

Infectious Rous Sarcoma Virus and Reticuloendotheliosis Virus DNAs

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An efficient and quantitative assay for infectious Rous sarcoma virus and reticuloendotheliosis virus DNAs is described. The specific infectivities of viral DNA corresponded to one infectious unit per 10^5 to 10^6 viral DNA molecules. Infection with viral DNA followed one-hit kinetics. The minimal size of infectious Rous sarcoma virus DNA was approximately 6 million daltons, whereas the minimal size of infectious reticuloendotheliosis virus DNA was larger, 10 to 20 million daltons.

The hypothesis of a DNA intermediate, the DNA provirus, in the replication of Rous sarcoma virus (RSV) has been directly confirmed by the demonstration of infectious RSV DNA from RSV-transformed cells. The original observation of infectious RSV DNA from RSV-transformed nonvirus-producing rat cells (10, 11) has been confirmed and extended to RSV- and avian myeloblastosis virus-transformed virus-producing chicken cells (18, 23, 38), and to murine sarcoma virus-transformed mouse and hamster cells (17). The progeny virus produced after transformation of chicken cells with infectious RSV DNA had the subgroup antigenicity of the parental virus (11, 12, 15, 23), and temperature-sensitive progeny were obtained when the infectious RSV DNA was extracted from cells transformed with a temperature-sensitive mutant of RSV (12).

In this paper, we describe a more efficient and quantitative assay for infectious RSV DNA. In addition, we extend the observation of infectious DNA to the reticuloendotheliosis viruses (REV), a recently described group of avian RNA viruses (30) with a virion DNA polymerase (16, 21, 29). The quantitative assay of infectious DNA was used to study the kinetics of infection with RSV and REV DNAs and to determine the minimum size of RSV and REV DNAs required for infectivity.

A preliminary report of this work was presented at the Cold Spring Harbor Symposium on Quantitative Biology, June 1974.

MATERIALS AND METHODS

Cells and viruses. Cells were grown by standard techniques in modified Eagle minimum essential medium containing 20% tryptose phosphate broth (ET medium) and supplemented with calf and fetal

bovine serum. Fertile chicken eggs were purchased from Spafas, Norwich, Conn. Chicken embryo fibroblasts were C/E, avian leukosis virus negative, avian leukosis virus group specific antigen negative, and chick helper factor negative. Ringneck pheasant eggs were obtained from the Poynette Game Farm, State of Wisconsin Department of Natural Resources. Fertile Muscovy duck eggs were obtained from a local farmer. Rat cells transformed by B77 virus [R(B77V)cells] were previously described (2). REV-T-spleen cells were cultured from the enlarged spleen of a chicken infected with reticuloendotheliosis virus (strain T) (H. M. Temin and V. K. Kassner, manuscript in preparation).

Trager duck spleen necrosis virus (TDSNV) and Schmidt-Ruppin RSV subgroup D (SR-RSV-D) were grown as previously described (21). RSV was assayed by focus formation. TDSNV and REV-T were assayed by their cytopathic effect on chicken or duck cells (40).

Extraction of infectious DNA. The procedure used was a modification of that described by Marmor (20). Cells were washed twice with phosphate-buffered saline and were suspended in SSC (0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) at a concentration of approximately 2×10^7 cells/ml. Sodium dodecyl sulfate was added to a final concentration of 0.5%, and Pronase (self-digested for 2 h at 37 C) was added to a final concentration of 250 μ g/ml. The lysate was incubated for 30 min at 37 C, cooled, and extracted three times with an equal volume of chloroform-isoamyl alcohol (24:1). Two volumes of 95% ethanol were layered on top of the final aqueous phase, and the DNA was collected by winding on a glass rod. The DNA was dissolved in $0.1 \times$ SSC at a concentration of approximately 200 μ g/ml, and 0.1 volume of $10 \times$ SSC was added. RNase A (heated for 10 min at 100 C) was added to a concentration of 100 μ g/ml, and the extract was incubated for 30 min at 37 C. Pronase (250 μ g/ml) was added, and the extract was incubated an additional 30 min at 37 C, cooled, and extracted four to six times with chloroform-isoamyl alcohol (24:1) until no denatured protein was visible. Two volumes of 95% ethanol were added, and

the DNA was collected by winding on a glass rod. The DNA was sterilized in 70% ethanol overnight at 4 C, dissolved in sterile $0.1 \times$ SSC, and brought to a final concentration of SSC by the addition of 0.1 volume of sterile $10 \times$ SSC. The DNA concentration was determined by absorbance at 260 nm (A_{260}). The $A_{260}:A_{280}$ ratio of the DNA preparations was 1.8 to 1.9. DNA was stored at -70 C for up to 3 months without loss of infectivity.

[3 H]labeled DNA was extracted from infected cells which had been incubated for 24 h in ET medium with 2% fetal bovine serum containing $2.5 \mu\text{Ci/ml}$ of [3 H]thymidine (17 Ci/mmol). The specific activity of [3 H]DNA preparations was approximately $5,000$ counts per min per μg of DNA.

DNA extracted from cultures in which 100% of the cells were virus-infected is referred to as virus DNA.

Assay of infectious DNA. Tertiary or later passages of chicken embryo fibroblasts were plated in 5 ml of ET medium at 6×10^6 cells per 60-mm dish. Four hours after plating, fetal bovine serum (4%) was added. Twenty-four hours later the medium was removed, and the cells were washed with 3 ml of Tris-sodium chloride (0.05 M Tris-hydrochloride- 0.14 M NaCl, pH 7.4). Two milliliters of DEAE-dextran ($50 \mu\text{g/ml}$) in Tris-sodium chloride was added, and the cells were incubated for 30 min at 37 C. The DEAE-dextran was removed, 0.4 ml of DNA in Tris-sodium chloride was added, and the cells were incubated for 45 min at 37 C. The DNA was removed, and the cells were washed once with ET medium supplemented with 3% fetal bovine serum and were incubated in 5 ml of the same medium at 37 C. Two days later the medium was changed to ET medium containing 2.5% fetal bovine serum, and subsequently the medium was changed at 2-day intervals to ET medium containing 2% fetal bovine serum.

Foci of transformed cells appeared on the original plates 6 to 12 days after treatment with RSV DNA. Cultures which did not show transformation on the original plate after treatment with RSV DNA were further tested for RSV infection either by transferring the cells or by assaying a sample of the culture fluid on chicken cells. Cultures which had been treated with REV DNA were tested for infection with REV 8 to 12 days after treatment with DNA by assaying samples of the culture fluid on chicken or duck cells and looking for typical REV-induced cytopathic effect (40).

The assay of infectious virus DNA was made quantitative by end-point dilution of the DNA. Usually quadruplicate recipient cultures were treated with serial twofold dilutions of the donor DNA preparations. ID_{50} values were determined by the method of Reed and Muench (31).

Treatment with more than $10 \mu\text{g}$ of DNA was toxic to recipient chicken cells. In addition, cells of some chicken embryos were extremely sensitive to treatment with DNA. These embryos were not used as recipient cells in further experiments.

Sedimentation analysis of DNA. DNA was centrifuged to equilibrium in CsCl solutions (starting density 1.71 g/cm^3 in SSC) at 28,000 rpm for 65 h in a Beckman SW 50.1 rotor. The fractions containing

DNA were identified by A_{260} . The DNA was precipitated with 95% ethanol and sterilized in 70% ethanol before analysis of its infectivity.

Velocity sedimentation of [3 H]labeled DNA was on 5 to 20% linear sucrose gradients in Tris-sodium chloride. Gradients were centrifuged at 45,000 rpm in a Beckman SW 50.1 rotor. Molecular weights were estimated relative to the sedimentation velocity of [3 H]labeled λ DNA (36).

Electrophoresis of DNA. Electrophoresis of [3 H]labeled DNA was performed in 0.7% agarose gels containing ethidium bromide as described by Sharp et al. (34). Unlabeled $\lambda\text{cb}2$ DNA and $\lambda\text{cb}2$ DNA fragments prepared by digestion with *Escherichia coli* R1 restriction endonuclease were incorporated into each gel as internal markers. The positions of the marker DNA bands were determined by fluorescence, and the gels were cut into fractions and counted to determine the distribution of radioactivity. The molecular weights of the $\lambda\text{cb}2$ DNA fragments were taken from Allet et al. (1).

Chemicals. DEAE-dextran, average molecular weight 5×10^5 , was purchased from Pharmacia. Pronase B was purchased from Calbiochem. RNase A and DNase I (electrophoretically pure) were purchased from Worthington Biochemical Corp. S1 nuclease was purified by the method of Sutton (37) and assayed as described previously (16). [3 H]labeled M13 DNA and RNA-DNA hybrids, prepared by annealing [125 I]labeled TDSNV RNA to TDSNV-infected chicken cell DNA (16a) were provided by C.-Y. Kang. $\lambda\text{cb}2$ DNA was kindly donated by W. Szybalski. *E. coli* R1 restriction endonuclease, purified by the method of Mulder and Delius (24), was a generous gift of B. Weisblum.

RESULTS

Assay of infectious viral DNAs. The results of representative experiments in which quadruplicate cultures of chicken embryo fibroblasts were treated with twofold serial dilutions of DNA extracted from chicken cells infected with either SR-RSV-D or TDSNV are presented in Fig. 1. The ID_{50} values in these experiments were $0.12 \mu\text{g}$ of SR-RSV-D DNA and $0.08 \mu\text{g}$ of TDSNV DNA. The infectious DNA assay was highly reproducible; ID_{50} values for a DNA preparation agreed within a factor of two in several independent assays.

Treatment of the recipient cells with DEAE-dextran was required for efficient assay of infectious viral DNAs. Omission of the DEAE-dextran pretreatment reduced the infectivity of SR-RSV-D and TDSNV DNAs to ID_{50} values greater than $5 \mu\text{g}$ of DNA.

All cultures which were transformed by DNA extracted from cells infected with SR-RSV-D produced infectious RSV. The progeny RSV was infectious for chicken cells (C/E), but not for ringneck pheasant cells (RPh/BD) (6), consist-

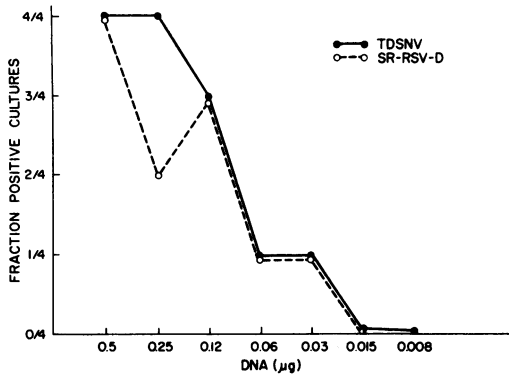


FIG. 1. Assay of RSV and REV DNAs. Quadruplicate cultures of chicken embryo fibroblasts were treated with twofold serial dilutions of DNA extracted from chicken cells infected either with SR-RSV-D (○) or with TDSNV (●).

ent with the progeny RSV being subgroup D. The morphology of cells transformed by the progeny RSV was similar to the morphology of cells transformed by the parental virus. The cytopathic effect of the progeny TDSNV on chicken and duck cells was also similar to that of the parental TDSNV.

No transformation or production of cytopathic or transforming virus was observed after treatment of recipient chicken cells with up to 15 µg of DNA extracted from uninfected chicken cells. Furthermore, no transformation was observed in cultures treated with REV DNA, and no production of cytopathic virus was observed in cultures treated with RSV DNA.

Similar levels of infectivity were observed for DNA extracted from chicken cells in the chronic and acute stages (40; H. M. Temin and V. K. Kassner, manuscript in preparation) of infection with TDSNV, from pheasant cells in the chronic stage of infection with TDSNV, and from chicken spleen cells transformed by REV-T (Table 1). The infectivity of DNA from RSV-transformed non-virus-producing rat cells [R(B77V cells)] was also similar to that of DNA from SR-RSV-D-transformed virus-producing chicken cells (Table 1).

Pheasant and duck cells were also sensitive to infection with DNA extracted from SR-RSV-D- and TDSNV-infected chicken cells (Table 2). Duck cells were almost as sensitive to infection with viral DNA as chicken cells, whereas pheasant cells were approximately 10-fold less sensitive to infection than chicken cells. Pheasant cells were also less sensitive than chicken cells to infection with DNA extracted from TDSNV-infected pheasant cells (data not shown).

The infectivity of DNA extracted from either SR-RSV-D- or TDSNV-infected chicken cells was completely inactivated by treatment with DNase, but was not affected by treatment with RNase in 0.1 × SSC (Table 3). More than 99% of the RNA in an RNA-DNA hybrid was hydrolyzed to acid-soluble material by an identical RNase treatment (data not shown). Denaturation of the DNA with alkali reduced its infectivity approximately 10-fold (Table 3). There was no significant difference in the infectivity of DNA preparations treated with 0.1 N NaOH for 1 min at 25 C or for 30 min at 37 C. [³H]labeled DNA preparations denatured under either condition were as sensitive to hydrolysis by S1

TABLE 1. Infectivity of virus DNAs extracted from different donor cells^a

Donor DNA	ID ₅₀ (µg of DNA)
Ch(TDSNV) 3 DAI ^b	0.1
Ch(TDSNV) 14 DAI	0.1
Ph(TDSNV)	0.1
Ch spleen (REV-T)	0.2
Ch(SR-RSV-D)	0.1
R(B77V)	0.1

^a DNA was extracted from TDSNV-infected chicken cells 3 days after infection (acute infection) and fourteen days after infection (chronic infection) (40; Temin and Kassner, manuscript in preparation). DNA was extracted from TDSNV-infected pheasant cells 14 days after infection. Chicken spleen (REV-T) DNA was extracted from cells cultured from the enlarged spleen of a chicken infected with REV-T (Temin and Kassner, manuscript in preparation). SR-RSV-D DNA was extracted from transformed virus-producing chicken cells. R(B77V) DNA was extracted from non-virus-producing rat embryo fibroblasts transformed with the B77 strain of avian sarcoma virus. The infectivity of the DNA preparations was determined by treatment of quadruplicate cultures of chicken embryo fibroblasts with twofold serial dilutions of DNA.

^b DAI, days after infection.

TABLE 2. Infection of pheasant and duck cells with RSV and REV DNAs^a

Recipient cell	ID ₅₀ (µg of DNA)	
	SR-RSV-D	TDSNV
Duck	0.1	0.2-0.5
Pheasant	5	2

^a DNA was extracted from chicken cells infected with either SR-RSV-D or TDSNV. Duck and pheasant embryo fibroblasts were treated with DNA in the same manner as chicken embryo fibroblasts. Pheasant cells were transferred once before scoring for transformation after treatment with RSV DNA.

TABLE 3. Sensitivity of infectious RSV and REV DNAs to treatments with DNase, RNase, or alkali^a

Treatment	ID ₅₀ (μg of DNA)	
	SR-RSV-D	TDSNV
None	0.1	0.2
DNase ^b	>10	>10
RNase ^c	0.1	0.2
NaOH ^d	1.0	1.0

^a Quadruplicate cultures of chicken embryo fibroblasts were treated with DNA extracted from chicken cells infected with SR-RSV-D or with TDSNV.

^b Incubated for 60 min at 37 C with 100 μg/ml of DNase I in 0.01 M MgCl₂, 0.01 M CaCl₂, and 0.01 M Tris-hydrochloride, pH 7.4.

^c Incubated for 60 min at 37 C with 100 μg/ml of boiled RNase A in 0.1 × SSC.

^d DNA preparations in SSC were adjusted to 0.1 N NaOH for 1 min at 25 C or for 30 min at 37 C. The DNA was neutralized by diluting 50-fold into Tris-sodium chloride.

nuclease as [³H]labeled single-stranded M13 DNA (92 to 94% hydrolysis to acid-soluble material) (data not shown).

DNA extracted from TDSNV-infected chicken cells was further purified by isopycnic centrifugation in a CsCl gradient. The peak DNA fractions, which banded at a density of 1.71 g/cm³, were recovered from the gradient, and the infectivity of the DNA was determined. The DNA was infectious with an ID₅₀ value of 0.05 μg of DNA. Similar results, demonstrating the association of infectivity with DNA in CsCl gradients, have been reported by others for RSV DNA (11, 39).

Kinetics of infection with viral DNAs. To determine whether viral DNA consisted of a single infectious unit or of multiple unlinked subunits, we analyzed the kinetics of infection with viral DNAs. The data from several different experiments in which quadruplicate recipient cultures were treated with serial twofold dilutions of DNA were normalized according to the ID₅₀ value for each titration. The percentage of total positive cultures is plotted as a function of ID₅₀ units for RSV and REV DNAs in Fig. 2 together with theoretical curves for one-, two-, and three-hit kinetics. The cumulative assay data for both RSV and REV DNAs closely follows the theoretical curve for one-hit kinetics, indicating that the viral DNAs were transferred as single infectious units.

To evaluate the statistical significance of the data presented in Fig. 2, the sums of squares of the deviations of the experimental data from the theoretical curves for one-, two-, and three-

hit kinetics were calculated, and the F test (5) was used to determine the statistical significance of the agreement between the experimental data and the theoretical curve for one-hit kinetics (Table 4). The difference between one- and two-hit kinetics is significant at the $P < 0.01$ level for RSV DNA and at the $P < 0.05$ level for REV DNA. For both RSV and REV DNAs, the difference between one- and three-hit kinetics is significant at the $P < 0.01$ level.

To rule out the possibility that the infectious DNA consisted of nonspecific aggregates, we prepared DNA from chicken cells which were doubly infected with both SR-RSV-D and TDSNV (Temin and Kassner, manuscript in preparation). Cultures treated with this DNA were scored for infection with SR-RSV-D and with TDSNV to determine the frequency of simultaneous infection with both viral DNAs. Most recipient cultures were infected with only one virus (Table 5), as would be predicted for independent segregation of the two viral DNAs. In a further experiment, 19 parallel recipient cultures were exposed to 0.1 μg of DNA ex-

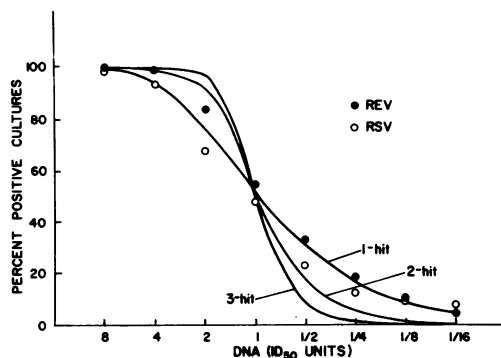


FIG. 2. Kinetics of infection with RSV and REV DNAs. Data from several experiments in which quadruplicate cultures of chicken embryo fibroblasts were treated with serial twofold dilutions of DNA extracted from cells infected with either RSV (○) or REV (●) were normalized according to the ID₅₀ value for each titration. Eleven experiments with RSV DNA and ten experiments with REV DNA were included. The data points represent the total percentage of positive cultures plotted as a function of ID₅₀ units of DNA. Each point represents 12 to 44 recipient cultures treated with DNA, with an average of 30 cultures per point. The lines are theoretical curves for one-, two-, and three-hit kinetics of infection calculated from the Poisson distribution. The theoretical percentage of positive cultures is equal to $1 - e^{-m}$, where m is equal to 0.69 multiplied by the dose of DNA (one-hit), by the square of the dose of DNA (two-hit), or by the cube of the dose of DNA (three-hit), with the dose of DNA expressed in ID₅₀ units.

TABLE 4. *Statistical analysis of kinetics of infection with RSV and REV DNAs^a*

Viral DNA	Sum of squares			F ratio		Significance level	
	One hit	Two hit	Three hit	Two hit to one hit	Three hit to one hit	Two hit to one hit	Three hit to one hit
RSV	140	990	1500	7.1	10.5	$P < .01$	$P < .01$
REV	180	710	1300	3.9	7.2	$P < .05$	$P < .01$

^a The sums of squares of the deviations of the experimental data points from the theoretical curves were calculated from the data presented in Fig. 2. F ratios are the ratios of the sums of squares. Significance levels were determined from a table of critical values of F with 8 degrees of freedom (5).

TABLE 5. *Segregation of RSV and REV in DNA extracted from doubly infected cells^a*

DNA (μg)	Fraction of infected cultures ^b			
	SR-RSV-D	TDSNV	SR-RSV-D + TDSNV	Neither virus
1.0	1/4	0/4	3/4	0/4
0.5	1/4	2/4	1/4	0/4
0.25	1/4	1/4	1/4	1/4
0.12	3/4	0/4	0/4	1/4
0.06	0/4	0/4	0/4	4/4

^a DNA was extracted from chicken embryo fibroblasts infected with both SR-RSV-D and TDSNV (H. M. Temin and V. K. Kassner, manuscript in preparation). Single cell plating of the infected cells demonstrated that more than 75% of the cells were producing both viruses.

^b Quadruplicate cultures of chicken embryo fibroblasts were treated with twofold serial dilutions of DNA. The recipient cultures were scored for infection with SR-RSV-D by transformation and by production of transforming virus assayed on chicken cells. Infection with TDSNV was scored by production of cytopathic virus assayed on chicken and duck cells. Results are presented as the fraction of recipient cultures infected with SR-RSV-D alone, with TDSNV alone, with both SR-RSV-D and TDSNV, or with neither virus.

tracted from the doubly infected cells. Three cultures were infected with TDSNV, one was infected with RSV, and the remaining 15 were uninfected. None of the recipient cultures were infected with both viruses. We conclude that the two viral DNAs segregated independently in DNA prepared from doubly infected cells, indicating that nonspecific aggregation of infectious DNA did not occur to a significant extent.

Minimal size of infectious viral DNAs. Since infection with RSV and REV DNAs proceeded by one-hit kinetics, the minimal size of viral DNA required for infectivity is a measure of its complexity. To determine the size of DNA in the infectious DNA preparations, we

extracted [³H]labeled DNA from chicken cells infected either with SR-RSV-D or TDSNV. The DNA extracted from both types of infected cells sedimented between 30 and 60S in neutral sucrose gradients (Fig. 3). The average molecular weight of this DNA was about 60×10^6 (36). The DNA which sedimented between 30 and 60S was recovered from the gradients and assayed for infectivity. Both RSV and TDSNV DNA preparations were infectious with ID_{50} values of 0.1 μg of DNA.

Preparations of [³H]DNA extracted from chicken cells infected with RSV and TDSNV were sheared by passage through syringe needles of different diameters. The molecular weights of the sheared [³H]DNA preparations were determined by electrophoresis in agarose gels (Fig. 4). From the electrophoretic mobility of the DNA in each sheared DNA preparation, we determined the average molecular weight of the DNA, and the fractions of DNA with molecular weights less than 6×10^6 and less than 20×10^6 . These molecular weights correspond, respectively, to the molecular weights of double-stranded DNA equivalent to the molecular weight of one subunit of the 60-70S viral RNA (3, 4, 9, 22) and to the molecular weight of the entire viral RNA, as estimated from its sedimentation coefficient of 60-70S (9, 19, 32).

The sensitivity of the infectivity of SR-RSV-D DNA to shearing is illustrated in Fig. 5. The infectivity of the sheared DNA preparations is plotted as a function of their average molecular weights together with the fraction of DNA in each preparation with molecular weights less than 6×10^6 and less than 20×10^6 . SR-RSV-D DNA sheared to an average molecular weight of 9×10^6 retained nearly the full infectivity of unsheared DNA. SR-RSV-D DNA preparations sheared to average molecular weights of 6×10^6 and 5×10^6 retained 20 and 5% of the infectivity of unsheared DNA, and the infectivity of SR-RSV-D DNA was inactivated by shearing to an average molecular weight of

3×10^6 . These results indicate that the size of infectious SR-RSV-D DNA was approximately 6×10^6 daltons. Since infectious RSV was produced by chicken cells transformed with the sheared DNA preparations, it appears that DNA of this molecular weight contained the genetic information required for virus replication in chicken cells as well as for transformation.

However, the infectivity of TDSNV DNA was significantly more sensitive to shearing than was RSV DNA (Fig. 6). TDSNV DNA sheared to an average molecular weight of 11×10^6 retained only 10% of the infectivity of unsheared DNA, and the infectivity of TDSNV DNA was inactivated by shearing to an average molecular weight of 6×10^6 . These results indicate that infectious TDSNV DNA was significantly larger than RSV DNA. The minimal size of infectious TDSNV DNA appeared to be approximately 20×10^6 daltons.

The difference between the minimal molecular weights of infectious RSV and TDSNV DNAs was further substantiated by shearing DNA extracted from chicken cells doubly infected with both SR-RSV-D and TDSNV (Fig. 7). The infectivity of TDSNV DNA was significantly more sensitive to shearing than was the infectivity of SR-RSV-D DNA, even when both viral DNAs were extracted from the same doubly infected chicken cells. The minimal molecular weight of infectious SR-RSV-D DNA was approximately 6×10^6 , whereas the minimal molecular weight of infectious TDSNV DNA was approximately 20×10^6 .

DISCUSSION

In this paper, we describe an efficient and quantitative assay for infectious avian ribodeoxyvirus DNA. Ribodeoxyvirus is the name of the virus group whose virions contain RNA and a DNA polymerase (H. M. Temin, *Annu. Rev. Genet.*, in press). Only a single exposure of the recipient chicken cells to viral DNA was required for demonstration of infectivity, and extensive cultivation of the cells after treatment with DNA was not necessary. The assay was made quantitative by end-point dilution of DNA, and ID_{50} values of $0.1 \mu\text{g}$ of DNA were reproducibly observed. This ID_{50} represents an efficiency which is 100- to 1,000-fold higher than that reported by earlier workers (12, 23, 38), and approximately 10-fold higher than that reported by Svoboda et al. (39) after pretreatment of the recipient cells with 5-bromodeoxyuridine. Pretreatment of the recipient chicken cells with 5-bromodeoxyuridine did not increase the efficiency of our assay of infectious RSV DNA (unpublished data). Assuming that there are between two and twenty copies of viral DNA per infected cell (16a, 25, 33, 41, 43), the specific infectivity of viral DNA in our assay corresponded to 1 infectious unit per 10^6 to 10^8 viral DNA molecules. This efficiency is comparable to the specific infectivities of DNA extracted from virions of papova viruses (28, 44) and herpes simplex virus (8, 35), and is higher than the specific infectivity of adenovirus DNA (7, 27).

The infectious material from cells infected

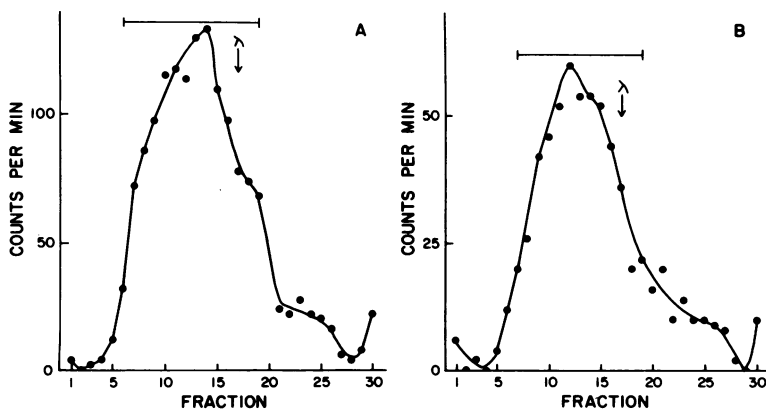


FIG. 3. Sucrose gradient sedimentation of DNA from infected cells. ^3H -labeled DNA extracted from chicken cells infected with SR-RSV-D (panel A) or with TDSNV (panel B) was centrifuged in 5–20% linear sucrose gradients in Tris-NaCl (pH 7.4) for 150 min. at 45,000 rpm in an SW 50.1 rotor. ^3H -labeled λ DNA marker was centrifuged in a parallel gradient. Sedimentation was from right to left. The fractions indicated by brackets were pooled for determination of infectivity (see text).

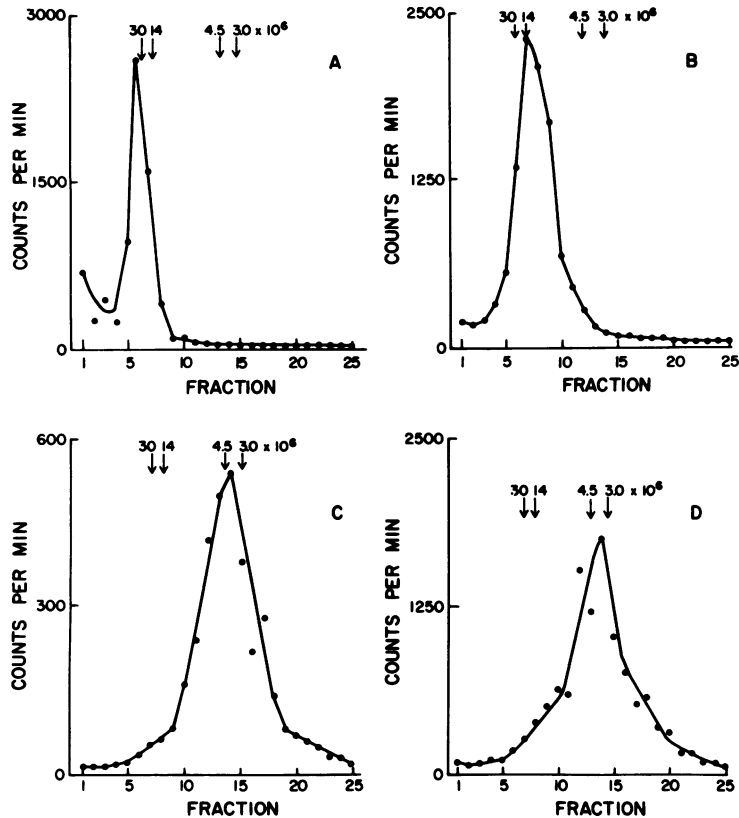


FIG. 4. Electrophoresis of DNA from infected cells. [^3H]labeled DNA preparations extracted from SR-RSV-D-infected chicken cells were subjected to electrophoresis for 3 h at 60 V in 0.7% agarose gels containing ethidium bromide. Unlabeled λ DNA and fragments of λ DNA prepared by digestion with *E. coli* R1 restriction endonuclease were incorporated into each gel as internal markers. The positions of the markers were determined by fluorescence and are indicated by arrows with the molecular weight of the marker DNA fragment (1). The gels were sliced and counted to determine the distribution of the [^3H]labeled DNA. The band width of the λ DNA markers was less than one gel slice. The SR-RSV-D [^3H]DNA preparations were unsheared (A) or sheared by 20 forceful passages of the DNA (200 $\mu\text{g}/\text{ml}$) through 25 (B), 27 (C), or 30 (D) gauge syringe needles.

with avian ribodeoxyviruses was shown to be DNA by its sensitivity to DNase, its resistance to RNase, its partial resistance to alkaline hydrolysis, and its banding with DNA in CsCl equilibrium density gradients. Denatured DNA was infectious, as previously reported by Hillova et al. (14). However, the specific infectivity of denatured DNA was approximately 10-fold lower than that of native DNA, which might account for the apparent lack of infectivity of denatured DNA reported by Svoboda et al. (39).

The infectivity of DNA extracted from REV-infected avian cells demonstrates that REV replicates via a DNA intermediate. This conclusion is consistent with the effect of inhibitors of DNA synthesis on the replication of REV (40) and confirms the demonstration of REV DNA in

infected cells by nucleic acid hybridization (16a).

Although Svoboda et al. (39) reported that Khaki Campbell duck cells were not sensitive to infection with RSV DNA, we found that Muscovy duck cells and pheasant cells were sensitive to infection with both RSV and REV DNAs. Since duck cells do not contain DNA hybridizable to either RSV or REV RNA (16a, 42), it does not appear that the presence of endogenous viral nucleotide sequences in the recipient cell DNA is necessary for infection with viral DNAs.

Infection with RSV and REV DNAs proceeded by one-hit kinetics, indicating that the viral DNA is integrated into host DNA (42) as a single unit of DNA rather than as two or more unlinked subunits. Furthermore, the high spe-

cific infectivity of viral DNA is incompatible with an unlinked subunit structure, since multiple independent events would then be required for infection. Since RSV and REV DNAs segregated independently in infectious DNA prepared from cells doubly infected with both viruses, it appears that the RSV and REV DNAs were not closely linked in the doubly infected donor cells. The independent segregation of the two viral DNAs also excluded the possibility that the one-hit kinetics of infection with viral DNA was an artifact of nonspecific DNA aggregation.

Since viral DNA was transferred as a single infectious unit, we were able to estimate the size of RSV and REV DNAs by determining the infectivity of viral DNA preparations sheared to different molecular weights. The minimal molecular weight of infectious RSV DNA was approximately 6 million. This finding is in agreement with earlier studies of infectious RSV DNA (13, 14, 23) and with recent data indicating that the complexity of RSV 60-70S RNA is approximately 3 million daltons (Duesberg, P. H., Cold Spring Harbor Symposium, 1974; C. Weissmann, Cold Spring Harbor Symposium, 1974). In contrast, the minimal molecular weight of infectious REV DNA was significantly larger than that of RSV DNA. The accuracy of absolute size determinations by this method is limited; therefore, we estimate that the minimal molecular weight of infectious REV DNA was 10 to 20×10^6 .

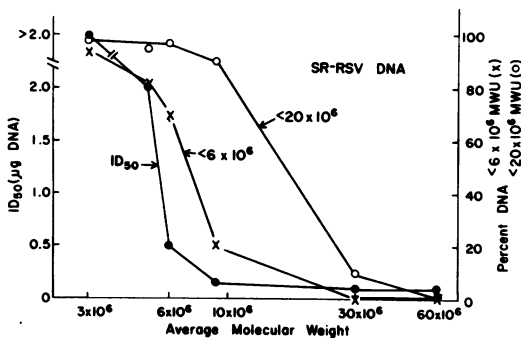


FIG. 5. Relationship of infectivity to molecular weight of SR-RSV-DNA. [³H]DNA was extracted from chicken cells infected with SR-RSV-D and sheared by passage through 22, 25, 27, and 30 gauge syringe needles. The average molecular weight of each DNA preparation and the fractions of DNA with molecular weights less than 6×10^6 (x) and less than 20×10^6 (o) were determined by electrophoresis of DNA in agarose gels as illustrated in Fig. 4. The infectivities of the unsheared and sheared DNA preparations were determined by assay on chicken embryo fibroblasts and are expressed as ID₅₀ values (●).

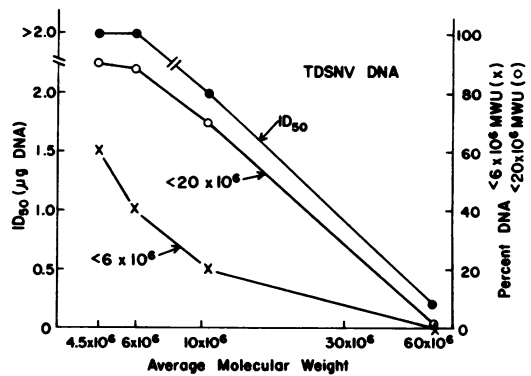


FIG. 6. Relationship of infectivity to molecular weight of TDSNV DNA. [³H]DNA was extracted from chicken cells infected with TDSNV and sheared by passage through 25, 27, and 30 gauge syringe needles. The ID₅₀ of each DNA preparation (●) and the fraction of DNA in each preparation with molecular weights less than 6×10^6 (x) and less than 20×10^6 (o) are plotted as a function of the average molecular weight of the DNA as described in the legend to Fig. 5.

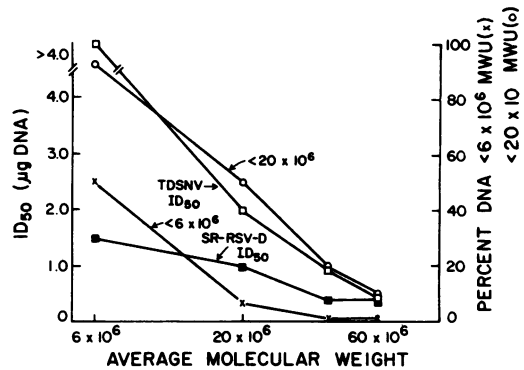


FIG. 7. Relationship of infectivity to molecular weight of SR-RSV-D and TDSNV DNAs extracted from doubly infected cells. [³H]DNA was extracted from chicken cells infected with both SR-RSV-D and TDSNV as described in Table 5. The DNA was sheared by passage through 22, 25, 27, and 30 gauge syringe needles. The ID₅₀ of each DNA preparation for SR-RSV-D (■) and for TDSNV (□) and the fraction of DNA in each preparation with molecular weights less than 6×10^6 (x) and less than 20×10^6 (o) are plotted as a function of the average molecular weight of the DNA, as described in the legend to Fig. 5.

The REV's replicate efficiently in both chicken and duck cells (40; Temin and Kassner, manuscript in preparation), whereas the avian leukosis-sarcoma viruses replicate efficiently in chicken, but not in duck cells (6; D. Zarlring, personal communication). Uninfected chicken cells contain nucleic acid sequences homologous to at least 70% of the RNA sequences of a non-

transforming avian leukosis-sarcoma virus (16a, 26), but to only 10% of the RNA sequences of the REVs (16a). Uninfected duck cells have no detectable nucleic acid homology to the RNA of either the avian leukosis-sarcoma viruses or the reticuloendotheliosis viruses (16a, 42). It is possible that replication of the avian leukosis-sarcoma viruses is complemented by the virus-related information present in uninfected chicken cells (6). However, the genome of the REVs may contain all of the information required for virus replication and consequently be larger than the genome of the avian leukosis-sarcoma viruses. In this case, one would predict that the complexity of REV 60-70S RNA is 5 to 10 million daltons.

Alternatively, the larger size of infectious REV DNA might be due to a requirement for cellular nucleotide sequences adjacent to infectious REV DNA. Such sequences might be necessary for integration of infectious REV DNA in the recipient cells. Further work will attempt to distinguish between these alternatives.

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