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Inhibition of Viral Transcriptase by Immunoglobulin Directed Against the Nucleocapsid NS Protein of Vesicular Stomatitis Virus

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In search of an anti-transcriptase, antibody was raised in rabbits to partially purified, soluble NS protein present in cytoplasmic extracts of cells infected with the Indiana serotype of vesicular stomatitis (VS_{Ind}) virus. This antiserum gave specific reactions of identity by agar immunodiffusion with both cytoplasmic and virion NS protein. NS antiserum also preferentially precipitated NS ^{3}H-labeled protein from infected cytoplasmic extracts, whereas anti-whole VS_{Ind} virion serum also precipitated N ^{3}H-labeled protein from extracts both of infected cytoplasm and virion nucleocapsids. Transcriptase activity of VS_{Ind} cytoplasmic or virion-derived nucleocapsids was effectively inhibited by ribonuclease-free immunoglobulin prepared from homologous NS_{Ind} antiserum or from anti-whole vesicular stomatitis virus serum. Transcriptase activity of heterologous New Jersey serotype (VS_{NJ}) nucleocapsids and virions was not appreciably affected by anti-NS_{Ind} or by anti-whole VS_{Ind} virion gamma globulin. Anti-NS gamma globulin immediately switched off RNA synthesis by actively transcribing VS_{Ind} nucleocapsids, a finding which suggests that NS antibody inhibits RNA chain elongation.

RNA-dependent RNA polymerase (transcriptase) is an integral component of the nucleocapsids of vesicular stomatitis (VS) virions (1) of both the Indiana (VS_{Ind}) and New Jersey (VS_{NJ}) serotypes, as well as other rhabdoviruses (6). This transcriptase activity is also associated with intracellular nucleocapsids of VS virus-infected cells (10). The two envelope proteins G and M are not involved in transcription (3, 7, 22). All three nucleocapsid proteins of VS virus are probably required for transcription. The major nucleocapsid (N) protein possesses no known enzymatic activity per se but serves at least an important structural function because naked viral RNA can not function as a template by reconstitution with transcriptase (7). The very large L protein, on the other hand, is indispensable; transcriptase activity is lost when the L protein is stripped off in a high-salt environment and is restored by reconstitution of the L protein and its nucleocapsid template (8). The companion paper by Emerson and Yu (9) demonstrates the essential role in transcription of the third nucleocapsid protein, designated NS, in association with the L protein and its nucleocapsid template. The NS protein is specifically phosphorylated (11, 16, 21) and is present as a major component in the virus-infected cell. Unlike all other VS viral proteins, most of the NS protein is not incorporated into the virion but is found in the cytoplasm where it remains soluble (24).

The experiments reported herein represent a first attempt to produce an antibody which can specifically neutralize the transcriptase activity of VS viral nucleocapsids. We decided to direct our attention initially to NS antibody because the NS protein can be obtained in large amounts from the infected cell relatively free of other viral proteins. Most of these experiments were performed with cytoplasmic nucleocapsids as templates, which do not require detergent for activation of polymerase. This preliminary report illustrates the feasibility of using a specific anti-transcriptase as a probe for studying the component parts of the VS viral transcription complex.

MATERIALS AND METHODS

Cell cultures and virus. L cells and BHK-21 cells were cultivated as previously described, except that BHK-21 cells were grown on BHK-21 medium rather

than Eagle BME (11). The origin and methods of cultivating and purifying the Indiana (VS_{Ind}) and New Jersey (VS_{NJ}) serotypes of VS virus have also been described (11, 14).

Production of antisera. Anti-NS serum was prepared by immunization of rabbits with NS protein purified by DEAE-cellulose chromatography of cytoplasmic extracts of 1.5×10^8 infected L cells. The antigen was suspended in complete Freund adjuvant and injected intramuscularly. Two equivalent antigenic boosters were given, one intramuscularly at five weeks in complete Freund adjuvant and one intravenously at eight weeks. Ten days after the final booster, the rabbits were bled from the ear.

As previously described (12), anti-whole VS_{Ind} virus and monospecific anti-VS_{Ind} glycoprotein (both provided by J. M. Kelley and S. U. Emerson) were raised in rabbits immunized by multiple intradermal injections of each antigen in complete Freund adjuvant. Boosters were given five weeks later by intravenous injection, and the rabbits were bled the following week.

Preparation of gamma globulin fraction from serum. The ribonuclease content of rabbit serum completely obviated its use in transcriptase reaction mixtures. Therefore, gamma globulin free of RNase was prepared by the method described by Palacios et al. (17). Each serum was diluted with an equal volume of buffer (10 mM $NaPO_4$ and 15 mM NaCl, pH 7.5) and saturated with $(NH_4)_2SO_4$ to 40%. After 10 min at room temperature, the precipitate was collected, dissolved in a volume of buffer equal to half of the original volume of serum, and dialyzed against two changes of 800 volumes of buffer for 5 h. The dialyzed material, equivalent to 5 ml of serum, was applied to a column of 3 ml of *O*-(carboxylmethyl)cellulose stacked over 3 ml of DEAE-cellulose. The gamma globulin fraction eluted at the buffer front, and its concentration was monitored by measuring spectrophotometric absorption at 280 nm.

Preparation of viral nucleocapsid fractions. Fifteen monolayer cultures (100-mm plates) of BHK cells were infected with VS_{Ind} virus (multiplicity of infection = 15) or VS_{NJ} virus (multiplicity of infection = 50). After adsorption for 1 h, 5 ml of BME was added. At about 5.5 h postinfection, cytoplasmic extracts of the cells were prepared by the method of Penman et al. (18). This extract was fractionated by centrifugation in a 0 to 60% discontinuous sucrose gradient as described by Caliguiri and Tamm (4). The gradient was prepared by successive layering of the following sucrose solutions (wt/wt) in reticulocyte standard buffer, pH 8.0: 2.5 ml of 60%, 6.5 ml of 45%, and 6.5 ml of 40% sucrose; 9 ml of the cytoplasmic extracts (from 15 cultures) made 30% with respect to sucrose; 6.5 ml of 25% sucrose; and finally 1.5 ml of reticulocyte standard buffer. The gradient was centrifuged in the SW25.1 rotor at $155,000 \times g$ for 16 h at 5 C. Band 3 of the four light-scattering bands contains predominantly VS viral nucleocapsids and was collected by side puncture of the tube. Wagner et al. (23) previously described the composition of the four bands fractionated by density centrifugation of VS virus-infected cell extracts monitored by electron microscopy, marker enzymes, and protein electropherograms.

RNA polymerase assay. RNA polymerase activity was assayed by measuring the incorporation of ^3H from [^3H]UTP into trichloroacetic acid-precipitable material by using the incubation conditions of Emerson and Wagner (7) with minor modifications. A standard polymerase incubation mixture in a volume of 0.1 ml contained: 25 mM Tris-hydrochloride, pH 8.0; 75 mM NaCl; 0.625 mM dithiothreitol; 4 mM $MgSO_4$; 0.7 mM ATP; 0.7 mM CTP; 0.7 mM GTP; and 0.009 mM [^3H]UTP (600 μCi/μmol). Assays were performed at 31 C in duplicate. Samples were processed for ^3H incorporation as previously described (7).

Polyacrylamide gel electrophoresis. Samples for gel electrophoresis were prepared by precipitating proteins with an equal volume of cold 20% trichloroacetic acid or with 6 volumes of methanol (11). Trichloroacetic acid precipitates were washed with acetone. All precipitates, including indirect immunoprecipitates, were dissolved in 10 mM *N*-2-hydroxyethyl-piperazine-*N*-2'-ethanesulfonic acid buffer (pH 7.4), 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol, and 6 M urea. Samples were placed in a boiling water bath for 2 min prior to layering on gels. As previously described (11), electrophoresis at 3.5 mA per gel for 18 h was performed by using 7.5% acrylamide gels containing 0.1% sodium dodecyl sulfate and 100 mM sodium phosphate (pH 7.4). After electrophoresis, the gels were frozen and cut into 1.25-mm slices. Protein was extracted from each slice by incubation for 2 h at 50 C in 0.5 ml of Nuclear-Chicago solubilizer-water (9:1). Ten milliliters of toluene-based scintillation fluor was added, and the samples were counted in a scintillation spectrometer.

RESULTS

Preparation of NS protein. NS protein was extracted and purified from L cells infected with VS_{Ind} virus. Twenty to thirty monolayer cultures of $\sim 2 \times 10^7$ L cells each were pretreated overnight with 5 ml of medium containing 0.25 μg of actinomycin D per ml to decrease incorporation of radioactive amino acid label into nonviral proteins. The cells were then infected with VS_{Ind} virus at a multiplicity of 15. After adsorption for 1 h at 37 C, 4.5 ml of medium was added; after 2 additional h, 5 μCi/ml of [^3H]leucine was added. Six hours postinfection, cytoplasmic extracts of the cells were prepared by Dounce homogenization by the method of Penman et al. (18). The cytoplasmic extract was centrifuged for 2.5 to 3 h at $100,000 \times g$ in a Spinco type 50 Ti rotor. The supernatant fraction was dialyzed overnight against 600 volumes of 50 mM Tris-hydrochloride (pH 7.5) and applied to a DEAE-cellulose column (1.1 by 11 cm) equilibrated with dialysis buffer. Proteins were eluted from the column by a linear gradient of 0 to 0.35 M NaCl in 180 ml of

dialysis buffer. Peak fractions of ^{3}H-labeled protein eluted in this salt gradient were analyzed by polyacrylamide gel electrophoresis.

Figure 1 shows that the peak ^{3}H-labeled protein fraction eluted from the DEAE-cellulose column in 0.20 to 0.25 M NaCl contains NS protein and is free of other detectable VS viral proteins. However, Coomassie blue staining of these sodium dodecyl sulfate-polyacrylamide gels revealed the presence of approximately 10 cellular proteins in addition to the viral NS protein (data not shown). These preparations of cytoplasmic NS protein partially purified by DEAE-cellulose chromatography served as the antigen for raising anti-NS serum (see Materials and Methods).

Specificity of anti-NS serum as determined by immunoprecipitation. The degree to which the anti-NS gamma globulin reacts with VS viral proteins was determined by double diffusion in agar and by indirect immunoprecipitation of soluble antigen-antibody complexes.

Figure 2 shows a diagram of precipitation lines in agar after diffusion of rabbit anti-NS serum against purified NS protein, a fivefold dilution of NS protein and the proteins of

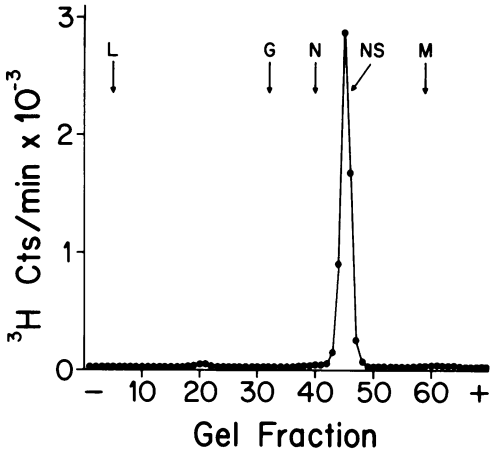

FIG. 1. *Electropherogram of purified NS_{Ind} protein. The 100,000 × g supernatant fraction of VS_{Ind} virus-infected L cell cytoplasmic extract labeled with [^{3}H]leucine was fractionated on a DEAE-cellulose column (1.1 by 11 cm) by elution with a linear gradient of 0 to 0.35 M NaCl in 50 mM Tris-hydrochloride, pH 7.5. The fractions eluted in 0.2 to 0.25 M NaCl, constituting the major radioactive peak, were combined. Protein precipitated by 10% trichloroacetic acid was suspended in 100 μl of buffer and co-electrophoresed with VS viral ^{14}C-labeled protein marker on a 7.5% sodium dodecyl sulfate-polyacrylamide gel. Arrows mark the positions of marker L, G, N, NS, and M ^{14}C-labeled proteins.*

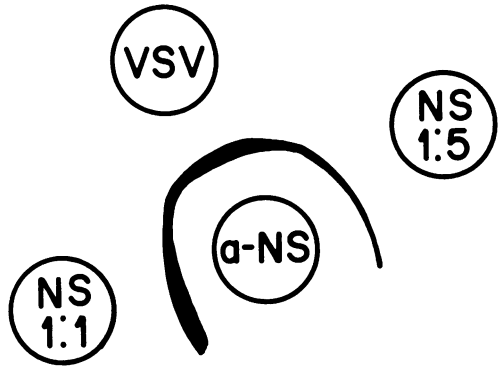

FIG. 2. *Sketch of immunodiffusion plate showing the precipitin reaction of NS antiserum with purified NS_{Ind} protein and disrupted VS_{Ind} virions. NS protein was purified by DEAE-cellulose chromatography of the supernatant fraction of a cytoplasmic extract of VS_{Ind} virus-infected L cells. Purified VS_{Ind} virions were disrupted by treatment with 1% Triton X-100 and 1% sodium deoxycholate for 30 min at room temperature. Purified NS protein undiluted (NS 1:1) and diluted 1:5 (NS 1:5) and detergent-disrupted VS_{Ind} virions (VSV) were placed in wells cut in 0.5% agarose containing 0.1% sodium deoxycholate and 0.001% sodium azide buffered with 75 mM sodium phosphate (pH 7.1), and were allowed to diffuse against anti-NS serum (a-NS) for 72 h at room temperature. The agar plate was fixed, washed, and stained with Coomassie blue.*

purified, whole VS_{Ind} virions disrupted with sodium deoxycholate and Triton X-100. Precipitin lines of identity can be seen between the NS protein preparations and a protein, presumably NS, liberated from VS_{Ind} virions by detergent disruption. Not shown in the diagram was a faint line seen only in stained preparations near the disrupted VS virion well, which could be due to poorly diffusing residual nucleocapsids. However, NS antiserum showed no capacity to form precipitin lines with VS viral G and N proteins, which are readily detected by double diffusion of solubilized VS virion proteins against whole VS virion antiserum. The paper by Kelley et al. (12) illustrates the agar precipitin reaction of G and N proteins with the same anti-VS viral serum used in these studies. It seems clear, therefore, that the antiserum prepared by immunization with NS protein exhibits no significant immunoprecipitin reaction in agar with the much larger amounts of G and N proteins of VS virions.

Indirect precipitin tests with sheep anti-rabbit serum were also used to determine which viral proteins in extracts of infected cell cytoplasm and virion nucleocapsids formed com-

plexes with either the anti-NS rabbit serum or with anti-whole VS viral rabbit serum. L cells infected with VS_{Ind} virus were labeled with [^{3}H]leucine, and a 150,000 $\times$ g supernatant fraction of the cytoplasmic extract was prepared from 6-h cultures. Anti-NS or anti-whole viral serum was incubated with the infected cytoplasmic supernatant fraction for 1 h at 37 C. The resulting rabbit gamma globulin-^{3}H-labeled protein complexes were then precipitated by adding 200 μl of sheep anti-rabbit serum, an amount capable of precipitating all the gamma globulin in 25 μl of serum. The precipitate was then washed, dissolved in buffer containing sodium dodecyl sulfate, urea, and 2-mercaptoethanol, and analyzed for viral ^{3}H-labeled proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Table 1 summarizes the electropherogram data on comparative amounts of N and NS ^{3}H-labeled proteins precipitated by sheep anti-rabbit serum from VS_{Ind} virus-infected cytoplasmic extract in the presence of anti-NS_{Ind} and anti-whole VS_{Ind} virion sera. As noted, the antigen-combining activity of anti-NS serum was predominantly directed against NS protein, whereas anti-whole VS virion serum reacted primarily with N protein. The NS protein in the cytoplasmic extract was in fourfold excess of N protein. The antibody in anti-VS virion serum also specifically combined with NS protein but to a lesser extent than with N protein. The explanation for 5% precipitation of N protein by anti-NS serum may well be due to co-precipitation of N protein trapped in NS protein–anti-NS complexes brought down by sheep anti-rabbit serum. However, these studies do not rule out completely some capacity of NS anti-serum to react with N protein.

Table 1 also summarizes data on the comparative combining ability to N protein of anti-NS and anti-whole VS virion sera. In these experiments, nucleocapsids prepared by Suzanne Emerson from purified VS_{Ind} virions by treatment with Triton X-100 in 0.72 M NaCl were then banded in CsCl by isopycnic centrifugation, which completely removes L and NS proteins (9). N ^{3}H-labeled protein solubilized by ultrasonic vibration was then reacted with rabbit anti-NS and rabbit anti-whole VS virion sera prior to immunoprecipitation with sheep anti-rabbit serum. The results (Table 1) again show the specificity of anti-whole VS virion serum for N protein and the minor degree of N protein co-precipitation in the presence of rabbit anti-NS serum.

These immunoprecipitation data indicate that the anti-whole VS_{Ind} serum contained a major antibody reactive with N protein and a somewhat lesser amount of antibody reactive with NS protein, as well as antibodies to G protein (12) and possibly to L protein. On the other hand, anti-NS serum either reacts specifically only with NS protein and co-precipitates N protein, or has minimal antibody reactivity with N protein.

Comparative transcriptase activity of VS_{Ind} and VS_{NJ} nucleocapsids. Prior to testing the effect on nucleocapsid transcription of antibody directed against whole VS_{Ind} virus and its NS protein, it was necessary to standardize the transcription reactions. Most studies on VS viral transcription have been performed with detergent-treated VS virions but comparable RNA synthesis can be demonstrated with cytoplasmic VS_{Ind} nucleocapsids in the absence of detergent (10). No similar reports have been published on the transcriptase activity of cytoplasmic VS_{NJ} nucleocapsids; detergent-activated transcription of VS_{NJ} virions is considerably less active than that of VS_{Ind} virions (6). VS_{NJ} nucleocapsids, as well as VS_{NJ} virions, seemed likely to provide good comparative controls for the anti-VS_{Ind} transcriptase specificity of antibody directed against VS_{Ind} viral proteins. Therefore, transcriptase systems were designed for parallel experiments with VS_{Ind} and VS_{NJ} nucleocapsids, as well as the respective purified virions.

To prepare cytoplasmic nucleocapsids, BHK-21 cells were infected with VS_{Ind} virions or VS_{NJ} virions (see Materials and Methods). Cells from 15 monolayer cultures infected with each virus were disrupted by Dounce homogenization, and the nuclei-free cytoplasm was fractioned by density centrifugation on discontinuous 0 to 60% sucrose gradients. Equivalent light-scattering bands (band 3 at the 40 to 45% sucrose interface) were harvested by side puncture of the gradient tubes, and the respective nucleocapsids were assayed for transcriptase activity at 31 C in a 0.1-ml reaction mixture containing: 25 mM Tris-hydrochloride, pH 8.0; 75 mM NaCl; 4 mM $MgSO_4$; 0.625 mM dithiothreitol; 0.7 mM each of ATP, CTP, and GTP; and 0.009 mM [^{3}H]UTP (600 μCi/mmol). No detergent was present. The reaction was stopped at intervals after incubation by addition of 0.5 ml of 67 μM sodium pyrophosphate as well as 0.1 ml (200 μg) of yeast RNA as carrier. RNA was precipitated by adding 0.5 ml of 25% trichloroacetic acid and collected on 0.45-μm membrane filters (Millipore); precipitated RNA was dissolved in 0.3 N NaOH and

TABLE 1. *Comparative indirect radioimmunoprecipitation by anti-NS_{Ind} and anti-whole VS_{Ind} virion sera of N and NS proteins from cytoplasmic extract and of N protein from nucleocapsid extract*

Immune serum	Cytoplasmic extract[a] (% ^3H precipitated)		Nucleocapsid[b] (% ^3H precipitated N protein)
	N protein	NS protein	
Anti-NS	5	65	6
Anti-VS virion	21	11	48

[a] L cells infected with VS_{Ind} virus were labeled with [^3H]leucine 3 to 6 h postinfection. Cytoplasm of 3×10^8 infected cells Dounce homogenized in reticulocyte standard buffer was centrifuged at 150,000 × *g* for 2.5 h in an SW50L rotor. Fifty-microliter amounts of the supernatant cytoplasm were incubated for 1 h at 37 C either with 40 μl of rabbit anti-NS_{Ind} serum or 40 μl of anti-whole VS_{Ind} virion serum. Then 200 μl of hyperimmune sheep anti-rabbit serum was added, and incubation was continued for 2 h at 37 C. The immune precipitates were collected by centrifugation at 800 × *g*, washed three times with phosphate-buffered saline, and dissolved in *N*-2-hydroxyethyl-piperazine-*N'*-2'-ethanesulfonic acid buffer with sodium dodecyl sulfate and 2-mercapto-ethanol. A portion of the VS viral cytoplasmic supernatant fluid was precipitated with cold methanol rather than antibody to determine the absolute precipitable amount of each VS viral protein potentially precipitable by antibody. Each sample was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the concentration of N and NS ^3H-labeled protein was determined as the area under each peak. The data are expressed as the percentage of protein precipitated by each antiserum, based on the total amount of methanol-precipitable protein. The amount of methanol-precipitable NS protein in the VS virus-infected cytoplasmic extract was four times greater than that of the N protein.

[b] Nucleocapsids were prepared by stripping envelopes off purified VS_{Ind} virions labeled with [^3H]leucine by treatment with 2% Triton X-100 in 0.72 M NaCl followed by centrifugation at 150,000 × *g* for 90 min in the SW50L rotor. The pelleted nucleocapsids were suspended in 4.63 ml of 50 mM Tris-hydrochloride, pH 7.5, containing 1.47 g of CsCl and banded by centrifugation at 150,000 × *g* at 22 C for 16 h. As described in the accompanying paper (9), these procedures remove all detectable L and NS protein, leaving pure N protein complexed with RNA. The material collected in a light-scattering band was dialyzed overnight against phosphate-buffered saline, and the nucleocapsids were disrupted by probe ultrasonification at maximal energy. Remaining particulate material was removed by sedimentation at 800 × *g* for 10 min. The supernatant N protein in 50-μl portions was treated with either rabbit anti-NS_{Ind} or rabbit anti-whole VS_{Ind} virion serum, followed by incubation with hyperimmune sheep anti-rabbit serum. The immune precipitates, as well as aliquots of trichloroacetic acid-precipitable N protein, were collected by centrifugation and washed three times on Whatman GF/C glass fiber before counting. Calculations of the percentage of immunoprecipitation of N ^3H-labeled protein were based on average values of 3,842 counts/min of trichloroacetic acid-precipitable total N protein and 502 counts/min of nonspecifically precipitable N protein in the presence of pre-immune normal rabbit serum.

TABLE 1.—*Continued*

counted by scintillation spectrometry.

RNA synthesis in this endogenous transcription reaction was linear for both VS_{Ind} and VS_{NJ} nucleocapsids. Comparative [^3H]UMP incorporation was 11,490 counts/min at 30 min and 21,920 counts/min at 60 min for VS_{Ind} nucleocapsids compared with 2,980 counts/min at 30 min and 6,050 counts/min at 60 min for VS_{NJ} nucleocapsids. Similar data are plotted as nucleocapsid transcriptase controls in Fig. 3A (VS_{Ind}) and Fig. 3B (VS_{NJ}).

Effect of anti-NS gamma globulin on transcriptase activity of VS_{Ind} and VS_{NJ} nucleocapsids. The availability of VS_{Ind} and VS_{NJ} nucleocapsids which contain all the template and enzyme requirements for RNA synthesis at a predictable rate enabled us to test the inhibitory effect on their transcription activity by various antisera. In these experiments antibody prepared against purified VS_{Ind} NS protein, purified VS_{Ind} G protein and preimmunized normal rabbit serum were tested for their effect on VS_{Ind} and VS_{NJ} nucleocapsid transcription. In each case purified ribonuclease-free gamma globulin (for preparation, see Materials and Methods) was used, because preliminary experiments had shown that RNase present in whole normal or immune rabbit serum completely degraded all newly synthesized RNA in the standard nucleocapsid reaction mixture.

Nucleocapsid fractions prepared by sucrose gradient centrifugation of VS_{Ind}- or VS_{NJ}-infected BHK-21 cell extracts were incubated with various dilutions of RNase-free anti-NS_{Ind} gamma globulin at 31 C for 30 min. Complete transcriptase reaction mixture containing all four nucleoside triphosphates and [^3H]UTP was then added to each nucleocapsid preparation previously exposed to 0, 0.75, 3, 15, or 75 μg of anti-NS gamma globulin. [^3H]UMP incorporation at 31 C was measured in each sample after stopping the transcription reactions at 30 and 60 min after initiation.

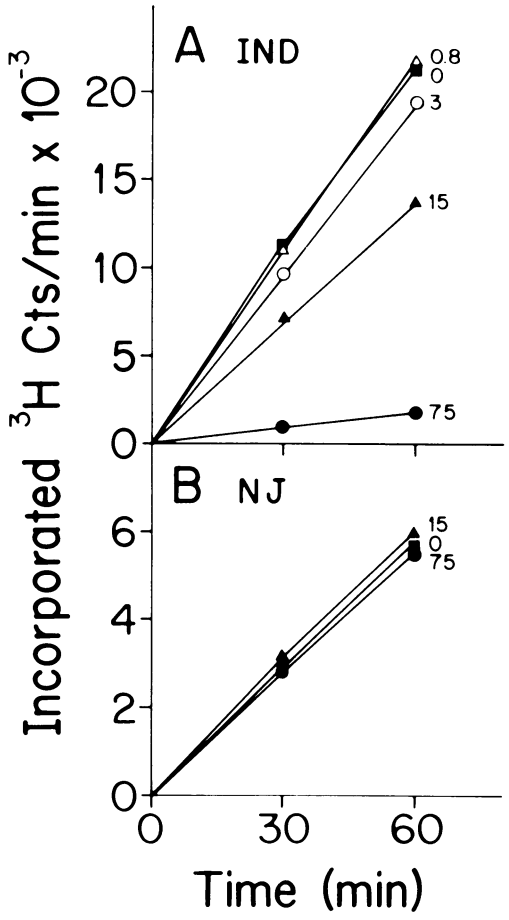

FIG. 3. *Effect of anti-NS_{Ind} gamma globulin on VS_{Ind} (A) and VS_{NJ} (B) nucleocapsid RNA polymerase activity. Twenty-five microliters (10 μg of protein) of either VS_{Ind} or VS_{NJ} sucrose gradient-fractionated nucleocapsids prepared from infected BHK cells were incubated for 30 min at 31 C with buffer alone, 0.8 μg, 3 μg, 15 μg, or 75 μg of RNase-free anti-NS gamma globulin in 25 μl of 10 mM sodium phosphate, 15 mM NaCl (pH 7.2). RNA synthesis was then induced by adding 50 μl of a nucleoside triphosphate reaction mixture (see Materials and Methods) prepared to provide optimal incubation condition for nucleocapsids diluted to 100 μl to initiate RNA polymerase activity. Samples were processed after 30 and 60 min of incubation to determine [^{3}H]UMP incorporation into trichloroacetic acid-precipitable RNA. The amounts (micrograms) of anti-NS_{Ind} gamma globulin are indicated for each reaction.*

Figure 3 shows the comparative transcriptase activity of VS_{Ind} and VS_{NJ} nucleocapsids after exposure to anti-NS_{Ind} gamma globulin. Anti-NS_{Ind} gamma globulin (75 μg) inhibited VS_{Ind} nucleocapsid transcription by about 92% of the baseline (control) activity (Fig. 3A). This anti-transcriptase activity was proportionately reduced by progressive dilution of the antiserum preparation. In marked contrast, 15 μg of anti-NS_{Ind} gamma globulin did not reduce the transcriptase activity of VS_{NJ} nucleocapsids, and 75 μg resulted in only 5% less RNA synthesis, which is probably not a significant effect (Fig. 3B).

Gamma globulins prepared from hyperimmune anti-G_{Ind} serum and from normal rabbit serum were also tested for their effect on VS_{Ind} nucleocapsid transcriptase; neither had any effect on RNA synthesis. Therefore, these data reveal considerable specificity of the anti-transcriptase activity of anti-NS gamma globulin and considerable selectivity for VS_{Ind} nucleocapsids compared with VS_{NJ} nucleocapsids.

Several attempts were made to reduce the anti-transcriptase activity of anti-NS_{Ind} gamma globulin by absorption with NS protein. Partially purified NS protein from 15 cultures of infected L cells was concentrated by lyophilization or was linked by cyanogen bromide to Bio-Gel A-5m. The soluble NS protein and the Bio-Gel-linked NS protein did reduce the anti-transcriptase titer of anti-NS_{Ind} gamma globulin but only to a limited extent. We can only conclude from these experiments that anti-transcriptase activity of anti-NS_{Ind} gamma globulin has a low affinity of binding for NS protein, the concentration of which was probably very low anyway. Moreover, no anti-transcriptase activity whatsoever could be removed from anti-NS_{Ind} gamma globulin by absorption with much larger concentrations of homologous nucleocapsids derived from equivalent numbers of VS virus-infected cells or from purified virions ($\sim 10^{11}$ nucleocapsids). It would appear, therefore, that the N protein and perhaps even the L protein of the nucleocapsid are incapable of absorbing out this anti-transcriptase activity.

Effect of anti-VS_{Ind} virion gamma globulin on VS_{Ind} and VS_{NJ} nucleocapsid transcriptase. Since anti-whole VS_{Ind} virion serum was shown to possess homologous anti-N and anti-NS immunoprecipitation activity (Table 1), and could perhaps also contain anti-L protein reactivity in addition to previously demonstrated anti-G antibodies (12), it was of interest to compare its anti-transcriptase activity with that of monospecific anti-NS_{Ind} serum. Therefore, four equivalent dilutions of RNase-free anti-VS_{Ind} gamma globulin were tested as described above for their effect on the RNA-sythesizing capacity of cytoplasmic VS_{Ind} and VS_{NJ} nucleocapsids prepared from infected BHK-21 cells. Sucrose gradient-fractionated nucleocapsids were incubated at 31 C for 30 min

with dilutions of anti-VS_{Ind} gamma globulin before adding nucleoside triphosphates in the standard transcriptase reaction mixture.

The transcriptive activity of VS_{Ind} and VS_{NJ} nucleocapsids after exposure to anti-VS_{Ind} gamma globulin were compared (Fig. 4). As noted in Fig. 4A, VS_{Ind} nucleocapsid transcription was inhibited to about the same extent by varying concentrations of anti-whole VS_{Ind}

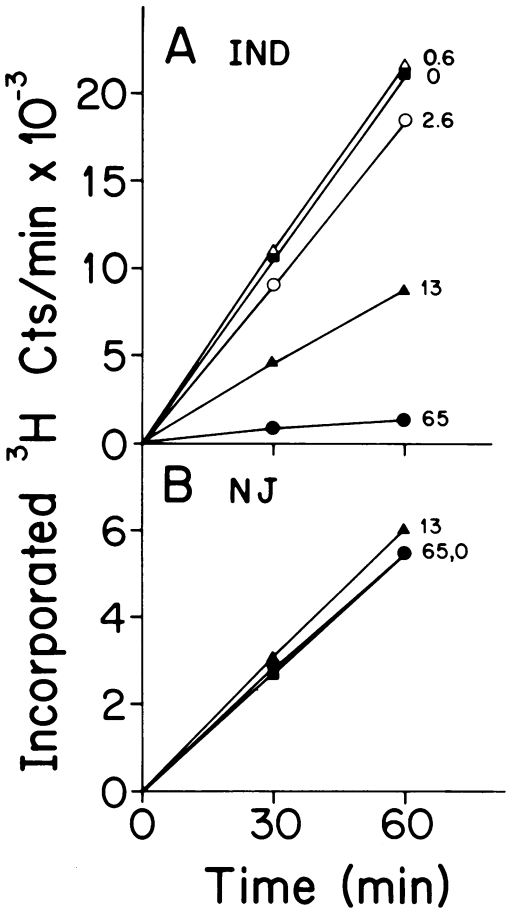

FIG. 4. *Effect of anti-whole VS_{Ind} virion gamma globulin on VS_{Ind} (A) and VS_{NJ} (B) nucleocapsid RNA polymerase activity. Twenty-five microliters (10 μg of protein) of either VS_{Ind} or VS_{NJ} gradient-fractionated cytoplasmic nucleocapsids was incubated with buffer alone, 0.6 μg, 2.6 μg, 13 μg, or 65 μg of RNase-free anti-VS_{Ind} virion gamma globulin in 25 μl of 10 mM sodium phosphate, 15 mM NaCl (pH 7.2) at 31 C for 30 min (see legend to Fig. 3). Then 50 μl of nucleoside triphosphate reaction mixture was added to each preparation to initiate RNA polymerase activity. Samples were processed after 30 and 60 min of incubation to determine trichloroacetic acid-precipitable [³H]UMP incorporation. The amounts (micrograms) of anti-whole VS virus gamma globulin are indicated for each reaction.*

gamma globulin as it was by equivalent amounts of anti-NS_{Ind} gamma globulin (compare with Fig. 3A). Similarly, anti-VS_{Ind} serum had no effect on transcription by VS_{NJ} nucleocapsids, which once again emphasizes the specificity of the antibody (Fig. 4B).

Antibody effect on transcriptase activity of VS virion nucleocapsids. The foregoing experiments on anti-transcriptase susceptibility of cytoplasmic nucleocapsids were repeated with nucleocapsids derived from purified VS_{Ind} and VS_{NJ} virions. This was done for the following reasons: (i) to rule out any possibility of unusual antibody reactivity of cytoplasmic nucleocapsids; (ii) to provide a basis for quantitating the nucleocapsid concentration by protein determination, which is possible with pure VS virions but not with impure cytoplasmic fractions; and (iii) to insure that the differential effect of the antibodies on VS_{Ind} and VS_{NJ} transcription was not due to differences in protein concentration. Therefore, the effects of anti-NS_{Ind} and anti-whole VS_{Ind} virus gamma globulin preparations were tested for their antipolymerase activity on nucleocapsids derived from purified VS_{Ind} and VS_{NJ} virions.

VS_{Ind} and VS_{NJ} virions were each grown in 15 cultures of BHK-21 cells and purified by rate zonal and differential centrifugation, as previously described (11). The envelopes of purified virions were removed by the procedure of Emerson and Wagner (7) in which virions are exposed to 2% Triton X-100 and 0.43 M NaCl, which results in solubilization of almost all the G and M proteins and liberates the intact nucleocapsids. The virion nucleocapsids of each virus were then pelleted, suspended in reticulocyte standard buffer, and used in the transcriptase reaction to test the effect of anti-NS_{Ind} and anti-VS_{Ind} gamma globulin.

Table 2 summarizes the results of an experiment in which samples of VS_{Ind} and VS_{NJ} virion-derived nucleocapsids containing equal amounts of protein were assayed for RNA polymerase activity in the presence or absence of anti-NS_{Ind} gamma globulin and anti-VS_{Ind} virion gamma globulin. As noted, transcriptase activity of VS_{Ind} virion nucleocapsids was inhibited by both antisera to almost the same extent as were cytoplasmic nucleocapsids. On the other hand, transcriptase activity of VS_{NJ} virion nucleocapsids of the same protein concentration was completely unaffected by either anti-NS_{Ind} or anti-VS_{Ind} gamma globulin. These data emphasize the selectivity of antisera for transcriptase of the specific serotype regardless of whether the nucleocapsids are derived from intact virions or cytoplasm of infected cells. It is also of

TABLE 2. *Effect of anti-NS_{Ind} and anti-whole VS_{Ind} virion gamma globulin on RNA polymerase activity of VS_{Ind} and VS_{NJ} virion-derived nucleocapsids*[a]

Nucleocapsid serotype	Inhibition of transcriptase (%)	
	Anti-NS gamma globulin	Anti-VS_{Ind} virion gamma globulin
Indiana	82	89
New Jersey	0	0

[a] Purified VS_{Ind} and VS_{NJ} virions in reticulocyte standard buffer were treated with equal volume of 3.74% Triton X-100, 1.2×10^{-3} M dithiothreitol, 18.7% glycerol, and 0.86 M NaCl for 60 min at 5 C. The mixture was centrifuged at 150,000 × *g* for 90 min in a Spinco SW50L rotor. The pellet was suspended in reticulocyte standard buffer. Twenty-five microliters of pellet fraction containing 2.5 μg of protein was incubated at 31 C for 30 min with either anti-NS gamma globulin (75 μg of protein) or anti-whole VS_{Ind} virion gamma globulin (65 μg of protein) in 25 μl of 10 mM sodium phosphate, 15 mM NaCl, pH 7.2. The enzyme reaction was then started by adding 50 μl of reaction mixture which yielded in a final incubation volume of 0.1 ml: 25 mM Tris-hydrochloride, pH 8.0; 75 mM NaCl; 4 mM $MgSO_4$; 0.625 mM dithiothreitol; 0.7 mM ATP; 0.7 mM CTP; 0.7 mM GTP; and 0.009 mM [^{3}H]UTP (600 μCi/mmol). After incubation for 60 min, the samples were processed to determine [^{3}H]UMP incorporation into trichloroacetic acid-precipitable RNA. Control transcriptase activity in the absence of gamma globulin was 5,720 counts per min per μg of protein for VS_{Ind} virion nucleocapsids and 980 counts per min per μg of protein for VS_{NJ} virion nucleocapsids. Protein was measured by the method of Lowry et al. (13) with bovine serum albumin as a standard.

interest that considerably greater antibody against N protein in the anti-whole VS virus serum (Table 1) did not significantly enhance its anti-transcriptase activity.

Effect on RNA synthesis of anti-NS gamma globulin added to nucleocapsids after initiation of transcription. Preliminary experiments were performed to gain some insight into whether anti-NS gamma globulin acts by blocking initiation and reinitiation of RNA synthesis or by blocking chain elongation of strands already initiated or, of course, both initiation and elongation. One would anticipate that if antibody acts only on the initiator molecules, RNA synthesis would continue for a time, owing to elongation of already initiated chains. If, however, antibody acts only on chain elongation, one would expect rapid switch-off of all RNA synthesis when antibody is added during any stage of the transcriptase reaction.

To determine whether anti-NS antibody actively inhibits RNA synthesis after transcription has already begun, samples containing 25 μl of VS_{Ind} nucleocapsids plus 50 μl of the transcription reaction mixture were incubated at 31 C for 0, 15, and 30 min. At these intervals after mixing the nucleocapsids and the transcription reaction mixture, either 25 μl of undiluted anti-NS gamma globulin in phosphate buffer or 25 μl of phosphate buffer alone was added to duplicate samples of the preincubated nucleocapsid-transcription mixtures. Samples removed at successive 15-min intervals after adding antibody or buffer were analyzed for acid-precipitable [^{3}H]RNA.

Figure 5 demonstrates that anti-NS gamma globulin added to transcribing nucleocapsids at each stage after the reaction is initiated completely stops further progression of RNA synthesis. These data indicate that anti-NS gamma globulin probably acts by interrupting RNA chain elongation and possibly re-initiation.

DISCUSSION

The data reported here strongly suggest but do not prove conclusively that the anti-tran-

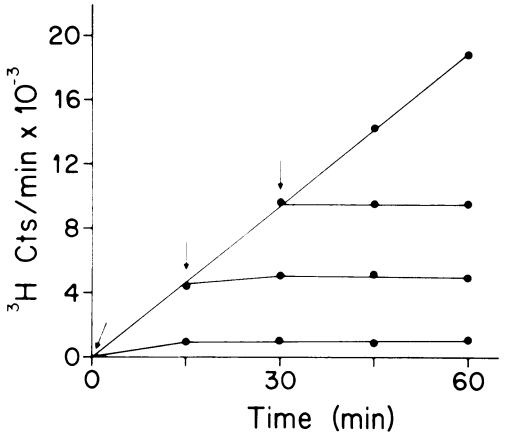

FIG. 5. *Effect of addition of anti-NS gamma globulin to actively transcribing nucleocapsids. Duplicate 25-μl preparations of gradient fractionated VS_{Ind} nucleocapsids were incubated with 50 μl of nucleoside triphosphate reaction mixture for 0, 15, or 30 min at 31 C. After each preincubation period, either 75 μg of anti-NS gamma globulin in 25 μl of standard buffered phosphate or 25 μl of buffer alone was added, and incubation was continued for a total of 60 min. Samples were removed every 15 min for determination of [^{3}H]UMP incorporation into trichloroacetic acid-precipitable RNA. Arrows mark times when anti-NS gamma globulin or buffer was added to preincubated nucleocapsid transcription mixtures.*

scriptase activity of anti-NS_{Ind} serum is directed specifically against the action of the NS protein. Anti-NS serum reacted quite strongly with NS protein by immunodiffusion and by radioimmunoprecipitation but also coprecipitated some N protein from a VS virus-infected cell extract. Precipitation of N ^3H-labeled protein by anti-NS serum could well be nonspecific and, in any case, anti-NS serum precipitated proportionately much less N ^3H-labeled protein than did anti-whole VS virus serum. Absorption of anti-NS gamma globulin with N protein in the form of nucleocapsids did not result in diminution of anti-transcriptase activity. On the other hand, soluble NS protein or Bio-Gel-linked NS protein reduced the anti-transcriptase activity of anti-NS serum to only a limited extent, possibly because the NS protein was used at too low a concentration and has a low affinity for binding anti-transcriptase gamma globulin.

Although these experiments do not rule out the possibility, it seems unlikely that contaminating antibody to N protein plays a major role in inhibition of RNA synthesis by anti-NS gamma globulin. The anti-transcriptase activity of anti-whole VS_{Ind} virion gamma globulin was no greater than that of anti-NS_{Ind} gamma globulin despite a much higher titer of anti-N precipitin activity per microgram of gamma globulin. Some attempts were also made by Norman Moore in our laboratory to raise specific antibody to N protein, which is both difficult to prepare and to separate from NS protein (unpublished data). Although the anti-N titer of these sera were quite low, they did not inhibit transcriptase activity of VS_{Ind} nucleocapsids (unpublished data). Also, only abortive attempts have been made to produce anti-L protein serum because sufficient quantities of undenatured L protein have not yet been available. An obvious extension of the present studies is to make a concerted effort to raise monospecific anti-L and anti-N serum to compare their anti-transcriptase activity with that of anti-NS gamma globulin, particularly for use in reconstituted enzyme-template transcription systems (9).

The absent or negligible effect of anti-NS_{Ind} gamma globulin and anti-whole VS_{Ind} virus gamma globulin on transcription by VS_{NJ} nucleocapsids provides an important control by ruling out ribonuclease as the serum transcriptase inhibitor. The VS_{Ind} and VS_{NJ} viruses are antigenically related by group-specific, complement-fixing antigens (5). These cross-reacting antigens of VS_{Ind} and VS_{NJ} viruses are attributable to nucleocapsid protein, almost undoubtedly the major N protein. Therefore, if anti-N gamma globulin possesses anti-transcriptase activity, one might anticipate inhibition of VS_{NJ} transcriptase as well as the VS_{Ind} enzyme. Therefore, it is quite striking that anti-whole VS_{Ind} virus gamma globulin, which contains high-titered antibody for VS_{Ind} N protein (12) in addition to anti-VS_{Ind} transcriptase activity, was completely without effect on VS_{NJ} nucleocapsid transcription. It seems unlikely, therefore, that anti-N gamma globulin is the anti-transcriptase.

The anti-transcriptase specificity of anti-NS_{Ind} gamma globulin and anti-whole VS_{Ind} virus gamma globulin only for VS_{Ind} transcriptase is consistent with other genetic and physiological properties of VS_{Ind} and VS_{NJ} viruses. The genomes of VS_{Ind} and VS_{NJ} viruses exhibit little RNA base-sequence homology (20) and *ts* mutants of these viruses share no genetic complementation groups (19). Moreover, Bishop et al. (2) were unable to demonstrate cross-complementation of VS_{Ind} and VS_{NJ} transcription by heterologous reconstitution of the nucleocapsid templates and transcriptase enzymes of the two viruses. It would appear, therefore, that the transcriptases of VS_{Ind} and VS_{NJ} viruses are physiologically as well as immunologically unrelated. Similar immunological specificity is exhibited by the reverse transcriptases of RNA tumor viruses (15).

More definitive studies on selective inhibition of the three proteins potentially involved in transcription of various rhabdoviral nucleocapsids will require a major undertaking to produce large amounts of highly purified L, N and NS proteins for use as specific antigens. Monospecific antibodies to each of these antigens from various rhabdovirus species may provide incisive probes to identify which nucleocapsid proteins regulate initiation, elongation, and termination of transcription.

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