been used as a bioassay for various biochemically characterized forms of SNV DNA (7). The majority of unintegrated linear SNV DNA molecules have been shown here to contain ribonucleotides. A small fraction (<5%) of − and + strand molecules do not contain ribonucleotides. The functional significance of ribonucleotide-containing viral DNA molecules was investigated by determining whether these molecules are infectious.

The experimental approach utilized the specificity of two enzymes, chicken embryo RNase H and S1 nuclease. Chicken embryo RNase H will act endonucleolytically at ribonucleotides in a DNA duplex. Treatment of SNV DNA with this RNase H would digest ribonucleotides in

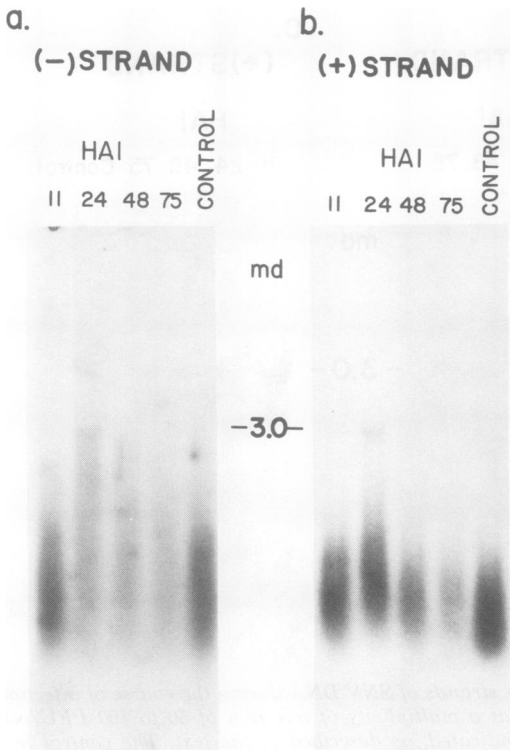


FIG. 6. *Presence of ribonucleotide linkages in − and + strands of SNV DNA during the course of infection. Viral DNAs were prepared at different times after infection as described in the legend to Fig. 5. The control lanes contain samples of DNA prepared as in Fig. 1 to 4. Portions of DNA from each time point were treated with RNase T_2 followed by treatment with glyoxal as described in the text. Duplicate portions of each treated sample were electrophoresed in parallel in a 0.7% agarose gel. The virus-specific − and + strand DNAs were detected as in Fig. 5. Fragments of lambda DNA generated by restriction enzyme digestion were included in each sample as internal molecular mass standards. The lambda DNA was not affected by the enzymatic treatment (data not shown). (a) Autoradiograph of RNase T_2 and subsequent glyoxal-treated DNAs hybridized with viral [^{125}I]RNA. (b) Autoradiograph of RNase T_2 and subsequent glyoxal-treated DNAs hybridized with viral [^{32}P]cDNA. HAI, Hours after infection.*

both strands of the viral DNA, leaving single-stranded nicks or gaps. Further treatment of the viral DNA with the single-stranded-DNA-specific nuclease S1 would result in cleavage of single-stranded regions of DNA generated by treatment with RNase H. If viral DNA molecules containing ribonucleotides are infectious, then treatment of the DNA with RNase H followed by S1 should abolish infectivity of the molecules. The extent of enzymatic digestion was monitored by gel electrophoresis of samples and quantitation of relative amounts of linear SNV DNA as described above.

The effect of the various enzymatic treatments is shown in Fig. 8. RNase H-treated samples had a mobility under nondenaturing condi-

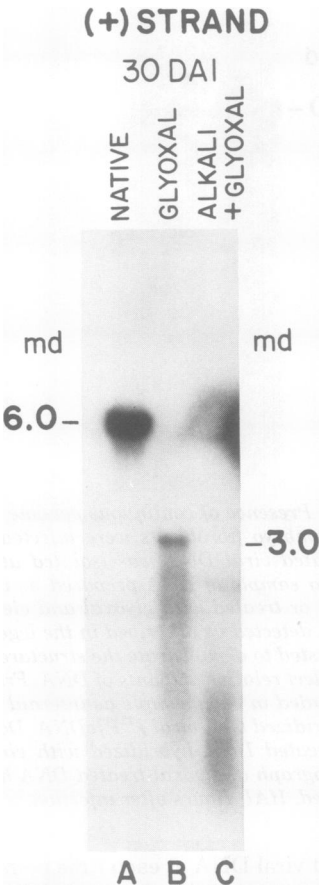


FIG. 7. *Presence of ribonucleotide linkages in + strands of SNV DNA isolated from chronically infected chicken cells. Unintegrated viral DNA was prepared 30 days after infection (DAI) as described in the text. Portions of this DNA were untreated (lane A), treated with glyoxal as described in the text (lane B), and treated with 0.1 N NaOH for 10 min at 80°C, neutralized, and treated with glyoxal (lane C). A portion of each sample was electrophoresed in parallel in a 0.7% agarose gel. The virus-specific + strand DNA was detected as in Fig. 1. Fragments of lambda DNA generated by restriction enzyme digestion were included in each sample as internal molecular mass standards. The lambda DNA was unaffected by the alkali treatment (data not shown). Molecular masses of double-stranded DNAs and single-stranded DNAs are to the left and right of the autoradiographs, respectively. The exposed region in lane C at the double-stranded molecular mass of 6.0 Md is from an overexposure of an adjacent lane.*

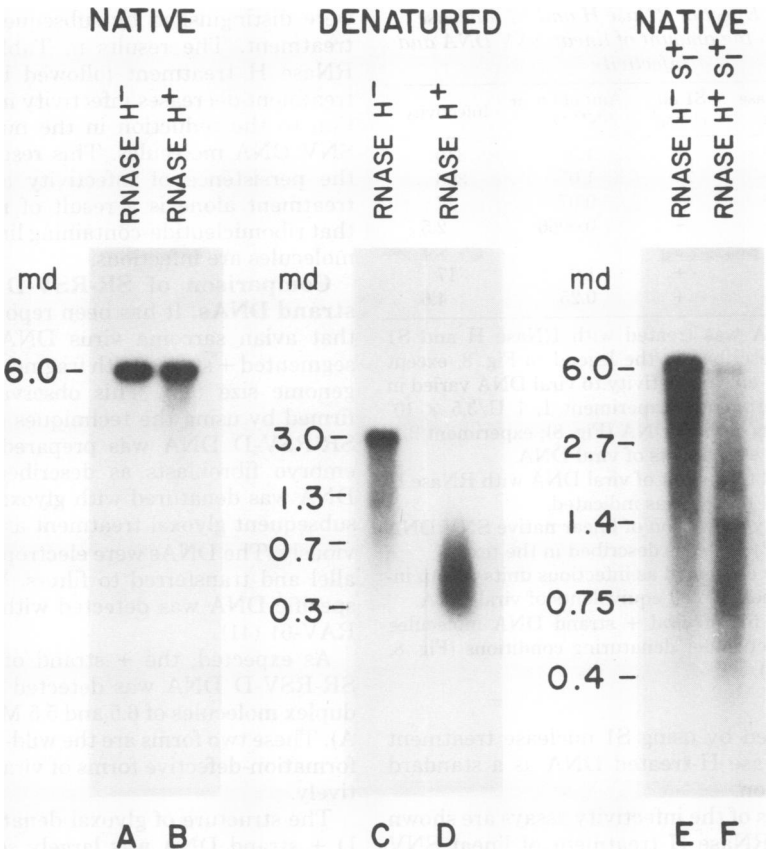


FIG. 8. *Treatment of viral DNA with RNase H and S1 nuclease. Unintegrated viral DNA was prepared 3 days after infection as described in the text. Portions of this viral DNA were either mock treated with RNase H (lanes A and C) or treated with RNase H as described in the text (lanes B and D). The reaction mixtures were then extracted twice with phenol and precipitated with ethanol. The precipitates were suspended in water. A portion of each reaction was subsequently treated with S1 nuclease as described in the text at a ratio of enzyme activity to viral DNA of 1 U/3.5 × 10^5 cell equivalents of SNV DNA (lanes E and F). Samples of each of the four reactions were treated as indicated (native or denatured by glyoxal) and subjected to electrophoresis in 0.7% agarose gels. The DNA was transferred onto nitrocellulose filters, and virus-specific DNA was detected by hybridization with viral [^{32}P]cDNA followed by autoradiography. Lanes A and B, C and D, and E and F reflect relative amounts of hybridization; otherwise, autoradiograph exposures were adjusted to demonstrate the structure of individual DNA samples. Fragments of lambda DNA generated by restriction enzyme digestion were included in each sample to monitor the effects of the various enzymatic treatments. The lambda DNA was unaffected by the RNase H treatment (data not shown).*

tions identical to that of mock RNase H-treated samples (compare lanes A and B). However, denaturation of the RNase H-treated samples with glyoxal demonstrated the hydrolysis of the ribonucleotide linkages (compare lanes C and D). The extent of this hydrolysis was the same as that caused by alkali or RNase T_2 (data not shown).

Optimal conditions were determined for maximum S1 nuclease digestion of RNase H-treated samples relative to S1 digestion of mock RNase H-treated samples. At high ionic strength (300 mM NaCl), less than 5% of RNase H-treated linear SNV DNA molecules were susceptible to S1 nuclease (data not shown). A lower ionic strength (75 mM NaCl) was sufficient to allow S1 nuclease to degrade approximately 50% of the molecules (data not shown). An ionic strength of 30 mM NaCl was sufficient to allow S1 nuclease to digest 75 to 90% of RNase H-treated SNV DNA, relative to S1 nuclease digestion of mock RNase H-treated DNA samples (Fig. 8, compare lanes E and F; Table 1). The latter ionic strength was chosen to ensure sufficient digestion of SNV DNA for infectious DNA assays. Nonspecific degradation by S1 nuclease

TABLE 1. *Effect of RNase H and S1 nuclease treatment on the amount of linear SNV DNA and infectivity*

Expt[a]	RNase H[b]	S1 nuclease[b]	Amt of linear SNV DNA[c]	Infectivity[d]
1	−	−	1.0	340
	+	−	1.0[e]	308
	−	+	0.07	27
	+	+	0.0066	2.5
2	−	+	1.0	17
	+	+	0.25	4.6

[a] Viral DNA was treated with RNase H and S1 nuclease as described in the legend to Fig. 8, except the ratio of S1 enzyme activity to viral DNA varied in different experiments. Experiment 1, 1 U/3.5 × 10^5 cell equivalents of viral DNA (Fig. 8); experiment 2, 1 U/6 × 10^5 cell equivalents of viral DNA.

[b] Sequential treatment of viral DNA with RNase H followed by S1 nuclease as indicated.

[c] Relative hybridization of linear native SNV DNA with SNV [^{32}P]cDNA as described in the text.

[d] Infectivity expressed as infectious units (mean infective dose) per 10^6 cell equivalents of viral DNA.

[e] Less than 5% of viral + strand DNA molecules remained intact under denaturing conditions (Fig. 8, lanes C and D).

was controlled by using S1 nuclease treatment of mock RNase H-treated DNA as a standard for comparison.

The results of the infectivity assays are shown in Table 1. RNase H treatment of linear SNV DNA did not significantly decrease the infectivity of SNV DNA, although more than 95% of the molecules had one or more ribonucleotide linkages hydrolyzed (Fig. 8, compare lanes C and D). DNA molecules which have single-stranded discontinuities in the − strand would not be expected to serve as a template for transcription of viral RNA and would consequently not be infectious. The observation that RNase H treatment does not reduce infectivity implies either that molecules containing ribonucleotides are not infectious and never expressed or that the transfected cells are capable of repairing the segmented molecules. These two possibilities were distinguished by subsequent S1 nuclease treatment. The results in Table 1 show that RNase H treatment followed by S1 nuclease treatment decreases infectivity in direct proportion to the reduction in the number of linear SNV DNA molecules. This result implies that the persistence of infectivity after RNase H treatment alone is a result of repair (34) and that ribonucleotide-containing linear SNV DNA molecules are infectious.

Comparison of SR-RSV-D and SNV + strand DNAs. It has been reported previously that avian sarcoma virus DNA consists of a segmented + strand with fragments smaller than genome size (38). This observation was confirmed by using the techniques described here. SR-RSV-D DNA was prepared from chicken embryo fibroblasts as described above. Viral DNA was denatured with glyoxal or alkali and subsequent glyoxal treatment as described previously. The DNAs were electrophoresed in parallel and transferred to filters. Viral + strand-specific DNA was detected with [^{32}P]cDNA to RAV-61 (41).

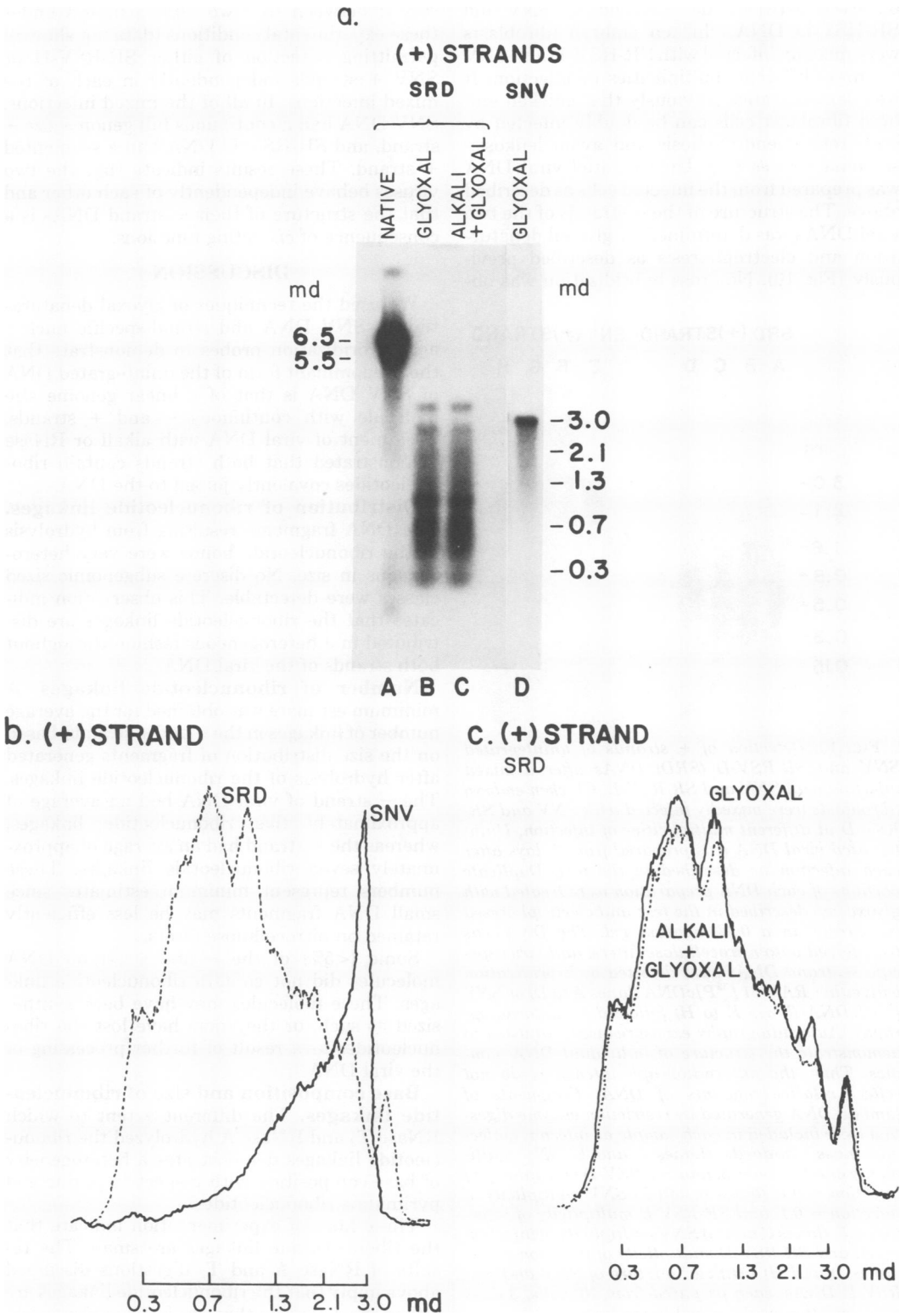
As expected, the + strand of nondenatured SR-RSV-D DNA was detected in the form of duplex molecules of 6.5 and 5.5 Md (Fig. 9a, lane A). These two forms are the wild-type and transformation-defective forms of viral DNA, respectively.

The structure of glyoxal-denatured SR-RSV-D + strand DNA was largely segmented (Fig. 9a, lane B), consistent with previous results. Previously unreported, discrete, subgenomic sized fragments were evident. For comparison, a sample of glyoxal-treated SNV + strand DNA is shown (Fig. 9a, lane D). The densitometry tracing demonstrates the difference in structure of SR-RSV-D and SNV + strand DNA (Fig. 9b).

Alkali treatment of SR-RSV-D DNA did not significantly reduce the size of SR-RSV-D + strand fragments (Fig. 9c). This lack of alkali-sensitive linkages contrasted sharply with the results obtained for SNV + strand DNA (Fig. 2 and 3).

To study further the difference in structure

FIG. 9. *Structure of the + strand of unintegrated SR-RSV-D (SRD) DNA. Unintegrated viral DNA was prepared 3 days after infection as described in the text. Portions of this DNA were untreated (a, lane A); treated with glyoxal as described in the text (a, lane B); and treated with 0.1 N NaOH at 80°C for 10 min, neutralized with HCl, and treated with glyoxal (a, lane C). For comparison, a sample of SNV DNA was treated with glyoxal as described in the text (a, lane D). All samples were electrophoresed in parallel in a 0.7% agarose gel. The DNA was transferred onto nitrocellulose filters, and virus-specific + strand DNA was detected by hybridization with either RAV-61 [^{32}P]cDNA or SNV [^{32}P]cDNA followed by autoradiography. Fragments of lambda DNA generated by restriction enzyme digestion were included in each sample as internal molecular mass standards. (a) Autoradiographs of SR-RSV-D DNA and SNV DNA hybridized with RAV-61 [^{32}P]cDNA (lanes A, B, and C) or SNV [^{32}P]cDNA (lane D), respectively. Molecular masses of double-stranded DNAs and single-stranded DNAs are to the left and right of the autoradiographs, respectively. (b) Densitometry tracing of (a), lanes B and D. (c) Densitometry tracing of panel (a), lanes B and C.*


a.
(+) STRANDS
SRD
SNV
NATIVE
GLYOXAL
ALKALI + GLYOXAL
GLYOXAL
md
6.5
5.5
md
3.0
2.1
1.3
0.7
0.3
A B C D
b. (+) STRAND
SRD
SNV
0.3 0.7 1.3 2.1 3.0 md
c. (+) STRAND
SRD
GLYOXAL
ALKALI + GLYOXAL
0.3 0.7 1.3 2.1 3.0 md

observed between the + strands of SNV and SR-RSV-D DNA, chicken embryo fibroblasts were mixedly infected with SR-RSV-D and SNV at three different multiplicities of infection. It was demonstrated previously that chicken embryo fibroblast cells can be doubly infected by both reticuloendotheliosis and avian leukosis-sarcoma viruses (37). Unintegrated viral DNA was prepared from the infected cells as described above. The structure of the + strands of the two viral DNAs was determined by glyoxal denaturation and electrophoresis as described previously (Fig. 10). No cross hybridization was observed between the two viral genomes under these experimental conditions (data not shown), permitting detection of either SR-RSV-D or SNV + strands independently in each of the mixed infections. In all of the mixed infections, SNV DNA had a continuous full genome size + strand, and SR-RSV-D DNA had a segmented + strand. These results indicate that the two viruses behave independently of each other and that the structure of their + strand DNAs is a consequence of *cis*-acting functions.

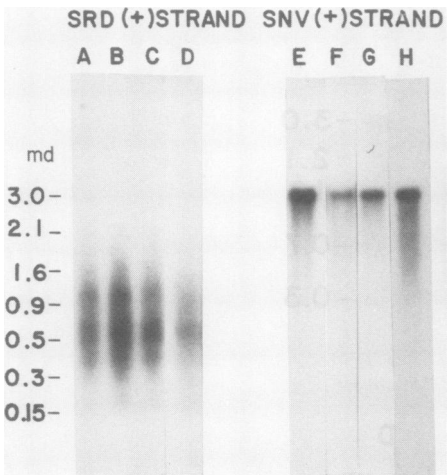


FIG. 10. *Detection of + strands of unintegrated SNV and SR-RSV-D (SRD) DNAs after a mixed infection with SNV and SR-RSV-D. Chicken embryo fibroblasts were mixedly infected with SNV and SR-RSV-D at different multiplicities of infection. Unintegrated viral DNA was prepared from 3 days after each infection as described in the text. Duplicate portions of each DNA preparation were treated with glyoxal as described in the text and electrophoresed in parallel in a 0.7% agarose gel. The DNA was transferred onto nitrocellulose filters, and virus-specific + strand DNAs were detected by hybridization with either RAV-61 [^{32}P]cDNA (lanes A to D) or SNV [^{32}P]cDNA (lanes E to H) followed by autoradiography. Autoradiography exposures were adjusted to demonstrate the structure of individual DNA samples. Thus, the autoradiograph intensities do not reflect relative amounts of DNA. Fragments of lambda DNA generated by restriction enzyme digestion were included in each sample as internal molecular mass standards. (Lanes A and E) SNV multiplicity of infection = 5, and SR-RSV-D multiplicity of infection = 0.1; (lanes B and F) SNV multiplicity of infection = 0.1, and SR-RSV-D multiplicity of infection = 5; (lanes C and G) SNV multiplicity of infection = 0.1, and SR-RSV-D multiplicity of infection = 0.1; (lanes D and H) artificial mixture of SNV and SR-RSV-D DNAs, each prepared from 10^6 cells, 3 days after infection as described in the text.*

DISCUSSION

We used the techniques of glyoxal denaturation of SNV DNA and strand-specific nucleic acid hybridization probes to demonstrate that the predominant form of the unintegrated DNA of SNV DNA is that of a linear genome size molecule with continuous − and + strands. Treatment of viral DNA with alkali or RNase demonstrated that both strands contain ribonucleotides covalently joined to the DNA.

Distribution of ribonucleotide linkages. The DNA fragments resulting from hydrolysis of the ribonucleotide bonds were very heterogeneous in size. No discrete subgenomic sized classes were detectable. This observation indicates that the ribonucleotide linkages are distributed in a heterogeneous fashion throughout both strands of the viral DNA.

Number of ribonucleotide linkages. A minimum estimate was obtained for the average number of linkages in the − and + strands, based on the size distribution of fragments generated after hydrolysis of the ribonucleotide linkages. The − strand of viral DNA had an average of approximately five ribonucleotide linkages, whereas the + strand had an average of approximately seven ribonucleotide linkages. These numbers represent minimum estimates since small DNA fragments may be less efficiently retained on nitrocellulose filters.

Some (<5%) of the − and + strand DNA molecules did not contain ribonucleotide linkages. These molecules may have been synthesized as such, or they may have lost the ribonucleotides as a result of further processing of the viral DNA.

Base composition and size of ribonucleotide linkages. The different extent to which RNase T_2 and RNase A hydrolyzed the ribonucleotide linkages demonstrates a heterogeneity of base composition with respect to purine and pyrimidine ribonucleotides.

Three lines of experimentation indicate that the ribonucleotide linkages are small. The results of RNase A and T_2 digestions discussed above imply that the ribonucleotide linkages are either short (one to three bases) or purine rich.

During the course of determining proper conditions of S1 nuclease digestion for the DNA transfection studies, it was found that a low ionic strength (30 mM NaCl) was required for digestion of 75 to 95% of the RNase H-treated linear SNV DNA molecules, relative to mock RNase H-treated DNA. This result indicates that most of the single-stranded regions resulting from RNase H digestion and, consequently, the ribonucleotide linkages themselves, are small.

The distribution of viral DNA in NaI density gradients indicates that the majority of viral DNA was of a homogeneous density. The absolute amount of ribonucleotides in viral DNA could not be determined from these experiments, since it was not determined whether the observed density of viral DNA was due to guanine-plus-cytosine content or ribonucleotide content. However, since five to seven short ribonucleotide linkages were present in each strand of viral DNA, the density of the viral DNA was most probably the result of guanine-plus-cytosine content only. Therefore, no viral DNA molecules containing a sufficient amount of ribonucleotides to increase the density significantly were detected.

Time course of infection. Ribonucleotide linkages were present in both viral DNA strands as early as 11 h after infection and as late as 30 days after infection. Furthermore, unpublished data show that ribonucleotides can be detected in incomplete + strand DNAs as early as 4 h after infection. These results indicate that the linked ribonucleotides are generated during viral DNA synthesis and are not the result of later additions or processing events.

Structure of avian sarcoma viral DNA. The structure of avian sarcoma viral DNA was characterized previously. The − strand is continuous and full genome size, whereas the + strand is segmented (38). We have confirmed the presence of a segmented + strand for SR-RSV-D DNA. Unlike SNV DNA, no alkali-labile linkages were detected.

The structure of SR-RSV-D + strand DNA observed here differed from previous observations in several ways. The SR-RSV-D + strand fragments observed here were considerably larger than previously reported. In one preparation, full genome size + strands were evident (Fig. 9). Also, discrete size classes of + strands smaller than genome size were observed. Other than a discrete 250- to 300-nucleotide + strand fragment, the size distribution of + strand fragments has been reported to be heterogeneous (38). The observed differences may reflect the different host cells used, the different times after infection at which viral DNAs were isolated for analysis, or the relatively higher resolution of the technique used here.

The results of the mixed infections with SNV and SR-RSV-D demonstrate that the structures of SNV and SR-RSV-D DNAs are the result of *cis*-acting functions. Since it is likely that several rounds of infection occurred in this mixed infection (41), the *cis*-acting functions may be the RNA-dependent DNA polymerase.

Possible significance of ribonucleotide linkages. Demonstration that the ribonucleotide-containing forms of DNA are infectious indicates that these molecules have the potential for viral expression and are not dead-end products of viral infection. Unfortunately, further inference about their in vivo role from these experiments is not possible until the mechanism of retroviral DNA transfection is understood.

Ribonucleotides that are covalently linked to DNA have been detected in several procaryotic and eucaryotic systems (1, 28, 32). In the cases of chloramphenicol-amplified ColE1 plasmid DNA, mitochondrial DNA, and cauliflower mosaic viral DNA, ribonucleotides have been demonstrated to be linked to DNA internally (11, 18; D. G. Blair, D. J. Sherratt, D. B. Clewell, and D. R. Helinski, Fed. Proc. **31**:442, 1972). SNV DNA constitutes another example of this class of linkage. It is generally hypothesized that ribonucleotide sequences covalently joined to DNA served as primers for the initiation of DNA synthesis.

Recent in vitro work has demonstrated a requirement for avian myeloblastosis virus RNase H activity during the synthesis of the "anti-complementary" (+) strand by avian myeloblastosis virus reverse transcriptase (24). This requirement is consistent with the hypothesis that fragments of viral RNA serve as primers for synthesis of the + strand DNA fragments observed in vivo and in vitro. Since SNV possesses a genome and provirus structure similar to those of other retroviruses, it is reasonable to expect basic similarities in the mechanism of viral DNA replication. If this is true, then the ribonucleotides detected in SNV + strand DNA may be remnants of primers utilized during DNA synthesis.

If the ribonucleotides in the − strand of SNV DNA are also remnants of primers, then their presence implies discontinuous synthesis of the − strand. Discontinuous synthesis has not been demonstrated for the − strand of avian sarcoma viral DNA. However, the possibility of RNA primers in addition to $tRNA^{Trp}$ (14) has not been excluded. Alternatively, the SNV RNA-dependent DNA polymerase may have other primer requirements. The RNA polymerase activity detected in reticuloendotheliosis virus virions (26) may be responsible for the generation of these primers.

An important corollary of the RNA primer hypothesis is that an RNA-DNA ligase is associated with the replicative machinery of SNV to generate the final, covalently joined structure observed.

The other possibility is that the ribonucleotides are present as a result of misincorporation of ribonucleotides instead of deoxyribonucleotides by SNV reverse transcription (25). In this case, the different number of ribonucleotide linkages between the − and + strands may reflect the fact that the templates for synthesis differ. The − strand is synthesized from an RNA template, whereas the + strand is synthesized from a DNA template.

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