Nascent Flavivirus RNA Colocalized in Situ with Double-Stranded RNA in Stable Replication Complexes

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INTRODUCTION

The flavivirus Kunjin (KUN) replicates in cytoplasmic sites comprising virus factories identifiable by immunofluorescence (IF) and by immunoelectron microscopy (Westaway et al., 1997a,b; Mackenzie et al., 1998). A replication model based on kinetic analysis of KUN RNA synthesis proposed that late in infection flavivirus RNA is synthesized asymmetrically and semiconservatively in a replicative intermediate (RI) that uses the double-stranded RNA (dsRNA) or replicative form (RF) as a recycling template (Chu and Westaway, 1985, 1987, 1992). The proposed dsRNA templates can be immunolabeled with antibodies to dsRNA (Ng et al., 1983) and late in infection are located in induced membranes, which form vesicle packets (Mackenzie et al., 1996). dsRNA is colocalized with most of the KUN nonstructural proteins by IF and by immunogold labeling of cryosections of infected cells (Westaway et al., 1997a,b; Mackenzie et al., 1998). The immunofluorescent sites labeled with anti-dsRNA antibodies are stable to disruption of membranes by Triton X-100 or by cytoskeletal disrupting agents (Ng et al., 1983; Westaway et al., 1997a,b), and the flavivirus RNA-dependent RNA polymerase (RDRP) activity of infected cell lysates has been shown to be largely unaffected by treatment with several ionic or nonionic detergents (Chu and Westaway, 1987, 1992; Grun and Brinton, 1988).

Because the flavivirus genome comprises one long open reading frame, all the gene products are translated sequentially and continuously throughout the cycle of infection. Of the seven nonstructural proteins KUN NS1, NS2A, NS3, NS4A, and NS5 appeared to be associated with the replication complex in biochemical assays and immunocytochemical analyses (Chu and Westaway, 1992; Westaway et al., 1997b; Mackenzie et al., 1998). NS3 and NS5 contain domains associated with putative helicase and RNA polymerase activities, respectively, in addition to serine protease (NS3) and methyl transferase (NS5) (Chambers et al., 1990a, 1991; Wengler and Wengler, 1991, 1993). Dengue-1 virus NS5 was shown to copy RNA nonspecifically using an in vitro assay (Tan et al., 1996), and NTPase activity has been reported for NS3 of several related flavivirus species (Wengler and Wengler, 1991; Cui et al., 1998, and references therein). The remaining nonstructural proteins have no recognizable domains associated with events of flavivirus replication. However, in addition to our results on immunolabeling in infected cells of NS1 and other nonstructural proteins associated with dsRNA in vesicle packets (Mackenzie et al., 1996; Westaway et al., 1997b), Lindenbach and Rice (1997) showed that yellow fever virus RNA with a large in-frame deletion of NS1 failed to replicate significant
amounts of RNA minus strands, but could be complemented in trans.

In our previous studies on KUN replication, the analyses involved identification of the nonstructural proteins associated with RDRP activity or with putative sites of replication by radiolabeling, membrane purification, IF, or immunogold labeling of cryosections (Chu and Westaway, 1992; Westaway et al., 1997a,b; Mackenzie et al., 1998). However, it was possible that the assumed sites of replication represented only sites of accumulation of dsRNA, rather than the actual sites of synthesis of viral RNA. Furthermore, because all the nonstructural proteins were continually being synthesized, it was not clear whether these newly synthesized proteins were required to maintain the active RDRP complex. In this paper we addressed these uncertainties by showing that nascent KUN RNA labeled during synthesis in situ was colocalized in foci with dsRNA and that these sites of replication retained replicative activity after all protein synthesis was inhibited.

RESULTS

Stability of immunofluorescent foci of KUN replication sites

Dual-labeled foci of NS3 (or of several other nonstructural proteins) and dsRNA were prominent by IF and by immunogold labeling of cryosections of KUN-infected cells by 24 h and were detectable at 16 h (Westaway et al., 1997b; Mackenzie et al., 1998). While such foci appeared to be stable, there are no data on whether they continue to function for long periods as replication sites or whether nonstructural flavivirus proteins in the replication complex remain active for many cycles of replication. We therefore continuously treated KUN-infected cells from 24 h with cycloheximide (CHXM) using a dose of $10^{-3.5}$ M (90 $\mu$g/ml), known to completely inhibit all protein synthesis (see Fig. 2), and examined the distribution by IF of the putative replication sites. We then measured the effects of CHXM treatment on viral RNA synthesis and yield of infectious virus.

Figure 1 shows that perinuclear foci dual-labeled with anti-NS3 and anti-dsRNA antibodies appeared similar to those in untreated infected cells after treatment with CHXM from 24 to 28 h (compare a, b and c, d) or from 24 to 40 h (compare e, f and g, h). Parallel cultures were also treated with CHXM from 24 to 40 h, the drug was removed, and the cells were reincubated in normal medium for 4 h (g, h). No CHXM was added to the control infected cultures harvested at 24 h (a, b) and 40 h (i, j). All cell monolayers were fixed in cold acetone and dual-labeled with rabbit and guinea pig antibodies to NS3 and dsRNA, respectively. The secondary antibodies were conjugated to fluorescein isothiocyanate or to Texas red, and dual label in d (only) shows coincidental foci as a yellow hue.
to 40 h (compare e, f and i, j). The extent of clear coincidence of label in major foci was striking. As reported previously, staining by anti-NS3 antibodies also extended from the perinuclear region into a pattern of dense endoplasmic reticulum (Westaway et al., 1997b).

We concluded that continuing protein synthesis (either viral or cellular) was not essential for preserving the apparent architecture of the replication sites and the colocalization of NS3 and dsRNA. Following CHXM treatment of cells from 24 to 40 h, removal of the drug caused no obvious rearrangement of the dual-labeled foci during the next 4 h (Figs. 1g and 1h). Similarly no apparent rearrangement of foci occurred after reversal for 3 h following CHXM treatment from 24 to 30 h as a reference sample. Virus-specified proteins are identified in lane 7. Electrophoresis was in a 12.5% SDS discontinuous–polyacrylamide gel. In (B), KUN-infected cells were radiolabeled from 24 to 28 h with [35S]methionine/cysteine in medium that included 10−3.5 M CHXM only for the cells represented in lanes 2 (mock) and 5 (KUN). Cells were either harvested in SDS immediately for gel electrophoresis (lanes 1 to 5) or chased for 3 h in medium with excess methionine/cysteine included, either unsupplemented (lane 7) or supplemented with CHXM (lane 6), and then harvested. Cells represented in lane 3 and lane 4 (shorter exposure time of dried gel than for lane 3) were radiolabeled continuously from 24 to 30 h as a reference sample. Virus-specified proteins are identified in lane 7. Electrophoresis was in a 12.5% SDS discontinuous–polyacrylamide gel. In (B), KUN-infected cells were radiolabeled from 24 to 28 h with [32P]orthophosphate in phosphate-deficient medium, and the phenol-extracted RNA was electrophoresed in a 1% agarose gel (lanes 1, 2) or in a 7 M urea–polyacrylamide gel (lanes 3, 4), as described previously (Chu and Westaway, 1985). The viral RNA species are replicative form (RF), replicative intermediate (RI), and genomic-sized RNA (vRNA). The RI is retained completely at the origin in the urea–polyacrylamide gel. Autoradiographs were prepared from dried gels.

To assess the effect of protein synthesis inhibition on viral RNA synthesis, KUN-infected cells were treated continuously from 23 h with ACD and CHXM and radiolabeled from 24 to 28 h either with [32P]orthophosphate in phosphate-deficient medium or with [35S]methionine/cysteine. SDS–polyacrylamide gel electrophoresis of 35S-labeled cell lysates showed that all protein synthesis was blocked by the CHXM treatment (Fig. 2A, lanes 2 and 5). The inhibitory effect on protein synthesis was fully reversible (not shown) and CHXM treatment had no effect on the gel profile of viral proteins radiolabeled prior to treatment (compare lanes 3, 4, 6, and 7 in Fig. 2A). RNA was extracted from 32P-labeled cells and electrophoresed in parallel with RNA from non-CHXM-treated cells (Fig. 2B); the gel profiles revealed only a small decrease in radiolabeled viral RNA synthesized in the CHXM-treated cells, in agreement with a measured decrease of about 25% in incorporated radiolabel. We concluded that viral RNA synthesis did not require continuing protein synthesis and, in view of the IF data in Fig. 1, that the viral replication complexes apparently continued to function at the same equivalent sites in CHXM-treated and untreated cells.

Supernatant culture fluids were also collected at the end of the radiolabeling periods and the virus yields were compared by plaque assay. As found for RNA synthesis, the inhibition of protein synthesis by CHXM treatment had a relatively small effect on virus yield, which was reduced relative to untreated control cultures (107 PFU/ml) by about 50 and 90% after 4 h (24–28 h) or 16 h (24–40 h) of treatment, respectively. However, some visible CPE appeared during the 16-h treatment. Presumably, the released virus represented virus preformed.
prior to CHXM treatment or newly assembled virus encapsidated by a pool of structural proteins that had accumulated prior to CHXM treatment.

**Subcellular location of nascent KUN RNA incorporating short pulses of BrU**

The bromo-substituted nucleotide 5-bromouridine 5′-triphosphate (BrUTP) can be incorporated into RNA during synthesis in cells and the incorporated analog can be detected in subcellular sites by IF using primary antibodies to 5-bromodeoxyuridine (BrdU; Jackson et al., 1993; Wansink et al., 1993; Pombo et al., 1994; Restrepo-Hartwig and Ahlquist, 1996; Van der Meer et al., 1998). This labeling procedure provided the opportunity to compare the site of incorporation of nascent flavivirus RNA with that of dsRNA, the proposed template, by dual-labeled IF. In order to allow entry of BrUTP into mammalian cells, it is essential to permeabilize them with streptolysin O (Jackson et al., 1993) or Triton X-100 (Wansink et al., 1993; Pombo et al., 1994) or to use cationic liposomes (Haukenes et al., 1997; van der Meer et al., 1998). The first two methods involve disruption of membranes, which may perturb any membrane sites of flavivirus nascent RNA synthesis, and the last entails a delay in uptake of BrUTP. In initial experiments we delivered BrUTP by transfection into KUN cells at 24 h postinfection using DOTAP (see Materials and Methods). Incorporation of BrUTP in the ACD-treated infected cells was detected by IF after 60 min of incorporation, and in dual-labeled cells many of the sites of incorporation were coincidental with foci labeled with anti-dsRNA (Fig. 3A, panels c–d). No incorporation was detected in cytoplasmic components of ACD-treated mock-infected cells after a 20-min incubation with bromouridine (BrU) and BrUTP (results not
shown), but if pretreatment with ACD was omitted, strong incorporation in newly synthesized cellular Br-RNA was detected in the nucleus (Fig. 3A, panels e, f). Interestingly, the nucleoplasm was clearly stained only when the nuclear membrane was permeabilized for antibody access using formaldehyde/methanol fixation (Fig. 3A, panel f), and the nucleolus was stained only after acetone fixation although in a rare possibly damaged cell the nucleoplasm was also stained (Fig. 3A, panel e). Similar patterns of nuclear staining were observed previously in KUN-infected cells using the same fixation conditions and antibodies to KUN core protein, which contains a bipartite nuclear localization signal and a possible nucleolar localization signal (Westaway et al., 1997a).

Because of the relatively slow uptake into cells of BrUTP over several hours using DOTAP (see Boehringer Mannheim 1998 Biochemicals catalogue) it was desirable to use a faster delivery of bromo-substituted uridine in order to detect its incorporation into viral Br-RNA during short pulses. We showed previously that the cycle of viral RNA synthesis to complete one full-length strand in KUN-infected Vero cells at 20 h is complete in about 15 min (Chu and Westaway, 1985), similar to the cycle for dengue-2 virus (Cleaves et al., 1981), and according to our model of RNA synthesis, the nascent and recently completed viral RNA remains bound in the recycling dsRNA template until displaced during the next round of RNA synthesis (Chu and Westaway, 1985). In such experiments, incorporation of [5-3H]uridine into RI and RF could be detected after a pulse of only 10 min. We reasoned that pulse incorporation of BrU in KUN-infected cells could be detected similarly by IF using anti-BrdU antibodies as recently employed for detection of newly made RNA in nuclei of HeLa cells (Jackson et al., 1998). Hence there would be a brief window of time during which truly nascent KUN RNA could be detected in replication sites in cells, without the delay or membrane disruption associated with use of BrUTP. Accordingly, infected cells were treated with ACD and incubated with BrU for 15- and 60-min pulses (Fig. 3B). These results showed unequivocal staining of nascent viral RNA by anti-BrdU antibodies after the 15-min pulse of BrU, and dual-labeling with anti-dsRNA antibodies showed that the foci containing Br-RNA were prominently and consistently coincident with the foci of dsRNA. After the longer 60-min pulse, a similar pattern of dual-staining was observed, confirming the proposed stability of the sites of replication. These results established that nascent RNA was incorporated directly into the proposed sites of flavivirus replication indicated by the IF pattern of dsRNA, of which most if not all were actively engaged in viral RNA synthesis. Because the pattern of IF after a pulse label with BrU specifically labels the sites of replicating viral RNA, the coincidence of label with that of dsRNA is superior to that involving nonstructural proteins such as NS3 engaged in other events of virus replication (compare the IF patterns in Fig. 3B with those in Fig. 1). Some diffuse staining of dsRNA probably represents early development of new sites of replication as observed during the latent period (Westaway et al., 1997b; Mackenzie et al., 1998) and that were beyond the detection limit of our antibodies to BrdU.

Confirmation that pulsed BrU is incorporated in an RNase-resistant form into foci coincident with double-stranded viral RNA

Our model of flavivirus RNA synthesis proposed that on average only a single strand of nascent RNA is bound to the putative dsRNA template and forms the RI (Chu and Westaway, 1985; see calculation below). However, if rapid reinitiation involving synthesis of several nascent strands on the same template is occurring (in conflict with our model), the period for which each nascent RNA is fully bound would be quite brief, hence exposing some or most of these nascent strands to possible RNase digestion as each is displaced by a newly initiated strand. We have established that KUN core protein is not present at the site of RNA synthesis, i.e., in vesicle packets (Westaway et al., 1997a,b), and hence it is unlikely to protect by encapsidation any partially released nascent RNA strands. The calculation of an average of only about one nascent strand of flavivirus RNA per active RI is based on application of the formula devised for poliovirus by Baltimore (1968): Percentage RNase-resistance in the totally labeled RI = (2/[2 + N/2]) × 100, where the average length of each nascent strand is assumed to be one-half of a complete molecule, N/2 is the average molecule equivalents of nascent strands N, and 2/[2 + N/2] represents the ratio of labeled complete (+) and (−) strands (2) to the total number of labeled strands (2 + N/2). However, in flavivirus RI only about 10% of incorporated radiolabel is in the RNA(−) strand late in infection (Cleaves et al., 1981), and virtually all of the RNA(+) strands would be labeled late in infection. The estimate for ratio of RNase-resistant label in flavivirus RI should therefore be 11/[11 + N/2]. Thus when the Baltimore formula is modified accordingly, the calculated N for number of nascent strands in flavivirus RI is 1.0 if RNase resistance of the RI is 70% as for KUN after a 60-min pulse (Chu and Westaway, 1985) and 70% also for dengue-2 virus after a 10-min pulse (Cleaves et al., 1981). Thus there is an expectation that RNase treatment of flavivirus replication complexes in situ would have very little if any observable effect on detectable BrU incorporated during a 15-min pulse in the IF foci of dsRNA late in infection, in accord with the cartoon of RI pulse-labeled with BrU in Fig. 4.

To test the above prediction, we pulsed infected cells with BrU, permeabilized these cells with Triton X-100 to allow penetration of subsequently added RNase, and
observed by IF the effects of RNase digestion on incorporated BrU (Fig. 5A). In the control cells (i.e., no RNase), BrU was incorporated after a 15- or 60-min pulse into IF foci dual-labeled by anti-BrdU and anti-dsRNA antibodies as previously (compare Fig. 5A, panels a, b and g, h, with Fig. 3B, panels a, b and g, h, respectively), and the Triton X-100 treatment had no apparent effect on Br-RNA labeling, indicating the stability to detergent of the newly labeled Br-RNA foci. RNase digestion under high-salt conditions also had virtually no discernible effect on the dual-labeled IF patterns of Br-RNA and dsRNA after a 15- or a 60-min pulse of BrU (Fig. 5A, panels c, d and i, j, respectively). The coincidence of BrU-labeled foci with most of the dsRNA foci was retained, and the size and intensity of the Br-RNA foci appeared to be unaffected by this RNase treatment. When all RNA including dsRNA was rendered susceptible to digestion by RNase in low salt, both patterns of IF labeling were virtually eliminated as expected (Fig. 5A, panels e, f, k, l). Also as expected, nascent single-stranded Br-RNA in mock-infected cells was totally digested by RNase under high- and low-salt conditions (Fig. 5A, panels n and o, respectively).

We therefore concluded that nascent KUN RNA labeled after short pulses of BrU was strongly protected from RNase digestion in situ where it was colocalized by IF with dsRNA and that these results are thus in accord with the above predictions based on our model of flavivirus RNA synthesis.

**Confirmation by IF of the persistence of viral RNA synthesis at BrU-labeled replication sites in the absence of protein synthesis**

In view of the earlier result showing that viral RNA synthesis continued in the absence of protein synthesis (Fig. 2) it was of interest to establish that under these conditions nascent RNA was incorporated in the previously identified foci of dsRNA rather than in other unsuspected sites. Therefore KUN-infected cells were treated at 24 h with CHXM for 4 h and pulsed with BrU, as previously, in order to detect by dual-labeled IF the location of nascent Br-RNA. The results were unequivocal. Foci of Br-RNA were readily identified after 15- and 60-min pulses of BrU, and these were coincident with the majority of foci labeled by anti-dsRNA antibodies (Fig. 5B, a–d). It was therefore concluded that viral RNA synthesis occurring after CHXM switch-off of all protein synthesis was associated with and located in the same subcellular location as the proposed dsRNA template employed during normal replication. These results confirm independently the biochemical assay showing continuation of viral RNA synthesis by radiolabeling after CHXM treatment (Fig. 2B).

**Reproducibility of the subcellular location of immunofluorescent foci containing nascent Br-RNA using a KUN replicon C20SDrep transfected in BHK cells**

C20SDrep RNA is a highly efficient KUN replicon derived from the full-length KUN cDNA clone FLSD (Khromykh et al., 1998) by deletion of the sequence of the structural genes (C.prM.E) except for the first 20 codons of the C gene and the last 21 codons of the E gene. Its replication was monitored initially by detection of expressed NS3 using IF at 24 h posttransfection (results not shown). Under the same conditions, the transfected BHK cells were pulsed for 15 min with 100 mM BrU and processed for IF using antibodies to BrdU and dsRNA, as previously (Fig. 5C). The observed foci showed virtually complete coincidence of the dual label and must represent the sites of synthesis of nascent replicon RNA in BHK cells. Because the replicon lacks the structural genes, these sites are completely dissociated from virus assembly and maturation. Thus this
result was obtained independent of the previous experiments, using a different cell line, a different RNA, and a different route for entry of RNA.

In summary, our results have established the suitability of BrU for pulse labeling nascent viral RNA and analysis by IF and shown that nascent KUN RNA colocalized
by IF in an RNase-resistant form with the putative dsRNA template in virus replication complexes.

**DISCUSSION**

The results presented have established that recently synthesized KUN RNA is associated with cytoplasmic foci identifiable by dual-labeled IF using antibodies to dsRNA and to incorporated BrU (Figs. 3 and 5). Our previous studies on the sites of KUN replication assumed that the presence of dsRNA in discrete foci, identified by both IF and immunogold labeling of vesicle packets in cryosections of infected cells, indicated the putative site of viral RNA synthesis late in infection (Westaway et al., 1997a,b; Mackenzie et al., 1998). Our present results are in accord with this assumption. IF foci were shown to contain BrU incorporated directly into truly nascent viral RNA synthesized during 15-min pulses and were precisely coincident with nearly all the dsRNA foci (Figs. 3B and 5). The resistance of this short-pulsed BrU-RNA in IF foci to RNase digestion in high salt (Fig. 5A) fits well with the prediction under Results based on our model of flavivirus RNA replication (Chu and Westaway, 1985, 1987). Furthermore, the IF sites of active replication were stable despite the complete block in translation by CHXM treatment (Figs. 1 and 5B) and were readily identified also by pulse-labeling with BrU of BHK cells transfected with KUN replicon RNA (Fig. 5C). Synthesis of viral RNA in the presence of CHXM (Fig. 2) indicates that continuing cell or viral protein synthesis is not required late in infection. Although newly synthesized viral RNA has been detected previously by IF in membrane-associated replication complexes after a 1-h pulse of Br-UTP in liposomes in BHK21 and RK13 cells infected with equine arteritis virus (Van der Meer et al., 1998), or after briefer pulses of BrUTF in brome mosaic virus-infected barley protoplasts (Restrepo-Hartwig and Ahlquist, 1996), we believe our results represent the first demonstration for RNA viruses of BrU incorporation in nascent viral RNA achieved using a short pulse of BrU.

Although minor amounts of uncleaved NS3-NS4A polyprotein have been reported for several flaviviruses (Chambers et al., 1991; Lobigs, 1992; Zhang and Padmanabhan, 1993), posttranslational cleavage of the flavivirus polyprotein appears to be otherwise complete in minutes rather than in hours, as well documented by kinetic studies of the processing of pulse labeled viral proteins for KUN and yellow fever virus infections (Schrader and Westaway, 1988; Chambers et al., 1990b). In view of this rapid proteolytic processing, and the lack of significant reduction in RNA synthesis during several hours of CHXM inhibition (Fig. 2), it seems unlikely that polyprotein precursors were involved in synthesis of KUN RNA and hence like the similar poliovirus strategy (Novak and Kirkegaard, 1994), coupling between translation of viral RNA and replication does not occur late in infection. The implication from the CHXM experiments must be that the viral replicase complex recycles the same protein components or that after each cycle of RNA synthesis the complex disperses and is reassembled from a relatively abundant pool of previously translated proteins. Several considerations favor the first alternative rather than the second. Thus the putative dsRNA template was recycled about every 15 min (Chu and Westaway, 1985, 1987), and RNA synthesis and the distribution of postulated dsRNA templates were not significantly affected by CHXM treatment (Figs. 1 and 2). Furthermore, the RDRP activity was shown to be unaffected by treatment of flavivirus-infected cell lysates with a range of detergents or by sedimentation away from soluble constituents (Grun and Brinton, 1987, 1988; Chu and Westaway, 1987, 1992). Dual enrichment of dsRNA and specific nonstructural proteins in or on individual vesicles within vesicle packets, as detected by immunogold labeling of cryosections of KUN-infected cells, indicates that the replicase complex is in a relatively stable arrangement or configuration (Westaway et al., 1997b; Mackenzie et al., 1998). Several of the components are probably bound strongly to specific membranes or located within the lumen of the ER, e.g., the hydrophobic proteins NS2A and NS4A, and glycoprotein NS1, respectively (Coia et al., 1988; Westaway et al., 1997b; Mackenzie et al., 1998). Membranes possibly act as a scaffold for assembly of the RDRP that remains cohesive and active even after solubilization (Chu and Westaway, 1992).

Characterization of the flavivirus replication complex is still incomplete. In addition to the present results and those showing the relationship between RF and RI (Chu and Westaway, 1985, 1987, 1992), our major contributions have been to establish that all the KUN nonstructural proteins except NS2B and NS4B are associated with dsRNA in induced vesicle packets, the postulated site of RNA replication (Westaway et al., 1997a,b; Mackenzie et al., 1998). We also showed previously that the structural proteins were not involved in the active KUN replication complex (Chu and Westaway, 1992; Khromykh and Westaway, 1997), as is again evident in the replicon experiment (Fig. 5C). An essential role for NS5 was evident because in transfected cells, KUN RNA with deletions in NS5 of the RNA polymerase motif GDD or the S-adenosylmethionine binding site was not viable, but was complemented in trans by KUN replicon RNA (Khromykh et al., 1998). In replication studies in vitro with other flaviviruses, purified dengue-1 virus NS5 copied nonspecific template RNA (Tan et al., 1996), NS3 and NS5 were implicated in flavivirus RNA replication by the demonstrated inhibitory effects of specific antibodies in the RDRP assay for dengue-2 and Japanese encephalitis viruses (Bartholomeusz and Wright, 1993; Edward and Takegami, 1993), and both proteins of the latter virus bound to the terminal stem loop in the 3′ UTR of genomic RNA (Chen et al., 1997). Despite this progress, the puta-
tive roles of NS3 and NS5 as RNA helicase and RNA polymerase, respectively, in specific synthesis of flavivirus RNA have not yet been directly demonstrated.

Our accumulated evidence on KUN replication strongly implicates the RF as the template for flavivirus RNA synthesis in RI, both being reactive with antibodies to dsRNA. RF and RI were pulse-labeled with [3H]U within about 10 min, radiolabeled genomic RNA became prominent during a 10- to 20-min chase period, and the labeled RF appeared to recycle rather than accumulate as a “dead end” product (Chu and Westaway, 1985). Similar results were obtained during in vitro RDRP assays (Chu and Westaway, 1987). Antibodies to dsRNA coprecipitated the same nonstructural proteins shown to be associated with dsRNA by cryo-EM in induced vesicle packets, the only cytoplasmic location where enrichment of dsRNA was observed (Westaway et al., 1997b; Mackenzie et al., 1998). In this paper, a 15-min pulse of BrU was incorporated in nascent RNA convincingly coincident with dsRNA in IF foci and was obviously resistant to RNase digestion, as predicted by our model. The source of the flavivirus-induced membranes and the precise relationships of the replicase components to one another have not yet been defined. Our present characterization of the postulated site of RNA synthesis and some of its properties should contribute significantly to future studies on flavivirus replication.

MATERIALS AND METHODS

Cells and virus

Vero cells were grown in Medium 199 (Gibco) supplemented with 5% fetal calf serum. In maintenance medium (Eagle’s minimum essential medium), serum was replaced with 0.1% bovine serum albumin. Cells were infected at 37°C using a m.o.i. of 2 to 10 and treated with 0.05% Triton X-100 on ice for 4 min. Cells were washed briefly in cold PBS, fixed in cold acetone, and incubated with 50 &mu;g/ml RNase A (Sigma Chemical Co.) for 2 h at 37°C in 0.01× SSC buffer (1× SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0) or in 2× SSC buffer, and then processed for IF.

Incorporation of bromo-substituted uridine in nascent RNA

Bromo-uridine (Sigma Chemical Co.) was added to infected cells at 24 h, as specified under Results. For incorporation using BrUTP (Sigma Chemical Co.), the procedure of Haukenes et al. (1997) was used. Briefly, BrUTP with or without BrU was incubated with an equal volume of DOTAP Liposomal Transfection Reagent (Boehringer Mannheim) for 10 min at 20°C, and 10 &mu;l of the mixture was added to 300 &mu;l MEM/ACD on coverslip cultures for specified periods.

Immunofluorescence and antibodies

Coverslip cultures were washed, fixed in acetone at −20°C for 30 s, and processed for IF as described previously (Westaway et al., 1997a,b). Rabbit anti-KUN NS3 antibodies have been described previously (Westaway et al., 1997b). Guinea pig anti-dsRNA antibodies were a generous gift from Dr. Jia Yee Lee (Macfarlane Burnet Centre for Medical Research, Melbourne, Australia). Mouse monoclonal IgG antibodies to BrdU, which can react with BrdU incorporated in DNA and cross-react with Br-RNA (Jackson et al., 1993; Pombo et al., 1994), were supplied by Boehringer Mannheim and by Sigma Chemical Co. Dual-labeling experiments used FITC- and Texas red-conjugated species-specific anti-IgG (Edward Keller, Australia). All photographs were taken with ASA 400 or ASA 1600 film, the color slides were scanned on an AGFA Arcus II Color Scanner, and images were processed for presentation using an IBM computer and Adobe and Powerpoint software. For ribonuclelease digestion of Br-RNA in situ cells on coverslips were pulse-labeled with BrU and immediately permeabilized by treatment with 0.05% Triton X-100 on ice for 4 min. Cells were washed briefly in cold PBS, fixed in cold acetone, and incubated with 50 &mu;g/ml RNase A (Sigma Chemical Co.) for 2 h at 37°C in 0.01× SSC buffer (1× SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0) or in 2× SSC buffer, and then processed for IF.

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