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Characterization of Kunjin Virus RNA-Dependent RNA Polymerase: Reinitiation of Synthesis *in Vitro*

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RNA-dependent RNA polymerase (RDRP) activity was characterized in a cytoplasmic extract of Kunjin virus-infected Vero cells at 24 hr. The activity was influenced, possibly indirectly, by the length of prior treatment of infected cells with actinomycin D; however, 6 μg/ml actinomycin D and 10^{-5} *M* α-amanitin in the RDRP assay had no effect. The replication complex was membrane-bound and Mg^{2+} was essential for RDRP activity. Incorporation was more dependent on exogenous UTP and GTP than ATP or CTP. The specific activity was low, and rate of incorporation of GMP decreased as the period of assay was increased; however, incorporation of label lasted for at least 60 min. RNA products were fractionated by LiCl precipitation, and kinetic studies showed that the sequence of accumulation of label was the same as that observed *in vivo,* viz., RI → RF → 44 S RNA; limited reinitiation was also observed. This sequence of labeling also indicated that the *in vitro* RDRP activity was due to an enzyme capable of elongation, release, and reinitiation of Kunjin RNA synthesis and not merely end labeling or elongating preexisting RNA molecules. No labeled bands in urea-polyacrylamide gels were observed using extracts from mock-infected cells and hence the three RNA products of assays were readily identified in a single gel. The replication complex was still active after treatment with nonionic detergent, but no labeled 44 S RNA was detected in gels, even in the presence of RNasin in the assay which inhibited some nuclease activity. Antibodies to flavivirus-specific nonstructural proteins were preincubated with infected cell extracts in the presence and absence of detergent but no inhibition of RDRP activity was observed. However, anti-dsRNA plus detergent blocked activity by as much as 78% and label was found only in RF.

INTRODUCTION

Kunjin virus is a member of the newly established virus family *Flaviviridae* (Westaway *et al.,* 1985). Flaviviruses possess a single-stranded (ss) 44 S RNA genome which has a type 1 cap at the 5′ end but is not polyadenylated at the 3′ end (Stollar *et al.,* 1967; Boulton and Westaway, 1972; Cleaves and Dubin, 1979; Wengler and Wengler, 1981). Extensive studies by pulse and pulse–chase experiments showed that there is no ss-subgenomic RNA detectable and the genome must therefore function as the sole mRNA for flavivirus replication (Boulton and Westaway, 1977; Chu and Westaway, 1985). Recent studies of Kunjin virus RNA synthesis in Vero cells after the latent period (Chu and Westaway, 1985) established that the replicative form (RF) is converted to the replicative intermediate (RI) RNA (Stollar *et al.,* 1967; Cleaves *et al.,* 1981) and that RF and RI are recycled as templates for synthesis of 44 S RNA. These results provided the basis for a model of flavivirus RNA synthesis (Chu and Westaway, 1985).

RNA-dependent RNA polymerase (RDRP) induced by flaviviruses has been detected in extracts from cells infected with Japanese encephalitis (JE) virus (Takehara, 1971; Zebovitz *et al.,* 1974), St. Louis encephalitis virus (Qureshi and Trent, 1972), dengue-2 virus (Cardiff *et al.,* 1973) and Kunjin virus (Chu and Westaway, 1985). In all of these studies, the properties and enzymology of the RDRP were not reported in any detail and conflicting results on the *in vitro* products were reported (see Westaway, 1980). An *in vitro* assay was employed recently for measuring the RDRP activity of extracts of *Aedes albopictus* cells infected with JE virus or Banzi virus but the only product, a "20–28 S peak of radioactivity" detected by sedimentation in sucrose gradients, was not characterized (Hommel and Schloemer, 1985). In this paper we have characterized the Kunjin virus RDRP activity in crude cell extracts obtained after the latent period and analyzed its products in gels. The results confirmed the *in vivo* studies showing that the RF is a recycling template (Chu and Westaway, 1985) and indicate that the polymerase is capable of reinitiation *in vitro.*

MATERIALS AND METHODS

Virus and cell cultures

The strain MRM61C of Kunjin virus was propagated in Vero cells as described previously (Boulton and

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Westaway, 1972) in the absence of antinomycin D (AMD; a gift from Merck, Sharp, and Dohme, Sydney) except as indicated in the text. Previous results (Chu and Westaway, 1985) showed that maximum polymerase activity was obtained at about 24 hr.

Preparation of crude enzyme extracts

Cells were infected at a m.o.i. of 25. Pelleted cells were disrupted by passing them through a 26-gauge needle in either 10 m*M* Tris–HCl, pH 8.0, containing 10 m*M* sodium acetate (TN buffer) or the same buffer containing 1.5 m*M* $MgCl_2$ (TNMg buffer) at 2×10^7 cells/ml as described previously (Chu and Westaway, 1985). The cell extract was centrifuged at 800 *g* for 7 min to obtain a cytoplasmic fraction (800 *g* supernate) and a nuclear-associated fraction (800 *g* pellet). The latter was resuspended in the same volume of buffer as the cytoplasmic fractions. All extracts were stored in small aliquots at −70° and retained enzyme activity for more than 12 months. Since previous studies (Chu and Westaway, 1985) showed that the cytoplasmic fraction synthesized a greater proportion of 44 S RNA, all optimization experiments were done using this fraction. Protein concentrations were determined on the extracts in a microassay using the Bio-Rad protein assay kit and bovine plasma albumin as standard.

RNA-dependent RNA polymerase assays

RDRP assays were carried out in duplicate on frozen-stored extracts using [α-^{32}P]GTP unless otherwise stated in the text. Before optimization, the standard assay (50 μl) contained 50 m*M* Tris–HCl, pH 8.0, 10 m*M* MgAc, 6 μg/ml AMD, 10 m*M* 2-mercaptoethanol, 5 m*M* phosphoenolpyruvate, 3 U/ml pyruvate kinase, 1 m*M* each of CTP, ATP, and UTP, 50 μ*M* GTP, 5 μCi [α-^{32}P]GTP and 25 μl enzyme extract containing 7.5–9 mg/ml protein. After optimization, the assays contained additional 7.5 m*M* KAc, 0.5 U/μl RNasin (Promega Biotech) and the ribonucleoside triphosphates were reduced to 0.5 m*M* for the unlabeled NTPs while GTP was reduced to 25 μ*M*. All assays were carried out at 37° for 60 min unless otherwise stated. After incubation, the reactions were stopped by addition of 0.5 *M* EDTA to 10 m*M*. The nucleic acid products were then extracted with phenol–SDS in TNE buffer and resuspended in 10μl H_2O per assay.

Estimation of incorporation of [^{32}P]GTP into RDRP products

Incorporation of [^{32}P]GTP into RDRP RNA products *in vitro* was estimated by two methods.

(a) TCA precipitation of labeled RNA. This was done as described by Maniatis *et al.* (1982) using sonicated DNA carrier, and TCA-insoluble radioactivity was determined by counting 2 μl aliquots of phenol-extracted RNA prepared from each duplicate assay.

(b) Gel electrophoresis. In vitro products were also analyzed in the aqueous-agarose gel, urea-polyacrylamide gel, and fully denaturing formaldehyde-agarose gel systems before and after fractionation in 2 *M* LiCl to identify RI, RF, and 44 S RNA of Kunjin virus using *in vitro* [^{3}H]uridine-labeled Kunjin viral RNAs and stained 28 and 18 S rRNAs as markers, as described previously (Chu and Westaway, 1985). After electrophoresis, gels were dried for autoradiography. Relative proportion of [^{32}P]GMP in RF, RI, and 44 S RNA in each lane was estimated using a LKB 2202 ultroscan laser densitometer and a LKB 2220 recording integrator (Chu and Westaway, 1985).

In all cases, the two methods gave results in agreement with each other. Thus the TCA data were used for comparing total incorporations while gel profiles were used to compare incorporations into the RF, RI, and 44 S RNA species as well as total incorporation.

RESULTS

Identification of the *in vitro* RDRP products

We obtained labeled products from the Kunjin virus *in vitro* polymerase assay and showed that when analyzed in urea-polyacrylamide gels as specified above (Fig. 1) the labeled virus-specified RNAs were the same as those produced *in vivo* (Chu and Westaway, 1985). These were 20 S RF RNA which fractionated only in the LiCl-soluble fraction, and RI plus labeled and released 44 S RNA which fractionated only in the LiCl-insoluble fraction as indicated by analysis of these fractions in the various gel systems (e.g., see Fig. 4C). Detection of the virus-specified products was facilitated by the absence of any significant incorporation of label in the RNA of mock-infected extracts (Figs. 1 and 3C) and their structural identities were confirmed by RNase digestions in 0.01 × SSC and 2 × SSC (1 × SSC = 0.15 *M* NaCl, 0.015 *M* Na citrate, pH 6.8) (results not shown, see Chu and Westaway, 1985).

Effect of treatment of infected cell with actinomycin D prior harvest

Effects of AMD and a requirement for a functional nucleus in flavivirus replication have been claimed previously (reviewed by Westaway, 1980). Recently we showed by indirect immunofluorescence that the dsRNA templates of Kunjin virus RNA synthesis are confined to the cytoplasm (Ng *et al.*, 1983).

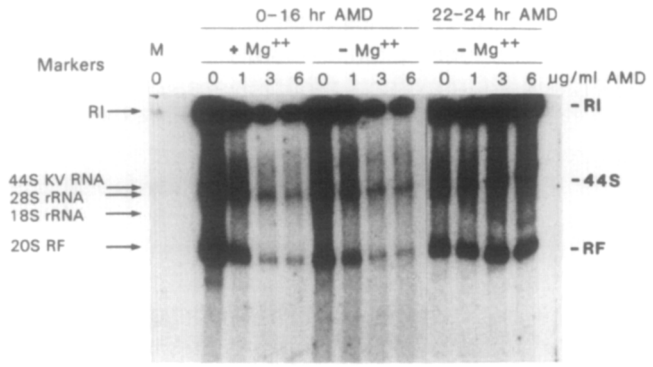

FIG. 1. Analysis in urea-polyacrylamide gel of *in vitro* RDRP products labeled using [^{32}P]GTP at 24 hr postinfection from cytoplasmic extracts (800 *g* supernate) prepared from cells pretreated with 0, 1, 3 and 6 μg/ml AMD from 0 to 16 hr or from 22 to 24 hr postinfection. All assays were carried out under optimized conditions at 37° for 1 hr as described under Materials and Methods. All assays included 10 m*M* Mg^{2+} and 0.5 U/μl RNasin. Note that no labeled products were detected in the typical mock-infected (M) lane while the RI, 44 S RNA, and RF are indicated for the Kunjin-infected (KV) lanes. The relative positions of the RNA markers (*in vivo* [^{3}H]uridine-labeled Kunjin RNAs and 28 and 18 S rRNAs detected by staining with toluidine blue) are indicated on the left. The extracts prepared in the presence of 1.5 m*M* Mg^{2+} or its absence, and the concentrations and times of AMD treatments, are indicated on the tops of the lanes. Similar results were obtained with the nuclear-associated fractions (800 *g* pellets). In the urea-polyacrylamide gels, the labeled materials on the top of the Kunjin lanes were identified as the RI (see text and Fig. 4C for details).

In order to determine whether AMD pretreatment of cells would have any effects on the *in vitro* polymerase activity, cells were pretreated with AMD prior to preparation of cell extracts at 24 hr, when no cytopathic effects were visible. Estimation of TCA-insoluble incorporation of [^{32}P]GTP and gel analysis of the *in vitro* polymerase products (Fig. 1) synthesized by these extracts showed that (i) pretreatment of cells from 0 to 16 hr (latent period) with only 1 μg/ml AMD reduced polymerase activity by more than 50%, and by more than 80% with 3 or 6 μg/ml AMD; (ii) after the latent period pretreatment of cells (22–24 hr) with up to 6 μg/ml AMD did not reduce the RDRP activity; (iii) there were no significant changes in the distribution of RDRP activity between the cytoplasmic and the nuclear-associated fractions in the various treatments (ratios were approximately 60:40), even after the inclusion of Mg^{2+} in the extraction buffer which also had no effect on the RDRP activity. This result suggested that exogenous Mg^{2+} was not required to maintain the integrity of the enzyme complex during its preparation and that the complex is strongly membrane-bound since RDRPs which are weakly associated with membranes are dissociated from the latter in the absence of Mg^{2+} in the extraction buffer (Rosenberg *et al.*, 1979; Dorssers *et al.*, 1983).

Only indirect conclusions could be drawn regarding the inhibitory effect of AMD pretreatment during the latent period. Cells treated with AMD from 0 to 16 hr disrupted more easily than untreated cells, or those treated from 22 to 24 hr. They also contained as much as 50% less rRNA (for 6 μg/ml AMD), measured by toluidine blue staining of cellular RNA in gels, than cells in other preparations. This reduction is possibly related to the long period of exposure to AMD (16 hr) and may have influenced the synthesis of the RDRP complexes and hence the *in vitro* RDRP activity.

Stability of the RDRP *in vitro*

The RDRP activity in crude extracts was found to be very stable; after one cycle of freezing (at −70°) and thawing, the activity was about 90% of the unfrozen control, but was reduced to 40–50% after two cycles. Storing the extracts at 4° for 2–3 days or at 18° for 1 hr, after a freeze–thaw cycle, also did not affect the enzyme activity.

When cell extracts were treated with 10 m*M* EDTA for 1 hr and then assayed for RDRP activity after neutralization of the EDTA with 20 m*M* Mg^{2+}, no inhibition of RDRP activity was observed. Preincubation of the extract with nonionic detergents, 0.1 to 2% NP-40, or 0.2% Triton X-100, did not completely inhibit RDRP activity, but partial degradation of labeled RI occurred and labeled 44 S RNA was not detected in gels (Fig. 2A). Inclusion of RNasin during preincubation with NP-40 prevented the degradation of RI but again labeled 44 S RNA was not detected (Fig. 2B). It was also observed that in the absence of detergent in the extracts, the RDRP activity in the cytoplasmic fraction was recovered in the heavy membrane-mitochondrial fraction (16,000 *g* pellet), but after addition of NP-40, the activity was found predominantly in the 16,000 *g* supernatant fraction (results not shown).

All these experiments again suggested that the RDRP complex in the cell extracts is bound to membranes by nonionic bonds and not by weak polar interactions. The apparent absence of labeled 44 S RNA in the *in vitro* products after solubilization of the membranes with detergents is probably due to loss of reinitiation factors and/or release factors in the replication complex, rather than to nuclease degradation.

Characterization of the RDRP assay

Experiments described below were carried out to determine conditions for the RDRP assays that would synthesize maximum amounts of labeled and released 44 S RNA relative to labeled RF and RI, and to interpret the effects of the various treatments on the RDRP ac-

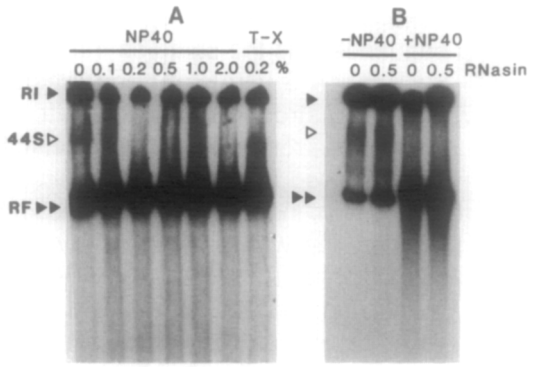

FIG. 2. Effects of nonionic detergents and RNasin on RDRP assays. In all these experiments RDRP activity was assayed using the optimized reaction conditions except that RNasin was not included unless as stated below. (A) Effects of detergent treatment on the *in vitro* polymerase products analyzed in a urea-polyacrylamide gel. Extracts were preincubated for 1 hr at 18° in the absence or presence of various concentrations of NP-40 or 0.2% Triton X-100 (T-X) prior to the polymerase assay at 37° for 1 hr. (B) Protective effects of RNasin (0.5 U/μl) on RI synthesized by extracts preincubated with 0.5% NP-40. The extracts were preincubated with or without NP-40 in the presence or absence of RNasin plus 10 m*M* 2-mercaptoethanol as indicated, and then assayed as in (A). In all the experiments above, the concentrations of RNasin, 2-mercaptoethanol, and detergents indicated were the final concentrations in the assays.

tivity, based on our model of Kunjin virus RNA synthesis. Thus, gel analysis of the products before (Fig. 3) and after LiCl fractionation (results not shown) showed that omission of 2-mercaptoethanol caused some reduction of label in RI (Fig. 3A), possibly as a result of inefficient reinitiation after release of labeled progeny 44 S RNA. Similarly, release of labeled 44 S RNA was apparently impaired when sources of energy (pyruvate kinase plus phosphoenolpyruvate), ATP and CTP were omitted (Fig. 3A), suggesting that high levels of these components were required for reinitiation of 44 S RNA synthesis. An even greater inhibitory effect was observed when UTP was omitted from the assay (Fig. 3A). This effect was also observed in other viral RDRP assays (e.g., Gilliland and Symons, 1968; Miller, 1984; Rohozinski *et al.*, 1986) and further work is needed to determine whether this is attributable to a low endogenous UTP pool, e.g., due to its conversion to CTP by amination (Lehninger, 1975) or a high K_m value of RDRP for UTP, relative to other substrates such as ATP or CTP. However, GTP was also a limiting substrate (see below), and free labeled 44 S RNA was also observed when [5,6-^{3}H]UTP was substituted for [α-^{32}P]GTP. Hence, true *in vitro* synthesis of 44 S RNA occurred rather than merely addition of 5′-caps by methylated radioactive GTP.

The reaction was found to be unaffected by 6 μg/ml AMD or α-amanitin at 10^{-5} *M*, showing that the enzyme activity is not due to a virus-induced host DNA-dependent RNA polymerase. The presence of 2 m*M* phenylmethylsulfonyl fluoride (PMSF), a protease inhibitor, did not increase the activity, while 0.5 U RNasin/μl in the assay appeared to increase the accumulation of 44 S RNA after 60 min indicating that the 44 S RNA released *in vitro* is partially susceptible to degradation by cellular nuclease. Addition of a nucleic acid preparation from Kunjin virus-infected Vero cells to 0.25 mg/ml caused a 83% reduction in synthesis (TCA-insoluble counts) rather than any increase, indicating that no labeling of endogenous viral RNA occurred by nontranscriptional mechanisms. Finally, the rate of reaction was not impaired when the temperature was reduced from 37° to 28° (nearer to the environmental temperatures for some arthropod vectors of flaviviruses).

Varying the concentration of exogenous unlabeled GTP in the assay indicated that GTP was a limiting substrate in the RDRP assay (Fig. 3B). Calculation of specific incorporation of GMP showed that higher concentrations of GTP increased the overall rate of incorporation of GMP into RF, RI, and 44 S RNA and that 25 μ*M* GTP was the best compromise between sensitivity and maximum synthesis of labeled 44 S RNA.

The RDRP activity and products obtained were similar over the range of pH 7.4 to 8.85; the midrange of pH 8.0 to 8.2 appeared most suitable (results not shown). In the absence of additional Mg^{2+} in the assay, no virus RNA was synthesized and maximum synthesis was observed at 10–12 m*M* Mg^{2+} (Fig. 3C). Gel analyses after LiCl fractionation of products showed that the same relative amounts of RF, RI, and 44 S RNA were synthesized over the range 5 to 20 m*M* Mg^{2+} (results not shown). In contrast, there was no absolute requirement for K^+ and total incorporation of [^{32}P]GMP was similar over the range 0 to 30 m*M* K^+; however, concentration greater than 15 m*M* appeared to reduce the amounts of incorporated [^{32}P]GMP into RI and 44 S RNA by up to 28% (30 m*M* K^+) indicating reduced reinitiation (results not shown).

Incorporation of labeled GMP into all the Kunjin RNA species increased linearly with the concentration of cell extract over the range 0 to 4.5 mg/ml protein. At 6.0 and 7.5 mg/ml, the reaction mixture was apparently saturated with enzyme and the relative amounts of RI (LiCl-insoluble) appeared to increase compared to RF (LiCl-soluble) (results not shown). Thus, in the standard assay which contained 7.5–9 mg/ml protein the RDRP enzyme was presumably not limiting. Calculations, based on TCA precipitation, showed that the specific activity of the RDRP in the extract was about 0.001 unit/mg protein (1 unit of activity is the amount of enzyme required for the incorporation of 1 nmol of GMP in 60 min at 37°).

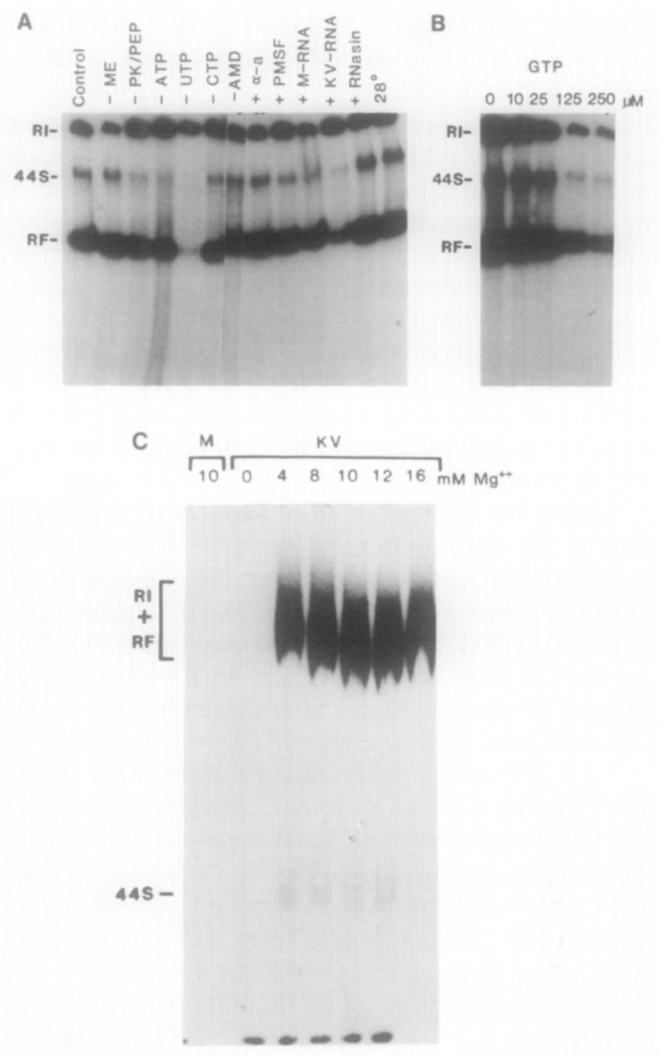

FIG. 3. Characterization and optimization of the *in vitro* reaction. The control assay was performed under conditions prior to optimization as described under Methods, and the others were modified as indicated below. (A) Effects of the absence or presence of the various reagents (as indicated on the top of each lane) on the *in vitro* products as analyzed in a urea-polyacrylamide gel. PK/PEP, pyruvate kinase/phosphoenolpyruvate; ME, 2-mercaptoethanol; α-a, α-amanitin; M-RNA and KV-RNA, 0.25 mg/ml final concentration of total nucleic acid extracted from mock or Kunjin virus-infected cells, respectively. (B) Effect of various concentrations of added GTP on the *in vitro* RNA products as analyzed in a urea-polyacrylamide gel. The concentrations of GTP added are as indicated on the tops of the lanes. (C) Analyses of the *in vitro* RNA products synthesized under various Mg^{2+} concentrations in an aqueous-agarose gel. Mock-infected (M) and Kunjin (KV) lanes and the levels of Mg^{2+} used in the assays are indicated on the tops of the lanes.

Kinetics of polymerase reaction

The kinetics of the incorporation of [^{32}P]GTP into the various RNAs was examined using the optimized conditions for the RNA polymerase assay as described under Methods (Fig. 4). Analysis of the total labeled RNA products in a urea-polyacrylamide gel (Fig. 4A), or of the LiCl-soluble (RF) and -insoluble (RI and 44 S RNA) products in an agarose gel (Fig. 4C), confirmed that the labeling sequence was the same as that reported *in vivo*, RI → RF → 44 S RNA (Chu and Westaway, 1985). RI was labeled strongly during the 0 to 5 min pulse, but RF was not prominently labeled until the 0 to 10 min pulse, followed by prominent labeling of 44 S RNA at about 20 min, indicating that reinitiation has occurred in accordance with our model. By 30 min, the proportion of label in RI decreased while RF and 44 S RNA accumulated, indicating that after the release of labeled 44 S RNA, RI was converted to RF. Subsequently, further incorporation of label occurred in RI, indicating a limited amount of further reinitiation. This was confirmed in the delayed pulse experiment (Fig. 4B) in which mainly RI and RF were labeled after delays of 30 and 40 min, respectively. Calculation of the rate of incorporation of labeled nucleotide showed a rapid decrease to 20% of the initial rate (0 to 20 min) in the period 20 to 40 min and to 9% during the period 40 to 60 min.

Effect of antibodies to flavivirus-specific proteins and to dsRNA on RDRP activity

RDRP assays were performed in the presence of rabbit antisera to Kunjin virus (shown by radioimmunoprecipitation to contain antibodies to all virus-specified proteins), to the largest nonstructural proteins NV4 and NV5 of dengue 2 virus (a gift from Dr. P. J. Wright) which were positive in indirect immunofluorescence tests in Kunjin virus infected cells, and to synthetic dsRNA (polyI:polyC; a gift from Dr. R. I. B. Francki). Also included in assays were monoclonal antibodies to JE virus NV4 which cross-reacts with Kunjin virus NV4 (a gift from Dr. E. A. Gould; Gould *et al.*, 1983). The RDRP assays were performed after preincubating the extracts containing RNasin with the virus-specific antibodies in the presence or absence of NP-40. None of the flavivirus-specific antibodies blocked RDRP activity either in the presence or absence of detergent (Fig. 5A); RI and RF were strongly labeled but as in Fig. 2 no labeled 44 S RNA was detected in the presence of detergent. However, anti-dsRNA was found to block RDRP activity only in the presence of NP-40, and the effect was antibody concentration dependent (Fig. 5B). At 1/10 dilution of this antiserum, the activity was inhibited by 78%, and only RF was detectably labeled after 60 min of incubation. No inhibition was observed by normal serum under the same conditions (see Fig. 5A).

DISCUSSION

The experiments reported here show for the first time that the same flavivirus RNAs and their sequence of

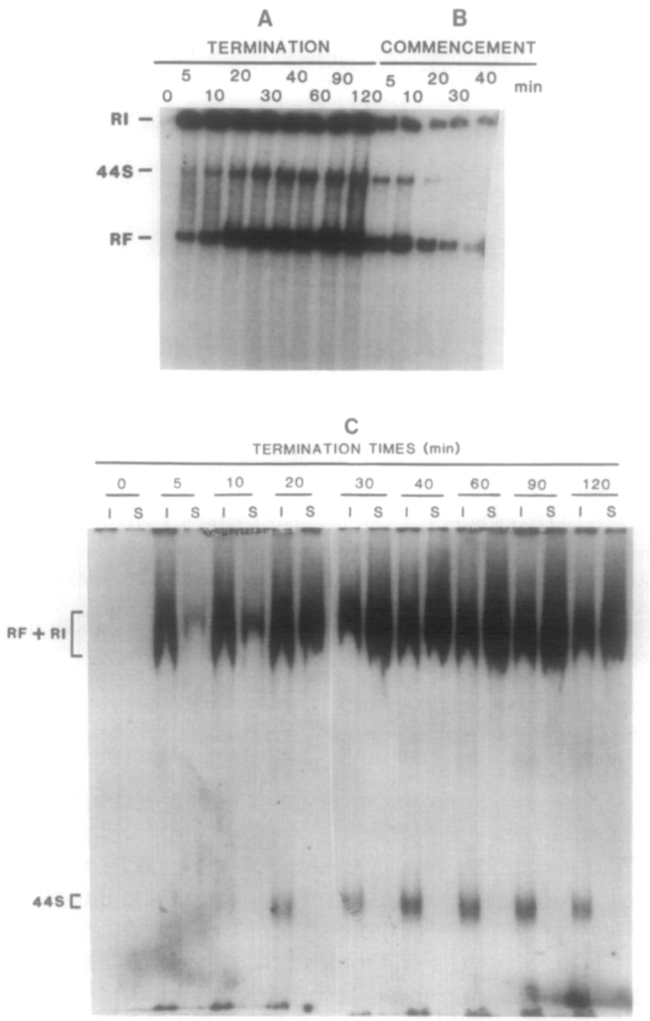

FIG. 4. Kinetic analyses of the *in vitro* polymerase reaction under optimized assay conditions containing RNasin as described under Methods. (A) Products synthesized and accumulated in RDRP reactions after various pulses of [^{32}P]GTP, as analyzed in a urea-polyacrylamide gel. The times of pulse in minutes are indicated on the tops of the lanes. (B) Products synthesized and accumulated at various times during the reaction. The experiment was done by delaying the addition of labeled GTP to the assays until the various times after the start of the reactions as indicated on the tops of the lanes and then allowing the reaction to proceed for 20 min from those times before extraction of the products. (C) Analysis of the RNA products from the same experiment described in (A) in aqueous-agarose gels after LiCl fractionation, showing the presence of only RI and 44 S RNA in the LiCl-insoluble (I) fractions, and only RF in the LiCl-soluble (S) fractions. The purity of the LiCl fractionated products was confirmed by analyses in urea-polyacrylamide gels and in denaturing formaldehyde-agarose gels (not shown).

synthesis observed *in vivo* can be reproduced in an *in vitro* system, and confirm the recycling role of RF and RI observed previously (Chu and Westaway, 1985). Kinetics of incorporation and accumulation of label into RI, RF, and 44 S RNA established that 44 S RNA was synthesized and released, and some reinitiation apparently occurred (Fig. 4).

The observed RDRP activity is distinct from that of any normal host enzyme because mock-infected extracts did not incorporate significant amounts of label from either [^{32}P]GTP or [^{3}H]UTP. Although the RDRP activity is dependent on GTP and UTP, it is also dependent on ATP and CTP to a certain extent. Furthermore, the sequential incorporation of [^{32}P]GTP into RI, RF, and 44 S RNA and the preferential labeling of RI and RF after 30 min when reinitiation is limiting (Fig. 4B) indicated that the RDRP activity is unlikely to be an RNA processing enzyme induced by viral infection that end labels preexisting viral RNA molecules. Such enzymes will be expected to label all RNA species concurrently and their activity would be short-lived *in vitro* (e.g., a few minutes). The use of [^{32}P]GTP instead of the other NTPs as the label is important because it has been reported that GTP can only be incorporated into RNA in crude extracts by actual transcription of new RNA (Manley, 1984). This is because it has been observed that all cellular RNAs synthesized *in vivo* are capped as soon as they are transcribed and that RNAs are not significantly labeled with GTP *in vitro* without prior transcription (Manley, 1984). UTP was essential for completion of 44 S RNA synthesis (Fig. 3A) and the fact that free 44 S RNA products were also labeled with [^{3}H]UTP showed that these molecules were not merely labeled by capping with methylated radioactive GTP after their synthesis. Hence, the RDRP assay is unique in that it represents a totally functional RNA replicase capable of initiation, elongation, release and partial reinitiation of 44 S RNA synthesis. The assay provides the essential basis for further investigation into the structure and composition of the flavivirus polymerase complex. Furthermore, the virtual absence of incorporation of label by mock-infected extracts enables all three species of viral RNAs synthesized *in vitro* to be identified and quantified after electrophoresis in a single urea-polyacrylamide gel.

Like the RDRP of other positive-strand RNA viruses, e.g., the alphavirus Semliki Forest (Gomatos *et al.*, 1980), the picornaviruses (Ehrenfeld *et al.*, 1970; Rosenberg *et al.*, 1972), and the coronavirus, murine hepatitis virus (Brayton *et al.*, 1982), the flavivirus polymerase complex is membrane associated (Westaway, 1980; and this investigation). Like some of these viruses, the RDRP labeled mainly RFs after solubilization of the membranes with detergents, e.g., cowpea mosaic virus (Dorssers *et al.*, 1983) and Semliki Forest virus (Gomatos *et al.*, 1980). In Kunjin virus infected Vero cell extracts, detergent solubilization of the RDRP-membrane complexes apparently inhibited reinitiation of RNA synthesis and/or some release as well since no labeled 44 S RNA was released in RDRP assays in the presence of NP-40 despite the addition of RNasin to block any degradation of 44 S RNA (Fig. 2). In con-

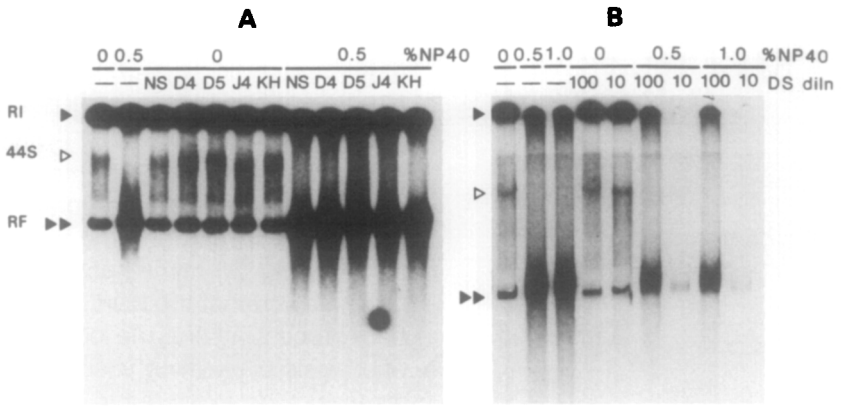

FIG. 5. Effects of antibodies on RDRP assays. (A) Analysis of the RDRP products synthesized after preincubating the extracts in the presence of viral antibodies with and without 0.5% NP-40 and then assayed as described in Fig. 2. All extracts were preincubated in the presence of 0.5 U/μl RNasin and 10 m*M* 2-mercaptoethanol under optimized conditions. The antibodies added to the extracts were 1/10 preimmune normal rabbit serum (NS); 1/10 rabbit sera to each of dengue-2 virus specific proteins NV4 and NV5 (D4 and D5, respectively), 1/20 monoclonal antibodies to JE virus-specified NV4 (J4), and 1/10 hyperimmune rabbit serum to Kunjin virus (KH). For lanes 1 and 2 no serum was included in the extract. (B) Inhibition of RDRP activity by anti-dsRNA. The extracts were preincubated with 1/10 and 1/100 dilutions of the antibodies (DS) in the presence or absence of NP-40 prior to the assay as in (A). Extracts in lanes 1 to 3 were preincubated and assayed in the absence of antiserum. In all the experiments above, the concentrations of RNasin, 2-mercaptoethanol, detergents and antibodies indicated were the final concentrations in the assays.

trast however, the specific activity of the Kunjin RDRP is very low (about 0.001 unit/mg protein, calculated from Figs. 3 and 4) compared with that of, for example, encephalomyocarditis virus (0.05 unit/mg protein) which requires the presence of Mg^{2+} in the extraction buffer to maintain its association with membranes pelleted at 20,000 *g* (Rosenberg *et al.*, 1972, 1979).

It is unlikely that the RDRP complex is composed of merely virus-induced host coded proteins since the RDRPs of all positive-strand RNA viruses observed so far have virus coded polymerase subunits (see Hall *et al.*, 1982; Strauss and Strauss, 1983). The inability of any of the flavivirus-specific antibodies to block RDRP activity probably suggests that the antigenic sites on the functional virus specified proteins of the replication complex still remain inaccessible after mild detergent treatment. Further inhibition studies with more purified enzyme complex may be required. Inhibition of the Kunjin RDRP activity by anti-dsRNA is in accord with our conclusion that the double-stranded RF functions as template in the RNA replication complex. It is conceivable that if anti-dsRNA prevents initiation of synthesis on the naked RF and the double-stranded parts of the RI, synthesis of nascent RNA might still continue on preexisting RIs from where they are complexed with the polymerase and any cofactors; labeled RF molecules would then accumulate as the nascent RNA strands were completed and the unlabeled 44 S RNA was released. In the absence of reinitiation on these labeled RF, no labeled RI or 44 S RNA would then be detected and the incorporated label would remain in the RF as observed in Fig. 5B. The outcome would thus again be in accordance with our model of flavivirus RNA synthesis.

These experiments have only investigated a limited aspect of the undoubtedly complex nature of flavivirus RNA replication. More work needs to be carried out to study RNA synthesis *in vivo* and *in vitro* during the latent period when virus RNA of negative polarity and proteins are first detected. For example, by using strand-specific probes, we hope to be able to determine the strandedness of the RNA templates and products of the Kunjin RDRP during both the latent period and time of maximum RNA synthesis.

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