Rabies mRNA Translation in *Xenopus laevis* Oocytes

WILLIAM H. WUNNER,* PETER J. CURTIS, AND TADEUSZ J. WIKTOR

The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104

Two rabies virus-specific mRNA species were identified by analysis of their encoded proteins after translation of the partially purified species in *Xenopus laevis* oocytes. One of these coded for the virion surface glycoprotein (G protein), and the other coded for the major structural protein of the virion nucleocapsid (N protein). The G-mRNA sedimented in a sucrose density gradient at about 18S, and the N-mRNA had a sedimentation coefficient of approximately 16S. Their respective translation products were identified in a radioimmunoassay with specific monoclonal antibody probes that recognized only G or N proteins. Immunoprecipitates formed between the radiolabeled viral antigens synthesized in programmed oocytes and their respective monoclonal antibodies were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The glycoprotein antigen translated from G-mRNA in oocytes migrated in the gel ahead of the virion G protein with a migration rate that was similar to that of nonglycosylated intracellular glycoproteins from virus-infected cells. The result suggested that the branched-chain carbohydrate of G protein was not required for recognition by the particular monoclonal antibody used. The nucleocapsid antigen translated from N-mRNA in oocytes migrated to the same position in the gel as marker virion N protein. Both the electrophoretic mobility of virus-specific antigens in sodium dodecyl sulfate-polyacrylamide gel and the antibody concentration dependence for immunoprecipitations were criteria for identifying the individual viral proteins encoded by the two rabies mRNA's.

Rabies virus, the prototype member of the genus *Lyssavirus* within the family Rhabdoviridae (3), replicates less efficiently than its counterpart member of genus *Vesiculovirus*, vesicular stomatitis virus. In suitable tissue culture systems, growth of rabies virus is normally slower than vesicular stomatitis virus at comparable multiplicities of infection, and virus yield (PFU per milliliter) is usually 2 to 3 log units lower (4). Estimates of replication efficiency based on the transcriptase activity in purified rabies virions (11, 13) and viral transcribing nucleoprotein complexes in vivo (22) compared with that of vesicular stomatitis virus suggest that the lower rate of transcription of the rabies viral genome RNA is directly related to the growth efficiency of the virus. Until now, detection of rabies virus-specific mRNA in virus-infected cells has been hindered by the low level of RNA transcription, which has been aggravated further by the presence of actinomycin D required to suppress host cell RNA synthesis (23). Consequently, complementary monocistronic mRNA molecules corresponding to each of the five structural genes (L, G, N, M₁, and M₂) of the rabies genome (10) have not yet been identified by gel electrophoresis. However, two size classes of RNA synthesized in vivo in the presence of actinomycin D, two-thirds of which

was complementary to the viral genome RNA (9), have been described after velocity sedimentation analysis in sucrose gradients. After binding this RNA to polyuridylic acid-Sepharose, 25% of the smaller RNA (8 to 25S) and 12% of the larger RNA (25 to 35S) contained polyadenylic acid [poly(A)] tracts which were indicative of rabies virus-specific mRNA (8). In one report (23) five actinomycin D-resistant RNA species were detected in denaturing polyacrylamide gel after extraction of RNA from persistently rabies virus-infected BHK-21 cells. Although molecular weights of the individual RNA species were approximately the size for the expected coding capacities of monocistronic mRNA's of the five structural proteins of the rabies virion, no attempt was made to identify these RNA species as viral protein-specific mRNA's or assign gene products on the basis of molecular size.

Assignments of viral protein to monocistronic mRNA can be made by comparing the coding capacity of each mRNA species with the size of known viral proteins (17, 19). The ultimate assignment would be better obtained by translation of individual mRNA species in vitro (2, 16). In this report we present evidence that two rabies mRNA's analyzed thus far show individual specificity for synthesis of: (i) the rabies surface glycoprotein (G protein) and (ii) the

nucleocapsid (N) protein, and that each of the mRNA's is correlated with its product of translation on the basis of a correspondence between RNA coding capacity and polypeptide size. We describe the use of *Xenopus laevis* oocytes for efficient translation of microinjected rabies mRNA's that have been partially separated by sucrose gradient centrifugation. The assay for mRNA is completed by the positive identification of translation products by selective immunoprecipitation with monoclonal antibodies of hybridoma origin.

MATERIALS AND METHODS

Cells and virus. All mRNA preparations were made from BHK-21/S13 cells infected with plaque-purified fixed ERA strain rabies virus. BHK-21 cell-adapted ERA rabies virus has been described previously (5).

Infection of cells and extraction of total cell RNA. We infected monolayer cultures of 2×10^6 BHK-21/S13 cells in roller bottles with stock virus (10^9 PFU/ml) at a multiplicity of infection of 25 PFU per cell. The virus was adsorbed at room temperature (20°C) for 1 h before infected cells were incubated at 37°C in the presence of [3 H]uridine (5 μ Ci/ml; 25 Ci/mmol) in modified Eagle medium containing 0.2% bovine serum albumin. The culture fluid was decanted after 20 h, and the infected cells were scraped into high-salt buffer containing 0.18 M NaCl in 0.01 M Tris-hydrochloride, pH 7.4, washed three times in high-salt buffer, and finally suspended in TE buffer (20 mM Tris-hydrochloride-1 mM EDTA, pH 7.5) to which was added 20% sodium dodecyl sulfate (SDS) and 20 mg of pronase (predigested) per ml to give final concentrations of 2.5% and 200 μ g/ml, respectively. After incubation at room temperature (20°C) for 30 min or 37°C for 10 min, total infected cell RNA was extracted twice in phenol (saturated with TE buffer), and RNA was precipitated from ethanol, dried under nitrogen, and resuspended in TE buffer. The RNA was extracted a third time with phenol and reprecipitated from ethanol. To remove DNA, the precipitated nucleic acid was first dissolved in water and then adjusted to 50 mM Tris-hydrochloride (pH 7.5), 10 mM $MgCl_2$, 2 mM $CaCl_2$, and 50 μ g of DNase (pretreated with sodium iodacetate) per ml. Incubation at 37°C for 5 to 10 min was sufficient to reduce drastically the viscosity of the solution, at which point the solution was adjusted to 20 mM EDTA and 0.2% SDS followed by extraction with an equal volume of phenol. The aqueous phase was dialyzed versus 20 mM Tris-hydrochloride (pH 7.5)-1 mM EDTA and stored at -20°C at a concentration of 1 mg/ml.

[35 S]methionine labeling and extraction of infected cell proteins. Monolayer cultures of 4×10^7 to 5×10^7 BHK-21/S13 cells in three T-75 flasks were infected with ERA strain rabies virus as described above, except that regular Eagle medium with 0.2% bovine serum albumin was replaced with methionine-free Hanks medium 199 (GIBCO Laboratories, Grand Island, N.Y.) to label infected cells from 4.5 to 24 h, 24 to 48 h, and 48 to 72 h postinfection and uninfected

cells for 24 h with [35 S]methionine (20 μ Ci/ml; >1,000 Ci/mmol). At the end of the labeling period, cells were scraped into the culture fluid and pelleted by low-speed centrifugation. The combined cells were washed three times in high-salt buffer and finally suspended in 0.8 ml of lysis buffer containing 0.5% Nonidet P-40, 0.15 M NaCl, 5 mM EDTA, and 200 μ g of phenylmethylsulfonylfluoride per ml in 50 mM Tris-hydrochloride, pH 7.4. The cell suspension was sonicated four times, 15 s each time, to break up DNA and clarified in Beckman cellulose nitrate microtubes by centrifugation in an SW 50.1 rotor at 12,000 $\times g$ and 4°C for 30 min. The clarified cell lysate (supernatant) was extracted from microtubes by side puncture with syringe and needle injected immediately above the cell debris pellet. In this way, most of the lipid that appeared at the top remained adsorbed to the wall of the tube. Extracted cell proteins were stored at -70°C for isolation of viral antigen.

Oligodeoxythymidylic acid-cellulose chromatography. Total infected cell RNA in TE buffer was heat denatured (100°C, 45 s) and adjusted to 2 $\times$ TNE (TNE: 0.02 M Tris-hydrochloride [pH 7.5], 0.15 M NaCl, 2 mM EDTA), 0.2% SDS, 0.2% polyvinylsulfate and applied to an oligodeoxythymidylic acid-cellulose column (1-cm diameter) previously equilibrated in 2 $\times$ TNE. The RNA on the column was washed through with 2 $\times$ TNE (20 bed volumes) at a flow rate of 0.5 ml/min. Poly(A)-containing [poly(A) $^+$] RNA that bound to the column was eluted by alternatively applying 1 ml of 10 mM Tris-hydrochloride (pH 7.4), 0.1% SDS, and water. RNA fractions were monitored by UV absorption, and pooled RNA fractions were precipitated twice with ethanol in 0.3 M sodium acetate, pH 5.5. The poly(A) $^+$ RNA concentration was adjusted to 1 mg/ml for oocyte injection and stored at -20°C.

Translation assay in *X. laevis* oocytes. Poly(A) $^+$ RNA (1 mg/ml) was microinjected into 10 oocytes so that each oocyte received 50 ng of poly(A) $^+$ RNA. The oocytes were incubated at room temperature (20°C) for 24 h in modified Barth medium (12). Oocytes were labeled with [35 S]methionine (>1,000 Ci/mmol), which was evaporated to dryness and dissolved in Barth medium at a concentration of 200 μ Ci/75 μ l per 10 oocytes. After incubation, oocytes were washed three times in cold Tris-glycine buffer (52 mM Tris, 52 mM glycine) and homogenized in 250 μ l of Tris-glycine buffer per 10 oocytes. Cell debris was removed by centrifugation for 5 min at 12,800 $\times g$ in the Eppendorf microcentrifuge, and the supernatant of clarified cytosol was stored at -70°C.

RIA and immunoprecipitation with monoclonal antibodies. A radioimmunoassay (RIA) was prepared in triplicate for the detection of viral antigen in 10 μ l of clarified oocyte supernatant. Selected monoclonal antibodies of hybridoma origin (HAB) with specificity for virion glycoprotein (HAB_G) or nucleocapsid protein (HAB_{NC}) were used according to described techniques (11a). 125 I-labeled rabbit anti-mouse (Fab')₂ fragments were kindly provided by W. Gerhard of the Wistar Institute.

Viral antigens were extracted from oocytes and rabies virus-infected cells by immunoprecipitation

with antigen-specific HAb produced in mouse ascites fluid (11a). The precipitates were collected by adsorption to a 10% suspension of formaldehyde-fixed *Staphylococcus aureus* (6) kindly provided by T. Dolby of the Wistar Institute.

Viral antigens were extracted from oocytes after the clarified cytosol was mixed with an equal volume of 2× lysis buffer and further clarified by centrifugation at $12,000 \times g$ as described for infected cell proteins. Aliquots (200 μ l) of clarified lysates from oocytes and infected cells were mixed with 10 μ l of HAb in undiluted ascitic fluid or serial 10-fold dilutions (with lysis buffer) for 30 min in ice before 25 μ l of 10% *S. aureus* suspension in lysis buffer was added to each antigen-antibody reaction tube. Incubation was continued in ice for 20 min with frequent (every 2 to 3 min) agitation on a Vortex mixer, and immune complexes adsorbed to *S. aureus* were pelleted by centrifugation for 6 to 8 s in an Eppendorf microcentrifuge. The supernatant was separated from the pellet and saved for further antigen extractions; the pellet was washed several times with 1 ml of cold wash buffer containing 5% sucrose, 1% Nonidet P-40, 0.5 M NaCl, 5 mM EDTA, and 15 mM Tris-hydrochloride (pH 7.4), resuspended by Vortex action, and repelleted by centrifugation for 20 s in an Eppendorf microcentrifuge. Removal of excess counts was monitored in 50 μ l of each wash. Finally, the antigen-antibody complexes were dissolved in 20 μ l of modified Laemmli sample-dissociating buffer (0.0625 M Tris-H₃PO₄, pH 6.8, 2% SDS, 0.2% dithiothreitol, 0.1% EDTA, 10% glycerol) (14) by heating at 95°C for 90 s for electrophoresis in an SDS-polyacrylamide gel. Samples were stored in 2 mM phenylmethylsulfonyl fluoride at -70°C.

SDS-polyacrylamide gel electrophoresis. Discontinuous Laemmli gels of 10% SDS-polyacrylamide were prepared essentially as described elsewhere (14), except slab gels were cast 1.5 mm thick and 13 cm long between glass plates (20). The 0.8-cm stacking gel of 4% acrylamide contained 14 sample slots. The stacking gel buffer system was modified from the Laemmli system by substituting Tris-H₃PO₄ for Tris-hydrochloride (21). Gels were loaded with 50 μ l of sample (1 to 5 μ g of protein; 20,000 to 150,000 cpm), and electrophoresis was performed at room temperature for 4 to 5 h at 20 mA until the migration front followed by bromophenol blue that was added to each sample before electrophoresis was 1 cm from the bottom of the gel. After electrophoresis, the gels were fixed and stained in 50% methanol-7% acetic acid containing 0.25% Coomassie brilliant blue. Gels were destained by gentle rocking in 50% methanol-7% acetic acid and photographed before drying under vacuum. Dried gels were autoradiographed by exposure to RP Royal X-Omat (Kodak) film at -70°C with Cronex lightning-plus intensifying screens. Exposed films were developed in Kodak liquid X-ray developer for 5 min, put in 25% acetic acid for 10 s, fixed for 5 min in Kodak rapid fixer, washed for 30 min, and dried.

RESULTS

Translation of rabies mRNA in *X. laevis* oocytes. Our initial assay of virus-specific mRNA translation in programmed oocytes was

performed with total poly(A)⁺ RNA (ERA-RNA) extracted from ERA rabies virus-infected cells. Similarly prepared RNA from uninfected cells served as the control. Viral antigens synthesized in oocytes injected with ERA-RNA were detected by RIA with HAb specific for virion glycoprotein and nucleocapsid antigens. Table 1 shows that a positive response was obtained, which suggested that the oocyte system was capable of faithfully translating rabies mRNA. The response with HAb_G was approximately eightfold, and HAb_{NC} was approximately twice the background levels observed with oocytes that were programmed with poly(A)⁺ RNA from uninfected cells (S13-RNA) or control uninjected (-RNA) oocytes. Whole virus antigen adjusted to a protein concentration of 2 μ g/ml at the same time gave a typical response with the HAb_G and HAb_{NC} in RIA (11a, 11b). Since the detection of viral antigen was at a low level, the RIA was repeated on undiluted and one-half and one-fifth dilutions of a second set of oocyte lysates containing viral antigens to demonstrate that the response, although slight, was real and concentration dependent. The results clearly showed that the detection of viral antigen in oocytes programmed with ERA-RNA was concentration dependent; the values for undiluted extract (190 and 266 cpm with HAb_G and HAb_{NC}, respectively) were reduced to approximately 50% (56 and 120 cpm) and 20% (23 and 57 cpm) in the one-half and one-fifth dilutions of the extract.

To increase the efficiency of translating rabies mRNA in oocytes, we fractionated the poly(A)⁺ RNA from virus-infected cells on a sucrose density gradient and collected different size RNAs (based on sedimentation coefficients) from the gradient in pooled pairs of adjacent gradient fractions. The RNA from each pool was concentrated to 1 mg/ml and microinjected into oocytes for translation. In Fig. 1, the response in separate RIAs with HAb_G and HAb_{NC} to translation products in oocytes programmed with individual pooled mRNA fractions is shown across the

TABLE 1. RIA of viral antigen produced in *X. laevis* oocytes programmed with total poly(A)⁺ RNA

Antigen source	¹²⁵ I cpm bound in the presence and absence of:		
	HS-PBS ^a	HAb _G	HAb _{NC}
Virus	292	5,910	5,250
Oocyte - RNA	12	39	27
Oocyte + S13-RNA	22	25	24
Oocyte + ERA-RNA	19	207	50

^a Ten percent γ -globulin-free horse serum (HS-PBS) was used as diluent for monoclonal antibodies.

FIG. 1. Detection of translation products in *X. laevis* oocytes programmed with poly(A)⁺ RNA from rabies virus-infected cells. Total infected cell RNA was separated on oligodeoxythymidylic acid-cellulose as described in the text. The poly(A)⁺ RNA was then fractionated in a 5 to 23% (wt/vol) sucrose density gradient by centrifugation at $76,400 \times g$ and 10°C for 17 h in an SW41 rotor. The RNA in pooled pairs of adjacent fractions was concentrated to 1 mg/ml and microinjected into oocytes for translations. The oocyte lysates were tested by separate RIAs for translation products with monoclonal antibodies against the glycoprotein or nucleocapsid protein, and the counts above background (38 cpm) are plotted for each pair of gradient RNA fractions. rRNA (28S and 18S) and 4S RNA markers were sedimented in a parallel sucrose gradient and detected by UV absorbance at 260 nm.

RNA gradient. Since the virion G protein has a molecular weight of approximately 70,000 (minus carbohydrate), the size of mRNA coding for this protein (G-mRNA) would be expected to be 1.8 kilobases long and sediment at about 18S. Similarly, the N protein with a molecular weight of 60,000 would be coded for by an mRNA (N-mRNA) of 1.6 kilobases sedimenting at about 16S. The response with HAb_G was greatest in oocytes that were programmed with mRNA that sedimented further into the sucrose gradient, whereas the response to HAb_{NC} was greatest in oocytes that were programmed with mRNA that sedimented more slowly. The results suggested that the rabies G-mRNA was larger than rabies N-mRNA, which is consistent with the hypothesis that the size of rabies monocistronic mRNA's and the size of the encoded polypeptide are directly related.

Extraction of viral antigen from oocytes programmed with rabies mRNA. *Xenopus* oocytes were injected with unfractionated poly(A)⁺ ERA-RNA or with pooled, fraction 7 mRNA (gradient fractions 13 and 14, Fig. 1); the latter showed a strong response in RIA to both HAb_G and HAb_{NC}. The injected oocytes were incubated for 24 h in the presence of [³⁵S]methi-

onine to label the translation products of exogenous mRNA. After translation, aliquots of the oocyte lysate were mixed with undiluted or serial 10-fold dilutions of ascitic fluid containing HAb_G, and antigen-antibody complexes were precipitated with formaldehyde-fixed *S. aureus*. Dissociated protein in the immunoprecipitate was analyzed in SDS-polyacrylamide gel. After electrophoresis, the stained gel was compared with the autoradiogram of the same gel. In both the stained gel and its autoradiogram, the background protein was minimal, thus making it possible to identify the virus-specific antigens. Ascites fluid protein, extracted with each immunoprecipitate in the stained gel (Fig. 2), decreased proportionally with each dilution of the monoclonal antibody fluid used until none was detected in the final (10^{-4}) dilution. Viral antigen labeled with [³⁵S]methionine was identified in Fig. 2 (autoradiogram) by the same dilution criterion, assuming that the amount of antigen interacting with antibody was proportional to the concentration of antibody present in the reaction mixture. A labeled polypeptide (G₀) corresponding to the nonglycosylated form of G protein (15) was detectable in diminishing amounts with antibody dilutions of 10^{-1} to 10^{-4} , suggesting specificity for HAb_G. Moreover, the specificity of the reaction was reflected in the increased amount of radiolabeled G₀ going from undiluted to a 10^{-1} dilution, which suggested that a 1:10 dilution of antibody-containing fluid was optimal for antigen-antibody interaction. All other labeled polypeptides appearing in the gel were detected in near equal quantities regardless of antibody dilutions, suggesting they were non-specifically adsorbed and co-precipitated.

The lysate supernatants that were saved after each reaction to extract antigen with HAb_G were subsequently mixed with HAb_{NC} in undiluted and serial 10-fold dilutions of ascitic fluid, respectively. The stained gel and its autoradiogram in Fig. 3 showed that ascites fluid proteins and labeled antigen, respectively, diminished with each dilution of antibody. The major [³⁵S]methionine-labeled polypeptide precipitated from oocytes also migrated coincidentally with virion N protein marker. Both observations provide evidence that N-mRNA was translated faithfully in oocytes. The apparent homogeneity of N antigen in the SDS-polyacrylamide gel was illustrated by the lack of shoulder peaks associated with the N protein peak in tracings of the autoradiogram (data not shown).

Extraction of viral antigen synthesized in rabies virus-infected cells. The comparison of putative viral antigens derived from rabies mRNA translation in oocytes with viral antigens

FIG. 2. SDS-polyacrylamide gel electrophoresis of immunoprecipitates formed between oocyte translation products and glycoprotein-specific monoclonal antibody (HAb_G). *X. laevis* oocytes were programmed to translate total (unfractionated) poly(A)⁺ RNA from rabies virus-infected cells or pooled fraction 7 (gradient fractions 13 and 14) of sucrose gradient-fractionated RNA and labeled with [³⁵S]methionine (Fig. 1). Translation products were mixed with HAb_G in undiluted ascites fluid (ND) or serial 10-fold dilutions of the ascitic fluid containing antibody and immunoprecipitated with *S. aureus* as described in the text. Protein in the immunoprecipitates was visualized after SDS-polyacrylamide gel electrophoresis in the stained gel (top) and the autoradiogram of the same gel (bottom). Ascites fluid proteins (AFP) and rabies virus structural proteins (G, N, M₁, and M₂) in lane 1 were visible in the stained gel. Presumptive nonglycosylated glycoprotein (G₀) migrated ahead of glycosylated virion protein (G).

synthesized in vivo was a necessary adjunct in the characterization of oocyte translation products. We labeled cells with [³⁵S]methionine between 4.5 and 24 h and from 24 to 48 h and 48 to 72 h after infection with ERA rabies virus. Uninfected cells were labeled with [³⁵S]methionine for 24 h as controls and processed in parallel to the infected cells for the extraction of viral antigens. Cell lysates were mixed first with HAb_G to extract the intracellular viral G protein in a procedure similar to that described for oocytes. Neither ascitic fluid nor *S. aureus* was preab-

sorbed with unlabeled uninfected BHK-21/S13 cell proteins in these experiments. Figure 4 shows the result of SDS-polyacrylamide gel electrophoresis of HAb_G-specific antigen extracted from infected cells by immunoprecipitation with *S. aureus*. A single polypeptide, designated G₀, was extracted in decreasing amounts as antibody dilutions decreased from 10⁻¹ to 10⁻⁴, suggesting specificity between G₀ polypeptide and HAb_G. Another polypeptide that co-extracted with the G₀ polypeptide was visible between G₀ and N in the autoradiogram. The electrophoretic mobility

FIG. 3. SDS-polyacrylamide gel electrophoresis of immunoprecipitates formed between oocyte translation products and nucleocapsid protein-specific monoclonal antibody (HAb_{NC}). *X. laevis* oocyte lysates that were used to extract rabies virus-specific antigens with HAb_G (Fig. 2) were similarly mixed with undiluted (ND) or serial 10-fold dilutions of ascitic fluid-containing HAb_{NC} . Immunoprecipitates were collected and proteins were visualized after SDS-polyacrylamide gel electrophoresis in the stained gel (top) and the autoradiogram (bottom). Protein markers are as described in Fig. 2.

of this polypeptide corresponded to that of a cellular polypeptide from *S. aureus* in the subsequent immunoprecipitation with HAb_{NC} (see Fig. 7) and in immunoprecipitations with HAb_G from uninfected cells (Fig. 5). These nonspecific polypeptides were extracted in quantities that were independent of antibody concentration.

The detection of a HAb_G -specific intracellular antigen, which migrated in SDS-polyacrylamide gel faster than purified virion G protein marker, suggested that the intracellular polypeptide was the immature (not fully glycosylated) form of the virion surface G protein (15). We repeated the extraction with HAb_G and two extended growth cultures of rabies virus-infected cells (multiplicity of infection = 25) which were labeled with [^{35}S]methionine from 24 to 48 h and

48 to 72 h postinfection in an attempt to extract the fully glycosylated (mature) G protein from these cells. The electrophoretic mobility of the HAb_G -specific antigen (G_0) extracted as late as 48 to 72 h (Fig. 6) was identical to that of the G_0 polypeptide obtained 24 h postinfection.

The supernatants of the infected and uninfected cell lysates once extracted with HAb_G were re-extracted, respectively with HAb_{NC} , ND, and 10^{-1} to 10^{-4} dilutions. An analysis of the immunoprecipitates from 24-h-infected cells is shown in Fig. 7. In the autoradiogram of the SDS-polyacrylamide gel, a ^{35}S -labeled polypeptide was detected with an electrophoretic mobility which corresponded to that of virion N-protein marker, and it diminished in quantity as antibody concentration was decreased. An ad-

FIG. 4. Immunoprecipitation of ³⁵S-labeled BHK-21 cell-derived viral antigens with HAb_G. Rabies virus-infected cell lysates were prepared 24 h after infection and mixed with HAb_G in undiluted ascites fluid (ND) or in serial 10-fold dilutions of the ascitic fluid. The antigen-antibody complexes were precipitated with prefixed *S. aureus* and analyzed by SDS-polyacrylamide gel electrophoresis as described in the text. Ascites fluid proteins (AFP) and rabies virus proteins (G, N, M₁, and M₂) were seen clearly in the stained gel (top). The autoradiogram (bottom) showed nonglycosylated virus-specific glycoprotein (G₀) which migrated ahead of the virion structural glycoprotein (G).

ditional labeled polypeptide corresponding to virion M₁ protein also decreased with serial dilutions of HAb_{NC}. Detection of both N and M₁ antigens by this criterion was in marked contrast to the other detectable polypeptides in both the

stained gel and autoradiogram. That these other polypeptides were of cellular origin appearing in near constant amounts regardless of antibody (HAb_{NC}) concentration was corroborated by the uniform recovery of cellular proteins from uninfected cells (data not shown). The co-extraction of N and M₁ antigens from infected cells with HAb_{NC} suggested that HAb_{NC} either had dual specificity for the soluble antigens or was monospecific for N protein and co-precipitated

FIG. 5. Immunoprecipitation of ³⁵S-labeled uninfected BHK-21 cell proteins with HAb_G. Uninfected cells were labeled with [³⁵S]methionine, and cell lysates were mixed with HAb_G in ascites fluid as prepared in Fig. 4. The immunoprecipitates were resolved on SDS-polyacrylamide gel and visualized in the stained gel (top) and in the autoradiogram (bottom) along with ¹⁴C-labeled proteins of purified rabies virions.

FIG. 6. Microdensitometer tracings of autoradiograms showing virus-specific antigen (G_0) from rabies virus-infected BHK-21 cells immunoprecipitated with HAb_G . Top tracing is of ^{14}C -labeled virion (G , N , M_1 , and M_2) protein markers. Each tracing below the top is an immunoprecipitate in which virus-specific antigen synthesized and labeled from 48 to 72 h postinfection interacted with HAb_G in undiluted (ND) or serial 10-fold dilutions of ascites fluid.

with it the M_1 protein as an integral part of the nucleocapsid structure. Experiments are in progress to rule out the unlikely possibility of dual specificity by reacting HAb_{NC} with antigen translated from purified M_1 -mRNA microinjected into oocytes.

DISCUSSION

Two rabies virus-specific mRNA species have been identified as monocistronic RNA transcripts, each coding for distinct viral polypeptides. This was done by using monoclonal antibodies specific for rabies mRNA translation

products in *Xenopus* oocytes and by correlating the size of mRNA and that of its encoded polypeptide.

The resolution of rabies virus-specific G-mRNA and N-mRNA by sucrose gradient centrifugation coincided with the sedimentation of purified vesicular stomatitis virus-specific G-mRNA and N-mRNA of comparable molecular weights, respectively, that were previously sep-

FIG. 7. Immunoprecipitation of ^{35}S -labeled BHK-21 cell-derived viral antigens with HAb_{NC} . Rabies virus-infected cell lysates (24 h postinfection) were mixed with HAb_{NC} after the same lysates were extracted with HAb_G (Fig. 4). Antigen-antibody complexes formed with HAb_{NC} in undiluted (ND) and serial 10-fold dilutions of the ascitic fluid were precipitated and analyzed by SDS-polyacrylamide gel electrophoresis as described in Fig. 4. The protein designations are described also in Fig. 4 for the stained gel (top) and autoradiogram (bottom).

arated by polyacrylamide gel electrophoresis (18). Separation of poly(A)⁺ RNA into G-mRNA- and N-mRNA-rich fractions resulted in a 5- to 10-fold greater response in the RIA compared with unfractionated poly(A)⁺ RNA. The synthesis of viral antigen (1 to 4 ng) in individually programmed oocytes possibly varied due to the actual amounts of mRNA injected into each oocyte and the "recruitment" rate of different mRNA's for translation (1). These factors are reflected in the increased RIA response when oocytes were programmed with sucrose gradient fractionated mRNA's.

The isolation of immunochemically pure viral antigens from programmed oocytes by sequential immunoprecipitation with monoclonal antibodies demonstrated that single preparations of mixed mRNA species could be analyzed in this system. We noticed that monoclonal antibody specific for virion G protein interacted with a polypeptide, G₀, which is synthesized in both *Xenopus* oocytes and BHK-21 cells. This polypeptide migrated in SDS-polyacrylamide gel electrophoresis ahead of the G protein marker, suggesting that G₀ represents the immature (not fully glycosylated) form of rabies virion G protein (15). Since the G₀ polypeptide reacted with the monoclonal antibody, we concluded that the carbohydrate moiety of the mature G protein was not part of the critical antigenic determinant. The absence of an oocyte HAb_G-specific polypeptide with the same electrophoretic mobility as mature G protein indicated either that G₀ polypeptide was not synthesized on membrane-bound polyribosomes where it might have been glycosylated or that glycosylation was not sufficient to produce a molecule equal to the molecular size of the virion G protein. The extent of glycosylation of foreign proteins in oocytes has not been determined. Nevertheless, the oocyte translation system was not expected to perform the same post-translational modifications on the rabies-specific glycoprotein that take place in virus-infected mammalian cells (7). If glycosylation did occur in the oocyte translation system, it would presumably modify transmembrane molecules destined for export from the oocyte, and such proteins would probably not be found in clarified oocyte lysates. We attribute absence of rabies virus-specific glycopolypeptide with electrophoretic mobility of G protein in virus-infected cells to fractionation; i.e., such a glycopolypeptide has been detected previously when whole cells were analyzed (15). We conclude that the mature glycopolypeptide is membrane associated and therefore is not present in our clarified cell lysates.

Xenopus oocytes provide a suitable *in vivo*

translation system for the identification of rabies virus-specific monocistronic mRNA's. The assay system coupled with the RIA with monoclonal antibodies has been an integral part of our program to clone the rabies genes in bacterial plasmids.

ACKNOWLEDGMENTS

We gratefully acknowledge the excellent technical assistance of Sally Shane, Erik Whitehorn, and Lynn Clompus.

This work was supported by Public Health Service research grants AI-09706 from the National Institute of Allergy and Infectious Diseases and RR-05540 from the Division of Research Resources.

ADDENDUM IN PROOF

Pennica et al. (Virology 103:517-521, 1980) reported the identification of five rabies virus-specified mRNA species in acid-urea-agarose gels. Four of the mRNA species were translated *in vitro* into products that were identical to authentic virus proteins. The polypeptide coding assignments of the individual mRNA species showed a relationship between sizes of mRNA and translation product which corresponds to the results that we report in this paper.

LITERATURE CITED

1. Berridge, M. V., and C. D. Lane. 1976. Translation of *Xenopus* liver messenger RNA in *Xenopus* oocytes: vitellogenin synthesis and conversion to yolk platelet proteins. *Cell* 8:283-297.
2. Both, G., S. A. Moyer, and A. K. Banerjee. 1975. Translation and identification of the mRNA species synthesized *in vitro* by virion-associated RNA polymerase of vesicular stomatitis virus. *Proc. Natl. Acad. Sci. U.S.A.* 72:274-278.
3. Brown, F., D. H. L. Bishop, J. Crick, R. I. B. Francki, J. J. Holland, R. Hull, K. Johnson, G. Martelli, F. A. Murphy, J. F. Obijeski, D. Peters, C. R. Pringle, M. E. Reichmann, L. G. Schneider, R. E. Shope, D. I. H. Simpson, D. F. Summers, and R. R. Wagner. 1979. Rhabdoviridae. *Intervirology* 12:1-7.
4. Clark, H. F. 1980. Systems for assay and growth of rhabdoviruses, p. 23-41. In D. H. L. Bishop (ed.), *Rhabdoviruses*, vol. 1. CRC Press, Inc.
5. Clark, H. F., and T. J. Wiktor. 1972. Rabies virus, p. 177-182. In S. A. Plotkin (ed.), *Strains of human viruses*. S. Karger, Basel.
6. Cullen, S. E., and B. D. Schwartz. 1976. An improved method for isolation of H-2 and Ia alloantigens with immunoprecipitation induced by protein A-bearing staphylococci. *J. Immunol.* 117:136-142.
7. Dietzschold, B. 1977. Oligosaccharides of the glycoprotein of rabies virus. *J. Virol.* 23:286-293.
8. Ermine, A., and P. Atanasiu. 1978. Polyadenylate (PolyA) sequences associated with rabies virus intracellular ribonucleic acid (RNA) species, p. 437-441. In B. W. J. Malry and R. D. Barry (ed.), *Negative strand viruses and the host cell*. Academic Press Inc., London.
9. Ermine, A., and A. Flamand. 1977. RNA synthesis in BHK-21 cells infected by rabies virus. *Ann. Microbiol. Inst. Pasteur* 128A:477-488.
10. Flamand, A., and J. F. Delagneau. 1978. Transcriptional mapping of rabies virus *in vivo*. *J. Virol.* 28:518-523.
11. Flamand, A., J. F. Delagneau, and F. Bussereau. 1978. An RNA polymerase activity in purified rabies

- virions. *J. Gen. Virol.* **40**:233-238.
- 11a. **Flamand, A., T. J. Wiktor, and H. Koprowski.** 1980. Use of hybridoma monoclonal antibodies in the detection of antigenic differences between rabies and rabies-related viral proteins. I. The nucleocapsid protein. *J. Gen. Virol.* **48**:97-104.
 - 11b. **Flamand, A., T. J. Wiktor, and H. Koprowski.** 1980. Use of hybridoma monoclonal antibodies in the detection of antigenic differences between rabies and rabies-related viral proteins. II. The glycoprotein. *J. Gen. Virol.* **48**:105-109.
 12. **Gurdon, J. B.** 1968. Changes in somatic cell nuclei inserted into growing and maturing amphibian oocytes. *J. Embryol. Exp. Morphol.* **20**:401-414.
 13. **Kawai, A.** 1977. Transcriptase activity associated with rabies virion. *J. Virol.* **24**:826-835.
 14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
 15. **Madore, H. P., and J. M. England.** 1977. Rabies virus protein synthesis in infected BHK-21 cells. *J. Virol.* **22**:102-112.
 16. **Morrison, T., M. Stampfer, D. Baltimore, and H. Lodish.** 1974. Translation of vesicular stomatitis messenger RNA by extracts from mammalian and plant cells. *J. Virol.* **13**:62-72.
 17. **Mudd, J. A., and D. F. Summers.** 1970. Polysomal ribonucleic acid of vesicular stomatitis virus-infected HeLa cells. *Virology* **42**:958-968.
 18. **Rose, J. K., and D. Knipe.** 1975. Nucleotide sequence complexities, molecular weights and poly(A) content of the vesicular stomatitis virus mRNA species. *J. Virol.* **15**:994-1003.
 19. **Stampfer, M., and D. Baltimore.** 1973. Identification of the vesicular stomatitis virus large protein as a unique viral protein. *J. Virol.* **11**:520-526.
 20. **Studier, F. W.** 1973. Analysis of bacteriophage T7 early RNAs and proteins on slab gels. *J. Mol. Biol.* **79**:237-248.
 21. **Tyrrell, D. L. J., and E. Norby.** 1978. Structural polypeptides of measles virus. *J. Gen. Virol.* **39**:219-229.
 22. **Villarreal, L. P., and J. J. Holland.** 1974. Transcribing complexes in cells infected by vesicular stomatitis virus and rabies virus. *J. Virol.* **14**:441-450.
 23. **Villarreal, L. P., and J. J. Holland.** 1976. RNA synthesis in BHK-21 cells persistently infected with vesicular stomatitis virus and rabies virus. *J. Gen. Virol.* **33**:213-224.