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The Ends of La Crosse Virus Genome and Antigenome RNAs within Nucleocapsids Are Base Paired

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The three La Crosse virus genomes are found as circular structures in the electron microscope, and the RNA ends of at least the small (S) and medium (M) segments are highly complementary. When examined for psoralen cross-linking, about half of the S, at most 1 to 2% of the M, and none of the large (L) nucleocapsid RNAs could be cross-linked in virions or at late times intracellularly, under conditions in which each free RNA reacted completely. For the S segment, genomes and antigenomes first detected intracellularly could not be cross-linked at all, and their cross-linkability increased gradually with time. Antigenomes behaved similarly to genomes in all respects. It appears that the majority of all three segments are base paired at their ends and that the limited cross-linkability reflects the accessability of the RNA within nucleocapsids to psoralen. The gradual increase in cross-linkability may be important in persistent mosquito cell infection, in which it correlates with decreased S mRNA synthesis rates, and may be part of the mechanism which this infection becomes self-limiting. The implications of double-stranded RNA panhandles within nucleocapsids are discussed.

Bunyaviruses are trisegmented negative-stranded RNA viruses with lipid envelopes. The three genome segments, labeled S (small), M (medium), and L (large), are found in virions and intracellularly in separate nucleocapsids (NCs), which are thought to have helical symmetry (for reviews, see references 5 and 16). The genomic RNAs here are very tightly complexed with the nucleocapsid protein N, as they band in CsCl density gradients without prior fixation, a property of most viral NCs, but not of any cellular RNA-protein complexes. Their buoyant density here (1.31 g/ml), which is very similar to that of Sendai virus and vesicular stomatitis virus NCs, as well as tobacco mosaic virus, suggests that these structures are also composed of 96% protein and 4% RNA by weight.

When viewed in the electron microscope (EM), bunyavirus NCs were found to be circular (14, 21, 24), even though their RNAs contain 5′ triphosphate and 3′ hydroxyl ends (14). This circularity could be due to the terminal complementarity of each RNA segment. For example, the first 11 nucleotides (nt) at both ends of all La Crosse virus (LAC) segments are conserved and complementary. The next 15 to 20 nt, although not conserved between segments, are nevertheless highly complementary within each segment (1, 6, 10), and double-stranded (ds)RNA panhandles of ca. 30 base pairs are isolated in excellent yields from the deproteinized RNA of each segment by RNase A digestion (20; J. Patterson, unpublished data). Conservation of complementarity without conservation of sequence at the ends of the genome segments would seem to make sense only if the RNA ends could interact within the NCs. However, it is unclear whether these ends can in fact base pair in the presence of such an excess of the tightly associated N protein, which might sterically hinder helix formation. In this case, the circularity might be due to protein-protein interactions between the ends of the NCs, similar to that which is observed with tobacco mosaic virus dimers or the occasional circular structure seen with paramyxovirus NCs.

A similar conservation of complementarity without strict conservation of sequence also exists for the eight segments of influenza virus, another segmented negative-strand RNA virus. Hsu et al. (11) have recently used psoralen cross-linking to demonstrate that the ends of the genome RNAs within intracellular and virion NCs are base paired. They found that cross-linked forms predominated early in infection but were a minority at later times. Since the termination signal for influenza mRNA synthesis directly abuts the panhandle, they suggested that a panhandle in the genome RNA was a *cis* regulatory signal promoting mRNA rather than antigenome synthesis. Antigenomes (and genomes) could then be made from linear templates and mRNAs from circular templates. As the cross-linked forms again predominated in virions, which do not contain antigenomes, this suggested that the panhandle was also important during virion assembly.

This report extends this study to LAC NCs, and we also examined the cross-linking of antigenomes as well as genomes, which for technical reasons is difficult to do with influenza virus.

MATERIALS AND METHODS

Preparation of LAC NCs and virions. BHK-21 cells and C6/36 mosquito cells were grown in minimal essential medium plus 5% fetal bovine serum at 37 and 33°C, respectively, and infected with 25 to 50 PFU per cell, and both infections were maintained at 33°C in 2% fetal bovine serum. Cells were harvested by scraping into phosphate-buffered saline, recovered by centrifugation, and lysed with TNE buffer (150 mM NaCl, 10 mM Tris chloride [pH 7.4], 1 mM EDTA) containing 0.5% Nonidet P-40. The lysates were centrifuged for 10 min at 10,000 × *g*, and the cytoplasmic supernatants obtained were used for psoralen cross-linking within 2 h. Intracellular NCs were prepared by overnight centrifugation of the supernatants through 20 to 40% CsCl density gradients. The visible NC band was removed with a syringe and recovered by pelleting onto a 50% glycerol cushion (18, 22). Purified virions were prepared as described previously (19).

Psoralen cross-linking and analysis. Cross-linking reactions were carried out in Eppendorf tube lids in a total volume of 150 μl of TNE containing the NC sample. The 4-aminomethyltrioxalen (AMT) derivative of psoralen was added to

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20 μg/ml, incubated for 2 min, and irradiated at 366 nm for 40 min. Another 20 μg of AMT per ml was then added, and irradiation continued for a further 30 min. The samples were then diluted to 400 μl with 1% sodium dodecyl sulfate and phenol extracted, and the RNAs were ethanol precipitated. Cross-linking reactions on cultured cells in situ were done in 6-cm dishes containing 2 ml of medium to which the AMT was added as above. Cytoplasmic extracts were then prepared with Nonidet P-40, and the RNAs were recovered as above.

The RNA samples were dissolved in 80% formamide containing 10 mM Tris chloride (pH 7.4) and 1 mM EDTA, heated for 2 min at 90°C, and separated on 1.5-mm-thick 2.8% (S RNA) or 2.3% (M and L RNAs) polyacrylamide gels (22). The gels were soaked in 50 mM NaOH for 15 min, washed with water, equilibrated with transfer buffer (22), and then electroblotted onto Hybond-N (Amersham Corp., Arlington Heights, Ill.) membranes and probed with riboprobes as described previously (22).

Primer extension analysis. Bands representing the linear and cross-linked forms of the S genomic RNAs, as well as S mRNA, were isolated from the gel by using markers of known relative mobility, electroeluted, phenol extracted, and ethanol precipitated. A 5'-end-labeled synthetic DNA primer representing positions 82 to 99 of the negative-strand genome was annealed to the RNAs and extended by reverse transcriptase (22). The reaction products were phenol extracted, ethanol precipitated, and analyzed on a 6% sequencing gel.

RESULTS

Psoralens are photoreactable nucleic acid-cross-linking agents which cross biological membranes and are activated by wavelengths (366 nm) which are absorbed by little else in cells. They can thus be used in situ on cultured cells as well as in vitro (7). Evidence of cross-linking can be obtained either in the EM or by electrophoresis on denaturing gels on which the cross-linking retards migration (band shifting).

We first examined the reactivity of the intracellular S segment RNAs with AMT because their length (983 nt) allows them to be followed easily by band shifting and because probes which detect antigenomes but not S mRNAs are available. When LAC-infected BHK-21 cells were treated in situ and their RNAs were extracted and analyzed by Northern (RNA) blotting, approximately 40% of the genome and antigenome RNAs migrated as a sharp band with reduced mobility, which was also identical for genomes and antigenomes (Fig. 1). This band shift was dependent on the presence of AMT (Fig. 1) as well as the irradiation (data not shown). Essentially the same result was obtained when either total cytoplasmic extracts or pure CsCl density gradient-banded NCs were reacted in vitro (Fig. 1). Little material appeared to be lost during reaction, since the sum of the cross-linked and the linear forms in the presence of AMT is similar to the amount of linear molecules in its absence. In several experiments, S antigenomes were always cross-linked to the same extent as genomes.

The sharpness of the shifted band suggested that it contained a unique cross-link (7, 28). To determine whether this cross-link was within the panhandle region, we recovered both the linear and the cross-linked RNAs from the gel as well as the S mRNA, and a negative-strand primer complementary to positions 82 to 99 of the antigenome was extended on them (Fig. 2). The primer extended to positions −12 to −18 on the mRNA and position +1 on the linear form

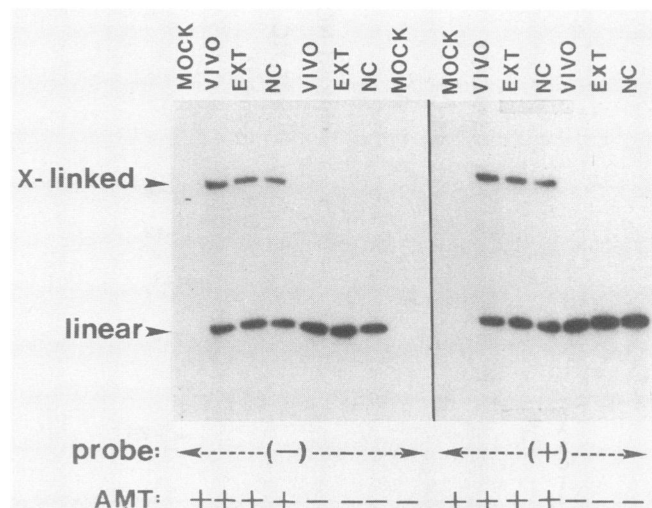

FIG. 1. AMT cross-linking (X-linking) of S genomes and antigenomes. Infected BHK-21 cells in situ (VIVO), total cytoplasmic extracts (EXT), or CsCl density gradient-banded NCs (NC) were irradiated in the presence or absence of AMT. The RNAs were examined on duplicate blots by Northern analysis with riboprobes containing positions 890 to 983 as plus (+) or minus (−) strands as indicated. The minus-strand probe reacts with antigenomes but not mRNA which terminates at position 886. MOCK refers to mock-infected BHK-21 cells reacted in situ.

of the antigenome, as found previously (19, 22). However, the primer extended only to position +24 on the cross-linked antigenome. The predicted ds panhandle of the antigenome RNA in which the preferred cross-linkable sites (opposite uridines separated by a single base pair) are also indicated is shown in Fig. 6. The G at position +24 is precisely the last base before the predicted cross-link at the inside end of the panhandle. No other cross-link within the first 80 nt of the antigenome was detected, nor was there any evidence that the unencapsidated S mRNA, which lacks the 3' half of the panhandle, was cross-linkable when the blots in Fig. 1 were reprobed (data not shown). The RNA bases at the ends of the antigenome RNA within NCs are thus base paired as they are in free RNA in at least 40% of the NCs.

We next examined the cross-linkability of the S-segment NCs as a function of the time after infection in both BHK-21 cells and *Aedes albopictus* (mosquito) cells. LAC grows to similar titers in both cell types, but in mosquito cells virus replication takes place more slowly and without cytopathic effect (23a). Although these are different infections with very different outcomes, a convenient point of reference between them is the intracellular accumulation of viral NCs. These rise steeply between 2 and 8 h in BHK-21 cells and 5 and 24 h in C6/36 cells and then level off (23a). At the earliest times examined (2.5 h for BHK-21 cells, 5 h for C6/36 cells), we could not find any cross-linked genomes or antigenomes, even with overexposures of the blots in Fig. 3 and 4. At the next time point (6 h for BHK-21 cells, 23 h for C6/36 cells), when genome and antigenome accumulation is mostly over, ca. 20% of the NC RNAs were cross-linked, and this level gradually rose to 40% at 20 h in BHK-21 cells and at 68 h in C6/36 cells. Thus, in both cell types, genomes and antigenomes are first made in a non-cross-linkable form. When the Northern blots are quantitated (lower panels, Fig. 3 and 4), it can be seen that the two infections are remarkably similar except for the absolute time course and that genomes and antigenomes are indistinguishable in both infections. The

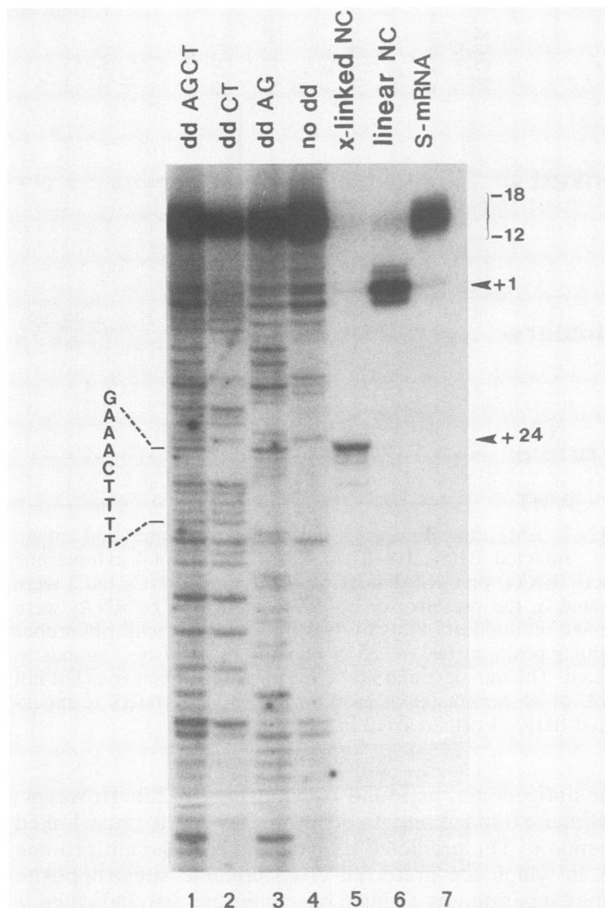

FIG. 2. Location of the cross-link in the S antigenome. Lanes 5 to 7 show the results of the primer extended on the various S RNAs isolated from a gel as described in the legend to Fig. 1 (Materials and Methods). Lanes 1 to 4 show the results of primer extension on unseparated CsCl pellet RNA in the presence of various combinations of dideoxynucleoside triphosphates (dd, dideoxy) as indicated above to provide a sequence ladder. The positions of the extended primers are indicated on the right; position +1 represents the 5′ end of the antigenome. The sequence shown on the left is that immediately preceding the cross-link at position +24.

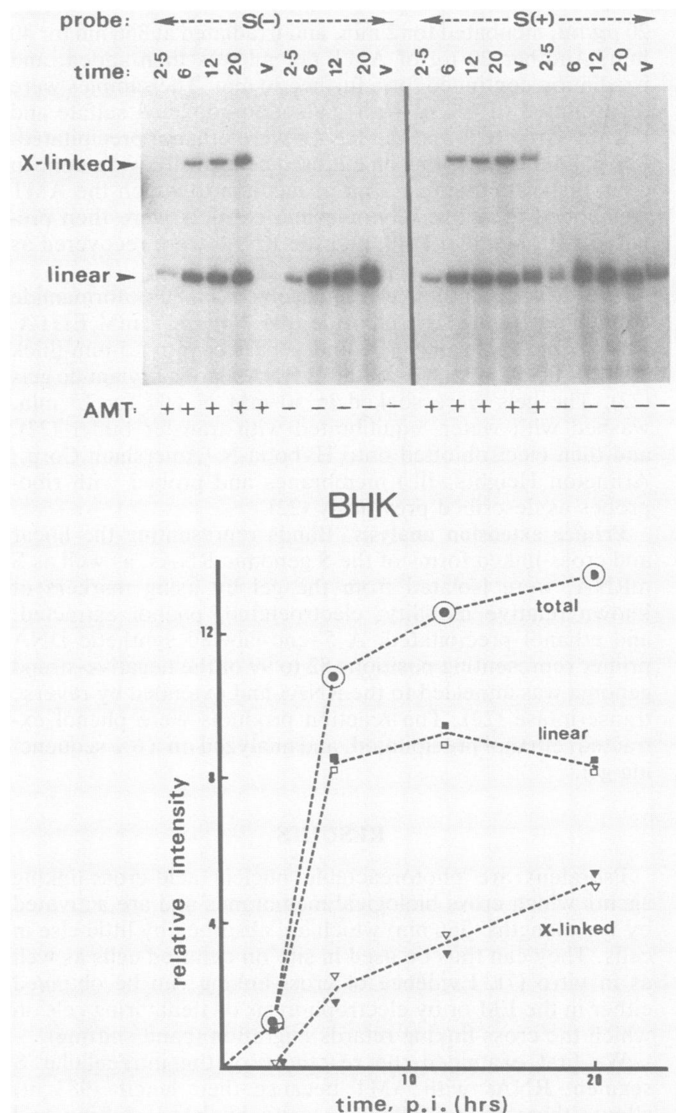

FIG. 3. Time course of S segment cross-linkability (X-linked) during BHK-21 cell infection. LAC-infected BHK-21 cell monolayers were irradiated in the presence or absence of AMT at the times (hours postinfection [p.i.]) indicated. Cross-linking of the S RNAs was monitored by Northern blotting as described in the legend to Fig. 1 (top panel). Lanes V refer to virions purified from the medium at 20 h postinfection. The autoradiograms were quantitated by densitometry, and the results are plotted in the bottom panel. Genomes (□, ▽) and antigenomes (■, ▼) were normalized to each other by using the values of the total amount of each species present at 20 h.

non-cross-linkable forms first accumulate rapidly but then level off sharply. The cross-linkable forms, on the other hand, increase gradually throughout this time. Thus, either the cross-linkable forms are made more frequently with time or the NCs are always first made in the non-cross-linkable form, but gradually mature to the cross-linkable form. In any event, the cross-linkability of the LAC S genomes during infection evolves quite differently from that of influenza virus (11).

The virus liberated into the medium at the end of the last time point in the above infections was recovered and also examined. In the case of the BHK-21 virions, which contained little if any antigenomes (Fig. 3, lanes V) and which can be detected in the medium as early as 8 h (23), the S genomes were only slightly more cross-linkable than those in the cell at 20 h (42% versus 37%). However, as these virions matured from 8 to 20 h, there may have been a slight preferential maturation of the cross-linkable form. This slight preference was also seen in mosquito cells (40% versus 33%; Fig. 4). Unexpectedly, the mosquito virions contained considerable amounts of antigenomes as well, whose cross-linkability was twice that of those in the cell at 68 h (66% versus 29%; Fig. 4). Thus, although cross-linkability may play a minor role in the packaging of genomes into virions, it appears to play no role in the mechanism that excludes antigenomes during maturation. Curiously, only in mosquito cells, in which antigenomes are not excluded from mature particles, are the cross-linkable forms clearly packaged preferentially.

The EM studies mentioned above indicated that all the virion NCs are circular, yet only about one-half of the S segment RNAs are cross-linkable under our conditions. Increasing the concentration of AMT or the time of irradia-

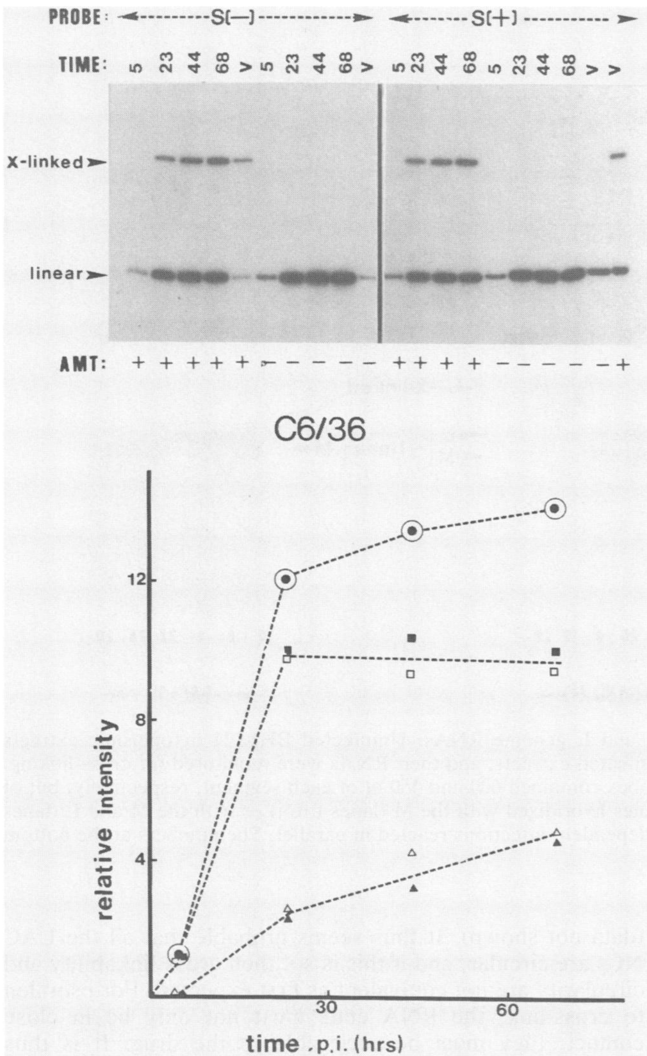

FIG. 4. Time course of S segment cross-linkability (X-linked) during C6/36 cell infection. The experiment is identical to that described in the legend to Fig. 3, except that the time course is longer. Lanes V refer to virions purified from the medium at 68 h. Genomes and antigenomes were normalized as described in the legend to Fig. 3, but with the 68 h values. The symbols are the same as in Fig. 3.

tion did not further increase the fraction of cross-linked molecules (data not shown). This suggests that the incomplete cross-linking is not the result of incomplete reaction conditions. To examine this point more carefully, we did the following reconstruction experiment. Free RNA was isolated from BHK-21 virions and added to uninfected BHK-21 extracts in the proportion normally found as NCs in infected extracts. Both the infected and reconstituted extracts were then examined in parallel for cross-linkability of their genome RNAs. Under conditions in which again only 40% of the genome NCs were cross-linked, the overexposure of the blot in Fig. 5 shows that all the RNA in the reconstituted extract had reacted, even at one-half the concentration of AMT normally used. The cross-linked unencapsidated genome RNA, however, migrated as a much broader band. Underexposure of the blot showed that it is composed of several bands within a smear, whereas that from the infected extract was a sharp single band as before. This heterogeneous migration presumably was due to additional cross-links formed in the absence of the attached N protein. Further, the extent of cross-linking did not increase when the reactions were done with pure CsCl-banded NCs, which contain only the N protein, rather than cytoplasmic extracts or intact cells (Fig. 1). The partial reaction is therefore unlikely to be due to the presence of another protein attached to the genome ends which prevents reaction. The incomplete cross-linking of the RNA within NCs would thus appear to be due to the NC structures themselves and not an artifact of the conditions used.

Cross-linkability of M and L NC RNAs. The M and L genome RNAs are considerably longer than the S RNA (4.6 [10] and 8 to 9 kilobases, respectively) and do not separate on the 2.8% polyacrylamide gels used for the S genome. Agarose gels allow better separation of larger RNAs, but it is unclear whether linear and cross-linked RNAs can be separated here because of the higher porosity. Band shifting owing to cross-linking has been reported only on polyacrylamide gels, and we have also been unable to see band shifting on agarose gels. However, when the polyacrylamide gel concentration is lowered to 2.3% and the gels are run more slowly for longer times, the M and L RNAs can be distinguished by mobility alone (Fig. 5, lanes k and l). To determine whether the cross-linked forms can be distinguished from the linear RNAs, we examined the reconstituted extracts as before. AMT reaction again led to a band shift of slower mobility (Fig. 5, lanes i and j, n and o), although again this band was a broad smear. However, the presence of this smear and the complete disappearance of the linear form upon reaction indicated that all the unencapsidated M and L genome RNA can also be cross-linked in the absence of the attached N protein.

We next examined whether the M and L NC RNAs could be cross-linked. Using intracellular extracts and virions in which 40% of the S NCs were cross-linkable, we could find no evidence of cross-linking of the M and L NC RNAs (Fig. 5, lanes f and g, k and l) in three separate experiments with nine different samples. Only in one experiment (Fig. 5, lanes p to u) could a band shift of the M genome dependent on AMT reaction just be detected, but this band represented only 1 to 2% of the RNA, although it was as sharp a band as the linear RNA. It is possible that we are underestimating cross-linking of the M and L segments because of their size. For example, the larger RNAs are better targets for nicking, and cross-linked molecules might be nicked faster than linear ones; the nicked circles could conceivably then comigrate with the linear forms. However, we saw little evidence of degradation in most of our experiments and, more importantly, no obvious loss of the genomes from the reconstituted extract upon their complete conversion to the cross-linked form. Our results would therefore indicate that the M and L genomes and antigenomes, unlike the S RNAs, can only be cross-linked to very small extents, if at all.

DISCUSSION

The ability of AMT to cross-link the ends of the S RNAs within NCs indicates that these ends are as close to each other as they are in free dsRNA. We find this base pairing remarkable in a structure composed of 96% protein which is stable enough to survive CsCl density gradient centrifugation. For example, in Sendai virus defective interfering NCs, which are structures of similar size, composition, and stability and in which there is a perfect complementarity of at least 120 nt at the ends (12), circular NCs were never seen in

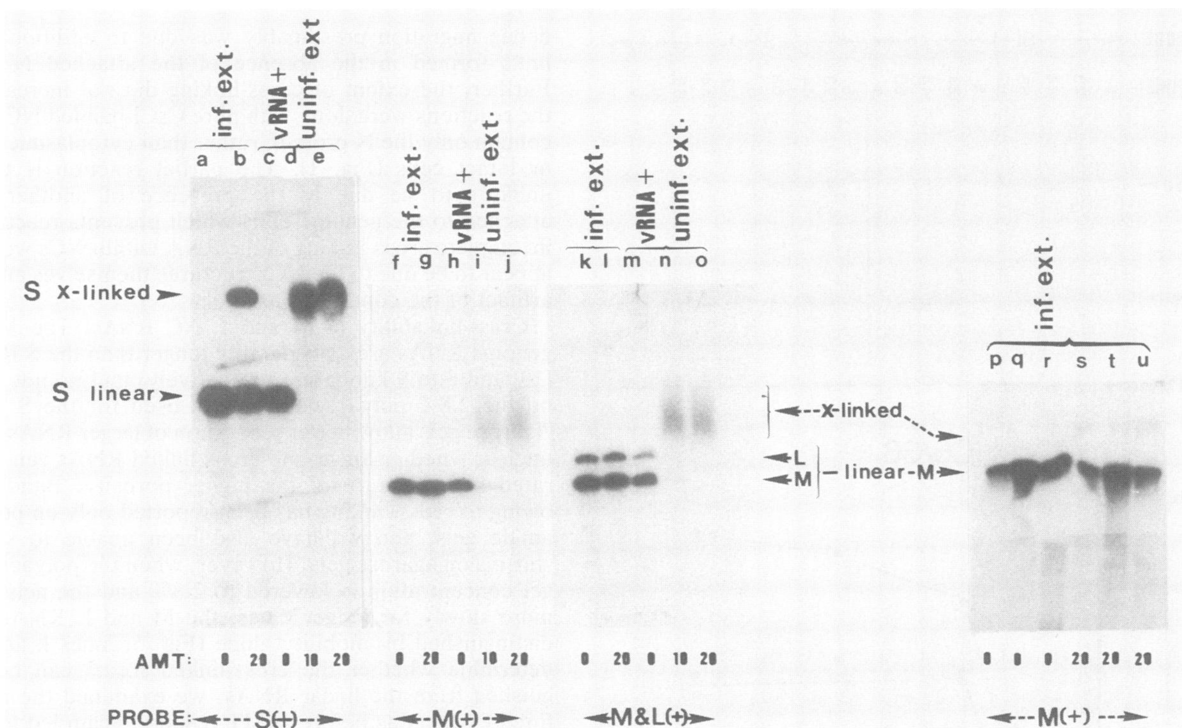

FIG. 5. Cross-linkability (X-linked) of free and encapsidated S, M, and L genome RNAs. Uninfected BHK-21 cytoplasmic extracts containing phenol-extracted virion RNAs were reacted in parallel with infected extracts, and their RNAs were monitored for cross-linking. The S(+) riboprobe contained positions 890 to 983; the M and L riboprobes contained 600 and 450 nt of each segment, respectively, but of unknown position (23a). The middle panel represents duplicate membranes hybridized with the M (lanes f to j) or with the M and L (lanes k to o) probes. Lanes p to r and s to u represent the extracts of three independent infections reacted in parallel. The numbers at the bottom refer to the AMT concentration in micrograms per milliliter.

the EM although they were searched for exhaustively (unpublished data). AMT cross-linking, furthermore, is not the only indication of the ability of the RNA within NCs to base pair. It has been previously shown that some oligodeoxynucleotides complementary to genome RNA induce the specific cleavage of the RNA within NCs in the presence of RNase H (4). It is unclear whether during this base pairing the protein must be displaced from the RNA or whether its relatively large mass could remain attached to the ribose-phosphate backbone without interfering with base pairing. In any event, the N protein would not be expected to dissociate because of protein-protein interactions.

Within virions or at late times intracellularly, we were able to cross-link only about 50% of the S, at most 1 to 2% of the M, and none of the L genome RNAs. These results contrast with those obtained with influenza virions, in which all eight segments were equally cross-linked (30 to 50%) as judged by EM and 60 to 75% of the NS, HA, and NA segments were cross-linked by band shifting (11), even though we were using the same psoralen and reaction conditions. All three bunyavirus segments, however, are considered to be circular. This is based on EM studies in which the NCs appeared first as very condensed and convoluted structures. For Uukuniemi virus (21), conditions were obtained in which the structures were unwound and all the segments appeared as circles, no free ends being visible. For LAC and Lumbo viruses, it was not possible to open the structures completely. Nevertheless, in those structures whose contours were clear, all three segments appeared as circles, and they were twisted or supercoiled as well (14, 24). We have also examined our preparations in the EM, with similar results (data not shown). It thus seems probable that all the LAC NCs are circular, and if this is so, then cross-linkability and circularity are not equivalent as first expected. For psoralen to cross-link, the RNA ends must not only be in close contact, they must be accessible to the drug. It is thus possible that a majority of all three segments are held together at their ends by base pairing and that the limited cross-linkability reflects the accessability of psoralen to the RNA. In this view, S genomes would represent a mixed population in which the N protein would exist in more than one conformation relative to the RNA, although not necessarily at all locations. Similar conclusions can be drawn from the finding that oligonucleotides which can anneal to the S genomes never anneal to more than half of them at equilibrium, using identical cell extracts (4). The notion of a mixed population is thus supported by an experimental approach not dependent on cross-linking.

All segmented negative-strand RNA viruses (influenza virus, bunyaviruses, and arenaviruses) contain genome segments with complementary ends which are in stable contact (3, 15, 17, 21, 26). For influenza viruses and bunyaviruses, at least, they also share a basic replication strategy in that mRNAs are initiated by primers (cap snatching) and at different rates for the various segments, determining in large part the differential gene expression. Genomes and antigenomes, on the other hand, are initiated by triphosphates at equal rates, as required for virus maturation. For example, during LAC infection of BHK-21 cells, the three genome segments replicate in an equimolar fashion but are transcribed into mRNAs to very different extents (roughly 5 S, 0.5 M, and 0.05 L mRNAs per genome [23a]). The molecular

basis for these events is mostly unknown, and it is tempting to speculate that the circular nature of the NCs is important in their explanation. We had hoped that psoralen cross-linking would provide insight into how these events are controlled for LAC. However, interpretation of cross-linking experiments will be more complicated than expected, because psoralen apparently distinguishes not only linear and circular forms, but also circular forms which can and cannot react.

In this case, we can only correlate the replication cycle with cross-linkable and non-cross-linkable forms, without knowing whether the latter are linear or circular. As shown previously in more detail (22, 23, 23a) and also seen in Fig. 3 and 4, in both BHK-21 and C6/36 cells, genomes and antigenomes accumulate rapidly after a brief lag and then level off. All LAC RNA synthesis is maximal at early times, when cross-linkability of the S segment rose to only 20% or less. The structures exhibiting AMT cross-linkability thus appear to be irrelevant in the early stages of infection when the majority of the viral RNAs are made. However, the cross-linkability we measure may be relevant for mosquito cell infections. Unlike BHK-21 cell infections, which are highly cytopathic and in which all protein synthesis is shut down owing to an induced generalized mRNA instability (23), the C6/36 cell infection is totally asymptomatic and persistent, with no detectable effect on host protein synthesis. During the first 68 h, however, S mRNA synthesis rates in C6/36 cells gradually decrease by the same extent that cross-linkability increases (40%) (23a). When these infections were observed for 72 days, the S mRNAs, which were five times as abundant as their templates at day 1, became undetectable at day 16 even though their templates had decreased only by 50%. They then cycled from undetectable to roughly half as abundant as their templates. Template cross-linkability and the gradual decline in mRNA synthesis may therefore be related. It will be of interest to determine whether S genomes become more cross-linkable over this longer time span and vary according to mRNA/template ratios.

There is as yet no satisfactory explanation for how the mosquito cell infection passes from an acute phase in which the viral RNAs accumulate rapidly to a persistent phase in which viral RNA levels stop accumulating and then decline. As this evolution takes place in the absence of detectable defective interfering genomes (23a), a gradual change in structure at the ends of the NC which decreases the rate of initiation of mRNA synthesis on these templates may then be part of the mechanism by which this infection becomes self-limiting.

These experiments also make it clear that the ends of the RNAs within at least some of the LAC NCs are in a ds structure and that this structure itself has interesting implications for control. The panhandles predicted for the S and M genomes and antigenomes are shown in Fig. 6 (complete L sequences are unavailable), with mismatched and unmatched bases displaced from the helix axis. Within a given genome-antigenome pair, each helical stack is identical to the other (except where G · U pairs are present), while the mismatched or unmatched regions are clearly different. The A · C mismatch in antigenomes at position 9, which is an allowed U · G pair in genomes, takes on added significance in this context as it lies within the first 11 bases, which are strictly conserved in all segments of the bunyavirus genus reported to date. Besides LAC, these include the S and M segments of snowshoe hare virus (1, 8), Bunyamwera virus (13), and Germistan virus (9, 17a), as well as the S segment

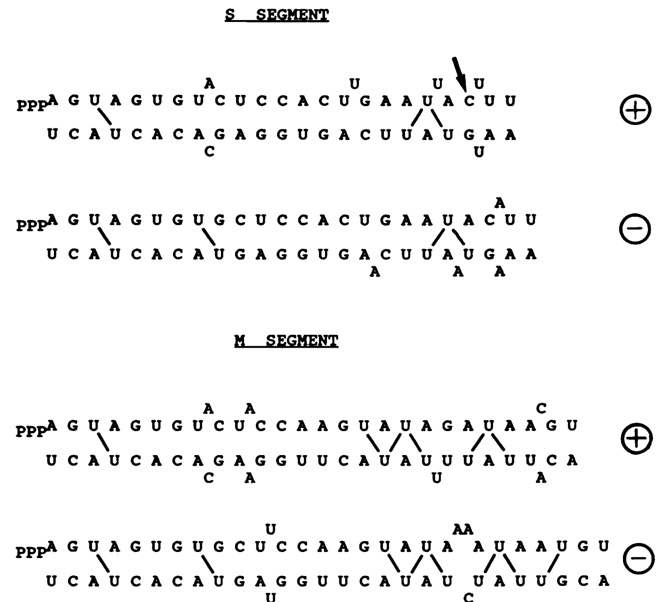

FIG. 6. Predicted RNA panhandle structures at the ends of the S and M genomes and antigenomes. The dsRNA structures are those predicted for the free RNAs; the 27-base-pair panhandle of the S genome has also been demonstrated (20). Base-pairing possibilities beyond the structures shown are much reduced. The most highly cross-linkable sites are indicated as diagonal lines linking U residues. The C residue at position +24 in the S antigenome, the complement of the last base incorporated by primer extension on the cross-linked RNA (Fig. 2), is indicated by an arrow. Plus and minus signs refer to antigenomes and genomes, respectively.

of Aino virus (2) and the L segment of Bunyamwera virus (R. Elliott, personnal communication). Unmatched and mismatched bases within ds regions are thought to play an important role in the recognition of RNA by proteins in several systems (27), and their replacement with other bases leads to loss of recognition (25). In this perspective, the ends of the LAC genome and antigenome NCs have significantly different structures even though their RNAs are highly complementary. The ability of proteins to distinguish these structures may then form the basis by which genomes and antigenomes are differentially transcribed and packaged into virions.

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