Comparison of the 3' Termini of Discrete Segments of the Double-Stranded Ribonucleic Acid Genomes of Cytoplasmic Polyhedrosis Virus, Wound Tumor Virus, and Reovirus

LEON J. LEWANDOWSKI¹ AND S. H. LEPLA²

Department of Molecular Biology and Virus Laboratory, University of California, Berkeley, California 94720

Received for publication 8 May 1972

The 3' terminal nucleosides of the isolated components of double-stranded ribonucleic acids of reovirus, wound tumor virus, and cytoplasmic polyhedrosis virus were determined by labeling with tritiated sodium borohydride. All wound tumor virus and cytoplasmic polyhedrosis virus components appear to contain approximately equal amounts of U_{OH} and C_{OH} termini. Reovirus segments have essentially only C_{OH} termini.

Previous studies in this laboratory (11) have shown that it is possible to oxidize in situ the 3' termini of the double-stranded ribonucleic acid (dsRNA) contained in structurally intact cytoplasmic polyhedrosis virus (CPV). The number of 3' termini subsequently found by reduction with tritiated sodium borohydride of the oxidized RNA extract was equivalent to the number observed in RNA isolated prior to oxidation and reduction. We were able to conclude that the dsRNA genome of CPV, like that of reovirus (12), exists in the virion superstructure in 10 distinct polynucleotide segments present in equimolar amounts, and that the segmented nature of isolated CPV RNA is not an artifact of the extraction procedures.

When end-group analysis was performed on the total dsRNA of these two viruses, only C_{OH} 3' termini were observed in the total reovirus RNA hydrolysate (1), whereas a 1:1 mixture of U_{OH}-C_{OH} was present in the hydrolysate of the total genome of CPV (4). Furthermore, the 3' ends of the segments of both these dsRNA genomes have been shown to be nonphosphorylated (4, 11, 12). One is able to conclude, therefore, that in the case of reovirus, both strands of each subunit probably terminate in cytidine. With the genome of CPV, however, the presence of more than a single species of 3' termini in the total RNA

hydrolysate makes the exact disposition of these termini among the 10 double-stranded segments less obvious. Three major possibilities seem to exist: (i) there is a random distribution of U and C termini; (ii) five of the duplex molecules contain exclusively U, the other five exclusively C termini; and (iii) each of the 10 duplex molecules contains one strand terminating in U_{OH} and an antiparallel strand terminating in C_{OH}. The present communication suggests that it is this last possibility which appears to represent the distribution of 3' termini in the individual segments of the CPV genome.

A third dsRNA-containing virion, wound tumor virus (WTV), has also been shown to possess a segmented genome, electrophoretically separable into distinct classes, and estimated to contain between 12 and 15 components (6, 14). The analysis of WTV termini presented below shows that, as with CPV, each dsRNA segment appears to possess approximately equal amounts of U_{OH} and C_{OH} termini.

MATERIALS AND METHODS

CPV was grown in *Bombyx mori* and purified as described in an earlier report (9). Reovirus was grown in L cells and purified by the method of Watanabe et al. (13). WTV-induced root tumor tissue from clover plants was kindly provided by L. M. Black, and virus was extracted and purified by the procedures of Kimura as outlined by Black and Knight (2). RNA was extracted from these purified virions with sodium dodecyl sulfate-phenol (6) and the dsRNA purified from nonnucleic acid contaminants and, in the case of reovirus, from single-stranded polynucleotides

¹ Present address: Department of Medical Microbiology, Stanford University School of Medicine, Stanford, Calif. 94305.

² Present address: Biomedical Center, Brown University, Providence, R.I. 02912.

by adsorption-elution from hydroxyapatite (10). Purified dsRNA was then oxidized with periodate and reduced with ^3H -sodium borohydride as previously described (8). ^3H -labeled sodium borohydride (NET 023 H, 15 Ci/mmol) was purchased from New England Nuclear Corp. In agreement with our previous findings [(11), and S. H. Leppla, Ph. D. thesis, Univ. of Wisconsin, 1969, table XVI], the efficiency of the oxidation-reduction reactions was generally found to be approximately 50% or greater for the RNA species examined. R17 RNA, a generous gift of W. Paranchych, was carried through the labeling and analysis procedures as a control on the specificity of the procedures. The terminally labeled dsRNA genomes were then separated into distinct segments by preparative polyacrylamide gel electrophoresis, under a modification of the conditions previously used, to estimate both the number and size of the subunits of each of the three genomes (6). After electrophoresis, the gels were stained with toluidine blue (0.025% in 1% acetic acid for 8 hr) and destained (1% acetic acid overnight in the cold). The electrophoretic resolution of the subunits of the three genomes can be seen in the comparative diagram (see Fig. 1) of the three stained gels. With WTV RNA, three bands stained more deeply than others and are represented in the diagram as doubly thickened lines. Quantitative isotopic and densitometric determinations have previously indicated that these bands contain two or more components of very similar molecular weights (6, 14).

Elution of the RNA segments after separation on gels, and subsequent enzymatic and alkaline digestions to release the trialcohols, were performed in the following manner. The gels were sliced into fractions of approximately 0.5 mm, and each dye-stained slice was perfused for 1 hr at room temperature, first with 0.5 ml of 0.3% NH_4OH to adjust the pH of the gels to between 6 and 8, and then with 0.5 ml of 0.005 M ethylenediaminetetraacetic acid. To make the dsRNA susceptible to ribonuclease, thereby facilitating polynucleotide diffusion from the gel, the tubes were heated to 100 C for 5 min and immediately cooled in ice. Each sample received 0.4 ml of pancreatic ribonuclease (20 $\mu\text{g}/\text{ml}$) and was reincubated for 12 hr at 37 C with shaking. The supernatant fluid was removed and saved, and the extraction process was repeated. The appropriate supernatant fluids were combined and lyophilized. The fractions resulting from the above procedures were hydrolyzed for 16 hr at 37 C in 200 μl of 0.5 M NaOH in a mixture of 20 μg of each of the four common hydroxymethyl diethylene glycol derivatives, conveniently called trialcohols, prepared by the method of Khym and Cohn (7). The digested samples were neutralized by passage through 0.5-ml columns of Biorex-70, NH_4^+ , and after lyophilization were solubilized in water.

RESULTS

Analysis of the radioactive content of each sample showed that approximately 85% of the total label applied to the gels was recovered. The presence of approximately twice as much label eluted from the three deeper staining bands of the

WTV genome showed that these bands were composed of either two distinct RNA components or twice as much of a single species. By analogy, from the equimolar amounts of each RNA subunit species present in the genomes of reovirus and CPV (11, 12), it is assumed that these bands contain distinct components of very similar molecular weights. This assumption is expressed in the numbers assigned to the bands in Fig. 1.

The ^3H -labeled terminal residues were identified by chromatographing the hydrolysates of the individual bands on 10-ml columns of Whatman phosphocellulose (P-11) and by comparing the position of the tritium to that of the trialcohol markers. Elution was with a linear gradient of ammonium formate (pH 3.85), 0.01 to 0.6 M (8). The absorbancy profile of the typical marker elution pattern is seen in the top panel of Fig. 2. "NTM" (nonterminal materials) denotes the position where proteins, carbohydrates, and mononucleotides elute (4, 8). Label appearing there indicates either that the sample contained traces of proteins or carbohydrates, or that a low level of labeling of internal nucleotides occurred. The radioactivity in nonterminal materials, which varied between 7 and 25%, did not interfere with identification or quantitative analysis of the RNA termini. "U", "G", "A", and "C" represent the elution positions of the four common trialcohols. The specificity of the labeling and analytical procedures was monitored with R17 phage RNA (bottom panel, Fig. 2), which is known to terminate at its 3' end in A_{OH} (3, 8).

FIG. 1. Electrophoretic separation of terminally labeled dsRNA subunits of cytoplasmic polyhedrosis virus, wound tumor virus, and reovirus. Migration was for 40 hr at 10 ma/tube in 4% polyacrylamide-bis acrylamide gels (14 by 0.7 cm in diameter) by using a running buffer composed of 0.04 M tris(hydroxymethyl)aminomethane, pH 7.4, 0.001 M ethylenediaminetetraacetic acid, and 0.005 M sodium acetate. Direction of migration is from left to right. Band positions were located by staining with toluidine blue prior to slicing the gel into 0.5-mm fractions.

FIG. 2. Identification of the ^3H -labeled termini of RNA from discrete electrophoretic bands of cytoplasmic polyhedrosis virus, wound tumor virus, and reovirus genomes. Terminally labeled polynucleotides were recovered from the distinct dye-stained fractions of dsRNA seen in Fig. 1 and digested as noted in the text in the presence of nonradioactive triacohol markers. The neutralized hydrolysates were applied to 10-ml columns of phosphocellulose (Whatman P-11) and eluted by linear gradients of ammonium formate (pH 3.85), 0.01 to 0.6 M in total volumes of 60 ml. The eluates were passed through an ultraviolet monitoring apparatus to record A_{260} (top panel) and collected directly in scintillation vials to determine ^3H (lower panels). Except for the omission of the polyacrylamide gel electrophoresis step, which was required to separate distinct dsRNA subunits, terminally labeled R17 RNA was handled in parallel.

The remaining panels present the elution patterns of labeled triacohol extracted from select components from the three genomes, component VII for reovirus, component VI for CPV, and the band representing components X and XI for WTV. The remaining bands from each gel were also individually analyzed, and each gave results approximately equal to those presented in the figure. The results are tabulated in Table 1. The

TABLE 1. Analysis of the distribution of label after oxidation of RNA followed by reduction with ^3H -sodium borohydride^a

RNA source	NTM (% total ^3H)	Percent of total ^3H in nucleoside triacohols ^b			
		U	G	A	C
Reovirus ^c ...	23 $\pm$ 2	3 $\pm$ 2		1 $\pm$ 0.5	96 $\pm$ 2
CPV ^c	22 $\pm$ 2	50 $\pm$ 2		2 $\pm$ 0.5	48 $\pm$ 2
WTV ^c	8 $\pm$ 1	52 $\pm$ 2	1.5 $\pm$ 0.5	6 $\pm$ 2	40 $\pm$ 2
R17.....	22	3	1.5	94	1.5

^a Abbreviations: NTM, nonterminal materials; CPV, cytoplasmic polyhedrosis virus; WTV, wound tumor virus.

^b U, G, A, and C represent the triacohol derivatives of uridine, guanosine, adenosine, and cytidine, respectively.

^c Each band of the three genomes seen in Fig. 1 was individually analyzed, but varied only within the limits noted.

CPV and WTV components all contain approximately equal amounts of U and C termini. The WTV components also contain small amounts of A and G termini.

When the reduction with ^3H -borohydride was performed on unoxidized samples of the viral RNAs, no incorporation of label into a triacohol derivative was observed. Moreover, pretreatment of the viral RNAs with alkaline phosphomonoesterase, which cleaves terminal phosphates, did not appreciably alter either the total level of incorporation per given amount of RNA or the relative amounts of ^3H incorporated into the individual fractions. This confirmed for the WTV genome what was already known for both reovirus and CPV (4, 11, 12); none of the 3' termini was phosphorylated, and each of the 10 electrophoretically separable dsRNA classes contained 3' hydroxyl termini. These results make it probable that the viral dsRNA molecules are replicated as discrete components, since RNA polymerase products are known to terminate in nonphosphorylated 3' termini. If these components were fragments produced from a single large RNA genome by the action of nucleases, they would be expected to have 3' monophosphate termini, the type resulting from the action of most of the commonly encountered endonucleases. In the case of reovirus and CPV, it has previously been demonstrated by oxidation of the RNA *in situ* that the 3' termini were present in the virion before extraction of the RNA. While *in situ* oxidation has not been performed on WTV, it is probable that WTV resembles reovirus and CPV in this respect also, and that the 3' termini preexist in the intact virion.

DISCUSSION

While the 3' termini of both strands of each reovirus segment are solely C_{OH}, the two strands of each segment of both CPV and WTV RNAs appear to terminate in different bases. It is possible that label which elutes with marker U may have arisen by deamination of terminal C_{OH}. However, when the marker triolcohols of cytidine and adenosine were individually subjected to digestion and analysis by column chromatography, no deamination to the uridine and inosine derivatives was detected. We conclude, therefore, that if deamination had occurred, it must have been *in situ*, at some stage prior to the oxidation-reduction steps. In such a case, one would further have to speculate that such deamination was restricted not only to the genomes of CPV and WTV (and not that of reovirus), but also to only one strand of each segment of each susceptible genome. We consider such a string of possibilities unlikely.

Finally, attention might be directed to the presence of minor amounts of terminal-specific ³H, most notably the labeled adenosine derivative present in the extracts of individual bands of WTV. The parallel analysis of R17 shows that the labeling technique is specific in that it does not introduce spurious termini. It follows that the minor amounts of A and G termini found must preexist in the sample. These could arise from either contaminating RNAs or from RNA chains in which nucleotides have been added to or removed from the 3' terminus, as might occur through enzymatic action. Examples of RNAs in which the terminus is metabolically unstable include transfer RNA and ribosomal RNA (S. H. Leppla, Ph. D. thesis, Univ. of Wisconsin, 1969). Alternatively, the presence of minor amounts of labeled A_{OH} and G_{OH} might indicate that the preparation of WTV used in the present study contained a small percentage of genomes which, though unaltered as to the relative size distribution of their segments, had been mutationally altered as to their 3' termini.

Regardless of the origin of the minority termini, it can be concluded that each RNA component of the WTV and CPV genomes contains predominantly almost equal amounts of U and C termini. It is probable that one strand terminates in C and the antiparallel strand terminates in U. To prove this distribution, it would be necessary to physi-

cally separate the strands. This must await improvement in techniques of strand separation in WTV and CPV.

ACKNOWLEDGMENTS

We are indebted to C. A. Knight and G. S. Stent for encouragement and support during the course of this research.

This investigation was supported by Public Health Service research grant AI 00634 from the National Institute of Allergy and Infectious Diseases, and grant GM 17866 from the National Institute of General Medical Sciences. L. J. L. was supported by contract no. 71-2173 from the Special Virus Cancer Program of the National Cancer Institute, and S. H. L. was supported by Public Health Service postdoctoral fellowship GM 41009 from the National Institute of General Medical Sciences.

LITERATURE CITED

1. Banerjee, A. K., R. Ward, and A. J. Shatkin. 1971. Cytosine at the 3'-termini of reovirus genome and *in vitro* mRNA. *Nature N. Biol.* 232:114-115.
2. Black, D. R., and C. A. Knight. 1970. Ribonucleic acid transcriptase activity in purified wound tumor virus. *J. Virol.* 6:194-198.
3. Dahlberg, J. E. 1968. Terminal sequences of bacteriophage RNAs. *Nature (London)* 220:548-552.
4. Furuichi, Y., and K. Miura. 1972. The 3' termini of the genome RNA segments of silkworm cytoplasmic polyhedrosis virus. *J. Mol. Biol.* 64:619-632.
5. Joklik, W. K., J. J. Skehel, and H. J. Zweerink. 1970. The transcription of the reovirus genome. *Cold Spring Harbor Symp. Quant. Biol.* 35:791-801.
6. Kalmakoff, J., L. J. Lewandowski, and D. R. Black. 1969. Comparison of the ribonucleic acid subunits of reovirus, cytoplasmic polyhedrosis virus, and wound tumor virus. *J. Virol.* 4: 851-856.
7. Khym, J. X., and W. E. Cohn. 1960. Characterizations and some chemical reactions of periodate-oxidized nucleosides. *J. Amer. Chem. Soc.* 82:6380-6386.
8. Lewandowski, L. J., J. Content, and S. H. Leppla. 1971. Characterization of the subunit structure of the ribonucleic acid genome of influenza virus. *J. Virol.* 8:701-707.
9. Lewandowski, L. J., J. Kalmakoff, and Y. Tanada. 1969. Characterization of a ribonucleic acid polymerase activity association with purified cytoplasmic polyhedrosis virus of the silkworm *Bombyx mori*. *J. Virol.* 4:857-865.
10. Lewandowski, L. J., P. C. Kimball, and C. A. Knight. 1971. Separation of the infectious ribonucleic acid of potato spindle tuber virus from double-stranded ribonucleic acid of plant tissue extracts. *J. Virol.* 8:809-812.
11. Lewandowski, L. J., and S. Millward. 1971. Characterization of the genome of cytoplasmic polyhedrosis virus. *J. Virol.* 7:434-437.
12. Millward, S., and A. F. Graham. 1970. Structural studies on reovirus: discontinuities in the genome. *Proc. Nat. Acad. Sci. U.S.A.* 65:422-429.
13. Watanabe, Y., S. Millward, and A. F. Graham. 1968. Regulation of transcription of the reovirus genome. *J. Mol. Biol.* 36:107-123.
14. Wood, H. A., and G. Streissle. 1970. Wound tumor virus: purification and fractionation of the double-stranded ribonucleic acid. *Virology* 40:329-334.