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The 3′-Terminal Nucleosides of the High Molecular Weight RNA of C-Type Viruses

([^{3}H]sodium borohydride/hamster/viper/mouse/cat)

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ABSTRACT **Tumor virus RNAs from several mammalian and one reptilian species were purified; their 3′-terminal nucleoside was identified by separation of the trialcohols produced by periodate oxidation followed by reduction and tritiation with NaB^3H_4. Each virus contained uridine as the predominant terminal nucleoside. Molecular weight estimations based on the tritiation reactions were consistent with a structure consisting of four subunits.**

Analysis of the 3′- and 5′-terminal nucleotide sequences of bacteriophages containing RNA has permitted considerable advancement in knowledge of the direction of translation of the viral genes, and has indicated possible relatedness of viruses that are otherwise quite distinct, e.g., R17 and Qβ (1, 2). Long, apparently untranslated, sequences showing a high degree of base homology (3) at the 3′ terminal are apparently important for binding of the viral RNA synthetase (3); thus there would be a high selection pressure for conservation of such sequences. It is evident that understanding of the oncogenic potential of RNA tumor viruses, and a definition in molecular terms of "oncogenes" (4), will depend on similar kinds of comparative studies. Determination of the 3′-terminal nucleoside of viruses from several mammalian and one reptilian species, reported here, indicate similar termini occur in viruses from each species. Molecular weight estimates based on these determinations are consistent with a molecular weight of about 2–3 × 10^6 for the viral RNA or its subunits.

MATERIALS AND METHODS

Viruses

The various C-type viruses used in this study were derived from chronically-infected tissue cultures (5).

3′-Hydroxyl terminus

Purified viral 70S RNA (6) was subjected to terminal tritiation at room temperature (7). Freshly prepared RNA (0.3 ml, containing 2–5 μg of RNA in saline–citrate buffer) was treated with 10 μl of 1.2 mM $NaIO_4$ in the dark for 1 hr. To this solution was added 10 μl of 0.1 M NaB^3H_4 solution (8 Ci/mmol, Amersham-Searle, in 0.1 N NaOH). The radioactive borohydride solution (final concentration, 6 Ci/mmol) was stored at −70°C until used. After incubation for 1.5 hr in the dark, 25 μl of 10 N acetic acid was added to stop the reaction and to remove excess NaB^3H_4. The solution was then lyophilized immediately.

The residues obtained were dissolved in 0.5 ml of water and 26.3 A_{260} units of *Escherichia coli* B tRNA (Schwarz, phenol treated) was added as a carrier. 3 Volumes of 95% ethanol containing 0.1 mM NaCl were added to precipitate RNA and this procedure was repeated three times. The final precipitate was obtained by centrifugation at 9000 rpm for 15 min and dissolved in water (final volume of 0.4 ml).

The ^{3}H-labeled viral RNA was then hydrolyzed, either enzymatically or by alkaline hydrolysis. Enzyme digestion was with a mixture of snake-venom phosphodiesterase and *E. coli* alkaline phosphatase, both obtained from Worthington Biochemicals, and Ribonuclease A from Sigma. The enzyme mixture contained per μl: 0.25 μg phosphodiesterase, 0.25 μg RNase, 0.2 μg phosphatase, 30 nmol of Tris·HCl (pH 7.5), and 10 nmol of $MgCl_2$. The amount of enzyme mixture added was dependent on the final concentration of carrier tRNA; thus, 50 μl of enzyme mixture was added per A_{260} unit of tRNA. Alkaline hydrolysis was by the addition of 1 volume of 0.6 N KOH to the RNA solution and incubation at 37°C for 16–18 hr. The solution was neutralized with cold $HClO_4$ and, after removal of the precipitate in the cold, the supernatant was analyzed.

The enzymic or alkaline hydrolyzate thus obtained was applied to a 20 × 20 cm thin layer sheet (Eastman-Kodak No. 6064 cellulose on plastic backing) with four unlabeled trialcohol markers and chromatographed with solvent E (ethyl acetate–*n*-butyl alcohol–isopropanol–7.5 N ammonia 3:1:2:2) (7) and/or solvent C (isopropanol–concentrated HCl–water 65:16.6:18.4) (8). Usually, 5–10 μl of the digest was spotted and two-dimensional ascending chromatography was done in solvents E and C, together with a 10- to 20-fold excess of carrier.

The four nucleoside trialcohol standards were prepared according to Khym and Cohn (9). 1 μmol of nucleoside was dissolved in 400 μl of water. 10 μl of 0.18 M $NaIO_4$ was added and the mixture was then kept in the dark at room temperature for 1 hr. After addition of 10 μl of freshly prepared 0.72 M $NaBH_4$ in 0.1 N NaOH, the reaction was allowed to proceed at room temperature for 1 hr. 40 μl of 10 N acetic acid was added and the solution was lyophilized. The residue was taken up in 30 μl of water and the solution was kept frozen. 5 μl of the solution was used for position markers.

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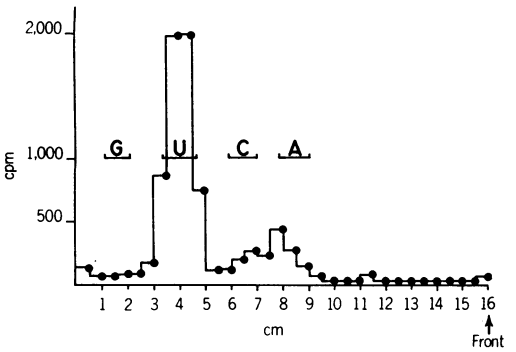

FIG. 1. ^3H distribution on TLC chromatogram. 10 μl of [^3H]hamster leukemia viral RNA, treated as described in *Materials and Methods*, was spotted on a No. 6064 cellulose sheet, together with unlabeled trialcohol markers. Development was with Solvent E for 2.5 hr in an Eastman Chromagram Chamber from Distillation Products Industries. 0.5-cm sections were assayed for radioactivity, which is expressed as cpm per 0.5-cm strip.

The UV-absorbing area corresponding to trialcohol markers in each chromatogram was scratched out and eluted at room temperature with 1.0 ml of 0.1 N HCl; a 0.5-ml aliquot was assayed for ^3H by counting in a Beckman liquid scintillation counter with 10 ml of Bray's solution. The elution of trialcohol markers was quantitative. ^3H was detected only in the four spots corresponding to each unlabeled trialcohol marker, while, if the extensive ethanol precipitation process was omitted, significant amounts of radioactivity were found at other positions. These reaction byproducts were eliminated by the ethanol precipitation step and were also completely separated by two dimensional development.

TABLE 1. *Molecular weight estimation of viral RNA by 3'-terminal tritiation*

Source of RNA	A_{260}	Trialcohol (pmol)	Estimated molecular weight
Viral 70S RNA			
AKR Mouse* leukemia	0.069	1.1	2.5×10^6
Feline leukemia	0.062	1.1	2.2×10^6
Hamster leukemia	0.047	0.65	2.9×10^6
Viper	0.102	2.5	1.6×10^6
E. coli tRNA	0.10	150	26.5×10^3

The various RNAs were tritiated at the 3' terminal as described in *Methods*. After removal of excess NaB^3H_4, RNA was precipitated sequentially three times with ethanol, and the final residues were dissolved in water. Aliquots were assayed for radioactivity and absorbance at 260 nm. The literature value for the molecular weight of tRNA was used as a reference point for calculation of the virus values as follows:

$$\frac{A_{260}\text{ viral RNA}}{A_{260}\text{ tRNA}}_{(0.1)} \times \frac{\text{pmole trialcohol from tRNA}}{\text{pmole trialcohol from viral RNA}}_{(150)} \times$$

26,500 = molecular weight of RNA.

If calculated on the basis of A_{260}(1 A_{260} unit = 45 μg of RNA), all values for molecular weight would be about 20% higher.

* A high-leukemic mouse strain, purchased from Jackson Laboratories, Bar Harbor, Me., is the normal host for this virus.

RESULTS

Molecular weight of viral RNA

The sedimentation constants of the various viral RNAs were all 65–70 S, corresponding to a molecular weight of 1–1.4 $\times$ 10^7. The 3'-terminal tritiation reaction permits an independent assessment of this value if we assume that the RNA remains intact during the sequential oxidation and reduction steps. With *E. coli* tRNA as a standard, calculations of molecular weight by terminal tritiation agree to within 20% of the literature value of 26,500 (10). With the tRNA data as a reference point, molecular weight calculations were made for viral RNAs (Table 1). These values are all consistent with molecular weights, about 25% of those based on sedimentation velocity calculations. As discussed below, this finding supports a subunit structure of the viral RNA suggested by others (11, 12).

3'-Terminal nucleosides

When unlabeled, purified viral RNA was subjected to terminal tritiation and subsequent hydrolysis, ^3H was seen only in the areas corresponding to the four nucleoside trialcohol markers on the chromatogram (Fig. 1). Table 2 shows the distribution of radioactivity in various viral RNA species after two dimensional chromatography in solvents E and C. All viral RNAs contained [^3H]uridine trialcohol as a unique or major component derived from the 3'-hydroxyl terminus. This result was obtained independently of the virus source or the method of hydrolysis.

Determinations of the 3'-terminal was made on three preparations of the same virus; on each occasion uridine accounted for 70–80% of the recovered activity. There was some variation in the percent activity seen with the other three nucleosides in separate experiments; thus, we consider it likely that some nonspecific breakdown of the viral RNA occurs during the treatment to expose new termini.

DISCUSSION

The C-type viruses contain a high molecular weight RNA with a sedimentation constant of about 70 S (13). This

TABLE 2. *Distribution of ^3H in nucleoside trialcohols after 3'-terminal tritiation of viral RNA*

Source of RNA	^3H-activity corresponding to each nucleoside Fraction of recovered activity			
	Guanosine	Uridine	Cytidine	Adenosine
70S Viral				
AKR Mouse leukemia	0.07	0.62	0.15	0.16
Feline leukemia	0.11	0.72	0.05	0.11
Hamster leukemia	0.03	0.75	0.07	0.15
Viper	0.03	0.69	0.07	0.21
E. coli tRNA	0.006	0.01	0.02	0.96

Average values from several experiments in which viral RNAs were degraded either by alkaline hydrolysis or enzymatically. No significant differences between the two methods were observed. Nucleoside trialcohols were separated by two-dimensional thin layer chromatography.

suggests a molecular weight of about 10^7, which is in agreement with results of electron microscopic measurements of RNA lengths (14, T. Kakefuda and M. Hatanaka, unpublished results). However, when the RNA is treated with heat or dimethylsulfoxide (11, 12), the molecular weight estimates obtained by sedimentation analysis decrease by a factor of 4. Since effects of the various treatments are irreversible, a subunit structure is suggested. The results of the terminal tritiation procedure were consistent with such a subunit structure. If indeed there are four subunits, three of these must have uridine as the 3′-terminal nucleoside. Since there was some variability from virus to virus on distribution of radioactivity among the other three nucleosides, although adenosine was predominant with hamster leukemia virus and the viper virus, it may well be that uridine is at the terminal of each subunit and the other activities resulted from some breakdown of the long polynucleotide chain. The 25–30% recovery of tritium in nucleosides other than uridine, even if an artifact, would not alter the conclusion that the molecular weight of the RNA is much less than that suggested by sedimentation analysis of freshly prepared material.

The finding of uridine at the 3′-terminal of C-type viruses from several mammalian species, the viper virus, and from avian C-type viruses (15) is strongly suggestive of a common ancestor for these viruses, as is suggested by their similarity in many other properties. Since the sequence at the 3′-terminal is presumably important for binding of the RNA-dependent DNA polymerase, this portion of the genome might be more similar among different C-type viruses than are other portions of the genome. This conclusion follows from the expectation that there should be strong selection pressure for retention of specific sequences in polymerase binding regions.

At this time, the mechanism of replication of C-type viruses *in vivo* is far from certain, although replication through DNA intermediates seems logical based on the presence of the appropriate polymerases in purified virions (16, 17). In this connection, studies of RNA-dependent DNA synthesis by C-type virions with γ-labeled deoxyribonucleoside triphosphates have revealed incorporation of dATP, at a faster initial rate than overall DNA synthesis, that rapidly reaches a plateau (unpublished data). This suggests that if this result was not because of a phosphate transfer reaction specific for dATP, that synthesis of the new DNA chain precedes in the 5′ to 3′ direction, with faithful replication of the 3′-terminal base. Whether this result is different than the case of the RNA bacteriophages, where the 3′-terminal is apparently not replicated (18) in the synthesis of viral minus strands, is not certain, since determinations of the penultimate 3′ base, which could also be uridine, have not yet been made.

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