TRANSFER RIBONUCLEIC ACID NUCLEOTIDYLTRANSFERASE ACTIVITY IN VIRIONS OF TYPE-C VIRUSES

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SUMMARY

A nucleotidyltransferase activity has been found associated with a number of mammalian and avian oncornaviruses. This activity catalyzes the incorporation of adenosine monophosphate and cytosine monophosphate into acid insoluble forms. The transferase activity from Rauscher murine leukemia virus has been characterized. The endogenous reaction is stimulated by various tRNAs particularly the 4S RNA isolated from Rauscher leukemia virus, whereas other RNAs have no effect. The product of the reaction is alkali and RNase sensitive, insensitive to DNase, and its size is similar to tRNA. Finally, the terminal nucleotide analysis of the product of the reaction indicates the presence of a CCA terminus. The properties of the activity found in the type-C viruses are in accord with those of known tRNA nucleotidyltransferases from other sources.

Following the discovery of reverse transcriptase, a number of enzymatic activities have been found associated with type-C RNA tumor viruses. Some of them are associated with the core and some with the outer envelope of the virion. The functional significance of many of these activities with regard to the viral life cycle and their precise origin is unclear. These activities include aminoacyl tRNA synthetase (1,2), DNA ligase (3), ribonuclease H (4,5,6) and a number of other nucleic acid metabolizing activities (for a recent review see reference No. 7).

We previously reported an enzymatic activity associated with R-MuLV, which is similar to a tRNA nucleotidyltransferase (8). A similar finding has also been reported by Faras et al. in avian type-C viruses (9). This observation has now been extended to several other type-C viruses and the activity from R-MuLV has been characterized.

MATERIALS AND METHODS

Viruses. R-MuLV, FeLV, RSV, K-MuLV, K-MuLV (K-MuSV) were obtained from Electro Nucleonics, Inc., Bethesda, Maryland. R-MuLV was grown in JLSV9 cells (10). SSV-1

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Abbreviations: R-MuLV, Rauscher murine leukemia virus; FeLV, feline leukemia virus; RSV, Rous sarcoma virus; K-MuLV, Kirsten murine leukemia virus; K-MuLV (K-MuSV), Kirsten murine leukemia-sarcoma virus; SSV-1, Simian sarcoma virus type 1.

was obtained from Pfizer, Inc., Maywood, New Jersey and has been shown to be associated with an excess of nontransforming helper virus (11). AMV from plasma was obtained from Dr. J.W. Beard, Duke University, Durham, North Carolina.

Enzymatic reactions. Transferase activity: Measurement of activity in virus preparations was carried out in the following mixture unless otherwise indicated: 0.05 M Tris HCl, pH 7.9, 0.04% Triton X-100, 10 mM magnesium acetate, 3.2 mM [^{3}H] ATP and/or 0.2 mM [^{3}H]CTP. Incubation was carried out for 1 hour at 30°C. The reaction was stopped by the addition of 10% cold trichloracetic acid containing 0.1 M sodium pyrophosphate. The precipitate was collected on millipore filters, which were washed extensively with 5% TCA and then with 80% ethanol. The filters were dried and the radioactivity was determined in a liquid scintillation counter.

Reverse transcriptase activity: Reactions were carried out as described previously (12).

Product analysis. 1) Extraction of transferase reaction product: The enzymatic reaction was stopped by the addition of one-fifth volume of cold 5% SDS and an equal volume of cold phenol-cresol-water solution (50:7:5, containing 0.1% 8-quinolinol and saturated with TNE buffer [10mM Tris HCl, pH 7, 0.1 M NaCl, and 1mM EDTA]). The mixture was gently shaken for 5 minutes at 4°C and the aqueous phase collected after centrifugation at 13,000 xg for 10 minutes at 4°C. The extraction was repeated twice. Nucleic acid in the aqueous phase was then precipitated by two volumes of absolute ehtanol in 0.4 M NaCl. The pellet was resuspended in deionized sterile water. 2) Analysis of product size: A 10-30% linear glycerol gradient was prepared in a buffer containing 0.01M Tris HCl, pH 7.9, 0.1 M KCl, 1 mM dithiothreitol, 0.1 mM EDTA. Siliconized polyallomer centrifuge tubes were used. Isolated product and marker molecules [tRNA from L1210 cells, 30S ribosome subunit (General Biochemicals)] were layered on the top of the gradients. The gradients were centrifuged in a SW41 rotor (Beckman Spinco ultracentrifuge) for 5 hours at 4°C at 200,000 xg. At the end of the centrifugation, fractions were collected and the density of each fraction determined by measurement of the refractive index. Protein concentrations were estimated by absorbance at 280 mu and aliquots of each fraction were taken for measurement of acid precipitable radioactivity.

Terminal nucleotide analysis. RNA, extracted from the transferase reaction mixture, was treated with 25 mM of cetyltrimethyl ammonium bromide in the presence of 400 ug of E. Coli tRNA and precipitated with ethanol (13). An aliquot containing 100 ug of E. Coli tRNA was treated with 0.2 N NaOH at 37°C for 18 hours together with 50 ug each of non-radioactive 3'-mononucleotides (Cp, Ap, Gp and Up) and nucleosides (C, A, G and U).

Fractionation of the nucleotides and nucleosides was performed using two-dimensional high voltage electrophoresis (K. Ohe in preparation). The sample treated with alkali was neutralized with HCl, spotted on Whatman No. 1 paper and subjected to electrophoresis at pH 3.5 using 5% acetic acid - 0.5% pyridine. After electrophoresis, the paper was divided at the origin into 2 portions, the portion toward the anode, containing nucleotides, and that toward the cathode, containing nucleosides Each portion was subjected to a second electrophoresis perpendicular to the direction of the first electrophoresis: at pH 3.5 for nucleotides and at pH 1.9 for nucleosides. With the above procedure, the ^{3}H-labelled nucleosides derived from the radioactive precursor incorporated at the 3'-terminus of RNA and the nucleotides from the precursor incorporated internally in the RNA chain were well separated from each other. They are identified as UV-absorbing spots, cut out and counted for the radioactivity in a toluene-based scintillation fluid.

RESULTS

AMP and CMP incorporation activity in the lysate of R-MuLV. R-MuLV contained an enzymatic activity that catalyzes the incorporation of CMP, AMP, and to a much

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Abbreviation: AMV, Avian myeloblastosis virus.

lesser degree, UMP into acid insoluble forms. No GMP incorporation was detected. This activity was dependent on virus concentration. The activity was stimulated by divalent cations. Another variable affecting the CMP and AMP incorporating activity was the intactness of the virions. We observed that nonionic detergent such as Triton X-100 enhanced this activity. A possible interpretation is that the enzyme activity is located inside the viral envelope (see below).

The optimal concentration of ATP was approximately 3.2 mM which is 16 fold higher than the 0.2 mM optimum of CTP. The presence of UTP or GTP in the reaction mixture did not interfere with either AMP or CMP incorporation.

Location of AMP and CMP incorporating activity in virions of R-MuLV. Similar to viral reverse transcriptase activity, AMP and CMP incorporating activity was enhanced by treatment with a nonionic detergent. This suggests that the AMP and CMP incorporating activity is an internal protein species. To test this possibility, the densities of intact virions and Triton X-100 partially disrupted virions were

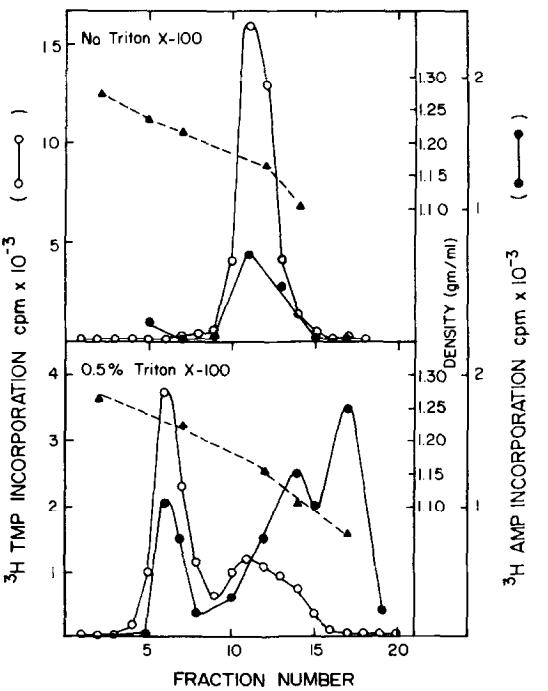

Fig. 1 Location of transferase activity in R-MuLV virions. The procedure for the preparation and analysis of intact virions and core structure in sucrose density gradients was described previously (6). The measurements of reverse transcriptase activity and transferase activity have also been described in Materials and Methods. Symbols: ●----●, [^{3}H]AMP incorporation which represents transferase activity; 0----0, [^{3}H]TMP incorporation which represents reverse transcriptase activity; and ▲----▲, density of gradient fraction.

analyzed in a sucrose density gradient. As shown in the upper panel of Figure 1 in intact viruses, AMP incorporating activity co-sedimented with reverse transcriptase activity at a density of 1.155gm/ml and the partially disrupted virions (lower panel) co-sedimented at density of 1.25 gm/ml with the endogenous reverse transcriptase activity. This indicates that the AMP and CMP incorporating activity is located in the core of the virions.

Product of endogenous reaction. The endogenous reaction of AMP and CMP incorporating activity was partially sensitive to ribonuclease treatment (pancreatic ribonuclease A and ribonuclease T_1) but insensitive to deoxyribonuclease treatment. Similarly, the product of the endogenous reaction became acid soluble by a treatment with RNases or by an alkali treatment but was unaffected by treatment with DNase. Therefore, the product is most likely RNA or associated with RNA. The size of this RNA was about 4S as indicated by co-sedimentation with tRNA (Figure 2). This suggests the possibility that AMP and CMP were incorporated into 3' end of the CCA terminal of 4S primer RNA (14). To test this possibility, the terminal nucleotide sequence of the product was analyzed.

Terminal nucleotide analysis. When the product synthesized in the presence of [^{3}H]ATP alone was hydrolyzed with alkali, a significant portion of the radioactivity was found in adenosine (A), not in 3' AMP (Ap), giving a ratio of A to Ap of more

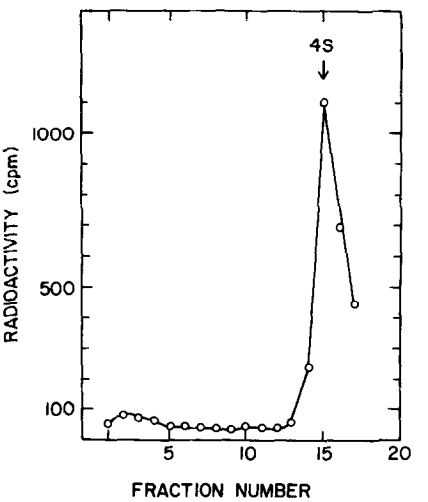

Fig. 2 Product size determination by a glycerol gradient. AMP and CMP incorporation reaction with R-MuLV was performed as described in Materials and Methods. The reaction was stopped by chilling and the addition of SDS to 1%. The product was then extracted by a pheno-cresol procedure described in Materials and Methods. The product was then layered on a 10-30% glycerol gradient and sedimented at 200,000 xg for 4 hours. Yeast tRNA was used as a marker for a determination of molecular size.

Table 1. Alkaline hydrolysis of the product of nucleotidyltransferase activity

Experiment number	Precursor				^{3}H(cpm) detected in monomers after alkaline hydrolysis [1]								Ratio	
	[^{3}H] ATP	Cold ATP	[^{3}H] CTP	Cold CTP	C	A	G	U	Cp	Ap	Gp[2]	Up[2]	$\frac{C}{Cp}$	$\frac{A}{Ap}$
1	+				27	444	25	29	32	17	26	223		26.1
2	+				1	304	5	3	1	18	13	295		16.9
3	+		+		481	457	6	40	3742	18	56	271	0.128	25.4
4	+		+		57	247	0	8	347	24	23	36	0.164	10.3
5	+			+	9	566	7	6	51	13	16	229		43.5
6	+			+	24	339	0	20	7	22	9	137		15.4
7			+		762	65	15	48	543	0	24	43	1.40	
8			+		1153	1	3	6	513	1	519	34	2.25	
9		+	+		180	47	1	9	1036	2	18	75	0.174	
10		+	+		193	7	11	18	970	16	14	30	0.199	

[1] ^{3}H radioactivity (cpm) of the nucleosides C, A, G and U and the 3' mononucleotides Cp, Ap, Gp and Up, fractionated on the two-dimensional electrophoretogram as described in Materials and Methods, is shown for each experiment performed with different combination of precursors. The ratio of the radioactivity of nucleoside to that of nucleotide is also listed for each experiment.

[2] Further electrophoretic analysis showed Gp to be CDP and Up to be ADP (see results).

than 15 (Table 1, Experiment Numbers 1 and 2). This indicates that the enzyme activity described here is incorporating adenosine residues predominantly at the 3' terminus of preexisting RNA molecules. This ratio of A to Ap remains unchanged when CTP is used in addition to ATP in the incubation mixture (Table 1, Experiment Numbers 3,4,5 and 6). However, when CTP was used alone as the precursor, ^{3}H radioactivity was found in both cytidine (C) and 3'CMP (Cp) (Table 1, Experiment Numbers 7 and 8). The ratio of the radioactivity of C to CP was 1.4 to 2.25, indicating that 60 to 70% of the cytidine residues were at the 3' terminus of the product without further addition of adenosine residues. This can be explained by hypothesizing that the enzyme

Table 2. Nucleotidyltransferase activity and reverse transcriptase activity in various type-C viruses

Virus [1]	Transferase activity [^{3}H]-CMP incorporation (pmole/mg protein/hr)	Reverse transcriptase activity [^{3}H]-TMP incorporation[2] (pmole/mg protein/hr)
R-MuLV	30	2678
K-MuLV	28	2357
K-MuLV (K-MuSV)	89	1288
SSV-1	55	591
FeLV	40	2357
RSV	39	31
AMV	41	5

[1] Amount of virus used for each assay was as follows: R-MuLV, 41 ug; K-MuLV, 14 ug; K-MuLV (K-MuSV), 96 ug; SSV-1, 33 ug; FeLV, 25 ug; RSV, 25 ug; and AMV, 58 ug.

[2] Divalent cation Mn^{++} (0.5 mM) was used for all assays except in cases of RSV and AMV in which Mg^{++} (10 mM) was used.

activity is adding CpC_{OH} and C_{OH} to the 3' terminus of RNA on a random basis, without further addition of A_{OH} under these experimental conditions. The cytidine residues are incorporated almost exclusively as internal residues in the RNA molecule when ATP is present in addition to [^{3}H]CTP in the incubation mixture (Experiment Numbers 3,4,9 and 10). Some products synthesized with [^{3}H]ATP showed ^{3}H radioactivity in the location equivalent to 3' UMP (UP) in electrophoretogram. However, upon further electrophoretic analysis, they were shown to be ADP. Similarly, ^{3}H radioactivity that is located in the electrophoretogram equivalent to 3' GMP (GP) in Experiment Number 8 was found to be CDP. These radioactivities became undetectable when more extensively purified products were analyzed. These results indicate that the enzyme activity is adding $CpCpA_{OH}$ to the 3' terminus of 4S RNA molecules, thus resembling a tRNA nucleotidyltransferase activity.

Substrate specificity. To test the substrate specificity of this transferase activity, various species of RNA were added to an endogenous reaction. The best stimulation, 2.2 fold, was obtained with purified 4S RNA from R-MuLV which mainly contains proline tRNA (W.K. Yang, personal communication). R-MuLV 70S RNA had only a slight stimulatory effect, 1.4 fold. Other tRNAs isolated from L1210 cells, chicken cells and E. coli gave a slight stimulation, 1.1, 1.2, 1.3 fold respectively. There was slightly more stimulation by yeast tRNA, 1.8 fold, and essentially no stimulation with 23S and 16S ribosomal RNA.

Nucleotidyltransferase activity in other type-C viruses. The presence of transferase activity is not unique to R-MuLV. As shown in Table 2, this activity was detected in other murine leukemia sarcoma viruses, simian sarcoma virus, feline leukemia virus and also avian viruses. There was no quantitative correlation between the transferase activity and the reverse transcriptase activity. This is probably due to the crudeness of the enzyme preparation and the method of measurement.

DISCUSSION

The repair of tRNA molecules deprived of their 3' terminal -pCpCpAOH sequence is catalyzed by tRNA nucleotidyltransferase. The specificity of this enzyme for its substrate has been well documented (15). We have reported its presence in RNA tumor viruses. The activity found in these viruses conforms to the characteristics reported for transferase from various sources. It catalyzes the specific incorporation of CMP and AMP into acid insoluble forms. A low level of incorporation is observed for UMP. This low level UMP incorporation was also reported by Faras et al.(9) for RSV nucleotidyltransferase and for rat and rabbit liver enzymes (15, 16). The concentration optimum is 3.2 mM for ATP and 0.2 mM for CTP. This higher concentration optimum for ATP over that of CTP is consistent with many other reports (17, 18, 19, 20). The endogenous reaction is stimulated by various tRNAs, particularly the 4S RNA isolated from R-MuLV, whereas other RNA's have no effect. The product of the reaction is

alkali and RNase sensitive and insensitive to DNase with its size being similar to that of tRNA. Finally, the terminal nucleotide analysis of the product of the reaction indicates the presence of a CCA terminus.

The primer 4S RNA for avian type-C viruses has been shown to be tryptophan tRNA and its sequence has also been determined (14). Obviously, these molecules have complete CCA termini (14). Initiation of DNA synthesis occurs exculsively on the hydroxyl group of the 3' terminal adenosine of these molecules (21). Thus, inclusion of tRNA nucleotidyltransferase in the virus core might assure the completion of 3' termini of primer molecules prior to final assembly into a 70S complex.

Alternatively, the presence of tRNA and terminal transferase activity could be related to the finding of some components in virions involved in translation such as ribosomes and aminoacyl tRNA synthetases. It is also possible that they are cellular components with no viral function but merely incorporated into the virus during viral assembly. Nevertheless, the finding of this transferase activity in virus cores and the consistency with which it is found in type-C virus preparations from various species lends credance to the fact that this enzyme may have a specific function in the type-C virus life cycle. This specific function of the tRNA nucleotidyltransferase enzyme may in fact be assuring the completion of the primer for transcription of DNA from the 70S genome of the virus. Therefore, the significance of this activity deserves consideration in light of the importance of a tRNA primer in transcription of oncornavirus RNA to DNA.

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