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# STRUCTURE OF AVIAN TUMOR VIRUS DNA INTERMEDIATES

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Summary: The mechanism of conversion of virion RNA to double-stranded DNA, in avian sarcoma virus-infected duck cells, has been investigated. The evidence presented here indicates a covalent linkage between the genomic RNA and the newly synthesized plus strand DNA. This suggests that plus strand synthesis is primed by the 35S virion RNA. Some data identifying the virus specific hybrid and linear double-stranded DNA molecules, which are probably intermediates, leading to the formation of supercoiled duplex viral DNA, has also been presented.

In the life cycle of RNA tumor viruses, the genomic RNA is converted to a covalently closed double-stranded supercoiled DNA, which appears to be a precursor for integration (1-4). Previously we have presented evidence for the synthesis of linear duplex viral DNA molecules, consisting of full length minus (-) strand, (complementary to virion RNA) and segmented plus (+) strand (same polarity as viral RNA) in the cytoplasm of avian sarcoma virus-infected duck and quail cells (5). That the cytoplasmic viral DNA is a precursor to nuclear circular DNA has clearly been established by pulse-chase experiments (6). Several in vitro studies have shown that the minus strand synthesis is initiated on a $tRNA^{trp}$ primer, which is located close to the 5' end of genome (7,8). The mechanism by which plus strand synthesis takes place remains to be understood. The results presented in this communication suggest that plus strand synthesis is primed by the genomic RNA of the virion; the data further identify some of the virus-specific nucleic acid structures which are probably intermediates in the synthesis of avian sarcoma virus supercoiled DNA (1,2).

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## MATERIALS AND METHODS

Cells and Viruses: Avian sarcoma virus (ASV), strain B77, was propagated in chick embryo fibroblasts (1). Pekin duck embryo fibroblasts were grown in medium 199 containing 5% calf serum, 10% tryptose phosphate broth, 0.2% sodium barcarbonate (9).

Isolation and Analysis of Viral Nucleic Acids: Duck embryo fibroblasts, infected with ASV, for 8 or 9 hours were collected by centrifugation and subjected to Hirt fractionation (10). The nucleic acids were extracted and centrifuged to equilibrium in CsCl-propidium diiodide ($PropI_2$) gradients (2). Fractions were collected from the bottom of the gradients, aliquots were hybridized to $^{32}P$-labeled DNA complementary to viral RNA (cDNA) (2). This probe detects only plus strand DNA. In order to detect minus strand, $^{32}P$ labeled viral RNA was used. Hybridizations were carried out in a 40 µl volume containing approximately 1000 cpm cDNA or $^{32}P$-RNA in 0.6 M NaCl, 10 mm Tris-HCl pH 8.0, 3 mM EDTA for 60 to 70 hours at 68°C. The extent of hybridization was monitored by S1 nuclease (2).

Covalently linked RNA-DNA hybrids were detected in $Cs_2SO_4$ gradients. The samples to be analyzed were dissolved in 3.1 ml Tris-EDTA buffer to which 2.3g $Cs_2SO_4$ was added. The solution was layered on a 1 ml saturated $Cs_2SO_4$ cushion and centrifuged for 60 to 70 hours in a SW 50.1 rotor at 30,000 rpm and 20°C. Fractions were collected from the bottom of the gradient. Nucleic acids were precipitated with CETAB (Cetylethyl Trimethyl ammonium bromide), collected by centrifugation and hybridized with appropriate radioactive virus-specific reagents. The size of viral nucleic acid intermediates was analyzed in velocity sucrose gradients. Appropriate conditions are given in the legends.

## RESULTS

Preliminary analysis of viral nucleic acid intermediates in velocity gradients revealed much heterogeneity. In order to identify various structures we found it necessary to first fractionate various viral structures in a CsCl-$PropI_2$ gradient. An example of such an experiment is illustrated in Fig. 1. Total virus specific nucleic acids, extracted from the Hirt supernatant, were subjected to equilibrium density gradient centrifugation in CsCl-$PropI_2$. Analysis of various fractions by hybridization to $^{32}P$-cDNA indicated that viral DNA structures spread all over the gradient with two distinct peaks. By contrast to the hybridization profile shown in this figure, if we pretreat nucleic acids with ribonuclease A, prior to banding, we observed only two major peaks. The DNA banding at high density is the supercoiled form and that at lighter density consists predominantly of linear DNA (2).

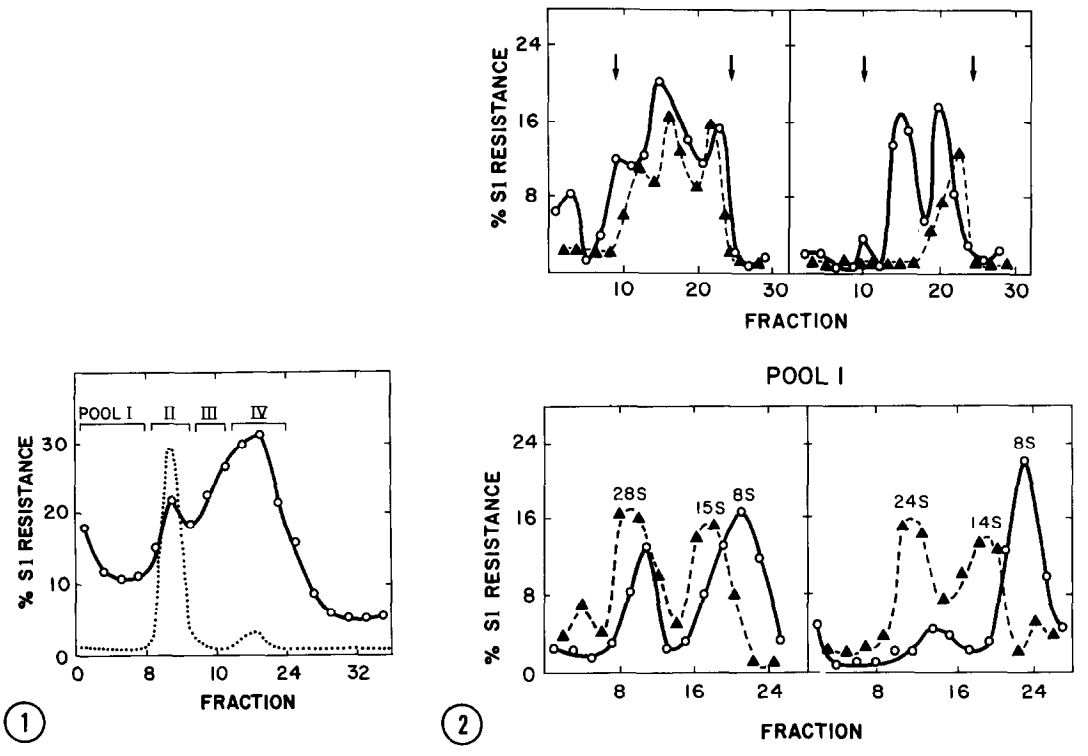


Figure 1. Preparative centrifugation of ASV nucleic acids in CsCl-Propi$_2$ gradients.

Duck embryo fibroblasts were infected by avian sarcoma virus, B77, for 9 hours at a multiplicity of infection of 3 to 4. Cells were collected and fractionated according to Hirt procedure as described before (2). Nucleic acids were extracted from Hirt supernatant and subjected to equilibrium density gradient centrifugation in CsCl-Propi$_2$. Fractions were collected from the bottom, without puncturing the tube, and assayed for virus specific DNA essentially as described earlier (2). Under these conditions of centrifugation, RNA and some hybrids pelleted, which were dissolved in buffer and analyzed as in figure 2. ●, Viral DNA; ●●● $^3$H PML 21 marker

Figure 2. Upper panel: Analysis of RNA and RNA Dna hybrids in $Cs_2SO_4$ equilibrium gradients.

RNA and hybrids that were pelleted in CsCl-Propi$_2$ gradients were resuspended in 10 mM Tris. HCl, pH 8.1, 10 mM EDTA and centrifuged as such or after heat denaturation in $Cs_2SO_4$ gradients as described in Methods. Left panel: native; right panel: heat denatured nucleic acids; Triangles represent hybridization of $^{32}$P labeled RNA to minus strand DNA and open circles represent cDNA plus strand DNA as detected by $^{32}$P labeled cDNA.

Lower panel: This figure and subsequent figures represent analysis of different pools in velocity gradients. In all cases left panels represent heat denatured nucleic acids and right panels represent the same after alkali digestion. Different pools from CsCl-Propi$_2$ gradient were denatured by heat or digested with alkali (0.3M NaOH, 1 hr 37°C), were layered on 5-20%, neutral sucrose gradients and centrifuged for 16 hours at 20,000 rpm and 20°C in a SW41 rotor. Fractions were collected from the bottom of the gradient and analyzed for minus and plus strand DNAs as above.
Hybridization to $^{32}$P RNA (▲), or $^{32}$P. cDNA (O).

Density Distribution of Pelleted Nucleic Acids: Analysis of the nucleic acids of the pellet was done in an isopycnic $Cs_2SO_4$ gradient in order to identify RNA-DNA hybrids (Fig. 2a). When native material was centrifuged to equilibrium and the fractions assayed for virus specific DNA by radioactive probes, after digestion with alkali to remove viral RNA, both minus and plus strands were found at hybrid density indicating the presence of DNA-RNA hybrids with various ratios of RNA to DNA. However, banding after denaturation by heating, showed a shift in the density of minus strands to DNA region, whereas plus strands remained as hybrids. Prior treatment of these preparations with alkali virtually eliminated the hybrid molecules. These results provide evidence for RNA-DNA duplexes consisting of DNA minus strands and virion RNA covalently linked to plus strand DNA. Sedimentation analysis further confirmed these results (data not shown; also see below). Similar hybrids were identified by Leis et al. (11).

Size Distribution of Nucleic Acids in Various Pools: Nucleic acids of pool I were analyzed in neutral rate zonal sucrose gradients after heat denaturation and after alkali digestion. The sedimentation profile showed that minus strand DNA consisted of molecules of complete length (28S) as well as 1/4 to 1/3 genome length (12 to 15S). The size distribution of these molecules remain unaltered after alkali digestion indicating that the minus strand DNA did not contain covalently linked RNA (2b). On the other hand, plus strands sedimented at positions corresponding to near genome size (24S) to 1/10 (8S). After alkali treatment, however, a homogeneous population of 8S only could be detected (2b). These results suggest that plus strands in this pool contain 8S DNA, some of which are covalently linked to parental RNA.

When similar analysis of pool III were performed, the results with respect to minus strand were essentially similar to pool I. However, plus strands consisted of predominantly 8S pieces (Fig. 2c and d). Fur-

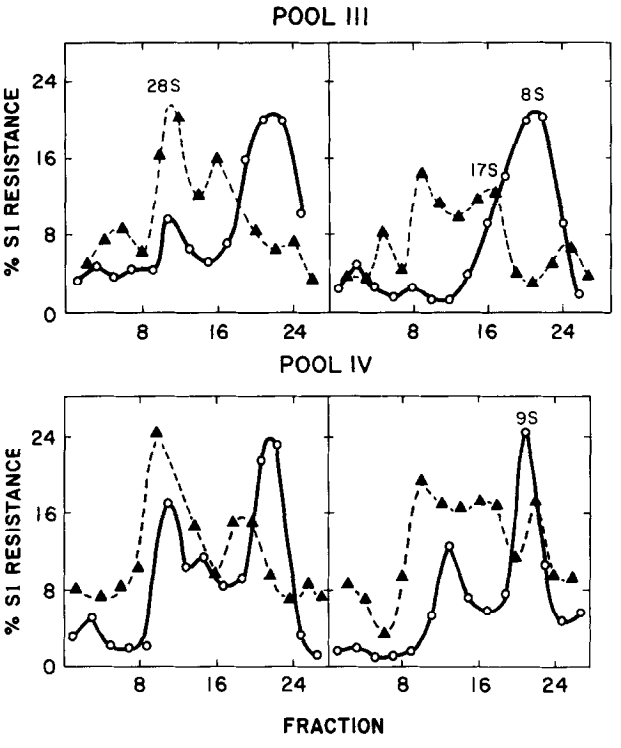


Figure 3. Velocity gradient centrifugation of pool III (upper panel) and pool IV (lower panel). Conditions of centrifugation and detection of virus specific DNA are as described in the legend to fig. 2 lower panel. Hybridization to $^{32}$P. RNA (▲), or $^{32}$P. cDNA (O).

ther analysis of this pool in NaI gradients indicated no free single-strand structures of either polarity (Richards and Guntaka, unpublished data). Some duplex molecules with single strand tails or gaps were also found.

Sedimentation analysis of pool IV DNA indicated that minus strands were of the same size as in all pools. Plus strands, however, comprised mainly 8 to 9S species with some sedimenting at 18 to 19S indicating approximately 1/2 the genome size. Alkali-treatment did not change the sedimentation pattern of either minor or plus strand DNA confirming that plus strands in this pool were entirely DNA pieces. We conclude that this pool contains duplex viral DNA of genome size as well as duplex DNA of 1/3 to 1/2 length minus strands base-paired with short plus strands.

## DISCUSSION

The results presented in this communication indicate a covalent linkage between virion RNA and newly synthesized plus strand DNA. This supports and further extends the results of Leis et al., (11) who demonstrated such a covalent hybrid in ASV-infected chick embryo cells. The heterogeneous nature of the covalent RNA-DNA hybrids and the fact that digestion of these hybrids with alkali results in a discrete size plus strand DNA (8S) suggest, but do not prove, that plus strand DNA synthesis initiates at several discrete positions.

The data presented here neither demonstrates the kinetics of appearance of plus strand synthesis, nor indicates any precursor-to-product relationship of these structures. Nevertheless the mere existence of these molecules consisting of various amounts of RNA to DNA and linear double-stranded DNA molecules consisting of longer minus strand and segmented plus strand suggest a logical relationship between these structures. Analysis of the kinetics of appearance of both minus and plus strand in alkali sucrose gradients (unpublished results; also Varmus et al., JMB in press) suggest that plus strand synthesis begins before the completion of minus strand. Further experiments with specific reagents are needed to elaborate the mechanism of viral plus strand DNA synthesis. Our recent experiments with purified viral preparations indicate a similar covalent linkage between virion RNA and plus strand DNA and that plus strand synthesis commences when the minus strand pieces reach a size of 300 to 400 nucleotides long (Guntaka, manuscript in preparation).

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