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ELECTRON MICROSCOPIC STUDIES ON THE STRUCTURE OF 60—70S RNA OF AVIAN MYELOBLASTOSIS VIRUS

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Summary. — Structural properties of the 60-70S RNA complex of avian myeloblastosis virus (AMV) were analysed in electron microscope after treatment under a set of non-denaturing, gently and strongly denaturing conditions. By selected denaturing conditions, the significant fraction of 60-70S AMV RNA molecules revealed partially unfolded structures either in a dimer or a more complex form and in a length corresponding to mol. wt. of 5.6×10^6. The typical dimers contained a characteristic central structure connecting the subunits and similar to those described for Rous sarcoma virus (RSV) and mammalian retrovirus RNAs. This dimer linkage in the AMV genome occurred at 384 ± 43 nucleotides from one end of each subunit. Besides partially unfolded complexes, collapsed structures and extended linear molecules were observed. The length of majority of the linear molecules had reached a half of that of the partially unfolded complexes corresponding to the mol. wt. of monomers estimated under conditions of strong denaturation to be 2.8×10^6. Based on our findings, we conclude that the genome of AMV shares the dimer structure with RSV and mammalian retroviruses. We also conclude that the secondary structure of AMV RNA molecule is more labile than that of RNA of mammalian retroviruses.

Key words: *AMV RNA*; *electron microscopy*; *structure of the 60-70S RNA complex*

Introduction

The RNA isolated from retroviruses has an unusual structure, occurring as a native high-molecular-weight 60—70S RNA and changing to 30—40S RNA when denatured. On the basis of biochemical investigations, two theories have been advanced to account for this behaviour. According to Duesberg (1968), the 60—70S RNA consists of subunits held together by intermolecular hydrogen bonds. Bader and Ray (1976) have considered the possibility that the 60—70S RNA may exist in a special conformation, which can be destroyed upon denaturation, producing the 30—40S RNA. Unlike to other

hairpin structures in conventional RNA species, the transition from the 60—70S RNA to the 30—40S RNA appears to be irreversible (Duesberg, 1968; Kung *et al.*, 1976).

It should be possible to decide between the dimer hypothesis and the conformational change hypothesis by visualizing the pertinent molecular forms of viral RNA by electron microscopy. In order to prevent artefacts, the RNA should be stabilized against uncontrolled renaturation or formation of the non-specific hydrogen bonds (Robberson *et al.*, 1971; Hsu et al., 1973; Delius *et al.*, 1974; Chi and Bassel, 1975). The first retrovirus RNA studied in detail by electron microscopy was that of the endogenous leukaemia virus, RD-114 (Kung *et al.*, 1975). The RNA had a dimer structure, in which the 20 kilobases (kb) molecules were formed by two 10 kb monomers non-covalently joined at their 5′-ends. Subsequent studies demonstrated the occurrence of dimers in the RNA of endogenous baboon virus (BKD), a sarcoma virus isolated from woolly monkey (WoMV) (Kung *et al.*, 1976), four naturally occurring murine retroviruses and reticuloendotheliosis virus (REV) (Bender *et al.*, 1978). In addition, these viral genomes (with the possible exception of REV) contained a large loop within each subunit.

Attempts to visualize the RNAs of some types of avian sarcoma viruses (ASV) have shown, however, that the linkage of subunits in these RNAs was considerably less stable in comparison to the RNA of the mammalian viruses mentioned above (Bender and Davidson, 1976; Kung *et al.*, 1976). Recently Murti *et al.*, (1981) observed that the partially unfolded 60—70S RNA of the Prague strain of RSV revealed a dimer structure very similar to genomes of mammalian retroviruses. This paper describes the electron microscopic visualization of AMV RNA exposed to non-denaturing, strongly denaturing and a variety of gently denaturing conditions, using the cytochrome c monolayer spreading technique (Davis *et al.*, 1971).

Materials and Methods

Virus. The avian myeloblastosis virus (AMV) was pelleted from 150 ml blood plasma of leukaemic White Leghorn chickens and purified as described previously (Korb and Heine, 1978).

Preparation of viral RNA. Total AMV RNA was extracted with phenol-sodium dodecyl sulphate (Spiegelman *et al.*, 1970). The 60-70S RNA was isolated by sucrose gradient centrifugation (Štokrová *et al.*, 1982a). Fractions were analysed at A_{260}; the material sedimenting at 60-70S was pooled, precipitated with ethanol, resuspended in TN buffer (0.1 mol/l NaCl, 0.05 mol/l-tris-HCl pH 8.0), and prepared for electron microscopy.

Treatment of the RNA. AMV RNA was treated before spreading under three kinds of conditions. (i) *Non-denaturing conditions.* The AMV RNA was suspended in 0.5 mol/l-ammonium acetate and 0.001 mol/l-EDTA buffer pH 7.5 to a final concentration of 0.2 μg/ml. The suspension was incubated at 20 °C for 30 s. In some cases, the heating at 50 °C for 3 min (Kakefuda and Bader, 1969) was used. (ii) *Gently denaturing conditions. Urea.* The AMV RNA in TN buffer pH 8.0 was incubated at a concentration of 0.2 μg/ml with either 4 mol/l — or 8 mol/l-urea at 37 °C for 10 min and cooled on ice. *Formamide.* Formamide (99% MCB, U.S.A.) was diluted with deionized water or buffer in such a way that the solution prepared from p volume formamide and 100—p volume aqueous solution is described as a p % formamide solution. The AMV RNA was denatured with aqueous 22% formamide at 0, 20, and 37 °C for either 30 s or 2 min, 44% formamide at 37 °C for 2 min, 77% formamide at 0, 37, and 65° C each for 2 min, and 98% formamide at 0 °C for 30 s. By 77% formamide the AMV RNA was denatured also in the pre-

sence of 0.1 mol/l-Tris and 0.01 mol/l-EDTA. *Urea-formamide.* The AMV RNA was denatured at a concentration of 0.2 μg/ml with a mixture of 3.9 mol/l-urea-78% formamide either at 20 °C for 5 min or 0° C for 30 s. (iii) *Strongly denaturing conditions.* The strong denaturation was achieved by incubation of AMV RNA either with 87% dimethyl sulphoxide (DMSO) at 55 °C for 5 min (Manjakov, personal communication) or in the 3.9 mol/l-urea-78% formamide mixture at 53 °C for 30 s (Robberson *et al.*, 1971).

Preparation of protein-RNA monolayer. The AMV RNA was prepared for electron microscopy by a modification of the basic protein film technique (Kleinschmidt and Zahn, 1959). Two microliters of cytochrome c (Sigma Type VI) stock solution (1.5 mg/ml) in TE buffer (1.5 mol/l-Tris, 50 mmol/l-EDTA pH 8.5) were added to 100 μl of non-denatured or denatured RNA solution. When the RNA was denatured without salts, the hyperphase contained 0.2 μg/ml RNA, 30 μg/ml cytochrome c, 0.03 mol/l-tris and 0.001 mol/l-EDTA. A 50 μl of the hyperphase was spread onto deionized water. In some cases, the 50% formamide in 0.01 mol/l-Tris and 0.001 mol/l-EDTA pH 8.5 was used as a hypophase. The cytochrome films were picked up on grids coated with parlodion, stained with uranyl acetate (Davis and Davidson, 1968) and rotary-shadowed with platinumpallasium alloy at an angle of 7°.

Length measurements. Preparations of AMV RNA were examined at a magnification of 10 000 in a Jeol 100 B electron microscope operated at 60 kV. The contour lengths of RNA molecules were measured after an 8-fold photographic enlargement, using a Hewlett-Packard 9864A digitizer equipped with a Hewlett-Packard 9830 calculator. The AMV RNA mol. wt. was calculated from the relationship between the length and mol. wt. of myeloblastic 18S rRNA (Korb and Heine, 1978).

Results

Monomer molecular length

To determine the lengths of subunits, the AMV RNA was visualized under strongly denaturing conditions using the urea-formamide mixture at 53 °C for 30 s (Robberson *et al.*, 1971, Heine *et al.*, 1975). The RNA showed a heterogeneous population of molecules ranging from 0.2 to 2.4 μm in length with preferential size classes of 0.46 ± 0.08, 0.75 ± 0.08, 1.03 ± 0.07-1.38 ± 0.09, and 1.76 ± 0.11 μ, into which fell 15, 22, 20, 29 and 11%, of the RNA molecules respectively (for details see Štokrová *et al.*, 1982*b*). Assuming a linear density of 1.6×10^6 daltons per micrometer calculated from the mol. wt. and length of 18S myeloblastic rRNA, the mean length of the longest AMV RNA molecules corresponded to mol. wt. of 2.8×10^6 daltons. We obtained practically the same results after denaturation of AMV RNA with 87% DMSO for 5 min at 55 °C. Under both types of strongly denaturing conditions the strands were completely stretched without any apparent secondary structures.

Structure of 60—70S AMV RNA

Non-denaturing conditions. Under non-denaturing conditions (see Materials and Methods) only highly collapsed molecules were observed (Fig. 1-I).

Gently denaturing conditions. To visualize the regions of 60—70S AMV RNA molecules with higher base-pairing stability, the RNA was exposed to a set of gently denaturing conditions, near its T_m. Urea, formamide and a mixture of both were used at various denaturation temperatures and salt concentrations.

Urea. We observed only collapsed structures when the AMV RNA was incubated with 4 mol/l or 8 mol/l urea in deionized water at 37 °C for 10 min and spread as described in Materials and Methods.

Formamide. We treated the AMV RNA with 22, 44, 77 and 98% formamide respectively at various temperatures and salt concentrations.

22% formamide. The AMV RNA, when incubated with 22% aqueous formamide at 0 °C for 30 s and at 20 °C for 2 min and spread onto deionized water, revealed only collapsed structures (not shown). Increasing denaturing temperature to 37 °C and the ionic stregth of the hypophase to 0.011 mol/l led to no substantial difference in the secondary structure of AMV RNA. Little extension of collapsed RNA molecules occurred when the RNA was spread without salts under the same conditions of denaturation.

44% formamide. After denaturation with 44% aqueous formamide at 37 °C for 2 min and spreading onto deionized water, the features of secondary structure could be recognized and many molecules were sufficiently extended (Fig. 1-II). The length of the molecules, which represented the RNA subunits, ranged from 1.28 to 1.76 μm, with a mean of 1.48 ± 0.17 μm. A detailed study of the location of base-paired regions showing higher stability has been described elsewhere (Štokrová *et al.*, 1982*c*).

77% formamide. After denaturation with 77% aqueous formamide at 0 °C for 2 min and spreading onto deionized water, only collapsed structures were observed (Fig. 1-III). Under the same conditions of spreading, an increase in denaturation temperature to 37 °C and of ionic strength to 0.11 mol/l, was accompanied by appearance of molecules with characteristic secondary structures. These features were similar to those observed after denaturation with 44% aqueous formamide. The longest molecules found were within the range from 1.3 to 2.1 μm with a mean length of 1.64 ± 0.32 μm. When the salt in the denaturing mixture was omitted, extended RNA molecules with one or two short secondary region were observed (Fig. 1-IV). Two types of the molecules within the range from 0.34 to 3.28 μm were found. Besides linear molecules (80%) with a mean length of 1.28 ± 0.15 μm, aggregated molecules appeared (20%) with a mean length of 2.23 ± 0.05 μm lacking any structural specificity.

A further increase in denaturation temperature to 65 °C and ionic strength to 0.11 mol/l during denaturation followed by spreading onto 50% formamide in 0.011 mol/l salt was accompanied by the presence of more than 95% of the linear molecules and less than 5% of aggregates. The length of both ranged from 0.30 to 2.21 μm. The use of deionized water as a hypophase, led to extension of all AMV RNA molecules (not shown).

98% formamide. The AMV RNA, denatured with 98% formamide at 0 °C for 30 s in the absence of salt and spread onto deionized water, revealed three types of structures: collapsed structures (80%), partially unfolded complexes (15%), and linear molecules (less than 5%). All types of the molecules revealed a high number of regions with secondary structure, which made the accurate legth measurement difficult.

Urea-formamide. To study the AMV RNA complex we selected 3.9 mol/l urea plus 78% formamide mixture under two conditions of incubation:

Table 1. Length of 60—70S AMV RNA after treatment under gently denaturing conditions

Denaturing conditions	Linear molecules			Partly unfolded complexes			Collapsed structure
	Yield**	Range*	Mean legth* (± S. D.)	Yield**	Range*	Mean length* (± S. D.)	Yield**
3.9 mol/l-urea + 78% formamide 0 °C/30 s AMV RNA	32	0.3—2.3	0.62 ± 0.14 1.15 ± 0.09 1.49 ± 0.12 1.97 ± 0.20	20	2.1—4.5		48
Main interval	70[a])	0.9—1.8	1.41 ± 0.21	70[b])	2.1—3.6	3.03 ± 0.40[c]) 2.89 ± 0.44[d])	
18S myeloblastic rRNA			0.336 ± 0.043				
Linear density $-1.99 \times 10^6/\mu m$ 3.9 mol/l-urea +78% formamide 20 °C/5 min AMV RNA	77	0.3—2.5	0.66 ± 0.14 1.04 ± 0.08 1.42 ± 0.13 1.87 ± 0.10	17	2.1—3.4		6
Main interval	63[a])	1.1—1.8	1.41 ± 0.17	70[b])	2.5—3.1	2.81 ± 0.20[c]) 2.88 ± 0.11[d])	
18S myeloblastic rRNA Linear density $-1.97 \times 10^6/\mu m$			0.340 ± 0.037				

a) Percentage of linear molecules falling into the main interval.
b) Percentage of all partially unfolded complexes falling into the main interval.
c) Mean length of all partially unfolded complexes.
d) Mean length of typical dimer structures only.
* in μm; ** per cent;

(i) at 0 °C for 30 s and (ii) at 20 °C for 5 min. The results were comparable to those obtained by denaturation with 98% formamide. However, the linear molecules and partly unfolded complexes were well extended and there types of structure were found in different proportions.

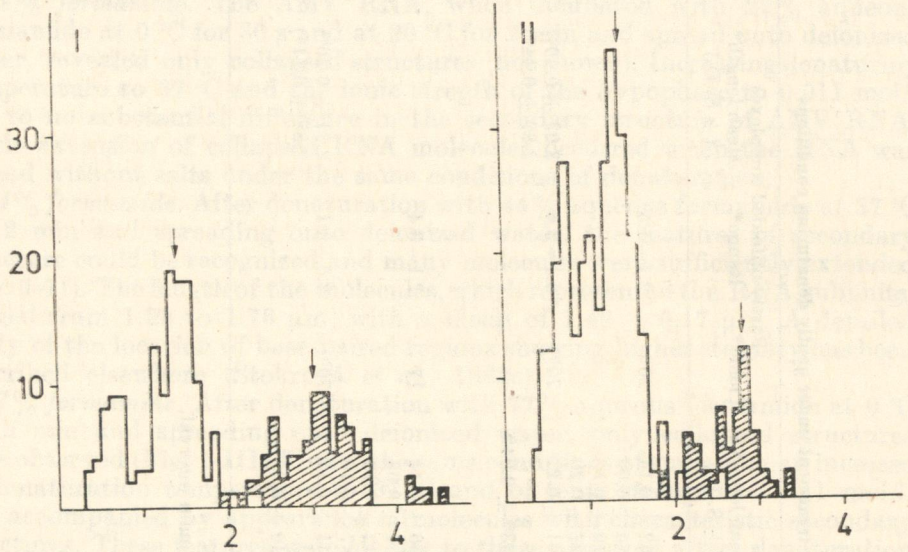

Fig. 4.
Histograms of the contour length of AMV RNA molecules
AMV RNA has been denatured with 3.9 mol/l-urea + 78% formamide mixture either at 0 °C for 30 s (I) or 20 °C for 5 min (II). Hatched blocks represent the partially unfolded complexes and open blocks illustrate the lengths of linear molecules in the same preparation. The mean lengths are indicated by arrows.

Abscissa: length of RNA molecules in μm; ordinate: number of molecules.

Under the conditions of incubation at 0 °C for 30 s, 48% collapsed structures (Fig. 2-I), 20% partially unfolded complexes (Fig. 2-II, 2-III) and 32% linear molecules (Fig. 2-IV) were observed. Linear molecules ranged in length from 0.3 to 2.3 μm with preferential size classes of 0.62 ± 0.14, 1.15 ± 0.09, 1.49 ± 0.12, and 1.97 ± 0.20 μm (Table 1). It can be estimated that over 70% of the molecules are in the range 0.9 to 1.8 μm with a mean length of 1.41 ± 0.21 μm (Fig. 4-I). When the length of 18S myeloblastic rRNA 0.336 ± 0.043 μm was used as a standard, the estimated mol. wt. of these linear molecules was 2.8×10^6. Partially unfolded complexes had lengths that ranged from 2.1 to 4.5 μm. Of these, 77% complexes revealed more than two ends (Fig. 2-II) and 23% revealed the typical dimer forms (Fig. 2-III) The majority of all partially unfolded complexes (70%) fell into the main interval from 2.1 to 3.6 μm with a mean length of 3.03 ± 0.40 μm (Fig. 4-I). The calculation of molecules with the typical dimer structure showed a mean length of 2.89 ± 0.44 μm (Table 1).

An increase in the denaturation temperature to 20° C led to a decrease in the number of collapsed structures below 6%; 17% partially unfolded complexes and 77% linear molecules were found (Table 1). The linear molecules (Fig. 3-IV) ranged in length from 0.3 to 2.5 μm and revealed preferential size classes with a means length of 0.66 ± 0.14, 1.04 ± 0.08, 1.42 ± 0.13, and 1.87 ± 0.10 μm (Table 1). More than 60% of the linear molecules had lengths from 1.1 to 1.8 μm, with a mean length of 1.41 ± 0.17 μm (Fig. 4-II) and a mol. wt. of 2.8×10^6, similarly as in the precendig case. Also with this type of denaturation, the partially unfolded complexes were either dimers (Fig. 3-III) (27%) or had more complex structures (Fig. 3-II) (73%). Nearly 70% of all these complexes ranged in length from 2.5 to 3.1 μm with a mean length of 2.81 ± 0.20 μm (Fig. 4-II). The typical dimer structures had a mean length of 2.88 ± 0.11 μm (Table 1).

In this study, the molecules revealing the dimer structure had four types of the characteristic linkage region (Fig. 5) which were located near the centre of the complex. The point of linkage betwen the subunits occurred at an average distance of 0.062 ± 0.007 μm (384 ± 43 nucleotides) from one end of each monomer.

Discussion

The dimer structure in the 60—70S RNA has been demonstrated by electron microscopy for several mammalian retroviruses (Kung *et al.*, 1975, 1976; Bender *et al.*, 1978) and just recently for RSV (Murti *et al.*, 1981). In this study we have demonstrated the structural features of AMV RNA using a set of non-denaturing and gently denaturing conditions. We preferred urea-formamide to glyoxal spreading (Kung *et al.*, 1975, 1976), because in our hands glyoxal led to a rapid dissociation of AMV RNA complex.

The observation of only collapsed structures of the 60—70S AMV RNA under non-denaturing conditions is in agreement with the results of others (Weber *et al.*, 1974; Chi and Basel, 1975). Among gently denaturing conditions the 3.9 mol/l urea — 78% formamide mixture at 0 or 20 °C revealed partly unfolded complexes in addition to linear molecules and collapsed structures. The stronger the denaturing power, the lower the percentage of collapsed forms. It suggests that dissociation of the 60—70S AMV RNA is not all-or--none process, which seemed to be different from mammalian retrovirus RNAs (Kung *et al.*, 1975, 1976).

The linear molecules, released from complexes under selected gently denaturing conditions, revealed length size classes, similar to those observed under strongly denaturation. The length heterogeneity of AMV RNA molecules was reported preciously (Chi and Basel, 1975; Heine *et al.*, 1975; Korb and Štokrová, 1980). Since the distribution of molecules was not dependent on either the method of RNA isolation or virus propagation (Štokrová *et al.*, 1982*b*) more labile regions in the viral genome, which are preferentially split, can be considered (Heine *et al.*, 1975; Korb and Štokrová, 1980). Slight size differences (5—10%) between genomes of defective AMV and its

helpers MAV-1, MAV-2 (Smith and Moscovici, 1969) have been recently reported (Bergman *et al.*, 1980; Chen *et al.*, 1980; Duesberg *et al.*, 1980; Souza *et al.* 1980). These, however, cannot influence in any case our length calculations. Therefore most of the linear molecules falling into the main peak and corresponding to a mol. wt. of 2.8×10^6 represent the AMV monomers released under gently denaturation.

From the point of view of structural arrangement of 60—70S AMV RNA, the partially unfolded complexes revealed the most interesting structural features. They were either dimers or exhibited more complex structures (more than two ends). The latter were very similar to those reported for partially denatured AMV RNA by heating in the presence of formaldehyde (Chi and Basel, 1975) or to the network structure for the RSV RNA after interaction with the bacteriophage T4 gene-32 protein (Mangel *et al.*, 1974). The dimers were very similar to those of the mammalian retrovirus and RSV RNAs (Kung *et at.*, 1975, 1976; Bender *et al.*, 1978; Murti *et al.*, 1981). However, unlike RSV RNA (Murti *et al.*, 1981), we did not observe any reproducible hairpins or loops on the AMV RNA molecules, except of the monomer linkage structure. The distance of the monomer linkage point from one end of each subunit was slightly different, too. Since most of the partially unfolded complexes revealed the length twice that of the linear molecules, representing the subunits in the same preparation, we can conclude that even the 60—70S AMV RNA is a dimer. The partially unfolded complexes having more than two ends and the length equal to the dimers might represent complexes in which the polynucleotide chains could be nicked in more labile regions already in the nucleo-protein complex. The nicked chains can be held together by hydrogen bonds.

Our structural studies of the 60—70S AMV RNA confirm and extend the results obtained by others with mammalian retroviruses (Kung *et al.*, 1975, 1976; Bender *et al.*, 1978) and quite recently with RSV (Murti *et al.*, 1981). The observations suggested that the dimer structure might well be a common feature of all retroviral RNAs being involved in the splicing process, as well as in the translation of viral RNA as discussed by Murti *et al.*, (1981). It seems probable that the dimer structure with the typical linkage at the 5′-end of both subunits plays an important role in transcription by facilitating two presumed transcriptional jumps (Taylor *et al.*, 1979; Omer *et al.*, 1981).

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Explanation of Electron Micrographs (Plates XLIV—XLV):

Fig. 1. Electron micrographs of the 60—70S AMV RNA molecules.
(I) RNA prepared by the aqueous non-denaturing procedure;
(II) RNA heated at 37 °C for 2 min in 44% formamide;
(III) RNA denatured with 77% formamide at 0 °C for 2 min;
(IV) RNA denatured with 77% formamide at 37 °C for 2 min. The bar represents 0.5 μm.

Fig. 2. Electron micrographs of 60-70S AMV RNA denatured with 3.9 mol/l-urea + 78% formamide mixture at 0 °C for 30 s.
(I) collapsed structures; (II) partially unfolded complexes having more than two ends; (III) typical dimer ; (IV) linear molecules. The bar represents 0.5 μm.

Fig. 3. Electron micrographs of 60-70S AMV RNA denatured with 3.9 mol/l-urea + 78% formamide mixture at 20° C for 5 min.
(I) collapsed structures; (II) partially unfolded complexes having more than two ends; (III) typical dimer ; (IV) linear molecules. The bar represetnts 0.5 μm.

Fig. 5. Examples of dimer linkage region of 60-70S AMV RNA shown at a high magnification. The bar represents 0.1 μm.