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Structure of the RNA inside the vesicular stomatitis virus nucleocapsid

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ABSTRACT

The structure of the viral RNA (vRNA) inside intact nucleocapsids of vesicular stomatitis virus was studied by chemical probing experiments. Most of the Watson–Crick positions of the nucleotide bases of vRNA in intact virus and in nucleoprotein (N)-RNA template were accessible to the chemical probes and the phosphates were protected. This suggests that the nucleoprotein binds to the sugar–phosphate backbone of the RNA and leaves the Watson–Crick positions free for the transcription and replication activities of the viral RNA-dependent RNA polymerase. The same architecture has been proposed for the influenza virus nucleocapsids. However, about 5% of the nucleotide bases were found to be relatively nonreactive towards the chemical probes and some bases were hyperreactive. The pattern of reactivities was the same for RNA inside virus and for RNA in N-RNA template that was purified over a CsCl gradient and which had more than 94% of the polymerase and phosphoprotein molecules removed. All reactivities were more or less equal on naked vRNA. This suggests that the variations in reactivity towards the chemical probes are caused by the presence of the nucleoprotein.

Keywords: chemical probing; protein–RNA interaction; protein–RNA template; replication; transcription; VSV nucleocapsid

INTRODUCTION

Vesicular stomatitis virus (VSV) is an enveloped virus with a single-stranded RNA genome of negative polarity, that is, complementary to mRNA sense (for a review, see Dietzschold et al., 1996). The viral RNA (vRNA) is completely covered by the nucleoprotein (N) with a stoichiometry of 9 nt per N monomer (Thomas et al., 1985) making an N-RNA complex. In the virus, the N-RNA complex is associated with some 50 copies of the viral RNA-dependent RNA polymerase (L for large protein) and 350–400 copies of the phosphoprotein (P), a polymerase cofactor (Thomas et al., 1985). L by itself does not bind efficiently to N-RNA but binding of L is helped or mediated by binding of P (Mellon & Emerson, 1978). The complex formed by N-RNA plus L and P is called the nucleocapsid (NC) and is transcriptionally active in vitro, either after opening up of the virus with detergent or after purification from other viral components. The N-RNA complex is the template for the polymerase activity rather than the naked RNA. N-RNA can be separated from P and L and transcriptional activity can be recovered by reconstitution of all components (Emerson & Wagner, 1972; Emerson & Yu, 1975; Emerson, 1982, 1987; Perrault et al., 1983; De & Banerjee, 1984; Helfman & Perrault, 1989; Canter et al., 1993). Inside the virus, the nucleocapsid is associated with the matrix protein, which condenses it into a tight helical skeleton that gives the virus particle its bullet shape (Newcomb & Brown, 1981; Newcomb et al., 1982; Barge et al., 1993).

There is a single site for transcription initiation on the vRNA at its very 3′ end (Emerson, 1982). After initiation, the polymerase transcribes a copy of the leader and then subsequently the individual mRNAs of the viral genes from 3′ to 5′ (Abraham & Banerjee, 1976; Ball & White, 1976; Wertz et al., 1998). There is a 30% attenuation of transcription at each gene boundary leading to a gradient of transcripts going from 3′ to 5′ (Iverson & Rose, 1981). The promoter for VSV transcription was defined as the first 3′ 15 nt of the viral genome by Smallwood and Moyer (1993), whereas Whelan and Wertz (1999), using a different approach, identified two distinct sequence elements to be important for tran-

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scription initiation, nt 19–29 and nt 34–46 from the 3′ end. P and L are more or less evenly distributed on viral and on intracellular NCs (Harmon et al., 1985). However, with the present knowledge about transcription and replication of VSV, there does not seem to be a functional necessity for any specific binding of P-L other than close to the 3′ terminus. It is not clear why the virus carries 50 copies of the polymerase, as only three to five P-L complexes appear to be active (Helfman & Perrault, 1989). It has been suggested that many of the bound P-L complexes are, in fact, complexes that were active in transcription when the nucleocapsid was packaged into newly made virus (Emerson, 1982, 1987).

Although the morphology of NC and the stoichiometry of 9 nt per N is known (Thomas et al., 1985), there is not much more known about the interaction of N with the nucleotides. The only studies that have been published have shown that N does not protect the N7 position of the guanines in the RNA against chemical modification with dimethylsulfate (Keene et al., 1981; Isaac & Keene, 1982). Our previous work on the structure of influenza virus ribonucleoprotein particles (RNP) has shown that influenza virus nucleoprotein binds to the sugar–phosphate backbone of the vRNA and exposes the bases of the nucleotides to the solvent (Baudin et al., 1994; Klumpp et al., 1997). We have suggested that, in this way, the bases were available to the polymerase and transcription could take place directly after cell entry, without the need to dissociate the nucleoprotein from the RNA. However, it is generally assumed that the RNA in VSV is entirely covered by the nucleoprotein and protected against the solvent and RNase attack. If that were true, then a structural change in the nucleocapsid or even dissociation of N from the RNA would be necessary before the viral polymerase could transcribe the vRNA, the first enzymatic activity in the replication process of negative-strand RNA viruses.

Here we show that the ribonucleotide bases inside the nucleocapsid of VSV are in general available for chemical modification both at their Watson–Crick positions and at the N7 positions of guanine. The RNA backbone is protected by N. This suggests that the interaction of N with RNA in VSV may be comparable to that of influenza virus nucleoprotein with RNA. We studied the reactivities of the nucleotide bases in the leader region, inside the gene for N, and at the N-P intergenic region and found quite a few single nucleotides or small regions in the viral genome that were poorly reactive towards chemical modification and others that were hyperreactive. One of the poorly reactive regions, between nt 16 and 30, was the same as found before by Keene et al. (1981) for the reactivities of the N7 positions of the guanine residues. In our study, removing at least 94% of P (and L) from the nucleocapsids did not make these sites reactive. After removing the nucleoprotein from the vRNA all nucleotides had similar reactivities, with only a few exceptions. This suggests that the variation in reactivity is not due to the presence of P and/or L but rather due to a specific structure or charge distribution imposed onto the RNA by the presence of N.

RESULTS

We have studied the solvent exposure of different atomic positions on the nucleotides of the vRNA inside the VSV nucleocapsid. We tested the phosphates of the backbone of the RNA, the Watson–Crick positions of adenine, cytosine, and guanine, and the N7 positions on G. The modified or cleaved positions on the vRNA were then revealed by reverse transcription using a primer located 3′ of the region under study (see Materials and Methods and Fig. 1).

In the RNP of influenza virus, the nucleoprotein binds to the sugar–phosphate backbone of the RNA and ex-

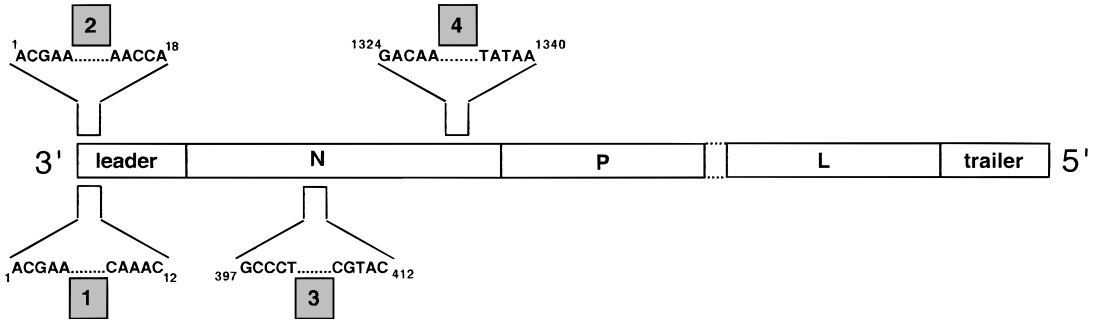

FIGURE 1. Schematic representation of the genome of VSV showing the leader and trailer regions and the genes for the nucleoprotein (N), the phosphoprotein (P) and the polymerase protein (L). The genes for M and G between P and L are not shown. Also indicated are the deoxyoligonucleotide primers that were used for reverse transcription to visualize the chemically modified positions on the RNA. The primers are complementary to the vRNA sequence; that is, primer 1 is complementary to nt 1–12 of the vRNA.

poses the bases to the solvent (Baudin et al., 1994; Klumpp et al., 1997). Therefore, we decided to test whether N of VSV also protected the RNA backbone against treatment with iron-EDTA. Fe(II)EDTA plus H_2O_2 produces hydroxyl radicals that react with and degrade the ribose ring in the RNA backbone (Hertzberg & Dervan, 1984; Tullius et al., 1987). The presence of protein bound to the RNA backbone lowers the reactivity of the ribose rings. Figure 2 shows a comparison of the sensitivity of naked vRNA with that of vRNA inside N-RNA. Whereas the backbone of the naked vRNA is cleaved by the treatment, the backbone of the vRNA in N-RNA is protected, like in influenza virus RNP.

In the following, we compare the reactivities of the bases of naked vRNA, of vRNA inside intact virions, and of vRNA in nucleocapsids after isolation and purification over a CsCl gradient (N-RNA). The preparation of N-RNA has lost most of its P and L. Figure 3 shows two Western blots of SDS-PAGE of serial dilutions of intact virus and CsCl-purified N-RNA using an anti-P antibody. The undiluted concentrations (1X lanes) were chosen such that the bands for N in the virus and in the N-RNA preparation had the same intensity on a Coomassie-stained gel. The blot shows that the intensity for the P-band in undiluted N-RNA was weaker than that in 16X diluted virus, meaning that N-RNA has less than 6% of the original complement of P in virus. The rationale behind comparing the three different preparations is that the purified N-RNA provides information about how the nucleoprotein binds to the vRNA and the experiment with intact virus may provide additional information on the binding sites for P and L. Initially, we compared the base reactivities of vRNA inside virus and in intact NC that was purified over a glycerol gradient and that still contained P and L. We never observed differences between modification of the RNA inside virus and the RNA inside intact NC, suggesting that the chemical reagents diffused efficiently through the viral envelope and labeled the vRNA inside virus as efficiently as in NC.

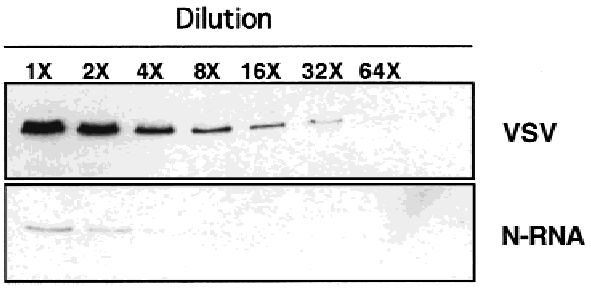

FIGURE 3. Western blot of serially diluted VSV virus and N-RNA using an antibody against P protein. The concentrations of nucleoprotein of the undiluted virus and N-RNA samples (lanes 1X) were the same as judged from the intensity of the bands on a Coomassie-stained SDS-PAGE. The undiluted virus lane contained about 10 μg of virus.

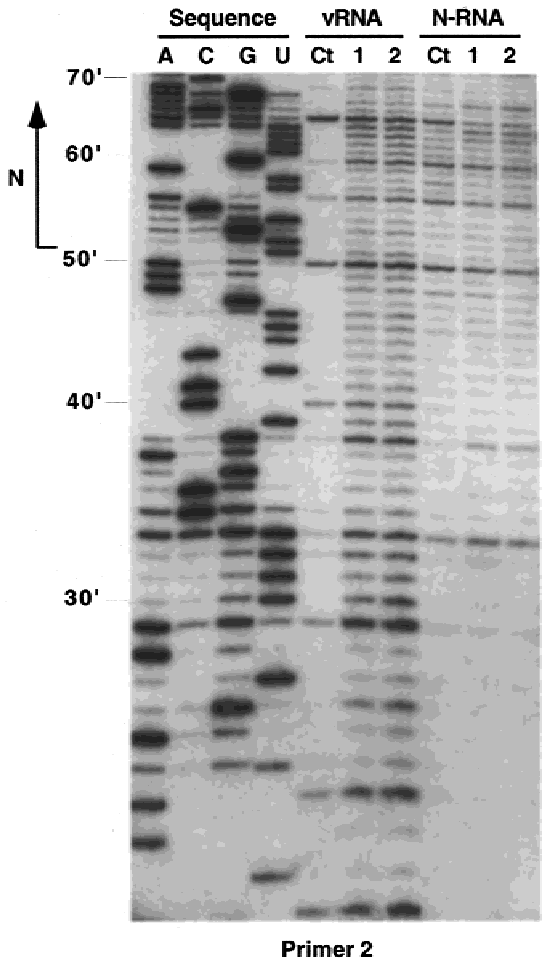

FIGURE 2. Twelve percent urea-PAGE autoradiogram of the cDNA fragments produced after reverse transcription of Fe-EDTA-treated naked viral RNA and RNA inside N-RNA using primer 2. Lane Ct is an incubation control of unmodified RNAs. Lanes 1 and 2 represent incubation of vRNA or N-RNA with 1 and 2 μL of reagents, respectively. Lanes A, C, G, and U represent the sequence of the vRNA under study (see Materials and Methods).

Figures 4–6 show the results of chemical modification of the 5′ half of the leader sequence and the beginning of the gene for N. Figure 4 shows the reactivities of the N7-G positions by dimethyl sulfate (DMS) labeling, which also labels A and C at their Watson–Crick positions. After DMS treatment, the vRNA is isolated and further treated with $NaBH_4$ and aniline to cleave the chain. Figure 4A shows the reactivities of N7-G inside intact virus as revealed by reverse transcription using primer 2. This panel shows that all G's are equally reactive except for G26, which is much less reactive than the other G's. The same experiment was performed on N-RNA, using primer 2 (Fig. 4B) and primer 1 (Fig. 4C). These two panels show the same result as was obtained with intact virus. When primer 1 is used,

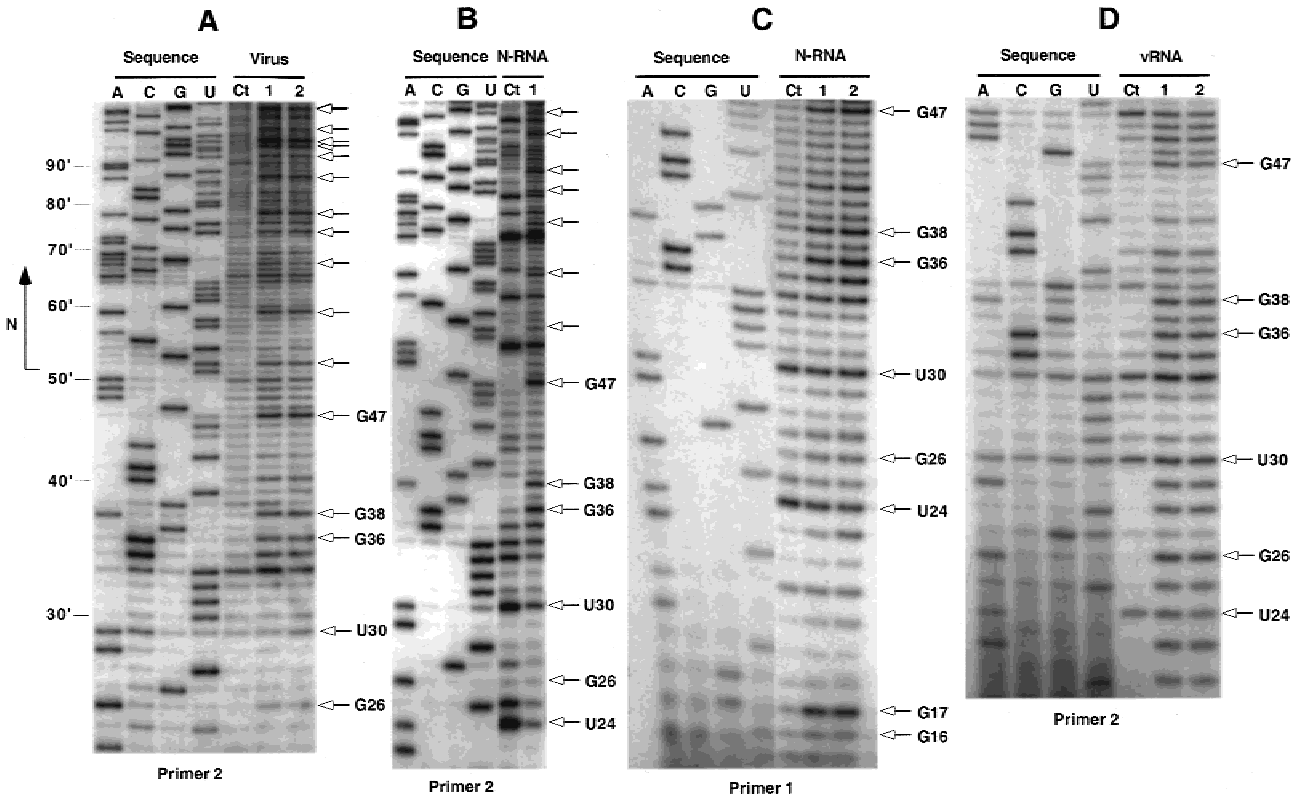

FIGURE 4. Twelve percent urea-PAGE autoradiograms of the cDNA fragments produced after reverse transcription of DMS-modified RNA, subsequently treated with $NaBH_4$ and aniline to reveal the modified N7-G positions, inside virus (**A**), in N-RNA (**B** and **C**) and naked vRNA (**D**). Note that the A and C residues are also modified. The gels show modified nucleotide positions in the leader region and the start of the gene for N (UUGU), indicated by an arrow. Lanes Ct are incubation controls of unmodified RNA (in virus, N-RNA, or naked vRNA) and lanes 1 and 2 represent incubations with increasing concentrations of DMS. Lanes A C G, and U are vRNA dideoxy-sequencing reactions of the VSV genome in the recombinant plasmid to identify the modified positions. The primers that were used to reveal the modified positions are indicated below the gels.

the low reactivity of G16 can also be noticed. Then we measured the reactivities on naked vRNA (Fig. 4D) and found that G26 was equally reactive as the other G's. This last reaction was done as a control to see if the variation in labeling was due to any intrinsic characteristic of the nucleotides or to any sequence effects.

Figure 5 shows the reactivities of G at its Watson–Crick positions towards labeling with kethoxal; Figures 5A and 5B in intact virus with primers 2 and 1 respectively, Figure 5C on N-RNA with primer 2 and Figure 5D on naked vRNA with primer 1. Although it is clear that all G's in virus and in N-RNA are reactive, some are clearly less reactive than others. It is also evident that the reactivity pattern of the G's in virus is the same as that of the G's in N-RNA. For instance, G38 is much less reactive inside virus and in N-RNA than its close neighbor G36, but in naked vRNA, G38 is as reactive as G36 or G47.

Figure 6 shows the reactivities of A and C at their Watson–Crick positions towards labeling with DMS. In this experiment the G's are not visible, as there was no subsequent treatment with $NaBH_4$ and aniline. The reactivities of A and C in intact virus (Fig. 6A) and in N-RNA (Fig. 6B) are rather variable, but again the results with virus and N-RNA are similar. Note, for instance, that A28 is not very reactive in virus and in N-RNA, whereas C35 is hyperreactive. The reactivities in naked vRNA are all more or less the same (Fig. 6C). Interestingly, U30 is also reactive towards DMS, both in virus and in naked RNA, although U's are not supposed to react with DMS and the chemistry of this reaction is not known. As the reverse transcriptase stops at the labeled U, labeling is probably at the N3 position. Labeling of a U base with DMS has also been observed for U7 at the conserved 3′ end of influenza virus RNA (in a mixture of the eight segments; Klumpp et al., 1997) and in other cases as discussed by Mayford and Weisblum (1989). This labeling of U30 is a characteristic of the RNA itself, as it was also reactive on naked vRNA (Fig. 6C) and not induced by the presence of N, P, or L.

The results presented in Figures 4–6 may be compared to those published by Keene et al. (1981) who treated NC with DMS/aniline to test for the reactivities

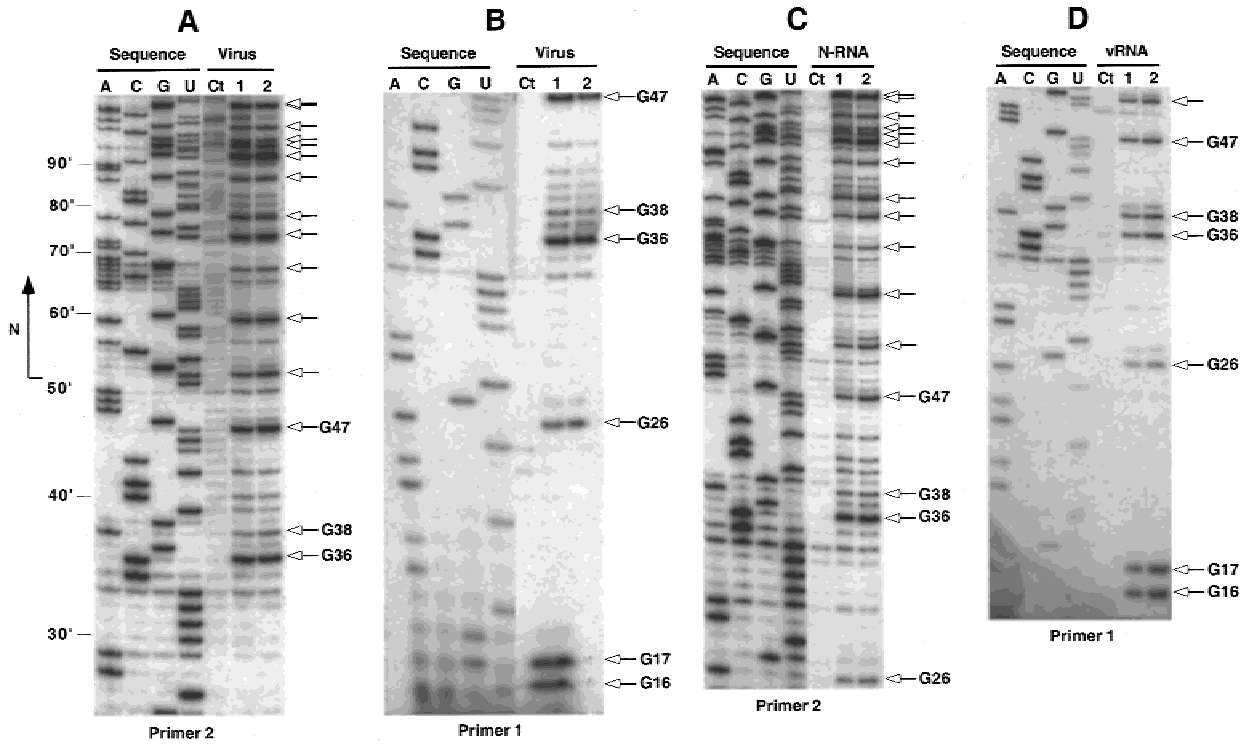

FIGURE 5. Twelve percent urea-PAGE autoradiograms of the cDNA fragments produced after reverse transcription of kethoxal modified RNA in virus (**A** and **B**) in N-RNA (**C**) and in naked vRNA (**D**) in the same region as shown in Figure 4. The arrows indicate modified G residues. Lanes Ct are incubation controls and lanes 1 and 2 represent shorter and longer incubation.

of the N7 positions of G. They found protection of the N7 positions of G16, G26, and G38 and enhancement of reactivity at U21, U24, and U30. In our experiments, enhancement of reactivity at U21 and U24 was difficult to assess, because the RNA was often broken in the controls (Figs. 4 and 5) and we did not detect protection at the N7 of G38. Keene et al. (1981) have stated that the protections and enhancements disappeared after removal of 94% of the P protein. Our preparation of N-RNA contained less than 6% of the amount of P found on nucleocapsids inside virus (see Fig. 3 and Materials and Methods), but we did not find that removal of P and L changed the reactivities of the bases. Our results suggest that the reactivities of the nucleotide bases inside virus are similar to the reactivities of the bases in N-RNA and the variation in labeling seems to depend on the presence of N rather than on the presence of L and P.

Figure 7 shows the reactivities of Watson–Crick positions of A and C in the middle of the gene for N, using primer 3 for the reverse transcription reaction. Here again, all Watson–Crick positions are accessible for modification but we observe large differences in the reactivities of the bases. For instance, A432 and C433 are very strongly modified, whereas neighboring A435, A436, and C440 are only weakly reactive. Towards the top of the gel, A463 is virtually nonreactive amid strongly reactive A's and C's. Figure 7 also shows the base reactivities of naked vRNA under two conditions, one in the same buffer as the experiments on virus and N-RNA and one in the absence of Mg^{2+} ions (as detailed in Materials and Methods). In the absence of Mg^{2+} ions, secondary RNA structures, which may influence the chemical modification experiments, are less stable. The results under the two conditions are strictly identical. With the exception of C440, which remained very weakly reactive in naked vRNA, all other reactivities have become similar, most notably A463, which is as reactive in naked RNA as its neighboring A and C bases.

The last region we studied was the end of the gene for N, the intergenic region, and the beginning of the gene for P, using primer 4 (Fig. 8). Again, all Watson–Crick positions of A and C are modified in the virus (Fig. 8A), but some more strongly than others. The Watson–Crick positions of G in the virus were modified with kethoxal (Fig. 8B). Here, the variation of intensity of labeling was extreme, with strong modification of G1371 but weak modification for G1368, 1388, and 1395 and total absence of modification for G1405. Next to G1405, G1407 and 1409 were strongly modified again. This pattern of labeling was again identical when N-RNA was treated (Fig. 8C). The position of the weakly modified residues was not restricted to the promoter region, as the unmodified G1405 is situated outside

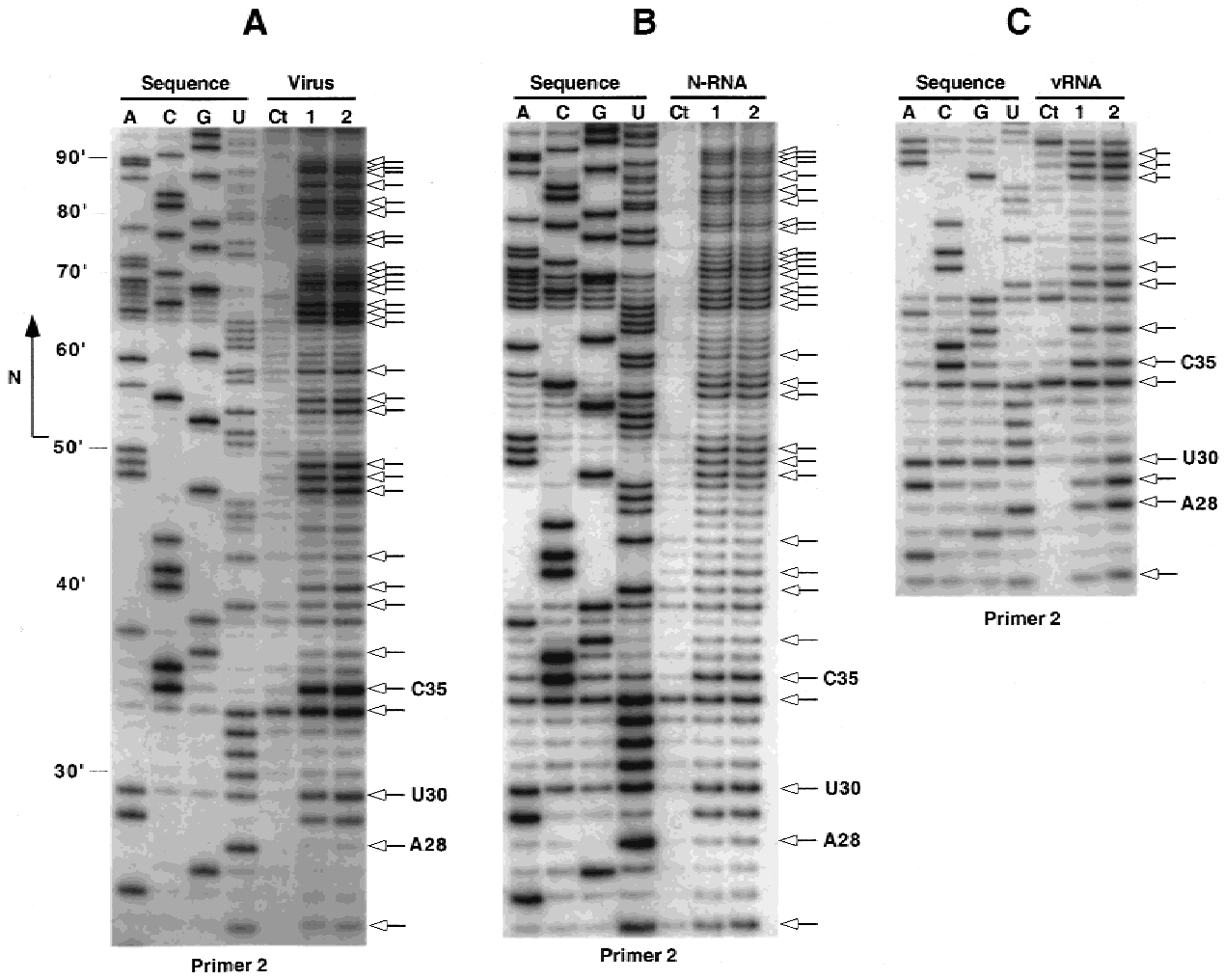

FIGURE 6. Twelve percent urea-PAGE autoradiograms of the cDNA fragments produced after reverse transcription of DMS-modified RNA in virus (**A**), N-RNA (**B**), and naked vRNA (**C**). The gels show DMS-modified N1-A and N3-C residues in the same region as Figs. 4 and 5.

this region and so is the weakly modified G1368. After removal of N, G's 1368, 1384, 1388, and 1395 in the naked vRNA are modified to the same extent as G1371. The 3 G's, 1405, 1407, and 1409, are also equally modified, but as a triplet reproducibly (in three independent experiments) less strong than the other G's in this region. We have no other explanation for this than that secondary structure formation involving these residues would prevent labeling to the same extent as the other G's, even in the absence of Mg^{2+} ions.

Note that on these gels, the C-residues give rise to weak bands upon kethoxal modification. This can also be observed in Figure 5. Further, the bands corresponding to the G's 3′ of the poly-U sequence have a tendency to be double. Doubling of bands was also seen 3′ of the poly-U sequence in the P-M and M-G intergenic regions (not shown). Therefore, this doubling of bands by the reverse transcriptase seems to correlate with the presence of a downstream poly-U sequence. Another case of doubling of bands by stuttering of the reverse transcriptase (but this time at strong stops and apparently independent of downstream sequences) has been described by Schlegl et al. (1997).

DISCUSSION

In the VSV nucleocapsid the nucleotide bases are exposed and the nucleoprotein protects the RNA backbone

The chemical modification experiments shown here indicate that, in VSV nucleocapsids, the bases are in contact with the solvent, but the backbone is protected by N. It also means that the vRNA inside the nucleocapsid is single stranded in the regions that we have tested. The function of N could be to present the vRNA as an extended single-stranded molecule to the viral polymerase complex. This protection of the RNA back-

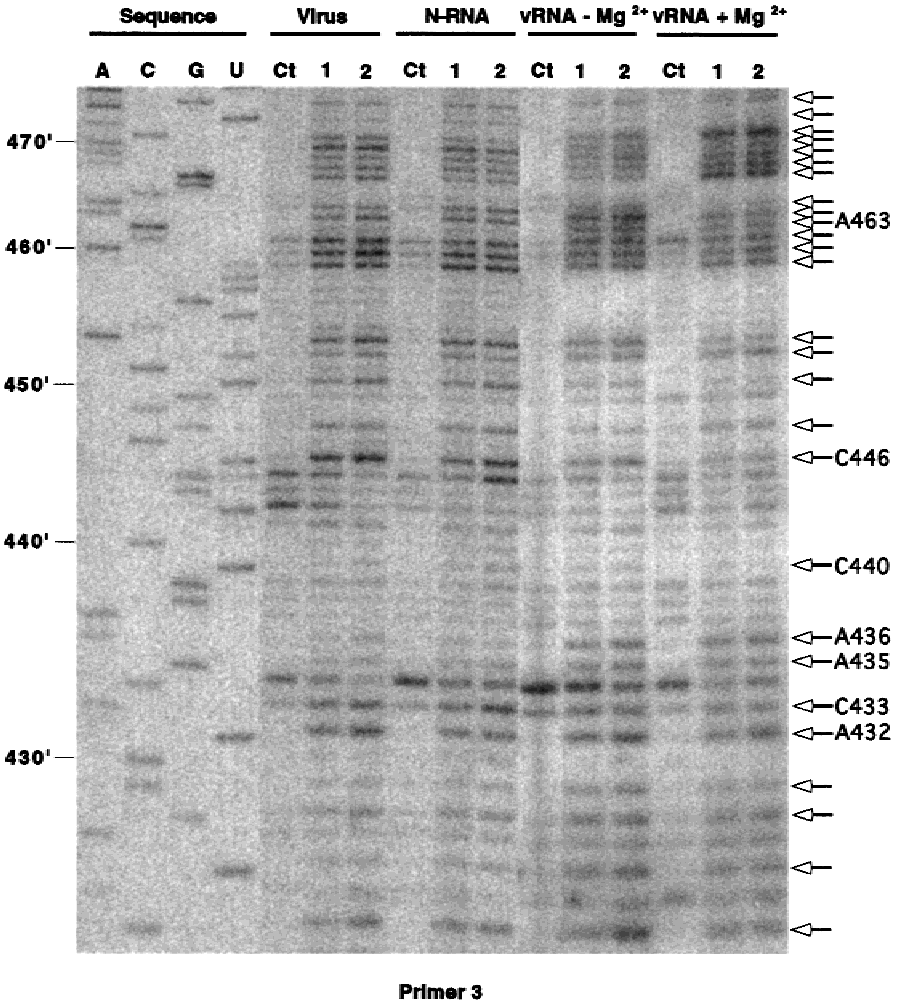

FIGURE 7. Twelve percent urea-PAGE autoradiogram of the cDNA fragments produced after reverse transcription of DMS-modified RNA in virus, N-RNA, and naked vRNA using primer 3. The probing reaction on naked vRNA was done once in the same buffer as the virus and N-RNA preparations ($+Mg^{2+}$) and once in the absence of Mg^{2+} ions. The gel shows DMS-modified N1-A and N3-C residues in the region between position 425′ and 475′ from the 3′ end, that is, in the middle of the gene for N.

bone and exposure of the nucleotide bases is the same as with the RNA in the ribonucleoprotein particles of influenza virus (Baudin et al., 1994; Klumpp et al., 1997) even though the morphologies, the RNA:protein ratios, and the RNase sensitivities for influenza virus and VSV nucleocapsids are very different. For influenza virus nucleoprotein (NP) we were able to show that, upon binding to double stranded RNA, NP melted the secondary structure elements and all RNA became single stranded in a reconstitution experiments of purified NP and a structured RNA probe (Baudin et al., 1994). For VSV we have not been able to perform such a reconstitution experiment so we do not know whether VSV N binds to the nascent vRNA when it is single stranded or whether it can also melt secondary structures. However, for rabies N expressed in insect cells, we have found that the protein is bound to cellular RNA with the same structure and stoichiometry as found in the viral N-RNA complex. One of the RNA species bound to N was sequenced and found to resemble a tRNA, suggesting that rhabdovirus N can also melt double-stranded RNA structures (Iseni et al., 1998).

The variation in intensity of chemical modification is caused by the presence of N but not by P and/or L

All the experiments that are shown in this work have been performed three times or more and the results have always been reproducible. Even unexplained findings like the doubling of bands for labeled G's in Figure 8, the weak reactivities for G1405–1409 in naked RNA (Fig. 8), or the absence of reactivity of C440 in naked RNA (Fig. 7) were reproducible with different virus preparations. It was also satisfying that we found a N7-G modification pattern similar to what Keene and coworkers reported almost 20 years ago (Keene et al., 1981). However, in contrast to these authors, we could not confirm that the protections and enhancements of modification were due to the presence of P and/or L, because all our experiments on intact virus gave results that were similar to those on N-RNA that had at least 94% of P removed. With the few exceptions mentioned above, the reactivities of the bases were more or less equal after removal of N from the RNA, sug-

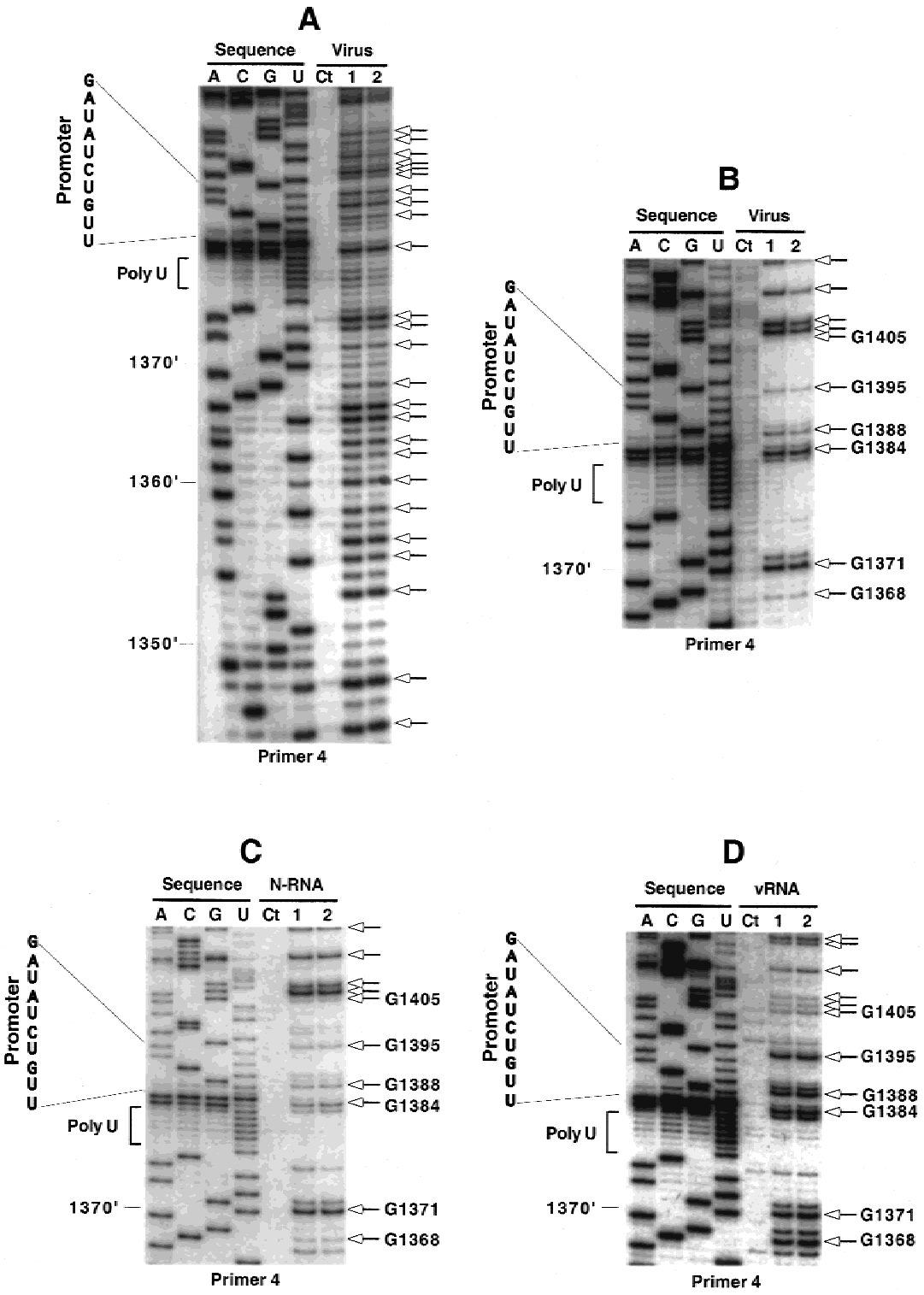

FIGURE 8. Twelve percent urea-PAGE autoradiograms of the cDNA fragments produced after reverse transcription of modified RNA in virus, N-RNA, and naked vRNA using primer 4, that is, at the end of the gene for N and the beginning of the gene for P. The gel in **A** shows DMS-modified N1-A and N3-C nucleotide positions on RNA inside virus. The gels in **B–D** show G residues modified by kethoxal inside virus (**B**), in N-RNA (**C**) and in naked vRNA (**D**). The poly-U sequence for the gene for N and the promoter sequence for the gene for P are indicated.

gesting that it is the presence of N that leads to the variation in reactivities of the nucleotide bases.

The gels in Figures 4–8 show chemical modification of the Leader/N-gene junction, of a region in the middle of the N-gene, and at the N-gene/P-gene junction. We have also determined nucleotide reactivities of G's in the intergenic regions between the P- and M-genes with kethoxal and reactivities of A and C between the M- and G-genes and at the end of the gene for L (shown schematically in Fig. 9). In total we determined the reactivities of some 400 nt. Regions or single nucleotides with very low reactivity or with hyperreactivity were found with an average of 4 per 100 nt. If this average were the same for the whole genome of VSV, then there would be some 440 protected/enhanced sites per genome, clearly too many for any remaining P on N-RNA. Apart from that, G1395, in the promoter of the gene for P (3′-UUGUCUAUAG-5′), is only very weakly reactive at its Watson–Crick positions in virus and N-RNA (Fig. 8) but the corresponding G's in the promoters of the genes for M and G are strongly modified (see schematics of Fig. 9). If P or L had caused the protection of G1395 by binding to the promoter, then similar protections would have been expected for all promoters.

Nevertheless, the observed protections of the Watson–Crick positions of A25, A28, and G38, the hyperreactive U30 and C35, and the protected N7 position of G26 in the leader region do coincide to some extent with the sequences that have been identified as being important for the regulation of transcription of VSV; nt 19–29 and 34–46 (Whelan & Wertz, 1999). However, the N7 position of G16 was also protected, whereas G16 is not important for transcription regulation (Smallwood & Moyer, 1993; Whelan & Wertz, 1999).

The fact that the modifications of NC inside virus or after isolation were the same as those of N-RNA also implies that we did not find a footprint for L or P. It should be noted that there are other chemicals that we could have used on isolated nucleocapsids but that do not diffuse properly through the viral membrane, such as DEPC, which labels the N7 positions of A. These positions are another possibility for contact sites with the polymerase. It is also possible that the P-L complexes on NC inside virus are evenly distributed on the genome but not on a specific site (Harmon et al., 1985; Emerson, 1987). Incubation of NC with ATP, under in vitro transcription conditions, which could have relocalized P-L complexes onto a promoter sequence, did not change the modification pattern (data not shown). It could also mean that the P-L complexes are bound at a specific site, but that there is a dynamic interaction even on this unique site. In that case the modifying chemicals could attack during the short intervals that certain positions on the bases were liberated. Recently, the binding site of HIV RT on the vRNA-tRNA$_3^{lys}$ complex was defined using a set of nucleotide- and RNA structure-specific RNases (Isel et al., 1999). In this article the authors comment on the fact that, whereas the binding site could be defined with the RNases, this had not been possible using chemical probing, probably because of a dynamic interaction of the protein with the RNA. Finally, with the method for revealing the modified bases that we used in this work, we were not able to obtain information on the first 15 nt that have been postulated by Smallwood and Moyer (1993) to contain the transcription promoter.

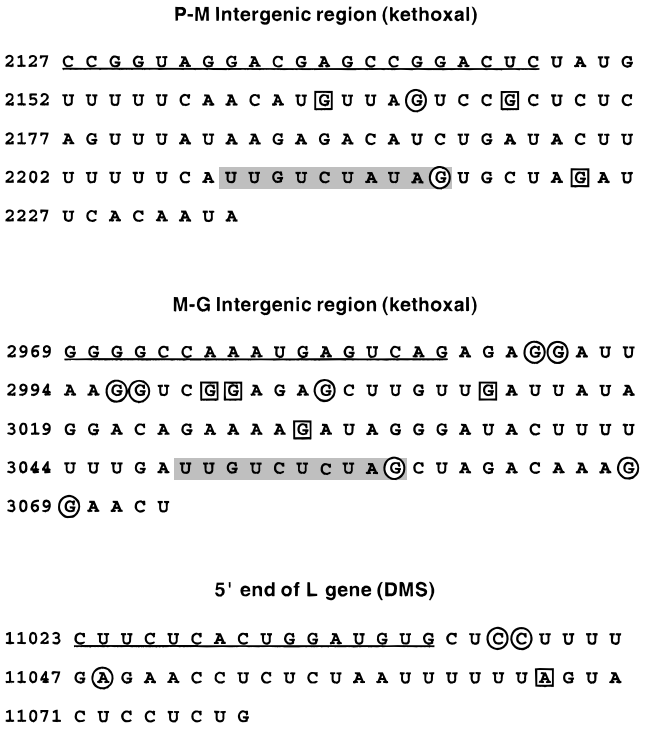

FIGURE 9. Schematic representation of the base reactivities in three regions of the VSV genome for which no gels have been shown. The titles above the sequences indicate the region under study and the probing chemical that was used. Underlined are the complementary sequences for the primers that were used for the revelation of the reactivities and shaded are the gene start sequences for the M- and G-genes, respectively. Circles indicate highly reactive residues and squares very poorly reactive or nonreactive nucleotides. The nucleotides that are not marked were normally reactive.

What could cause the variation in reactivities of the bases?

Sendai virus and some other paramyxoviruses and their defective interfering RNAs must have genomes with lengths that are multiples of six for efficient replication (Calain & Roux, 1993; Kolakofsky et al., 1998). Probably the Sendai virus polymerase only functionally recognizes the various promoter sequences and

other signals in the N-RNA template when the specific sequences are in the right "reading frame" on the protein. This requirement for genome length or the position of the starts of the genes in the context of multiples of nine is not needed for VSV (Kolakofsky et al., 1998). However, as we have found here that specific nucleotides in the N-RNA template are protected or hyperreactive, this implies that the nucleoprotein is always associated with these nucleotides in the same manner. That is, if rhabdovirus N has nine sites for the binding of nucleotides, then a specific nucleotide will always be at the same site on the protein. Therefore, we have tried to determine if the variation in reactivity in intact virus was regular, like whether every ninth residue was more or less reactive, or every ninth C or A, but we have not found such regularity. For example, G38 is not very reactive towards kethoxal but G47 is (Fig. 5). Neither could we find regularities for every eighth or tenth nucleotide. This probably means that the reactivities are not only influenced by N, but that the local nucleotide sequence is also important. Note that it is not only the base sequence that influences reactivity, as, with a few exceptions, the bases in naked vRNA all have more or less the same reactivities. In at least one case, U30, the nucleotide base itself seems to be chemically different in some manner that could have a signaling role inside the promoter for transcription. Note again that this result on U30 is not some strange result found only by us, but that it has also been described by Keene et al. (1981).

In the kind of chemical modification experiments that we have performed, protection against modification can occur when positions on the nucleotide bases are occupied in salt bridges and therefore not available for the chemical attack. These salt bridges could be with other nucleotides, with proteins, or with bivalent cations. It is also possible that the local nucleotide sequence plus the presence of the various charges on the nucleoprotein create a charge landscape that locally increases or decreases ion concentrations or that locally perturbs the water structure and that may have an effect on local concentrations of the chemical-modifying reagents. It may be that these local conditions also have an influence on the affinity of P and/or L for the vRNA. Another consideration could be that the helical coil structure of the nucleocapsid is locally under stress or has locally different helical parameters. At least for Sendai virus NC, it has been shown that the helical pitch may vary quite extensively (Egelman et al., 1989) and for VSV NC it has been shown that the normally helical NC can be stretched out into an almost linear state (Naeve & Summers, 1980; Naeve et al., 1980). If such variations occur locally, they could also have an influence on chemical modification of the bases or on binding of P and/or L.

MATERIALS AND METHODS

Virus, nucleocapsid, N-RNA, and vRNA preparation

The Indiana laboratory strain (Orsay) of VSV was propagated in BSR cells, a clone of BHK21 (baby hamster kidney cells) at 37 °C in Eagle's minimal essential medium supplemented with 2% calf serum. At 24 h postinfection, cell fragments were removed from the culture fluid by centrifugation, and virus particles were pelleted through 30% glycerol in 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, and resuspended in 25 mM Tris-HCl, pH 7.5, 0.7 mM Na_2HPO_4, 137 mM NaCl, 5 mM KCl, 10 mM EDTA.

Viral nucleocapsid containing L and P proteins was prepared from virus by treatment with Triton X-100 (1.85%) in 0.2 M NaCl, 10 mM Tris-HCl (pH 7.5), 0.6 mM DTT, 5% glycerol, and incubation at room temperature for 30 min. The detergent-solubilized nucleocapsids were centrifuged through 30% glycerol in 50 mM sodium cacodylate (pH 7.5), 5 mM $MgCl_2$, 150 mM NaCl (buffer A) onto a 100% glycerol cushion (SW 55 rotor, 45,000 rpm, 90 min, 4 °C) and dialyzed against buffer A.

N-RNA was isolated by detergent disruption of virus as above but with 1 M NaCl rather than 0.2 M. The solubilized nucleocapsids were then centrifuged over a 20–40% CsCl gradient (SW 55 rotor, 40,000 rpm, 90 min, 12 °C). N-RNA was recovered by punction of the visible band through the tube and dialyzing against buffer A. N-RNA was checked by SDS-PAGE to see if there was any remaining P or L, but even with a silver-stained gel, we did not detect these proteins (not shown). We then performed a Western blot of virus and N-RNA using a polyclonal antibody against P (Harmon & Summers, 1982; kindly provided by Dr. Joseph Curran, Geneva). The concentrations of virus and N-RNA were made the same in terms of band intensity for the N-protein in a gel stained with Coomassie blue. Then the samples were serially twofold diluted, run on a 10% SDS-PAGE, blotted with the antibody, and revealed with S^{35}-protein A. The two gels were blotted in the same apparatus at the same time and the blots were incubated with the antibody and other reactives in the same container and the autoradiography was done in the same cassette on a single film. As mentioned earlier, comparison of the intensities of the bands in the diluted samples suggested that 94% of P had been removed from N-RNA during the CsCl gradient centrifugation. The variation between different experiments was between 94 and >97% removal of P. A second CsCl gradient step did not remove more P, as observed in a similar blot (not shown) and the results of chemical probing experiments on singly and doubly CsCl centrifuged N-RNA were strictly identical (not shown).

Viral RNA was isolated from virus by phenol/chloroform extraction and precipitated with 100% ethanol in the presence of 0.3 M sodium acetate (pH 6.8). The precipitated vRNA was washed with 70% ethanol, vacuum dried, and dissolved in the appropriate buffer.

Cloning of the 3′ end of the VSV genome

vRNA was extracted from virus as described above. An oligodeoxyribonucleotide complementary to nucleotides 1–18

of the 3′ end of the viral genome was used for reverse transcription of the viral RNA into single-stranded DNA. The same primer and a primer complementary to nt 1521–1537 of the antigenome were then used for the synthesis of the second strand of the DNA. This double-stranded DNA of 1537 nt was directly ligated into the pTag vector (R&D Systems) according to the instructions of the manufacturer. The recombinant plasmid was amplified in *Escherichia coli.* This DNA was used for the sequencing reactions shown alongside the chemical probing experiments (see below).

Chemical probing experiments

The chemical reagents we used allowed us to test whether the Watson–Crick positions (N1-A and N3-C with DMS, N1-G and N2-G with kethoxal) were reactive or protected, giving information on the single- or double-stranded nature of the RNA or on the occupation of these sites by viral proteins. Probing the N-7 positions of G with DMS gives information on the tertiary structure of an RNA molecule or on the interaction of a specific nucleotide atom with a protein. The chemical probes and how they are used have been described by Ehresmann et al. (1987). For each condition (thus for each lane on the gel) we used 250 μg of purified intact virus, corresponding to about 1.3 pmol of RNA. Labeling reactions with N-RNA and naked vRNA were done with amounts of material per lane corresponding to what could be isolated from 250 μg virus. Chemical modification reactions were done at 37 °C in prewarmed buffer A. However, modification of vRNA was usually done in 50 mM sodium cacodylate (pH 7.5) plus 1 mM EDTA to destabilize formation of secondary structures (Baudin et al., 1994). In particular, the absence of Mg^{2+} ions could prevent possible double-strand formation between the complementary 3′ leader and 5′ trailer sequences. A control experiment comparing chemical probing of naked vRNA in the presence and absence of Mg^{2+} ions is shown in Figure 7, suggesting that doing the probing in the low-Mg buffer probably does not lead to artifacts.

DMS modification

DMS (0, 0.1, or 0.2 μL; conditions Ct, 1, and 2 in the respective autoradiograms) was added to purified virus, intact NC, N-RNA, or naked vRNA. Incubation time with DMS was 3 min at 37 °C. To detect the modification on N7-G, the methylated RNA was subsequently incubated with 10 μL of 1 M Tris-HCl (pH 8.3) plus 10 μL 0.5 M $NaBH_4$ for 20 min on ice in the dark. The ribose–phosphate chain was then split by treatment with aniline (pH 4.5) for 15 min at 60 °C in the dark.

Kethoxal modification

Five microliters kethoxal (20 mg/mL in 20% ethanol) were added to 250 μg of intact virus (or to NC, N-RNA, or vRNA) in buffer A. Incubation times with kethoxal were 15 and 30 min at 37 °C (conditions 1 and 2 in the respective autoradiograms). The control (Ct) was done by incubation for 30 min in the absence of the chemical. The reaction was stopped by addition of 20 μL 50 mM potassium borate (pH 7.0).

Cleavage of the phosphates

We added 0 μL, 1 μL, or 2 μL 50 mM $Fe(NH_4)_2(SO_4)_2$, 2.5% H_2O_2, 250 mM L-ascorbate, 100 mM EDTA to naked vRNA or N-RNA (conditions Ct, 1, and 2 in Fig. 3) and the incubation time was 10 min at 37 °C. The reactions were stopped by addition of 3 μL 0.1 M thiourea.

Detection and analysis of the modified positions

After chemical modification, the virus particles were centrifuged for 3 min at 150,000 × *g* in an Airfuge (Beckman). The pellet was resuspended in water and the modified RNA extracted with phenol/chloroform. Naked vRNA and vRNA from N-RNA were directly phenol extracted and precipitated.

The precipitated RNA was washed with 70% ethanol and vacuum-dried and dissolved in double-distilled water. Cuts in the RNA or modified positions were detected by the primer extension method using avian myeloblastosis virus reverse transcriptase, as described (Baudin et al., 1994; Klumpp et al., 1997). An oligodeoxyribonucleotide complementary to nucleotides located 3′ of the region of vRNA that we wanted to study (see Fig. 1) was labeled at its 5′ end with [γ-^{32}P]ATP and was used as a primer for reverse transcription. The RNA template was hydrolyzed after reverse transcription by addition of 3 μL 3 M KOH and incubation for 3 min at 95 °C followed by 1 h at 37 °C. The cDNA fragments were then precipitated and sized at nucleotide resolution by PAGE using 12% acrylamide/0.5% bis(acrylamide)/8 M urea slab gels at 1,500 V for 2 h. Dideoxy sequencing reactions using the same primers were carried out in parallel using the plasmid DNA corresponding to the 3′ end of VSV genome as the template for primer extension as described by Sanger et al. (1977). Incubation controls were run in parallel to detect nicks in the unmodified RNA or pauses of the reverse transcriptase due to secondary RNA structure. Note that with this analysis, a band in the control or a modified position in the treated RNA is found as a band one position below the corresponding position in the sequence due to the fact that the polymerase stops on the nucleotide preceding the modified residue.

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