Acta virol. **37:** 305–319, 1993

OKAZAKI FRAGMENTS, A CONSTANT COMPONENT OF AVIAN MYELOBLASTOSIS VIRUS CORE-BOUND 7 S DNA

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Received January 20, 1993; revised March 4, 1993

Summary. – We have shown that the unusual CsCl-buoyant density and velocity sedimentation properties of the isolated host 7 S DNA species associated with the core fraction of avian myeloblastosis virus (AMV) are made mainly by tight association of RNA pieces prevalently joined to the single-stranded portion of this material. It was shown indirectly on sedimentation patterns of [methyl-^{3}H]thymidine and [^{14}C]uridine double-labelled and glyoxylated total AMV DNA, and directly in phosphorylation experiments with T4 polynucleotide kinase performed on the single-stranded portion of AMV DNA that the RNA-DNA link in AMV DNA is of a covalent nature and that the 5'-terminal end of DNA at the RNA-DNA junction is occupied by all four common deoxyribonucleotides. This first evidence of the presence of Okazaki fragments in 7 S AMV DNA clearly indicates that this DNA does not represent a randomly fragmented host DNA included by chance into virions but special fragments of host DNA having the properties of DNA replicative structures with possible consequences for some viral function(s) including those involved in virus-cell interactions.

Key words: AMV core-bound DNA; Okazaki fragments; host nucleic acids

Introduction

The occurrence of small 7 S DNA molecules was detected in 1970, independently in virions of Rous sarcoma virus (RSV) (Levinson *et al.* 1970) and avian myeloblastosis virus (AMV) (Řím an and Beaudreau, 1970). Such DNA species were later found in other so far studied avian and mammalian retroviruses (Rokutanda *et al.*, 1970; Biswal *et al.*, 1971; Weber *et al.*, 1973; Puga *et al.*, 1974; Deeney *et al.*, 1976*a*). As shown in the case of AMV and MC29 virus, this DNA is mostly associated with the core fraction (Deeney *et al.*, 1976*b*). It is of host origin, as found by Levinson *et al.* (1972) who has demonstrated that RSV DNA does not hybridize with viral RNA but with chicken DNA. These findings led to a conclusion that this DNA represents randomly fragmented host DNA occlu-

ded into virions. Essentially similar results were obtained by analyzing the AMV DNA (Dvořák and Říman, 1980). In contrast to data showing that RSV DNA is completely double-stranded (Levinson *et al.*, 1972), the AMV DNA was found to be composed in part (up to 20 %) of single strands (Beaudreau, personal communication; Dvořák and Říman, 1980), and our extensive hybridization experiments indicated that this DNA was complementary to host myeloblast DNA unique sequences with low frequency and repetition. We also found differences in the kinetics of hybridization of this DNA when hybridized with myeloblast DNA and with DNA of chick fibroblasts, uninfected and infected with AMV, thus indicating a higher amount of these sequences in myeloblast DNA than in fibroblast DNA and its elevation in fibroblast DNA after AMV infection. Finally, we found that a great heterogeneity of this small DNA triggered strongly its renaturation abilities (Dvořák and Říman, 1980). In addition, the AMV DNA has other peculiar features. In isopycnic CsCl gradients its broad band has two main distinct peaks or shoulders with densities of 1.722 g/cm^3 and 1.709 g/cm^3, respectively (Říman and Beaudreau, 1970; Dvořák and Říman, 1980) simulating in these peak fractions a (G+C) content of 63 % and 43 %, respectively, while melting temperature-derived (G+C) values are for both peak fractions identical, i.e. 29 % (Dvořák and Říman, 1980). Moreover, this DNA reveals a pronounced labelling velocity and kinetics of incorporation into virions (Říman, 1971; Říman *et al.*, 1972) and its synthesis exhibits an unusual response to some inhibitors of RNA (Říman, 1971) and DNA (Křemen *et al.*, 1971) synthesis.

All the above mentioned properties of AMV DNA have indicated that it may represent some special fragments of host DNA, the features of which deserve a deeper elucidation, rather than randomly fragmented host DNA. Being convinced that this is true even now, after a decade of silence on this uneasy research topic, we have returned once more to studies of this DNA. In this paper, we present the first direct evidence that the core-associated AMV DNA contains components having properties of Okazaki fragments present in eukaryotic DNA-replicative structures, the features of which contribute to an anomalous physicochemical behaviour of this DNA. All results presented here were obtained by analyzing the AMV DNA isolated from the core fraction of 7 hours old virions produced *in vitro* by chicken leukaemia myeloblasts. Core fractions, checked electron microscopically, were completely void of virus particles (Korb *et al.*, 1993).

Materials and Methods

Chemicals and radioisotopes. The following detergents were used: Sterox (Monsanto, St. Louis, MD), Nonidet P-40 (LKB), and Sarcosyl (Serva). Dithiothreitol (DTT) and deoxyribomononucleotides were from Calbiochem, and CsCl from BDH Chemicals Ltd. Formamide and glyoxal (Merck) were before use purified according to Maniatis *et al.* (1982). Calf thymus DNA and yeast tRNA were from Calbiochem. All other reagents were of corresponding analytical grade. Both [methyl-^{3}H]

thymidine (^{3}H-mTdR, 1.5–2.0 TBq/mmol) and [^{14}C]uridine (^{14}C-UR, 14–17 GBq/mmol) were from UVVVR, Prague. [gamma-^{32}P]adenosine-5'-triphosphate ([gamma-^{32}P]ATP, 110 TBq/mmol) was from NEN.

Enzymes. Ribonuclease A (RNase A) Worthington Biochemicals was prior to use incubated in 10 mmol/l sodium acetate, pH 5.0, at 90 °C for 15 mins to eliminate endonuclease activity attacking the single-stranded DNA (Hirose *et al.*, 1973). Deoxyribonuclease I (DNase I, RNase free) was from Worthington Biochemicals. Snake venom phosphodiesterase (Pharmacia) was prior to use deprived of its 5'-nucleotidase activity according to Sulkowski and Laskowski (1971). T4 polynucleotide kinase was from Amersham and proteinase K (Merck) was thermally pretreated before use (Maniatis *et al.*, 1982).

Buffers. TNE buffer: 0.1 mol/l NaCl, 0.01 mol/l Tris and 0.001 mol/l EDTA, pH 7.5. TNED buffer (Weber *et al.*, 1973): 0.15 mol/l NaCl, 0.01 mol/l Tris, 0.001 mol/l EDTA and 0.005 mol/l DTT, pH 8.0. TE buffer and STE buffer were both according to Maniatis *et al.* (1982). Digestion buffer (1×): 0.02 mol/l Tris, 0.02 mol/l $MgCl_2$ and 0.005 mol/l $CaCl_2$, pH 7.4. Other concentrations of buffers are appropriately indicated (1/2, 2×, 10×).

Tissue cultures and isotopes labelling. White Leghorn chickens with myeloblastic leukaemia induced by AMV (Říman, 1964) were a source of leukaemic myeloblasts cultivated *in vitro* (Říman and Beaudreau, 1970). Cells (5–8×10^7/ml) in 60–85 ml of medium were labelled with ^{3}H-mTdR (1.4 MBq/ml) or double-labelled with ^{3}H-mTdR (1.4 MBq/ml) and ^{14}C-UR (96.2 KBq/ml) for 7 hrs, and for additional 7 hrs after medium change in the presence of the same isotope(s). Pooled supernatants, purified twice by centrifugation at 5000 xg for 45 mins at 4 °C, were used as starting material for virus isolation and purification.

Virus isolation. Virus was isolated from pooled culture supernatants by centrifugation through discontinuous (50 % and 20 %) sucrose gradients in TNE buffer at 57 580 xg for 90 mins, and the virus collected from the interphase was purified by isopycnic (50–20 %) sucrose gradient centrifugation at 57 688 xg for 12 hrs. The material with a mean sucrose density of 1.16 g/cm^3 was collected and sedimented in TNE buffer at 57 688 xg for 60 mins. All high rate centrifugations were done at 4 °C with a Beckman centrifuge L8 80 or L2 65B.

Isolation of AMV core fraction. Core fraction was isolated from ^{3}H-mTdR-labelled or ^{3}H-mTdR-plus ^{14}C-UR-labelled purified virus according to Stromberg and Litvak (1973) with the following minor modification shortening the contact of virions with the detergent and standardizing the core recovery. Discontinuous gradients supplemented with TNED buffer consisted of 48 % sucrose (1 ml), 40 % sucrose (0.5 ml), 20 % sucrose with 5 % Sterox (0.25 ml), 1 ml of virus suspension in 10 % sucrose and 2 ml of TNED buffer. Upon electron-optic evaluation each core fraction isolated in this way showed the presence of undamaged core structures free of virus particles (Korb *et al.*, 1993).

Isolation of labelled AMV DNA. Virus core fraction was extracted for 30 mins at 0 °C in 0.1 ml of TNED buffer with 0.25 % Nonidet P-40 and 0.5 % Sarcosyl, and subjected to isopycnic centrifugation in CsCl gradients performed according to Brunck and Leick (1969) at 147 000 xg for 22 hrs at 22 °C. Fractions of the gradient of a density from 1.740 g/cm^3 to 1.695 g/cm^3 were pooled, diluted four times with 1/2 TNE buffer and precipitated with ethanol in the presence of 1/20 volume of 3 mol/l sodium acetate, pH 5.2, for 24 hrs at –20 °C. Precipitated AMV DNA was further purified by (a) proteinase K treatment in the presence of 0.5 % SDS and phenol-chloroform extraction, (b) phenol-chloroform extraction, and (c) rebanding in CsCl gradients. No differences in AMV DNA properties were found using these various purification procedures. The total amounts of core AMV DNA, isolated from virus produced by 5×10^9 cells at two 7-hrs intervals and expressed in cpm of ^{3}H-mTdR radioactivity, were about 1×10^5, representing 70 % and 50 % of the total ^{3}H-mTdR activity originally bound with the core fraction and virus, respectively.

Hydroxylapatite chromatography. Hydroxylapatite chromatography was performed in the presence of 50 % formamide at 26 °C in thermostated water-jacketed 5 ml columns according to Goodman *et al.* (1973). Pooled phosphate molarity fractions containing the single- and double-stranded portions of AMV DNA were intensively dialyzed against 1/2 STE buffer prior to DNA precipitation by ethanol or isopropanol.

Denaturation and RNase treatment of AMV DNA. Thermal denaturation of AMV DNA samples was done in 1/2 TE buffer for 2 mins at 90 °C. Glyoxylation was performed in 0.5 mol/l glyoxal at

60 °C for 20 min. Sedimentation of glyoxylated AMV DNA was done in sucrose gradients containing 0.1 mol/l glyoxal and 20 mmol/l potassium phosphate, pH 7.4, according to Brun and Weissbach (1978). RNase A (50 μg/ml) treatment was for 15 mins at 37 °C.

Phosphorylation of AMV DNA. Phosphorylation experiments were done on the single-stranded portion of double-labelled AMV DNA, which prior to separation by hydroxylapatite chromatography was once more purified by isopycnic CsCl gradient centrifugation and then isolated as described above.

Masking of the free 5'-terminal hydroxyl groups of AMV DNA by T4 polynucleotide kinase phosphorylation with non-labelled ATP, a preparation of "masked" AMV DNA samples, was done essentially according to Hirose *et al.* (1973). Sedimented portions of single-stranded AMV DNA with a radioactivity of 1720 cpm of ^{3}H-mTdR and 1560 cpm of ^{14}C-UR were denatured at 90 °C for 2 mins in a volume of 20 μl of 1/2 TE buffer. The T4 polynucleotide kinase reactions were performed after addition of corresponding reaction mixture components in a final volume of 50 μl according to Maniatis *et al.* (1982), with following modifications: non-labelled ATP was added at a final concentration of 10 μmol/l, the T4 polynucleotide kinase 20 U at the beginning and 10 U in the middle of the 60 mins incubation period. In this case the reactions were performed at 0 °C to eliminate the phosphate exchange accompanying at higher temperatures the T4 polynucleotide kinase reaction (Okazaki *et al.*, 1975). After the arrest of the enzymatic reaction (Maniatis *et al.*, 1982) and complementation of the reaction mixture volume to 200 μl with 1/2 TE buffer, the samples were extracted with phenol-chloroform and twice with chloroform. The supernatants containing extracted AMV DNA separated by centrifugation (Eppendorf, 5414 S) were divided into equal parts and precipitated in the presence of 1/20 volume of 3 mol/l sodium acetate (pH 5.2) with two volumes of isopropanol at -20 °C for 24 hrs (further isopropanol precipitation only). The sedimented precipitates represented the "masked" AMV DNA samples to be used for further treatment.

Adjustment of "masked" samples for phosphorylation with [gamma-^{32}P]ATP: sedimented precipitates of "masked" AMV DNA were dissolved in 50 μl of cold 0.3 N NaOH, immediately neutralized with 1 N HCl and precipitated with 2 volumes of isopropanol. On the other hand, sedimented precipitates of "masked" samples to be used for exposure of 5'-terminal hydroxyl groups of DNA at the RNA-DNA junction for phosphorylation with [gamma-^{32}P]ATP were dissolved in 50 μl of 0.3 N NaOH, incubated for 16 hrs at 37 °C, cooled, neutralized with 1 N HCl and precipitated with isopropanol.

Phosphorylation reactions by T4 polynucleotide kinase with [gamma-^{32}P]ATP of "masked" as well as of alkali hydrolyzed „masked" samples were run in duplicates. The end volumes of reaction mixtures were 25 μl at a concentration of 70 μCi of [gamma-^{32}P]ATP per reaction mixture. Other conditions were the same, as already described. In this case, to get rid of non-incorporated ^{32}P, the enzymatic reactions were terminated by addition of 7 volumes of cold 0.5 mol/l sodium phosphate, pH 6.8 and precipitated in the presence of 30 μg of yeast tRNA per sample by addition of a cold mixture consisting of equal parts of 100 % trichloroacetic acid (TCA), saturated sodium orthophosphate and saturated sodium pyrophosphate (Weber *et al.*, 1971). Precipitates were collected on Synpor No. 6 filters, washed ten times with 5 ml of cold 10 % TCA, dried and counted in a scintillation liquid.

Analysis of deoxyribonucleotide distribution at the RNA-DNA link in single-stranded AMV DNA. Portions of single-stranded AMV DNA with ^{3}H-mTdR radioactivity of 1760 cpm and ^{14}C-UR of 1280 cpm were "masked", alkali hydrolyzed and phosphorylated as described above. After termination of the reactions by addition of 2 μl of 0.5 mol/l EDTA, pH 8.0, the reaction mixture (25 μl) was supplemented with 10 μl of 10× STE buffer, 5 μl (10 μg) of denatured calf thymus DNA dissolved in 1/2 TE buffer and with water to a final volume of 100 μl and extracted by the phenol-chloroform procedure. The separated supernatants were precipitated with isopropanol. Sedimented precipitates were dissolved in 100 μl of STE buffer and the ^{32}P-labelled DNA was separated from the unreacted [gamma-^{32}P]ATP by spun-column chromatography through Sephadex G 50 (Maniatis *et al.*, 1981). The eluates were precipitated with isopropanol. The separated sediments, dried in vacuum and dissolved in 5 μl of water, were supplemented with 10 μl of the digestion buffer (2×) with DNase I (5 μg) and 5 μl (5 μg) of denatured calf thymus DNA dissolved in 1/2 TE buffer, and incubated for 60 mins at 37 °C. Thereafter 2 μl of 1 mol/l glycine-KOH, pH 9.0 and 4 μl (2.5 μg) of snake venom phosphodiesterase was added and incubated for 45 mins at 37 °C. The reactions run in duplicates,

were stopped by addition of 2 μl of 0.5 mol/l EDTA, pH 8.0 and incubated 2 mins in a boiling water bath. After addition of 2 μl of a water solution of unlabelled all four common deoxyribonucleotide monophosphates (2 μg each) as markers, the reaction mixture was loaded on a Whatman 3MM paper and electrophoresed in 0.05 mol/l dihydrogen citrate, pH 3.8 at 1000 V for 60 mins. The paper discs with distinctly separated marker spots, visualized by UV-light and cut out from the dried paper, were counted in a toluene-based scintillation liquid.

Assays of ^{3}H-mTdR and ^{14}C-UR radioactivity incorporated into AMV DNA were performed using conventional TCA precipitation technique and counting the acid insoluble radioactivity remaining on filters (Synpor No. 6, Lachema) in toluene scintillation liquid. All radioactivity measurements were done with a Beckman liquid scintillation spectrometer LS 6000 SE.

Results and Discussion

Behaviour of denaturated AMV DNA treated und untreated with RNase in isopycnic CsCl gradients

Our first attempts were directed to the elucidation of the CsCl buoyant density properties of AMV DNA which in this case are not influenced strictly by the (G+C) content, as was apparent from our earlier data (Dvořák and Říman, 1980). Similarly, it was unlikely that the buoyant densities of this DNA might be so much influenced by its single-stranded DNA component, since the conversion of double-stranded DNA into single strands elevates the CsCl density by 15 mg/cm^3 only (Smith and Vinograd, 1972). Therefore we turned our attention once more to the possibility that the CsCl buoyant density properties of AMV DNA might be due to small RNA pieces tightly bound to some of the AMV DNA molecules. This possibility was earlier indicated by the observation of Weber *et al.* (1973) that the treatment of MC29 DNA with RNase prior to centrifugation in CsCl gradients shifts the density of a part of this material to lower density regions. However, the core AMV DNA treated in this way did not exert reproducibly similar changes (Dvořák and Říman, 1980). From both these observations we have now concluded that in the case of our AMV DNA preparations the RNA pieces, if present, may not be amenable properly to enzymatic digestion and that the AMV DNA therefore requires an appropriate pretreatment. Thermal denaturation appeared to be the pretreatment of choice.

Results summarized in Fig. 1 show that the treatment of thermally denatured ^{3}H-mTdR-labelled AMV DNA with RNase prior to centrifugation in isopycnic CsCl gradients leads to pronounced changes in its buoyant density radioactivity profile (Fig. 1A). The appearance of new distinct radioactivity peaks in the low-density region suggests that part of AMV DNA in its native state is attached to RNase-sensitive components that influence its buoyant densities.

The prominent new radioactivity peaks, at CsCl densities of 1.690 g/cm^3 and 1.670 g/cm^3, indicate that in this DNA deprived of RNA, DNA species of a lower (G+C) content and, consequently, of a higher content of (A+T) of 69 % and 80 %, respectively, occurred.

Some radioactivity still remains after RNase treatment in the higher-density regions due to either a higher RNase resistance of a minor portion of AMV DNA or its higher (G+C) content. In contrast, the buoyant density radioactivity

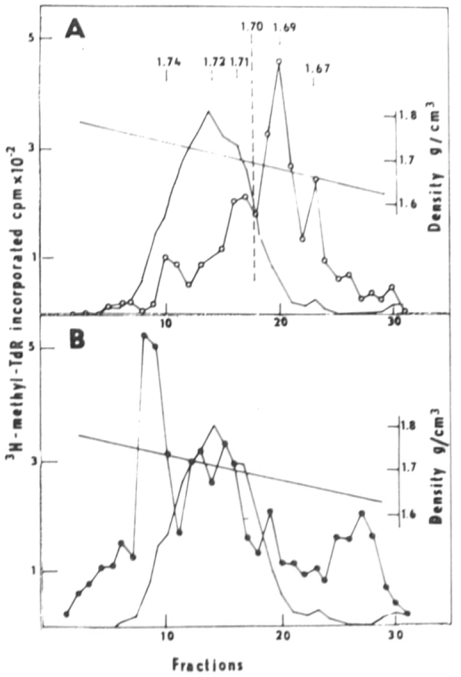

Fig. 1
CsCl buoyant density radioactivity profiles
A: Thermally denatured and RNase-treated AMV DNA (O——O) and native AMV DNA (———). Vertical line indicates density of nuclear myeloblastic DNA.
B: Thermally denatured native AMV DNA (●——●) and native AMV DNA (———).

profile of the denatured AMV DNA untreated with RNase (Fig. 1B) shows an opposite picture. Most of the radioactivity of this material is located in the higher-density region with its "core" bound to the density region of the native DNA and with a new prominent radioactivity peak at a density of 1.750 g/cm³, suggesting accumulation of a major part of RNA-containing DNA liberated from AMV DNA by denaturation. Similarly, the new radioactivity zones appearing in the low-density ragions may represent AMV DNA material more extensively fragmented by denaturation and so deprived of the attached RNA species. Thus, the behaviour of denaturated, RNase-untreated AMV DNA in isopycnic CsCl gradients supports additionally the conclusion that the anomalous CsCl buoyant density properties of native AMV DNA not corresponding to its actual base composition, are influenced mainly, besides other extraordinary properties of this DNA (Říman *et al.*, 1993; Korb *et al.*, 1993), by attachment of small pieces of RNA to this DNA. Such an interpretation of the results obtained here seems to be plausible, since the base composition values, like those tentatively depicted in Fig. 2, were revealed by evaluating the base composition of the individual cloned and sequenced AMV DNA molecules which were found to be prevalently AT rich (Říman *et al.*, 1993). In addition, the data on the base composition, determined according to Schildkraut *et al.* (1962) from CsCl buoyant density of

denatured, RNase-treated AMV DNA, corresponded in this case fairly well to those showing a mean (G+C) content of 29 % of native AMV DNA obtained previously (Dvořák and Říman, 1980) from the denaturation temperature according to Marmur and Doty (1962). However, some additional changes in buoyant densities based on base pair mismatching due to the presence of single-strands and AT-rich DNA molecules present in AMV DNA (Říman *et al.*, 1993) cannot be excluded (Paleček, 1991).

Sedimentation behaviour of native and after RNase treatment glyoxylated AMV DNA in velocity sucrose gradients

The demonstrated participation of RNase-sensitive components attached to AMV DNA in its CsCl buoyant density properties consequently needed to be complemented by an analysis of their possible influence on the sedimentation patterns of this DNA in velocity sucrose gradients. To get more standardized conditions for a final explanation of expected possible changes in sedimentation properties of AMV DNA treated with RNase, we denatured this material, native or enzymatically treated, with glyoxal prior to centrifugation. Results summarized in Fig. 3 show changes in sedimentation properties in velocity sucrose gradients of ^{3}H-mTdR-labelled native AMV DNA after various treatments. The radioactivity profile of native AMV DNA under the conditions used (Fig. 3A) shows a broader band with a peak at 7.5 S.

The glyoxylation of native AMV DNA, which converts the double strands to single strands and keeps them in extended configuration (McMaster and Carmichael, 1977), makes the radioactivity sedimentation profile more distinct, with appearance of inflections indicating the presence of molecular species of

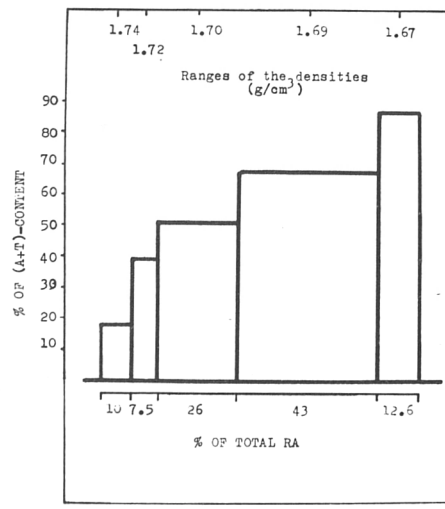

Fig. 2
Tentative (A+T) percent content of DNAs in 7S AMV DNA
Values estimated according to Schildkraut *et al.* (1962) from the percent ^{3}H-mTdR-radioactivity distribution in the respective CsCl density zones along the buoyant density profile of the thermally denatured and RNase-treated AMV DNA (Fig. 1A).

higher sedimentation rates when compared with the peak fraction which becomes sharper and is positioned at 6.5 S. The whole sedimentation profile of AMV DNA treated with RNase and thereafter glyoxylated shows a visible shift in velocity sucrose gradients to the lower sedimentation region with a peak fraction positioned at 5 S when compared with glyoxylated AMV DNA non-treated with RNase (Fig. 3A). In this case the inflections indicating the presence of molecules with higher sedimentation rates are even more prominent than in the case of glyoxylated native AMV DNA.

The sedimentation radioactivity profile of RNase-treated non-glyoxylated AMV DNA, when compared to that of non-glyoxylated native material, shows basically similar changes, i.e. a shift of radioactivity to the lower sedimentation rate region with the appearance of a distinct peak of radioactivity at 4 S (Fig. 3B). In contrast to the identically treated but glyoxylated AMV DNA, the remaining radioactivity spreads in this case also to the high sedimentation rate zones, probably due to an uncontrolled aggregation accompanying the sedimentation of RNase-treated, non-glyoxylated AMV DNA molecules.

The results obtained here clearly indicate that the RNase-sensitive components, apparently small in size and attached to the AMV DNA do contribute also to the properties of AMV DNA sedimentation patterns in its native state.

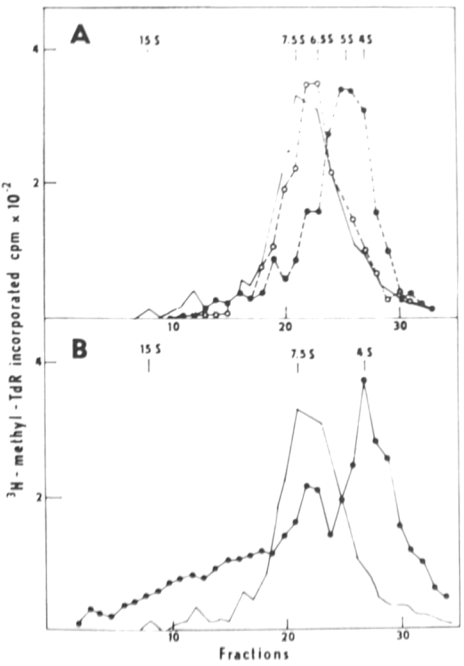

Fig. 3
Sucrose velocity gradient radioactivity profiles
A: Native AMV DNA (———), glyoxylated AMV DNA (O---O), and AMV DNA treated with RNase and thereafter glyoxylated (●---●).
B: Native AMV DNA (———) and RNase-treated AMV DNA (●———●). 50–20 % sucrose, 108 000 xg, SW 50.1, 14 hrs, 4 °C.

Sedimentation patterns of glyoxylated ^{3}H-thymidine- and ^{14}C-uridine-labelled AMV DNA in velocity sucrose gradients

To evaluate approximately the nature of attachment of RNA to DNA in AMV DNA, we characterized the radioactivity sedimentation patterns of glyoxylated AMV DNA, which was double-labelled for RNA and DNA detection, in velocity sucrose gradients. This was done because, in the case of glyoxylated RNA-DNA material, cosedimentation of RNA with DNA in velocity sucrose gradients was used as an indication of the covalent nature of attachment of RNA to DNA in the case of short DNA fragments appearing in the early phase of HeLa cell DNA synthesis *in vitro* (Brun and Weissbach, 1978).

Results obtained in this direction are shown in Fig. 4. From comparison of the CsCl buoyant density radioactivity profiles of ^{3}H-mTdR- and ^{14}C-UR-labelled AMV DNA it is evident that the distribution of RNA inside the total AMV DNA is not equal, exhibiting a greater or a lesser preference for certain buoyant density zones of AMV DNA (Fig. 4A). However, most of the ^{14}C-UR label is located in the higher-density region of this DNA.

AMV DNA isolated from pooled isopycnic gradient fractions, as indicated in Fig. 4A, was glyoxylated, and its radioactivity profiles obtained by velocity sucrose gradient centrifugation are shown in Fig. 4B. As evident in this case, both radioactivity profiles of glyoxylated double-labelled DNA reveal a cosedi-

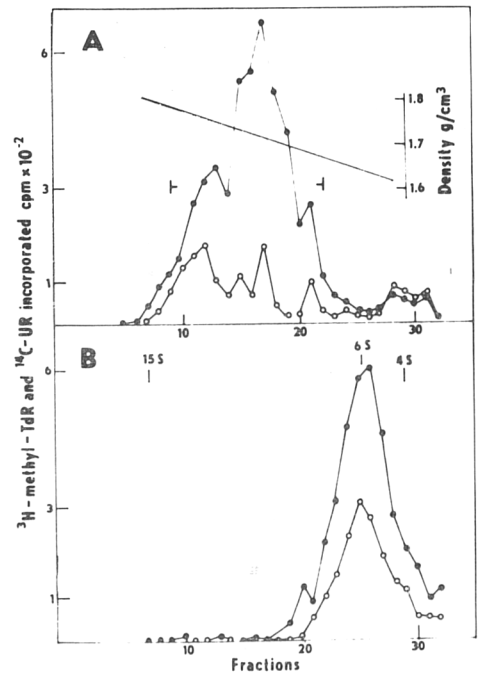

Fig. 4A
CsCl buoyant density radioactivity profiles AMV DNA double-labelled with ^{3}H-mTdR (●——●) and ^{14}C-UR (○——○), with indication (⊢ ⊣) of the pooling mode of fractions for isolation of AMV DNA to be used for glyoxylation and sucrose velocity gradient analysis.

Fig. 4B
Sucrose velocity gradient radioactivity profiles
Glyoxylated double-labelled AMV DNA. 50–20 % sucrose, 108 000 xg, SW 50.1, 14 hrs, 4 °C. See also the legend to Fig. 4A.

mentation pattern with a common peak of glyoxylated native AMV DNA (Brun and Weissbach, 1978; DePamphilis and Wassarman, 1980) at 6 S. This characterization indicates, as conceded, that the attachment of RNA to DNA in AMV DNA preparations may be of a covalent nature, as it is in the case of Okazaki fragments of replicative DNA structures of prokaryotes and eukaryotes (Ogawa and Okazaki, 1980).

Characteristics of RNA distribution in single-stranded and double-stranded components of AMV DNA

To answer the question which part of the secondary structure of AMV DNA, the single-stranded or the double-stranded, is more associated with RNA, we subjected the AMV DNA double-labelled with ^{3}H-mTdR and ^{14}C-UR to hydroxylapatite chromatography in the presence of formamide (Goodman *et al.*, 1973). Taking into account the unusual properties of this DNA, we checked, before using this methodical approach, in separate experiments on single- and double-strandedness, the AMV DNA material present in each of the eluted phosphate molarity fractions by S_1 nuclease treatment. We obtained very well reproducible identical elution patterns with AMV DNA isolated from three different AMV core samples (Fig. 5). Here, most (79 %) of the total ^{14}C-UR radioactivity is associated with the single-stranded DNA portion representing according to ^{3}H-mTdR radioactivity distribution 21 % of the total AMV DNA eluting from 0.10 to 0.18 phosphate molarity. However, a minor part (21 %) of ^{14}C-UR radioactivity is present also in all fractions of the double-stranded portion of AMV DNA which elutes in this case, due to the small size of its molecules ranging from 20 to 900 bp only (Říman *et al.*, 1993), from 0.22 to 0.30 phosphate molarity, in contrast to native *E. coli* DNA eluting not before 0.30 phosphate molarity (Goodman *et al.*, 1973). A sharp decline in ^{14}C-UR radioactivity at the elution boundary between single- and double-stranded AMV DNA

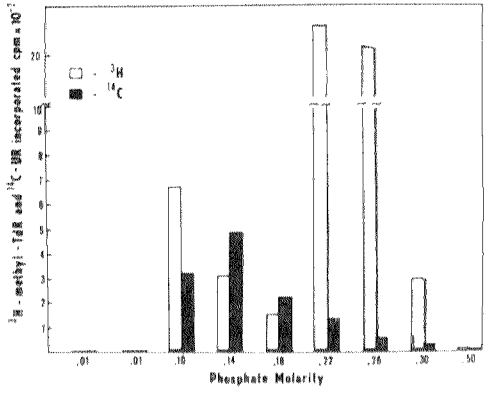

Fig. 5
Hydroxylapatite column chromatography of AMV DNA double-labelled with ^{3}H-mTdR and ^{14}C-UR
Phosphate molarity elution profiles in the presence of formamide at 26 °C.

molecules and a gradual decline in this radioactivity following elution conditions of longer AMV DNA molecules reminds the distribution of Okazaki fragments in replicative DNA structures of various length or stages of replication found by Eliasson and Reichard (1978) and others (for review see DePamphilis and Wassarman (1980)).

Direct evidence for the presence of Okazaki fragments in AMV DNA

The covalent nature of the attachment of RNA to DNA is the basic feature of Okazaki fragments. To analyze the nature of attachment of RNA to DNA in the putative Okazaki fragments present in AMV DNA, we used as starting material single-stranded ^{3}H-mTdR- and ^{14}C-UR-labelled AMV DNA characterized and separated by hydroxylapatite chromatography in the presence of formamide. To detect the covalent RNA-DNA links in AMV DNA, we used the approaches previously applied in defining Okazaki fragments isolated from prokaryotic (Hirose *et al.*, 1973) and eukaryotic (Pigiet *et al.*, 1974; Kaufmann *et al.*, 1977) DNA replicative systems. The principle of a direct detection of the covalent nature of RNA-DNA links resides in the ability of 5'-terminal hydroxyl groups of the DNA portion of RNA-DNA molecules exposed after alkaline hydrolysis to be phosphorylated with ATP by T4 polynucleotide phosphorylase.

In the case of single-stranded AMV DNA (Table 1), when such material, appropriately pretreated with an aim to mask its naturally occurring 5'-terminal hydroxyl ends, was alkali treated, it revealed a new ability to be strongly (up to 15–17 times more than the relevant control) labelled with [gamma-^{32}P]ATP by T4 polynucleotide phosphorylase. Phosphorylation of the liberated 5'-OH ends of AMV DNA under the conditions used (Okazaki *et al.*, 1975) represented a real [gamma-^{32}P]ATP transfer and not an exchange reaction.

Table 1. Phosphorylation of single-stranded RNA-DNA molecules of core-bound AMV DNA by T4 polynucleotide kinase. Evidence of covalent attachment of RNA to 5' end of DNA

Exp. No.		Sample treatment	^{32}P cpm		Sample treatment	^{32}P cpm
I	(1)	Phosphorylation with unlabelled ATP.	429	(1)	Phosphorylation with unlabelled ATP.	7061
				(2)	Alkaline treatment.	
II	(2)	Phosphorylation with [gamma-^{32}P]ATP.	459	(3)	Phosphorylation with [gamma-^{32}P]ATP.	7219

Each experiment was run in duplicate with samples having each 430 cpm of ^{3}H-mTdR- and 390 cpm of ^{14}C-UR-radioactivity.

Thus, the results obtained here provide the first direct evidence that the RNA-DNA link in single-stranded AMV DNA is of a covalent nature as in the case of Okazaki fragments.

The second important characteristics of Okazaki fragments in both prokaryotes (Ogawa and Okazaki, 1980) and eukaryotes (DePamphilis and Wassarman, 1980) is the representation of the nucleotides at the RNA-DNA junction. The discontinuous DNA synthesis of eukaryotes and their DNA viruses that were intensively studied especially in the last decade (De Pamphilis and Wassarman, 1980; Thömmes and Hübscher, 1990; Linn, 1991), in general, do not seem to be much affected at the RNA-DNA junction of Okazaki fragments or their precursors, the so-called primer DNAs (Nethanel *et al.*, 1988; Nethanel and Kaufmann, 1990; Bullock *et al.*, 1991), by sequence specificity, since all four common ribo or deoxyribonucleotides were found to be present at the RNA-DNA link in replicating DNAs of all so far studied systems. While the *in vitro* studied DNA replicating systems allow us to define at the RNA-DNA link the representation of nucleotides of both types, the *in vivo* replicating DNAs, as is the case of AMV DNA, allow us to determine at the RNA-DNA junction the representation of deoxyribonucleotides only. Consequently, to contribute to this characterization of Okazaki fragments present in AMV, we analyzed the deoxyribonucleotide representation at the RNA-.DNA link of AMV DNA molecules represented by the single-stranded portion of AMV DNA double-labelled with ^{3}H-mTdR and ^{14}C-UR. In this analysis we applied the methodical principles previously used in analyzing the same properties of Okazaki fragments isolated from prokaryotes (Hirose *et al.*, 1973) and eukaryotes (Pigiet *et al.*, 1974; Kaufmann *et al.*, 1977).

Results that are summarized in Table 2 show that, according to the percent radioactivity distribution of the 5'-terminally ^{32}P-labelled deoxyribonucleotides, the deoxyribonucleotide nature of the RNA-DNA link in RNA-DNA molecules of AMV DNA corresponds, in principle, to properties of eukaryotic Okazaki fragments because all four common deoxyribonucleotides are represented here at the RNA-DNA junction. However, the higher dAMP and dTMP representation found at the 5' ends of DNA of single-stranded RNA-DNA molecules of AMV DNA may suggest that the switch of RNA to DNA synthesis does not appear to be a completely random process, as supposed in the case of an analogous deoxynucleotide representation observed at the RNA-DNA junction of single-stranded polyoma replicative structures (Pigiet *et al.*, 1974). But, such a deoxyribonucleotide representation at the RNA-DNA link of AMV DNA molecules may reflect simply an AT enrichment of these molecules, as indicated in this paper and, consequently, an increase of probability of switches of RNA to DNA synthesis at these deoxyribonucleotides. In conclusion, both here presented basic properties of the nature of RNA-DNA attachment in single-stranded AMV DNA molecules provide the first evidence for the presence of Okazaki fragments in AMV DNA, as well as the evidence for their main responsibility for the anomalous CsCl buoyant density properties of AMV DNA.

Table 2. Phosphorylation of single-stranded RNA-DNA molecules of core-bound AMV DNA by T4 polynucleotide kinase. Percentage of deoxyribonucleotide distribution at the RNA-DNA junction

Exp. No.		Sample treatment	% dCMP	% dAMP	% dGMP	% dTMP	100 % in ^{32}P cpm
I	(1) (2)	Phosphorylation with unlabelled ATP. Alkaline treatment.	11.00	37.08	14.08	37.07	1272
____	(3)	Phosphorylation with [gamma- ^{32}P]ATP.	_____	_____	_____	_____	_____
II	(4)	Digestion: DNase I and phosphodiesterase.	10.45	41.61	13.42	34.45	1061

See the legend to Table 1.

In addition, the presence of Okazaki fragments in AMV DNA is also responsible for small sedimentation shifts toward lower S values in the range of 1.0 to 1.5 S, observed in the sedimentation pattern of glyoxylated AMV DNA digested prior to glyoxylation with RNase A, indicating that RNA attached to AMV DNA degraded with RNase A is small in size, as observed in similarly analyzed other eukaryotic replicative DNA structures (Brun and Weissbach, 1978).

To estimate tentatively this size, we used the principles of Eliasson *et al.* (1974) based on estimation of the length of RNA liberated from AMV DNA by DNase I digestion. We found (data not shown) in pilot experiments that single-stranded AMV DNA double-labelled with ^{3}H-mTdR and ^{14}C-UR digested by DNase I exhibits in 12 % PAG-urea electrophoresis the appearance of ^{14}C-UR radioactivity located at a short distance behind the internal marker of Bromophenol Blue and between the positions of synthetic deoxynucleotides, 8 and 14 bases in size, run in parallel and visualized by silver staining, as described elsewhere (Říman *et al.*, 1993). Thus, the tentatively estimated size 8–14 bases of RNA pieces covalently bound to AMV DNA corresponds to the size of 8–12 (mean 10) bases given for the finished initiator RNA (Reichard *et al.*, 1974; Tseng and Goulian, 1977), or the primer RNA (Thömmes and Hübscher, 1990) of the eukaryotic Okazaki fragments or their, so-called, primer DNA precursors (Nethanel *et al.*, 1988; Nethanel and Kaufmann, 1990; Bullock *et al.*, 1991). The findings presented in this paper show that the Okazaki fragments represent a constant component of the 7 S AMV DNA. This clearly indicates that this DNA is not a random fragmented host DNA but represents its special fragments that have properties of replicative DNA structures segregating from the host cell into virus core nucleoprotein complex. Consequently, this raises once again the question of possible involvement of this DNA in some retroviral functions.

Dedication. The first author dedicates this work in memory of the Czechoslovak Academy of Sciences based on J. E. Purkyne's ideas.

Acknowledgements. We are indebted for special instrumental and material help to Dr. K. Horská and Dr. V. Hořejší, respectively.

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