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Isolation of a Replication-Efficient Mutant of West Nile Virus from a Persistently Infected Genetically Resistant Mouse Cell Culture

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Flavivirus-resistant mouse embryofibroblasts (C3H/RV) that were infected with West Nile virus, strain E101 (WNV), yielded fewer infectious virions than did cultures of congenic susceptible cells (C3H/HE). Analysis of intracellular viral RNA synthesis indicated that the incorporation of [^{3}H]uridine into 40S genome RNA was markedly reduced in resistant cells, and about 100-fold less labeled 40S RNA was found in pelleted extracellular virions from resistant cultures than in those from susceptible ones. A non-temperature-sensitive mutant of WNV isolated from culture fluid of a persistently infected culture of genetically resistant mouse cells was found to produce higher yields of infectious virus than the parental WNV used to initiate the persistent infection. Analysis of intracellular actinomycin D-resistant RNA indicated that the mutant virus (WNV-RV) was more efficient at incorporating [^{3}H]uridine into 40S RNA in resistant cells than was the parental virus. WNV-RV also synthesized 40S RNA more efficiently than parental virus in congenic susceptible cells and in BHK cells. Analysis of the incorporation of [^{35}S]methionine into viral proteins was likewise enhanced in WNV-RV-infected cells. The WNV-RV mutant provides a tool for studying the regulation of transcription of flavivirus RNA.

Flaviviruses are small (50 to 60 nm in diameter), round, enveloped viruses, which contain a single-stranded 40S genome of plus-strand polarity (20). The replication of flaviviruses takes place entirely in the cytoplasm. Plus- and minus-strand 40S RNAs are both synthesized in low, but approximately equal, amounts during the 12- to 16-h latent period. Thereafter, the transcription of 40S plus-strand RNA predominates (13-15, 19, 20). Unfortunately, the components of the flavivirus polymerase have not been identified, and little is yet known about the regulation of 40S plus-strand RNA synthesis.

In mice, a host gene that codes for resistance to flavivirus-induced encephalitis has been demonstrated. This resistance is inherited as a Mendelian autosomal dominant allele (9, 10, 17). Mice that have this resistance gene support the replication of flaviviruses, but virus yields in their brain tissues are about 3 logs lower and the spread of the infection is slower than in susceptible mice (4, 5, 7). Cell cultures derived from various tissues obtained from resistant (C3H/RV) mice produce lower yields of flaviviruses than do comparable cultures of cells from congenic susceptible (C3H/HE) animals (3, 18). Flavivirus adsorption and penetration apparently occur equally well in resistant and susceptible cells, since the same percentage of cells shows perinuclear virus-positive immunofluorescence in both types of culture by 6 to 8 h after infection (3). The resistance gene product most likely acts on an intracellular flavivirus replication step.

Persistent West Nile virus (WNV) infections have been established in simian virus 40-transformed embryofibroblast cell lines prepared from resistant (C3H/RV) and susceptible (C3H/HE) mouse strains (M. A. Brinton, submitted for publication). A number of temperature-sensitive WNV mutants have been isolated from culture fluids harvested from these persistently infected cell lines during weekly subcultures. We report here the isolation and preliminary characterization of a non-temperature-sensitive mutant (WNV-RV) from culture fluid harvested from a persistently infected resistant cell culture at subculture 25. The incorporation of [^{3}H]uridine into parental WNV 40S virion RNA was found to be less efficient in resistant cells than in susceptible cells, and less infectious virus was produced. In contrast, the WNV-RV mutant replicated efficiently in resistant cells as assessed by [^{3}H]uridine incorporation into 40S RNA and the production of infectious virus. Further study

of the WNV-RV mutant should yield basic information about the regulation of flavivirus 40S plus-strand synthesis.

MATERIALS AND METHODS

Cells and cell culture. The congenic mouse strains, WNV-resistant C3H/RV and WNV-susceptible C3H/HE, were maintained as inbred colonies. Embryofibroblast cultures from RV and HE mice were prepared from 14- to 18-day-old embryos, propagated in Eagle minimal essential medium supplemented with glutamine (0.29 mg/ml), gentamicin (50 μg/ml), and 8% (vol/vol) fetal calf serum, and incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. Cells were used in experiments after the first, second, and third subcultures.

BHK-21/W12 cells (16) were used for plaque titration of WNV and also as a second susceptible cell type from another species for experiments comparing WNV RNA synthesis in resistant and susceptible cells.

Virus. The E101 strain of WNV was originally obtained from J. S. Porterfield and has been passaged approximately 100 times in the brains of newborn mice intermittently during the last 15 years. This virus was successively plaque-purified three times and inoculated intracerebrally into 60 3-day-old inbred Swiss mice (10^4 PFU per mouse). Brains were harvested when mice were moribund, and a clarified 10% (wt/vol) brain homogenate was prepared. This pool was arbitrarily designated WNV-103 and had a titer of 2.5×10^8 PFU/ml. A pool of threefold plaque-purified SAAR 339 strain Sindbis virus was prepared in BHK-21 cells. The titer of this pool was 1.1×10^9 PFU/ml.

The isolate, WNV-RV, was obtained from a plaque picked during titration of a persistently infected resistant culture fluid. After a second plaque purification step, a pool was made by infecting BHK cell monolayers at a multiplicity of infection of 0.5. Culture fluid was harvested 48 h after infection, clarified, aliquoted, and stored at −70°C. This pool had a titer of 3.8×10^8 PFU/ml.

Virus infectivity was assayed by plaque titration on confluent monolayers of BHK-21/W12 cells in six-well Linbro plates. After adsorption of virus for 1 h at room temperature, monolayers were overlaid with minimal essential medium supplemented with 2.5% fetal calf serum, glutamine, gentamicin, and 0.5% (wt/vol) agarose (Seakem). Plates were incubated at 37°C, and plaques were visualized 2 to 3 days later, after addition of a second overlay of the above agarose solution supplemented with neutral red (0.1 mg/ml). Infectivity titers were calculated as the average number of plaques from duplicate-endpoint wells.

Interferon and antibody to interferon. Rabbit anti-mouse interferon antibody was kindly provided by the National Institute of Allergy and Infectious Diseases Antiviral Substances Program. The neutralizing titer of this preparation was 6,000 against 10 U of mouse interferon. Mouse reference interferon was provided by the Research Resources Branch of the National Institute of Allergy and Infectious Diseases. Interferon levels in acid-treated culture fluids were assayed by a cytopathic effect-inhibition assay, using encephalomyocarditis virus and LF-2 cells.

Analysis of virus-specific intracellular RNA synthesis. At indicated times after virus infection (multiplicity of infection of 10), RV, HE, and BHK cell monolayers in T25 flasks were supplemented with 2 μg of actinomycin D per ml and, 1 h later, with 20 μCi of [5-^{3}H]uridine (28 Ci/mmol; New England Nuclear Corp., Boston, Mass.) per ml of culture fluid. After 1.5 h of incubation at 37°C, the culture fluid was discarded, and the monolayer was washed twice with serum-free minimal essential medium. Cells were then lysed with 2 ml of 10 mM Tris-hydrochloride (pH 7.2) containing 1% (wt/vol) sodium dodecyl sulfate, 2 mM vanadyl-ribonucleoside complex (Bethesda Research Laboratories, Rockville, Md.), and 20 μg of self-digested pronase (Sigma Chemical Co., St. Louis, Mo.). The lysates were incubated at 37°C for 15 min with repeated vigorous mixing, extracted three times with phenol, and precipitated with ethanol at −20°C. Precipitates were layered onto linear 15 to 30% (wt/vol) sucrose gradients made in a solution composed of 0.05 M NaCl, 10 mM Tris-hydrochloride (pH 7.2), 10 mM EDTA, and 0.5% (wt/vol) sodium dodecyl sulfate and centrifuged in an SW27 rotor at 22°C for 16 h. Fractions of 1.2 ml were collected by means of a density gradient fractionator and a recording UV analyzer (ISCO 640 and VA-2). Radioactivity incorporated into trichloroacetic acid-insoluble material was measured on glass fiber filters in a toluene-based scintillation fluid.

Isolation of virion RNAs. Confluent cultures of RV and HE cells in T75 flasks were infected with WNV-103 or WNV-RV at a multiplicity of infection of 10. Culture fluids were supplemented with 20 μCi of [^{3}H]uridine per ml 4 h after infection and harvested at 29 h. After clarification by low-speed centrifugation, virus was pelleted at 80,000 × *g* for 2.5 h. Pellets were resuspended in cold 10 mM Tris-hydrochloride (pH 7.2) containing 0.01 M NaCl and 2 mM vanadyl-ribonucleoside complex, and then sodium dodecyl sulfate was added to a final concentration of 1%. After incubation at 37°C for 15 min, the samples were centrifuged on 15 to 30% (wt/vol) sodium dodecyl sulfate-sucrose gradients as described above.

Uninfected control cultures were also labeled, and a small amount of labeled 18S and 28S RNA was observed in all pelleted samples. The amount of [^{3}H]uridine found in cellular RNA was negligible in comparison to that incorporated into extracellular virus particles from WNV-RV-infected cells, but was subtracted from WNV-103 gradient fractions (Fig. 3).

Analysis of intracellular proteins. Confluent RV and HE cultures were infected with WNV-103 or WNV-RV at a multiplicity of infection of 10. At various times after infection, the growth medium (minimal essential medium containing 8% fetal calf serum) was replaced with methionine-free, serum-free minimal essential medium containing 2 μg of actinomycin D per ml, and 4 h later, 50 μCi of [^{35}S]methionine (New England Nuclear Corp.; 1,140 Ci/mmol) per ml was added. After a 3-h labeling period, cell monolayers were washed several times with ice-cold phosphate-buffered saline. Cells were scraped into a small volume of cold phosphate-buffered saline containing 0.5% Nonidet P-40. Sodium dodecyl sulfate and 2-mercaptoethanol were added to final concentrations (wt/vol)

of 2 and 0.1%, respectively. Cellular DNA was sheared by passing the extracts through a 27-gauge needle. Proteins were electrophoresed on 10% polyacrylamide slab gels with a 5% stacking gel, prepared according to the method of Laemmli (8). The gels were fixed in 7% acetic acid, stained with 0.25% (wt/vol) Coomassie blue, dried, and autoradiographed by using Kodak X-Omat XR-5 film.

RESULTS

Isolation of the WNV-RV mutant. Plaques were picked from titration plates of culture fluids harvested weekly from cell lines persistently infected with WNV. Virus in picked plaques was grown for 72 h on BHK monolayers and then tested for ability to form plaques at both 32 and 40°C. One medium-sized plaque, isolated from culture fluid harvested at subculture 25 of a persistently infected resistant cell line which was originally infected with WNV-103, displayed an unusually high titer at both plaquing temperatures. This virus was designated WNV-RV. Titers of about 300 picked plaques had not exceeded 10^5 PFU/ml after growth for 72 h in BHK cells, but the WNV-RV isolate displayed a titer of 1.2×10^9 PFU/ml at both 40 and 32°C.

The WNV-RV mutant was neutralized by two preparations of WNV antibody: a mouse ascites fluid anti-WNV E101 antibody preparation prepared at Walter Reed Army Institute of Research, Washington, D.C., and kindly provided by Walter Brandt; and a rabbit antibody preparation raised against WNV-103. These data confirmed that WNV-RV was a WNV.

Comparison of WNV-103 and WNV-RV replication in resistant and susceptible embryofibroblasts and in BHK cells. Confluent monolayers in T25 flasks of second-passage RV and HE embryofibroblasts and BHK cells were infected with WNV-103 or WNV-RV at a multiplicity of infection of 10. Flaviviruses replicate efficiently in BHK cells; therefore, these cells were used as a susceptible cell from another species for comparison. Culture fluid samples (0.3 ml) were removed at the indicated times for plaque assay and replaced with 0.3 ml of fresh media. The yield of infectious WNV-103 virus from resistant cultures was always observed to be lower than that from comparable susceptible cultures (Fig. 1). However, virus replication was more efficient in BHK cells than in susceptible embryofibroblasts, as indicated by the faster time course of appearance of infectious virus and the increased peak titer of virus in the medium.

The WNV-RV replicated to a higher titer in all three types of cells than did the parental WNV-103 (Fig. 1A, B, and C). However, the most dramatic difference between the two viruses in the yield of infectious virus produced

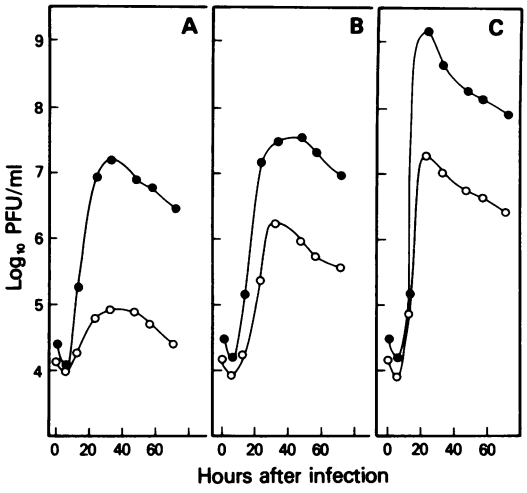

FIG. 1. *Comparison of replication of WNV-103 (○) and WNV-RV (●) in second-passage cultures of (A) resistant (RV) embryofibroblasts, (B) susceptible (HE) embryofibroblasts, and (C) BHK cells. Cultures were infected at a multiplicity of infection of 10.*

was observed in resistant cultures. A cytopathic effect was first noted in BHK cells infected with either WNV-103 or WNV-RV by 33 h and became extensive by 48 h after infection. WNV-103 caused no detectable cytopathic effect in either HE or RV cells. In contrast, a cytopathic effect was observed in WNV-RV-infected HE cultures by 33 h, and all cells were affected by 57 h. Only a slight cytopathic effect was noted in RV cells by 57 h after infection with WNV-RV. In preliminary studies in animals, the WNV-RV mutant was found not to produce disease or death in resistant C3H/RV mice, but was found to kill 100% of the susceptible C3H/HE mice. Four-month-old mice were infected intracerebrally with a 0.03-ml inoculum containing 10^7, 10^6, or 10^5 PFU.

The amount of interferon produced by resistant and susceptible cultures infected with either WNV-103 or WNV-RV (multiplicity of infection of 10) was measured in culture fluids harvested 48 h after infection. The interferon titer observed after WNV-103 infection was 10 U/ml in resistant cultures and 80 U/ml in susceptible cultures. After WNV-RV infection, resistant culture fluids contained 640 U/ml, whereas susceptible fluids contained 1,280 U/ml.

Analysis of intracellular viral RNA synthesis. In preliminary experiments, incorporation of [^{3}H]uridine into RNA in actinomycin D-treated cells was compared at different times after infection and was found to be maximal between 22 and 27 h after infection in both resistant and susceptible cells. Therefore, exper-

iments performed to compare the synthesis of intracellular viral RNA were carried out during this period. At 24 h after WNV-103 or WNV-RV infection of confluent monolayers of RV, HE, and BHK cells, actinomycin D was added to culture media 1 h before the addition of [^{3}H]-uridine. Uninfected cultures were treated similarly. After 1.5 h, cells were lysed and RNA was analyzed by rate-zonal centrifugation on sucrose gradients, as described in Materials and Methods. A representative series of RNA patterns is shown in Fig. 2. Although the total amount of uridine incorporated into actinomycin D-resistant RNA was approximately equal in cultures of WNV-103-infected resistant and susceptible mouse cells, the amounts of the particular species of RNA synthesized differed markedly. Clearly distinguished peaks of RNAs sedimenting at about 20S and 26S were observed in extracts from infected HE and BHK cells (Fig. 2B and C). In contrast, a single broad peak was observed in this region on gradients of RNA from infected resistant cells (Fig. 2A), and the amount of [^{3}H]uridine incorporated into genome size RNA (40S) in resistant cells was much reduced compared with the amount incorporated into 40S RNA in comparable HE and BHK cultures. The 20S RNA has been found to be the replicative intermediate, whereas the 26S RNA is a conformation variant of the 40S RNA (20).

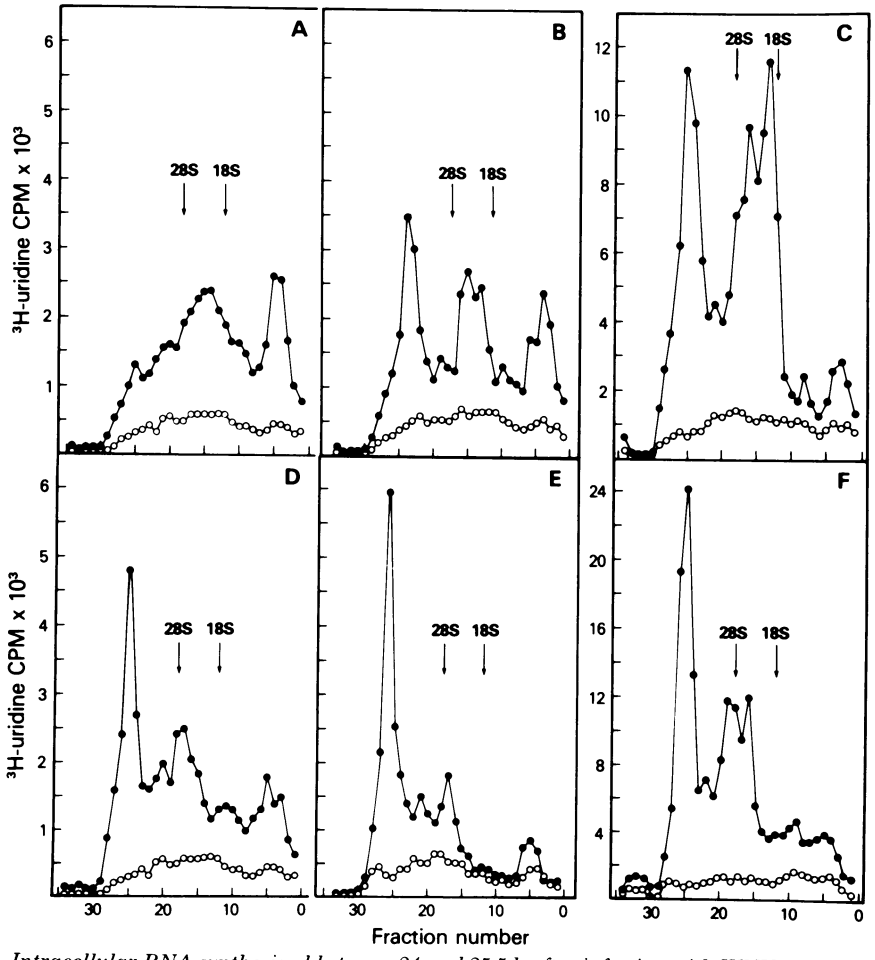

FIG. 2. *Intracellular RNA synthesized between 24 and 25.5 h after infection with WNV-103 in (A) resistant (RV), (B) susceptible (HE), and (C) BHK cells and after infection with WNV-RV in (D) RV, (E) HE, and (F) BHK cells. Actinomycin D (2 μg/ml) was added to culture fluids at 23 h, and [^{3}H]uridine (20 μCi/ml) was added at 24 h after infection. Cell extracts were prepared in sodium dodecyl sulfate-containing buffer, and RNA was extracted at 25.5 h as described in the text. RNA was analyzed by rate-zonal sedimentation in 15 to 30% (wt/vol) sucrose gradients. The positions of the 18 and 28S rRNA's were determined from absorbance profiles. Symbols: ●, infected; ○, uninfected control.*

RNA sedimenting at about 9S was observed in gradients from all of these cell types. The total amount of [^{3}H]uridine incorporated into 9S RNA was about the same in samples for the three types of cell cultures, but represented a much smaller proportion of the [^{3}H]uridine-labeled RNA in BHK cells.

Less uridine was incorporated into 40S RNA in WNV-103-infected, resistant cells at all times analyzed between 10 and 72 h after infection (data not shown). In RV cells the amount of [^{3}H]uridine incorporated into the 40S RNA peak varied between 5 and 14% of the total radioactivity incorporated into actinomycin D-insensitive RNA, whereas in HE cells the 40S RNA peak represented 22 to 31% of the total.

In all three WNV-RV-infected cell cultures, the 40S RNA was observed to be the predominant species of [^{3}H]uridine-labeled RNA (Fig. 2D, E, and F). Incorporation of uridine into 40S RNA was increased in WNV-RV-infected RV, HE, and BHK cultures compared with their WNV-103-infected counterparts. The percentage of the total amount of uridine incorporated between 24 and 25.5 h after infection into intracellular actinomycin D-insensitive RNA present in the 40S gradient peaks is given in Table 1. The amount of uridine incorporated into RNA in the 20S region of the gradients was markedly reduced in extracts from all three types of cells infected with WNV-RV compared with WNV-103-infected cells.

The mechanism by which incorporation of [^{3}H]uridine into 40S RNA is limited in resistant cells infected with WNV-103 is currently not known. However, several possible hypotheses have been ruled out. The possibility that an increased nuclease activity present in resistant cell cytoplasms was responsible for the lower amounts of [^{3}H]uridine-labeled 40S RNA observed in resistant cell extracts was examined by mixing cytoplasms from WNV-infected resistant and susceptible cells before extraction. In these experiments no vanadyl-ribonucleoside was added to the cell lysis buffer. The amount of [^{3}H]uridine-labeled 40S RNA observed in the mixed cytoplasmic extracts was equal to the sum observed in the unmixed RV and HE extracts (data not shown). The possibility that the decreased incorporation of [^{3}H]uridine into 40S WNV RNA in resistant cells might be due to a differing ability of RV and HE cells to transport uridine or to differences in nucleotide pool sizes in the two types of cells, or to both, was examined by comparing incorporation of uridine into viral RNA in Sindbis virus-infected RV and HE cells. The incorporation of uridine into Sindbis virus 26S and 42S RNA was found to be equally efficient in RV and HE cells (data not shown). The possibility of an enhanced effect of interferon on resistant cells was assessed with the aid of antibody to mouse interferon, which was added to culture media to a final concentration of 12 U/ml 24 h before infection and was present in the cultures throughout the rest of the experiment. After this treatment, the amount of incorporation of radioactivity into 40S RNA between 24 and 25.5 h after infection was observed to increase by about 2- to 2.5-fold in RV and 1.2- to 1.5-fold in HE cultures. However, the total amount of label incorporated into 40S RNA in resistant cultures remained 3- to 6-fold lower than that in susceptible cultures. The yields of infectious virus from RV and HE cells treated with antibody to mouse interferon were found to be 5- to 10-fold higher than the yields from untreated infected control cultures. For example, at 36 h the titer of culture fluid from an RV culture was 1.8×10^5 PFU/ml with antibody to mouse interferon treatment and 3.6×10^4 PFU/ml in control cultures. At the same time, the titer in a comparable HE fluid was 8.2×10^6 PFU/ml with antibody to mouse interferon treatment and 2.5×10^6 PFU/ml in control HE cultures.

TABLE 1. *Comparison of incorporation of [^{3}H]-uridine into actinomycin D-insensitive intracellular 40S RNA between 24 and 25.5 h after infection by WNV-103 and WNV-RV*

Cell type	Virus	Total cpm on gradient	cpm in 40S peak	(cpm in 40S/total cpm) × 100
HE	WNV-103	45,774[a]	13,832	30
	WNV-RV	32,105	15,276	48
RV	WNV-103	44,710	2,286	5
	WNV-RV	48,170	14,095	29
BHK	WNV-103	136,850	41,030	30
	WNV-RV	192,530	71,050	37

[a] Counts per minute incorporated into uninfected control cultures of the same cell type were subtracted.

Analysis of RNA in extracellular virus. To analyze the RNA species present in extracellular virions, virions were labeled with [^{3}H]uridine from 4 to 29 h after infection and then pelleted from culture fluids. RNA was extracted as described in Materials and Methods and sedimented through 15 to 30% sodium dodecyl sulfate-sucrose gradients. The titer of virus in the WNV-103-infected RV fluid from which the RNA was extracted was 6.9×10^5 PFU and that of the HE fluid was 4.1×10^7 PFU. The RNA contained in culture fluid pellets from WNV-103-infected HE cells was essentially all 40S (Fig. 3B). In contrast, the majority of the labeled

RNA in the infected culture fluid from RV cells (Fig. 3A) sedimented in the top and middle portions of the gradient. It seems unlikely that the RNA in these peaks was digested by nucleases during the extraction procedure, since vanadyl-ribonucleoside complex had been added to the samples before virus disruption. The amount of labeled 40S RNA present in culture fluids from WNV-infected RV cells was found to be about 100-fold less than that observed in fluids from infected HE cells. This correlated directly with the 100-fold-lower infectivity observed in the RV sample. Similar results were obtained with virus labeled between 24 and 48 h after infection (data not shown).

The predominant species of RNA in extracellular particles from both RV and HE cells infected with WNV-RV was 40S (Fig. 3C and D). The total infectivity of the RV sample from which RNA was extracted was 2.5×10^9 PFU, and the titer of the HE sample was 4.4×10^9 PFU. Other peaks of smaller RNAs were also observed in these gradients. The relative amounts of these RNAs in virus pellets from resistant and susceptible cells were similar.

Comparison of intracellular protein syn-

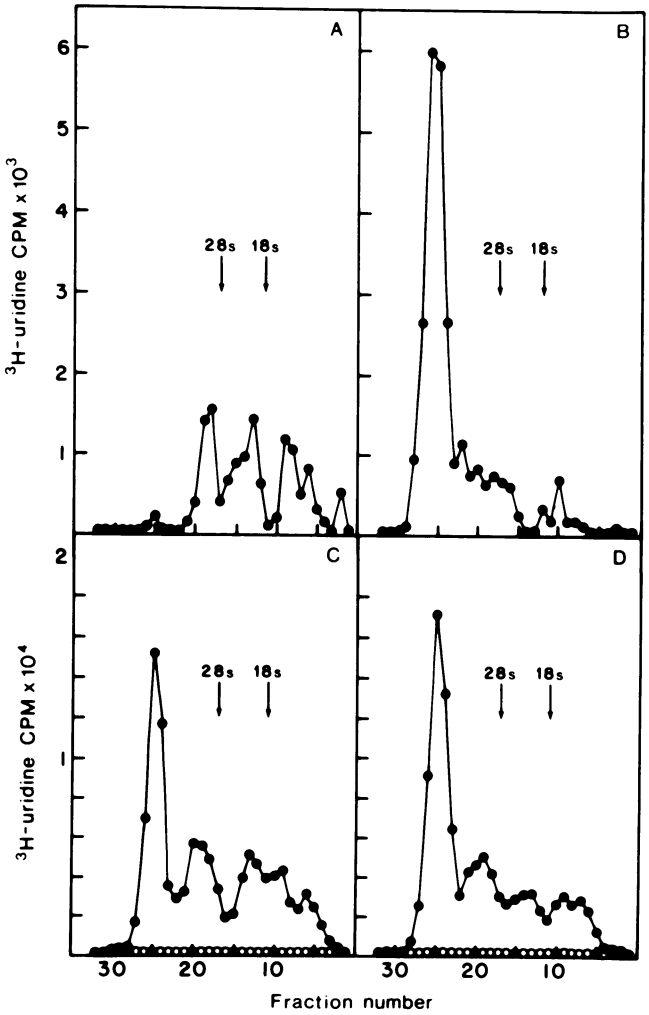

FIG. 3. *Rate-zonal sedimentation analysis of [^{3}H]uridine-labeled RNA in culture fluids from WNV-103-infected (A) resistant (RV) and (B) susceptible (HE) embryofibroblast cultures and WNV-RV-infected (C) RV and (D) HE cultures. Cultures were incubated with [^{3}H]uridine (20 μCi/ml) from 4 to 29 h after infection. Virions in harvested culture fluids were pelleted, extracted as described in the text, and sedimented through 15 to 30% (wt/vol) sucrose gradients. Unlabeled BHK rRNA's were used as markers. Symbols: ●, infected; ○, uninfected control.*

thesis in RV and HE cells infected with WNV-103 or WNV-RV. At different times after infection, the pattern of viral protein synthesis was compared in RV and HE cells infected with either WNV-103 or WNV-RV. Cells were incubated with actinomycin D in methionine-free media for 5 h before addition of [^{35}S]methionine to reduce the background of host protein synthesis. Figure 4 presents an autoradiogram of the proteins labeled between 24 and 28 h after infection. Bands appearing only in infected cell extracts are indicated by arrows. The molecular weights of these proteins were estimated to be 93,000, 65,000, 48,000, 36,000, 31,500, 26,500, 23,500, 22,500, and 19,700. The 48,000-dalton protein comigrates with the structural protein, V3 (data not shown), which is the envelope glycoprotein. The remaining two structural proteins, V2 (14,000) and V1 (8,500), were not resolved on these gels. Westaway (20) also has not observed these two structural proteins in cell extracts from flavivirus-infected cells. A 44,000-dalton protein that comigrated with a cell protein was visible only as a darkening of this band in infected cell extracts. This protein was not indicated with an arrow on the autoradiogram but is indicated on the graph. In general, incorporation of [^{35}S]methionine into the proteins appearing only in infected cells was more efficient in WNV-RV-infected cells than in WNV-103-infected cells. However, all bands were not uniformly darker. The 93,000-, 65,000-, and 19,700-dalton nonstructural proteins and the probable structural protein, V3, incorporated more [^{35}S]methionine than the other proteins (Fig. 4, cf. tracks 2 and 3). The same differential labeling pattern was observed when proteins were labeled with [^{3}H]leucine (data not shown). Resistant cells incorporated less radioactivity into virus-specific proteins than did susceptible cells. Also, less label was observed to be incorporated into the 93,000-, 65,000-, and 19,700-dalton proteins in the WNV-103-infected resistant cells than in WNV-RV-infected resistant cells (Fig. 4, tracks 5 and 6).

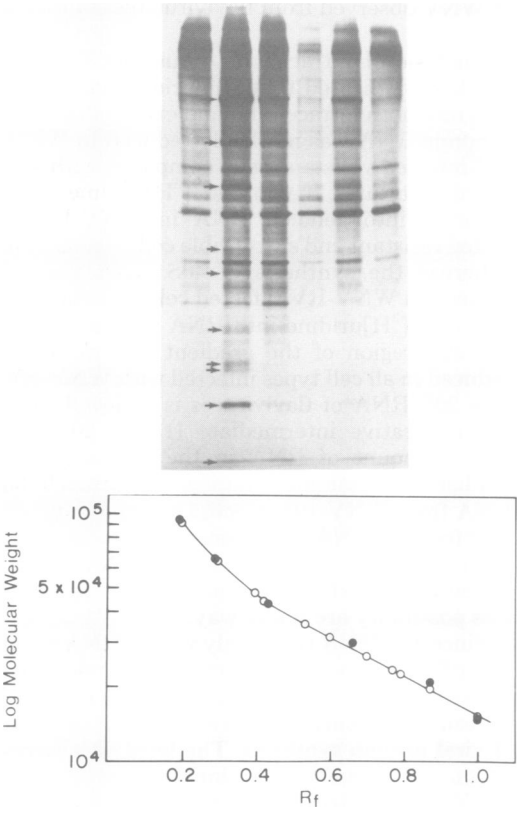

FIG. 4. *Viral proteins synthesized in resistant and susceptible cell cultures after infection with WNV-103 or WNV-RV. Cultures were incubated in methionine-free medium containing 2 μg of actinomycin D per ml beginning at 20 h and then with [^{35}S]methionine from 24 to 28 h after infection. Samples of cell extracts containing equal amounts of total cell protein were electrophoresed on a 10% polyacrylamide slab gel as described in the text. Cell extracts applied to the gel tracks were, from left to right: uninfected HE cells; HE cells infected with WNV-RV; HE cells infected with WNV-103; uninfected RV cells; RV cells infected with WNV-RV; RV cells infected with WNV-103. The autoradiogram exposure time was 7 days.*

DISCUSSION

We report here the isolation of a mutant of WNV strain E101, designated WNV-RV, which is able to replicate more efficiently in genetically resistant C3H/RV cells than is the parental virus. This mutant was isolated during screening for temperature-sensitive mutants from culture fluid of a genetically resistant, simian virus 40-transformed C3H/RV cell line persistently infected with WNV-103 (Brinton, submitted for publication). Infectivity titers produced by wild-type WNV E101 infections in resistant cells or in brains of resistant mice are routinely 2 to 4 logs lower than those from cells or brains of congenic susceptible C3H/HE mice (3, 18). The WNV-RV mutant can overcome this limitation placed on flavivirus replication by resistant cells. The mechanism by which the replication of flaviviruses is limited in resistant cells is not presently understood. Viral adsorption and penetration appear to occur normally in resistant cells (3). In contrast to what has been observed with mice that are genetically resistant to myxovirus-induced disease (6), interferon does not play a specific role in bringing about the reduced yields

of WNV observed from flavivirus-resistant cells (1).

Analysis of intracellular virus-specific RNA synthesis indicated that [^{3}H]uridine incorporation into both intracellular and extracellular 40S genomic RNA was markedly reduced in WNV-103-infected resistant cells compared with susceptible cultures. In contrast, [^{3}H]uridine incorporation into genome RNA in WNV-RV-infected resistant and susceptible cells was similar. Whereas the synthesis of 40S RNA was increased in WNV-RV-infected cells, the incorporation of [^{3}H]uridine into RNA sedimenting in the 20S region of the gradient was markedly reduced in all cell types infected with WNV-RV. The 20S RNA of flaviviruses is believed to be the replicative intermediate (13–15, 20). The larger amount of RNA in the 20S region of gradients containing samples of intracellular RNA from WNV-103-infected cultures suggests that free 40S RNA is not generated as efficiently from replicative intermediates in WNV-103-infected cells. Further experiments to investigate this possibility are under way.

Since 40S RNA is the only viral mRNA so far identified in flavivirus-infected cells, a decrease in the synthesis of this RNA would be expected to result in a concomitant reduction in the level of viral protein synthesis. The level of incorporation of [^{35}S]methionine into viral proteins in WNV-103-infected resistant cells was far below that observed in infected susceptible cells, and viral protein synthesis was markedly increased in WNV-RV-infected resistant cells (Fig. 4).

Because of the present lack of knowledge about the regulation of flavivirus transcription and translation, we can only speculate upon the nature of the alteration in the WNV-RV mutant which allows it to replicate more efficiently than the parental virus in resistant cells. Beginning at the end of the latent period, a shift occurs from equal transcription of both plus- and minus-strand 40S RNAs to the preferential transcription of plus strands (20). Westaway et al. (21) have postulated, but not demonstrated, that the attachment of the core protein, V2, to the 3′ end of nascent 40S plus strands could block the binding of polymerase molecules but allow RNAs to be translated or assembled (or both) into cores. Such a regulatory process would be autocatalytic. It is possible that the WNV-RV mutant may code for a polymerase with an enhanced capacity to bind to minus-strand templates. Data reported recently indicate that different polymerases transcribe the plus- and minus-strand 42S templates in alphavirus-infected cells (2, 10, 11). Whether two polymerases are also synthesized in flavivirus-infected cells or whether host components constitute a part of the functional polymerase complex(es) is not known. Resistant cells may restrict the production of a functional plus-strand polymerase. Alternatively, the 40S virion RNA of the WNV-RV mutant might contain an altered 3′ end that allows more efficient binding of V2.

Small-sized RNA species were observed to be the predominant RNAs associated with pelleted virions obtained from WNV-103-infected resistant cells. Preliminary information indicates that this RNA is virus specific. Although 40S RNA was the major species of RNA found in pelleted extracellular virus particles produced by both RV and HE cultures infected with WNV-RV, several species of smaller-sized RNAs were also consistently observed to be associated with these pelleted virions. It is not yet known whether the small RNAs observed in WNV-103 pellets from resistant cells and those in pellets from WNV-RV-infected cells are similar. If the small RNAs prove to be deleted forms of virion RNA, then in WNV-RV infections their autointerfering activity must be minimal since virus yields from both resistant and susceptible cells are unusually high.

The differences in the patterns of intracellular WNV-specific RNA and the amount of virus-specific proteins synthesized in RV and HE cells after infection with WNV-103 indicate that host cell factors influence the amount and species of viral RNA and virions synthesized. However, genes of the infecting virus are also important, as indicated by comparison of RNA and virion production in resistant cultures infected with WNV-103 or WNV-RV. Although the WNV-RV mutant has acquired an enhanced ability to replicate, it produces only a slight cytopathic effect in resistant embryofibroblasts and does not kill resistant animals (data not shown). This may be because the WNV-RV mutant was selected at subculture 25 of a persistently infected resistant culture. The WNV-RV mutant is a unique tool for use in further studies of the regulation of flavivirus transcription and the mechanism of genetically controlled resistance to flaviviruses.

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