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STUDIES ON THE STRUCTURE OF AVIAN MYELOBLASTOSIS VIRUS (AMV) RNA. III. ELECTRON MICROSCOPIC DEFINITION OF SECONDARY STRUCTURE

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Summary. — The secondary structure of avian myeloblastosis virus (AMV) RNA was characterized by electron microscopy under moderately denaturing spreading conditions. Under denaturation by aqueous 44% formamide or 77% formamide in the presence of salts, partly stretched RNA molecules with measurable double-stranded regions were observed. This approach allowed the localization from 5 to 11 regions of preserved secondary structure on AMV RNA molecules. Topographic analysis revealed a nonrandom occurrence of stable secondary structures in several prevalent regions. These regions with higher secondary structure stability revealed certain similarity to hairpin structures localized by electron microscopy on Rous sarcoma virus RNA or to highly structured regions found on this RNA by T1 ribonuclease oligonucleotide analysis.

Key words: avian myeloblastosis virus RNA; secondary structure; electron microscopy

Introduction

An important approach to the studies of the functional properties of retroviruses is the definition of the size and structure of their genomes. Retroviral RNA plays a decisive role in both transcription and translation processes. Therefore an elucidation of the secondary structure of this RNA is of particular importance as it is expected to provide some information about the molecular basis for the specificity of both these functions.

Investigations of retroviral RNA revealed two characteristic features of secondary structure: (*1*) a structure joining the non-poly(A) ends of monomer subunits and (*2*) symmetrically disposed loops on each subunit at an equal distance from the dimer linkage. Up to date a model composed of two identical 35 S RNA subunits forming the 60-70 S retroviral complex has been accepted by most authors (Mangel *et al.*, 1974; Riggin *et al.*, 1975; Kung *et al.*, 1975, 1976; Bender and Davidson, 1976; King, 1976; Dion *et al.*, 1977; Shine *et al.*, 1977; Darlix *et al.*, 1978; Bender et al., 1978). It was

clearly proved by electron microscopy in mammalian retrovirus RNA (Kung *et al.*, 1975, 1976; Bender *et al.*, 1978).

For avian retrovirus genomes, however, the structure of high-molecular-weight RNA has not been visualized with certainty, presumably because of the weakness of the helical linkage connecting their subunits (Bender and Davidson, 1976; Kung *et al.*, 1976). Just recently the dimeric structure of 60-70 S Rous sarcoma virus (RSV) RNA was visualized (Murti *et al.*, 1981). Nevertheless, this model has already been proposed by Delius *et al.* (1974) on the basis of the RNA length distribution analysis. The secondary structure of avian retrovirus genomic RNA and its 60-70 S complex have also been investigated by methods based on the susceptibility od f the RNA molecules to various nucleases or on partial digestion of RNA molecules by T1 ribonuclease and, after resolving the digest into its components by gel electrophore phoresis, on localization of these components on the basis of their T1 ribonuclease fingerprints. Isolation and localization of a large "hairpin" segment from the 35 S RNA of avian sarcoma virus was done by Perdue *et al.*, (1979).

Electron microscopy was only successful for a long time in the direct visualization of the secondary structure features in mammalian retrovirus RNA. These investigations revealed the characteristic presence of symmetrically localized loops on both subunits of RD-114, baboon and woolly monkey virus RNA (Kung *et al.*, 1975, 1976; Bender *et al.*,1978). Recently, similar loops on nearly 50% of the RSV RNA molecules were visualized (Murti *et al.*, 1981).

We tried to apply electron microscopy to define the secondary structure on RNA molecules isolated from AMV, a representative of avian retroviruses, for no electron microscopic report concerning dimeric linkage or secondary structure of AMV RNA subunits was published. AMV RNA was visualized by a modification of the basic protein film technique (Kleinschmidt and Zahn, 1959). Two quite different forms of RNA molecules can be observed depending on the conditions of denaturation (i) collapsed unmeasurable structures at very mild conditions and (ii) linear, extended molecules at strongly denaturing conditions. Under special mildly denaturing conditions, partly unfolded RNA structures can be obtained and measured. We studied the structure of AMV RNA under conditions which allowed the preservation of stable base-paired regions. In this paper we describe the presence of several preferential double-stranded (ds) regions with higher stability on 30-40 S AMV RNA molecules.

Materials and Methods

Virus and RNA preparations. The avian myeloblastosis virus (AMV) was purified and pelleted from 150 ml of blood plasma of leukaemic chicken (White Leghorn) as described (Korb and Heine, 1978). The RNA was extracted by phenol from sodium dodecyl sulfate- (SDS)-disrupted virions and the 60-70 S complex isolated as will be described elsewhere (Štokrová *et al.*, 1982*a*).

Denaturation of 60-70 S AMV RNA. AMV RNA preparation at a concentration of 0.2 μg/ml was denatured in a solution of aqueous 44% formamide (MCB) for 2 min at 37° C or in 77% formamide in the presence of 0.1 mol/l TRIS, pH 8.5, 00.1 mol/l EDTA for 2 min at 37° C. After denaturation, the RNA solution was chilled on ice and prepared for spreading.

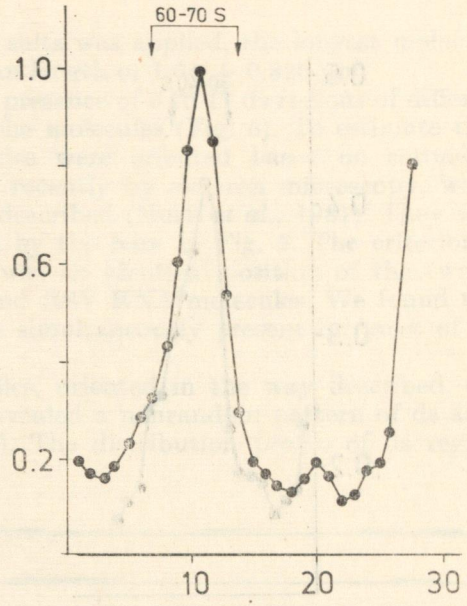

Fig. 1.
Sucrose gradient sedimentation of total RNA prepared from AMV
Linear sucrose gradient (10—30%) in TNE buffer, pH 7.5, with 0.1% sarcosyl was centrifuged at 37 500 rev/min for 130 min at 20° C in a Spinco SW 41 rotor. The 60-70 S RNA (arrows) was pooled and used for electron microscopic analysis of secondary structure.
Abscissa: fraction number; ordinate: absorbancy (A_{260} nm).

Spreading conditions. 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 solution (1.5 mg/ml) in TE buffer, pH 8.5 (1.5 mol/l TRIS, 50 mmol/l EDTA) was added to 100 µl of denatured RNA solution. A 50 µl of the mixture was spread on to a deionized water surface and the cytochrome film was picked up on grids coated with parlodion, stained with uranyl acetate (Davis and Davidson, 1968) and rotary-shadowed with Pt/Pd.

Length measurement and polarization of molecules. Preparation of AMV RNA was examined in a Jeol 100 B electron microscope at a magnification of 10 000 and at 60 kV. The lengths of AMV RNA molecules were measured as described (Štokrová *et al.*, 1982). Maps of the secondary structures were constructed and normalized as described (Korb and Heine, 1978). The molecules were oriented according to the position of two typical regions of secondary structure localized on RSV RNA (Murti *et al.*, 1981).

Results

The AMV RNA used for electron microscopic detection of secondary structures was prepared from plasma virus by SDS-phenol extraction and revealed a typical sedimentation pattern (Fig. 1). The 60-70 S RNA sedimented in the position indicated by the arrows. Slowly sedimenting components corresponded to 28, 18 and 4 S RNAs. The integrity of 60-70 S RNA was confirmed by heat dissociation (73° C for 3 min) and by sedimentation analysis (Fig. 2). The RNA representing a sharp peak of 30-40 S RNA with minimal shoulder in the position of the slowly sedimenting components was only used for electron microscopic analysis, assuming a minimal amount of single-strand (ss) breaks on the molecules.

Three types of electron microscopic visualization of AMV RNA were tried for secondary structure analysis:

a) When AMV RNA was prepared for electron microscopy under non-denaturing or mildly denaturing conditions (far below the T_m value), only

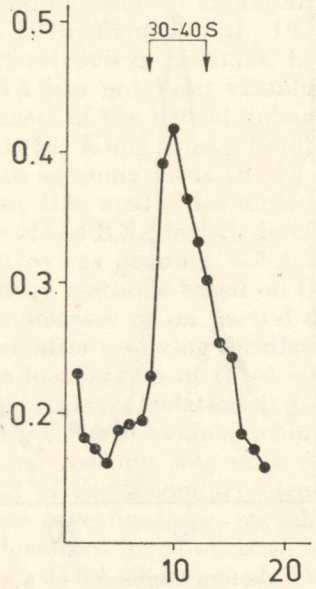

Fig. 2.
Sucrose gradient sedimentation of heat-treated high-molecular-weight AMV RNA
Part of 60—70 S RNA of Fig. 1 was heated (73° C, 3 min) and applied to a 20—30% linear sucrose gradient in 0.01 mol/l phosphate buffer, pH 7.2. Centrifugation was carried out in a Spinco SW 41 rotor at 31 500 rev/min for 13 hr at 4° C. Arrows indicate 30-40 S AMV RNA.
Abscissa: fraction number; ordinate: absorbancy (A_{260} nm).

collapsed "folded" structures were observed. They were unsuitable for secondary structure analysis (not shown).

b) Strongly denaturing conditions (e. g., 78% formamide, 3.9 mol/l urea, 53° C for 30 sec) revealed completely stretched molecules without apparent secondary structure, as described elsewhere (Korb and Štokrová, 1980; Štokrová *et al.*, 1982 *a*).

c) Only moderate denaturation of RNA (denaturation near T_m value) appeared to be satisfactory for analysis of residual secondary structure as both the linear and measurable aggregates were present in electron microscope preparations (Fig. 3). Details will be described elsewhere (Štokrová *et al.*, in preparation).

Two types of moderately denaturing conditions were preferred for the study of residual secondary structure in the AMV RNA molecules: (1) action of 44% formamide in deionized water at 37° C for 2 min; and (2) action of 77% formamide in 0.1 mol/l TRIS, pH 8.5, 0.01 mol/l EDTA at 37° C for 2 min.

Both types of denaturation had a similar effect and resulted in the presence of partly stretched linear RNA molecules. Single-stranded (ss) and ds regions were detected and easily measured on electron micrographs (Fig. 4). The length heterogeneity of AMV RNA molecules with this type of spreading was similar to that in other types of spreading (Štokrová *et al.*, 1982 b). The length of the longest molecules denatured by aqueous 44% formamide varied from 1.28 to 1.76 μm with a mean length of 1.49 ± 0.171 μm. When

77% formamide in the presence of salts was applied, the longest molecules were from 1.3 to 2.1 μm with a mean length of 1.64 ± 0.320 μm.

The RNA molecules revealed the presence of 5 to 11 ds regions of different length (from 0.04 to 0.25 μm) on the molecules (Fig. 5). To estimate their positions, the AMV RNA molecules were oriented based on secondary structures of RSV RNA estimated recently by electron microscopy, where two characteristic ds regions were described (Murti *et al.*, 1981). They were localized in the positions indicated by the bars in Fig. 5. The criterion of AMV RNA molecules orientation was an identical position of the two ds regions on AMV RNA molecules and RSV RNA molecules. We found that both characteristic structures were simultaneously present on most of the AMV RNA molecules measured.

The map of AMV RNA molecules, oriented in the way described, and carrying both ss and ds regions, revealed a nonrandom pattern of ds areas along the RNA molecules (Fig. 5). The distribution profile of ds regions

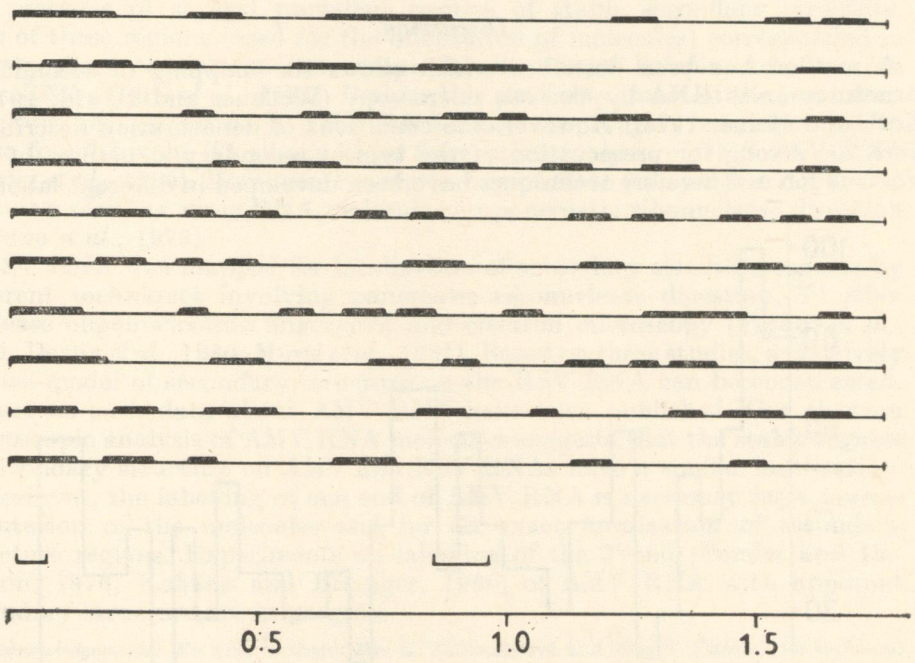

Fig. 5.

Secondary structure map of selected molecules of AMV RNA

The map, constructed as described in Materials and Methods, shows the length and localization of ds regions on ss AMV RNA molecules.

The bars indicate positions of hairpin structures localized on RSV RNA by electron microscopy (Murti *et al.*, 1981).

Abscissa: length in μm.

involving their position and length on the oriented RNA molecules measured is shown in Fig. 6. This histogram displays several peaks. Their position resembled the data published for highly structured regions on RSV RNA (Darlix *et al.*, 1980). The first two peaks from 0 to 0.15 and from 0.35 to 0.50 μm, respectively, may correspond to the highly structured regions described for the gag gene. The peaks from 0.65 to 0.75 and from 0.80 to 0.90 μm were localized in the positions of the regions with strong secondary structure of the pol gene. The broad area with peaks at the positions 1.05, 1.25 and 1.35 μm may involve the RSV regions near the ends of the pol and env genes. The area from 1.45 to 1.70 μm was comparable to the structured regions localized in the src gene of RSV. The present results strongly suggested the probable similarity in localization of the areas of secondary structure with high stability in AMV and RSV RNAs.

Similar results were obtained when AMV RNA was denatured with 77% formamide in the presence of salts. The size and position of ds regions corresponded to those observed under denaturation with aqueous 44% formamide (data not shown).

Discussion

A method has been described which allows the mapping of secondary structures on ss RNA by electron microscopy (Wellauer and Dawid, 1973; Korb and Heine, 1978). However, the conditions of denaturation described were too strong for preservation of the typical secondary structures of retrovirus RNA. Therefore techniques have been developed in different labora-

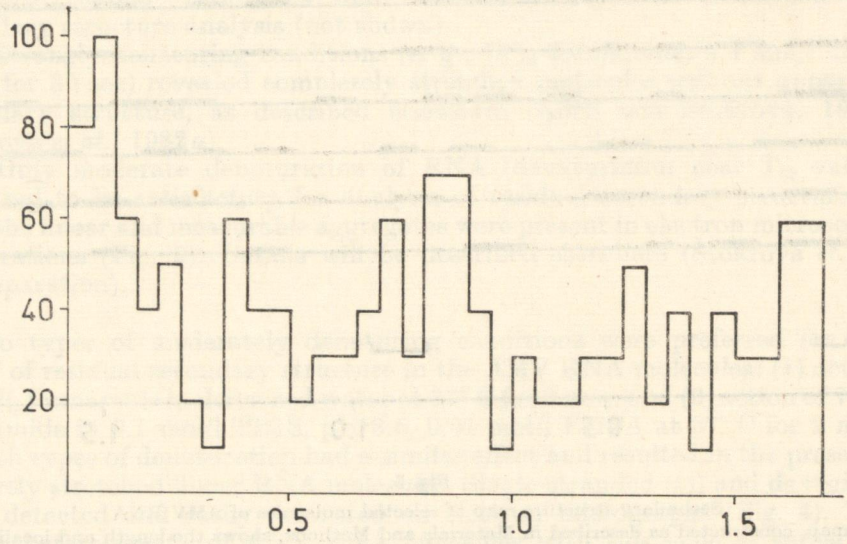

Fig. 6.
Frequency of occurrence of ds regions along the AMV RNA molecules
Abscissa: % of molecules with secondary structure region; ordinate: length in μm.

tories, resulting in stretching of viral RNA molecules with the secondary structure still preserved (Kung *et al.*, 1975, 1976; Bender *et al.*, 1978; Murti *et al.*, 1981). These techniques have been successfully applied in studies on the secondary structure of mammalian retrovirus RNA but they were less satisfactory with avian retroviruses (Kung *et al.*, 1976; Bender and Davidson, 1976) probably because of lower stability of the bonds. Just recently a successful mapping of RSV RNA was published (Murti *et al.*, 1981).

In studying AMV RNA secondary structure we used gently denaturing conditions which led to dissociation of the 60-70 S RNA complex into subunits but the regions with higher stability of secondary structure were still present. This approach allowed us to localize from 5 to 11 regions of preserved secondary structure on AMV RNA molecules. The postition and the length of these areas varied to some extent on individual molecules, but the histogram of their topographic localization revealed a nonrandom character with preferential sites (Fig. 6). For topographic analysis, the molecules were oriented according to the recent data on the secondary structure on RSV RNA (Murti *et al.*, 1981). AMV RNA molecules oriented in this way revealed the presence of several prevalent regions of stable secondary structure. Two of these regions (used for the orientation of molecules) corresponded in their position to electron microscopic localization of hairpin structures on RSV RNA (Murti *et al.*, 1981). Besides this similarity, most of the secondary structure regions on 35 S AMV RNA molecules roughly corresponded to those found by the T1 ribonuclease fingerprint analysis of 35 S RSV RNA (Darlix *et al.*, 1980). Moreover, the region from 0.80 to 0.90 μm was confirmed on avian sarcoma virus RNA molecules by pancreatic ribonuclease digestion (Perdue *et al.*, 1979).

RSV RNA was mapped for localization of secondary structure regions by different techniques involving pancreatic ribonuclease digestion, T1 ribonuclease oligonucleotide fingerprint and electron microscopy (Perdue *et al.*, 1979; Darlix *et al.*, 1980; Murti *et al.*, 1981). Based on these studies, a relatively precise model of secondary structure on the RSV RNA can be constructed. As yet no such data about AMV RNA have been published. Our electron microscopic analysis of AMV RNA molecules suggests that the stable regions of secondary structure on AMV and RSV RNAs have a similar localization.

However, the labelling of one end of AMV RNA is necessary for a precise orientation of the molecules and for an exact localization of secondary structure regions. Experiments on labelling of the 3'-end (Bender and Davidson, 1976; Kahana and Erlanger, 1980) of AMV RNA with apparent secondary structure are in progress.

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Explanation of Electron Micrographs (Plates III and IV):

Fig. 3. Electron micrograph of 60-70 S AMV RNA treated under moderately denaturing conditions (3.9 mol/l urea — 78% formamide, 0° C, 30 sec). The bar represents 0.5 μm.

Fig. 4. Electron micrographs of AMV 60-70 S RNA with residual secondary structure.
I — RNA prepared by denaturation with 44% aqueous formamide for 2 min at 37° C.
II — RNA prepared by denaturation with 77% formamide for 2 min at 37° C in the presence of 0.1 mol/l TRIS, 0.01 mol/l EDTA.
The bar represents 0.5 μm.