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AVIAN MYELOBLASTOSIS VIRUS CORE-BOUND 7 S DNA, A COLLECTION OF MINUTE REPLICATIVE HOST-CELL DNA STRUCTURES

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Summary. – The early replicative nature of avian myeloblastosis virus core-bound 7 S DNA (AMV DNA), indicated by our preceding findings (Říman *et al.*, 1993), has been confirmed using various experimental approaches. It has been shown by agarose and polyacrylamide gel electrophoresis that this DNA represents actually a collection of molecules the size of which is strongly reminiscent of the minute early replicative structures found in DNA of sea urchin embryos (Baldari *et al.*, 1978). With such a characteristic correspond, the sequence properties of the individual AMV DNA clones, the majority of which were found to be AT-rich with ARS-like motifs and stretches of A-residues carrying conformational requirements for bending. In comparative hybridization experiments, AMV DNA exhibited the highest homology with chicken leukaemic myeloblast scaffold-bound DNA. Compatible with high replicative activity of AMV DNA was also found its specific [methyl-^{3}H]thymidine radioactivity. The constancy of the virus content of this DNA and its virus age-dependent cleavage changes taking place inside the virus core structure open the question of possible significance of this special host DNA for the reaction machinery represented by the retroviral nucleoprotein core complex.

Key words: AMV core-bound DNA; minute replicative DNA structures; host nucleic acids; ARS-like sequences; DNA bending

Introduction

In our preceding paper (Říman *et al.*, 1993) we have demonstrated that the host cell 7 S DNA occurring in virions of AMV (Říman and Beaudreau, 1970) and associated with the virus core fraction (Deeney *et al.*, 1976; Dvořák and Říman, 1980*a*; Říman *et al.* 1993) contains Okazaki fragments present prevalently in its single-stranded portion. Simultaneously we have shown that a major part of this DNA is represented by AT-rich molecules. Accordingly these findings prompted us to characterize a set of other relevant properties of this DNA. Here we show that the samples of isolated virus core-bound AMV DNA

represent collection of DNA molecules that have properties of minute sequentionally heterogeneous replicative structures corresponding in size to basic replication units that descend from the regions surrounding the origins of cell DNA replication. Demonstrating a constancy of virus content of this DNA and its virus age-dependent cleavage changes that take place inside the virus core structure *in vivo*, we present the first indications of its possible functional significance for the multiplication cycle of at least AMV, since this kind of DNA has been found in all avian and mammalian retroviruses so far studied (Říman *et al.*, 1993). Finally, we open here also the question of the functional meaning of this special cell DNA for the life cycle of the proliferating cell alone, considering that such a closely similar DNA present in the cytoplasmic postmicrosomal fraction of chicken leukaemic myeloblast as well as of chicken embryo virus-free fibroblasts (Dvořák and Říman, 1980*b*) seems to be a common property of proliferating vertebrate cells.

Materials and Methods

Chemicals. Acrylamide (N', N'-methylenetriacetylamide), TEMED (N, N, N', N'-tetramethylene diamide), ammonium persulphate, phenylmethylsulphonyl fluoride (PMSF), Bromophenol blue and xylene cyanol FF were from Serva, and lithium 3', 5'-diiodosalicylate was from ICN. All other chemicals used were of the highest analytic purity.

Biomaterials. AMV DNA samples labelled either with ^{3}H-mTdR or double-labelled with ^{3}H-mTdR and ^{14}C-UR were prepared from the core fraction of virions up to 7 hrs old produced *in vitro*. Virus production, radioisotopes, isotopic labelling and isolation of AMV was described in detail in our preceding paper (Říman *et al.*, 1993). T4 DNA polymerase, T4 RNA ligase, T4 DNA ligase, Klenow fragment of DNA polymerase I and endonuclease *Eco*RI, were from Amersham, and proteinase K was from Merck. Synthetic oligodeoxyribonucleotides and *Alu*I pBR322 fragment markers were kindly provided by Dr. V. Vlček and Dr. L. Arnold, respectively, from this Institute.

Gel electrophoresis. In polyacrylamide (8 %) gels (PAG) in TBE buffer (Maniatis *et al.*, 1982) were analyzed specimens either of native, ^{3}H-mTdR- and ^{14}C-UR-double-labelled AMV DNA or its separated single- and double-stranded (ss, ds) portions obtained as described previously (Říman *et al.*, 1993), denatured prior to the analysis in 90 % formamide in TBE buffer for 2 mins at 100 °C. Applied amounts of native AMV DNA were about 9000 cpm of ^{3}H and 4600 cpm of ^{14}C radioactivity, respectively, per slot. Migration positions of native AMV DNAs were estimated by comparison with positions of *Alu*I pBR322 marker fragments run in parallel and visualized by silver staining (Blum *et al.*, 1987) of the lengthwise cut out gel strips. In the same way estimated positions of AMV ssDNA and dsDNA analyzed in PAG (8 %) under denaturing (7 M urea) conditions by means of synthetic deoxyribonucleotide markers (40, 14 and 8 bases in size). In this type of PAG analysis, Bromophenol blue (BPB) and xylene cyanol (XC) always served as internal markers (Maniatis *et al.*, 1982). Electrophoresis was run for 200 mins at 12.7 V/cm at room temperature in the case of native AMV DNA and for 90 mins at 36 V/cm at 4 °C in the case of the PAG run under denaturing conditions. For radioactivity determination, the gel strips were cut into slices (3 mm) which were treated and counted according to Eliasson *et al.* (1974). DNA samples analyzed by electrophoresis in NuSieve agarose (4 %) in TEB buffer with inclusion of ethidium bromide (Maniatis *et al.*, 1982) were represented by ^{3}H-mTdR-labelled AMV DNA (about 2500 cpm per slot) isolated as described previously (Říman *et al.*, 1993). As markers served *Alu*I pBR322 fragments run in parallel, detected by fluorescence in UV-light. DNA samples were electrophoresed for 60 mins at 15 V/cm at room temperature. The gel slices (2 mm) solublized at 65 °C were extracted with a phenol-chloroform mixture. The water phase of the individual extracted slices separated by centrifugation was used for determination of radioactivity in TCA precipitates collected on Synpor 6 filters as described

previously (Říman *et al.*, 1993). A Beckman LS 6000 SE liquid scintillation spectrometer was used.

Cloning and sequencing of AMV DNA. AMV DNA for cloning purposes was represented by total AMV DNA (CsCl buoyant densities from 1.740 to 1.700 g/cm^3) isolated as described previously (Říman *et al.*, 1993). This DNA (0.5 to 1.5 μg) was blunt-ended with T4 DNA polymerase (Maniatis *et al.*, 1982) and ligated overnight at 12 °C in 10 μl of reaction mixture with 100 ng of *Sma*I-digested pUC19 DNA (Perbal, 1988) in the presence of both T4 DNA (6 U) and T4 RNA (2 U) ligases (Maniatis *et al.*, 1982). Ligated DNA was extracted with a phenol-chloroform mixture, precipitated with ethanol and used for transformation of competent DH5 alpha F'cells (Woodcock *et al.*, 1989). Ampicillin-resistant colonies carrying vectors unable to complement the defective beta-galactosidase were isolated. Recombinant plasmid DNAs from 18 colonies were purified and sequenced by the dideoxy-mediated chain-termination method (Sanger *et al.*, 1977). Fluorescent primers, synthesis kit reagents and an A. L. F. Sequencer™ (Pharmacia LKB) were used.

Mathematical evaluation. The matrix analysis (Pustell and Kafatos, 1984) run on a Vax 780 computer was used to search for homology between nucleotide sequences of AMV DNA clones and ARS (Umek *et al.*, 1989), and topoisomerase (Topo) II consensuses (Sander and Hsieh, 1985), and ARS-carrying sequences of DNAs from the EMBL database (release 3, 1991).

Isolation of nuclear scaffold and scaffold-bound DNA. Starting material for scaffold isolation were pellets (about 150 to 300 mg w.w.) of chicken leukaemic myeloblasts cultivated as described previously (Říman and Beaudreau, 1970). Both preparations of nuclear scaffold were done according to Mirkowitch *et al.* (1984). According to the electrophoretic patterns in 10 % PAG-SDS elctrophoresis (Lebkowski and Laemmli, 1982) (data not shown), both scaffold preparations were similar to the type 1 of the non-histone nuclear structures composed, besides the three lamina proteins, of numerous high-molecular-mass species (Lebkowski and Laemmli, 1982).

Scaffold-bound DNA was isolated according to Mirkowitch *et al.* (1984) from scaffold samples digested with endonuclease *Eco*RI (1000 U/ml, 3 hrs at 37 °C) and deprived of solubilized DNA by sedimentation. *Eco*RI-treated, sedimented scaffold material, digested with proteinase K (100 μg/ml, 3 hrs at 55 °C) in the presence of SDS (0.5 %), was used for conventional extraction and isolation of scaffold-bound DNA. The amounts of 35 and 70 μg of this DNA recovered from 150 and 300 mg w.w. of chicken leukaemic myeloblast pellets, respectively, represented about 1 % of the total cell DNA in this case.

Reassociation and hybridization of AMV DNA. Phenol emulsion reassociation technique (PERT) of Kohne *et al.* (1977) is the only method so far known that permits reassociation of AMV DNA (Dvořák and Říman, 1980*a*). To enhance similarly the association of complementary sequences between AMV DNA and the considered DNA species, we used this technique for detection of the putative homology between AMV DNA and the myeloblastic scaffold DNA and, by comparison, the total DNA of chicken leukaemic myeloblasts and calf DNA. Individual experiments were done with samples containing in 50 μl of a mixture of Na_2HPO_4-NaH_2PO_4 (0.48 mol/l, pH 6.8) supplemented with phenol (9 % v/v) the respective DNA samples. The ^{3}H-mTdR-labelled AMV DNA probes (3550 cpm of each per sample) were represented in this case by the ds-portion of the AMV DNA (Říman *et al.*, 1993). The DNAs examined for homology with AMV DNA were added at 10 μg per sample. Reactions were run in Eppendorf tubes. Denaturation of DNAs was accomplished directly in the reaction mixture at 100 °C for 2 mins. After quick chilling, the tubes containing individual samples were fixed on a styrofoam platform and shaken for 22 hrs at room temperature at the maximum speed with a Tettlock shaker. Afterwards, samples diluted with 4 ml of 0.01 mol/l sodium phosphate, pH 6.8 solution containing formamide (50 % v/v) were subjected to hydroxylapatite column chromatography followed by determination of ^{3}H radioactivity in each of the phosphate molarity elution fractions accomplished as described previously (Říman *et al.*, 1993). The principles used in the evaluation of the results obtained are given in the legend to Fig. 7.

Protein, RNA and DNA determination in AMV. Starting material were pellets (150 to 180 mg w.w.) of highly purified AMV (sucrose buoyant density of 1.16 g/cm^3) (Říman *et al.*, 1993) derived from pools (180 to 200 ml) of blood plasma of chickens with virus-induced avian myeloblastosis (Říman, 1965). Pellets delipidated with ethanol (70 %), ethanol-ether (3:1) and ether were hydrolyzed with 0.1 N NaOH at 60 °C for 4 hrs in Eppendorf tubes. Aliquots from this step were used for protein determination (Lowry *et al.*, 1951). Alkali hydrolysates neutralized and supplemented with $HClO_4$

to a final concentration of 0.5 mol/l $HClO_4$ were left to precipitate for 18 hrs at 4 °C. Supernatants separated by centrifugation (30 mins, Eppendorf 5445) were used for determination of RNA spectrophotometrically as well as by the modified orcinol reaction according to Ogur and Rosen (1950). Separated sediments were used for determination of DNA according to Burton (1968).

Results and Discussion

Electrophoretic pattern of AMV DNA in polyacrylamide and agarose gels

To characterize the apparent molecular polydispersity (Říman *et al.*, 1993) of the core-bound 7 S AMV DNA, we analyzed this DNA double-labelled with ^{3}H-mTdR and ^{14}C-UR by electrophoresis in 8 % PAG. Not taking into account the presence of single strands (Říman *et al.*, 1993), this DNA migrates according to the ^{3}H-mTdR radioactivity profile of its electrophoretogram (Fig. 1) like dsDNA molecules of the size ranging from 20 to about 900 bp, with the main portion (about 60 % of the total ^{3}H-mTdR radioactivity) at 220 bp. The distribution of

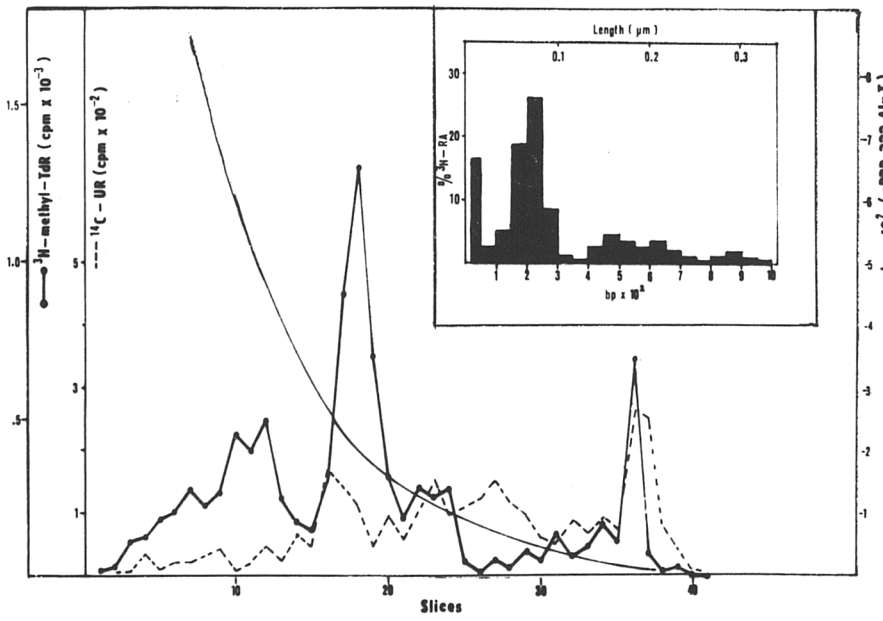

Fig. 1

Electrophoretic mobility patterns of native AMV DNA double-labelled with ^{3}H-mTdR and ^{14}C-UR analyzed in 8 % PAG

The insert shows a histogram of the size (bp) and length distribution of the molecules making up the 7 S AMV DNA. Size of *Alu*I pBR322 marker fragments in bp: 910, 657 (2 ×), 521, 403, 281, 257, 226, 100, 97, 63, 57, 49, 46, 19, 15 and 11.

the ^{14}C-UR radioactivity indicates variations in the amount of RNA attached to DNA in various DNA size classes. However, basically the major portion of ^{14}C-UR label is associated with DNA molecules of smaller size in the range from 100 to 20 bp. An evaluation of percent distribution of ^{3}H-mTdR radioactivity in the individual 50 bp zones of the electrophoretogram of this AMV DNA led to a histogram of base pair distribution of molecules that make up this DNA. Assuming that 1 μm of DNA corresponds to 3 kb (Baldari *et al.*, 1976), it is possible to assess simultaneously the length distribution of individual size classes present in this 7 S AMV DNA isolate. As shown in the insert in Fig. 1, the size of molecules ranges from 20 to 950 bp. However, the main portion (57.9 % of the total radioactivity) is represented by molecules 100–300 bp in size (200–250 bp in the uppermost column) with the corresponding calculated length ranging from 0.03 to 0.10 μm (0.06 to 0.08 μm in the uppermost column), respectively. This pattern is strongly reminiscent of the histograms of length- and consequently base pair size-distribution of electron microscopically evaluated minute replicative DNA structures, so-called "microbubbles" or „small eyes" that were found in allover replicating DNA of sea urchin embryos in the third S phase after fertilization (Baldari *et al.*, 1978).

In this case, as well as in ours, the fact that such DNA structures can be in part single-stranded was not taken into consideration, since the presence of single strands ubiquitously present in various DNA size classes of AMV DNA, as will be shown later, cannot profoundly change the basic character of the histogram pattern obtained here. This assumption was also confirmed by electron microscopic analysis (Korb *et al.*, 1993). As shown in Fig. 1, a minor portion of AMV DNA (about 20 % of the total ^{3}H-mTdR radioactivity) is represented by DNA molecules of higher size classes which, according to their sizes of 470, 610 and 875 bp in their peak fractions, are reminiscent of roughly multiples of two, three and four, respectively, of the 220 bp size found in the peak fraction of the main molecular size class portion.

Since a major part of AMV DNA appeared to be AT-rich (Říman *et al.*, 1993) and since elctrophoretic mobilities of small-size DNA pieces of a lower (G+C) content may exhibit in PAG (Allett *et al.*, 1974), in contrast to agarose gels (Perbal, 1988), unusual mobility properties, we characterized comparatively the electrophoretic patterns of AMV DNA in 4 % NuSieve agarose gel. In this case we analyzed a core 7 S AMV DNA preparation labelled with ^{3}H-mTdR only, which has been already characterized previously (Říman *et al.*, 1993) in isopycnic CsCl and sucrose velocity gradients.

As shown in Fig. 2, the radioactivity profile of the electrophoretogram of this AMV DNA analyzed in agarose gels reveals an analogous pattern with 5 distinct DNA size classes ranging in this case from 20 to about 700 bp, with a main portion (over 60 % of the total radioactivity) from 190 to 150 bp (mean 180 bp). Similarly, in this case of AMV DNA analysis a smaller portion (about 20 % of the total radioactivity) is represented by DNAs of higher size classes, which with the sizes of 320 and 520 bp at the peak positions of their components are reminiscent

again of roughly multiples, here of two and three, respectively, of the mean value of 180 bp of the main size class AMV DNA portion. Similarly, an evalution of the radioactivity distribution in the individual 50 bp zones along the electrophoretogram of this AMV DNA led, as shown in the insert in Fig. 2, to a histogram of the length- and base pair size-distribution of the DNA size classes that is closely similar to the one obtained by analysis of another AMV DNA sample in PAG (Fig. 1). In this case again the main portion (63.8 % of the total radioactivity) was represented by molecules 100–300 bp in size (150–200 bp in the uppermost column) with the corresponding calculated length ranging from 0.03 to 0.10 (0.05 to 0.065 μm in the uppermost column), respectively. Thus, on the basis of comparison of electrophoretic patterns in PAG and agarose gels, we can conclude that the 7 S DNA preparations of core-bound AMV DNA represent collections of host DNA molecules which, according to their base pair size- and length-distribution, are strikingly similar to minute replicative DNA structures visualized in replicating DNAs of various eukaryotes (Baldari *et al.*, 1976; Zakian, 1976; Burks and Stambrook, 1978). The assumption of the replicative nature of AMV DNA is supported here by direct evidence of the presence of

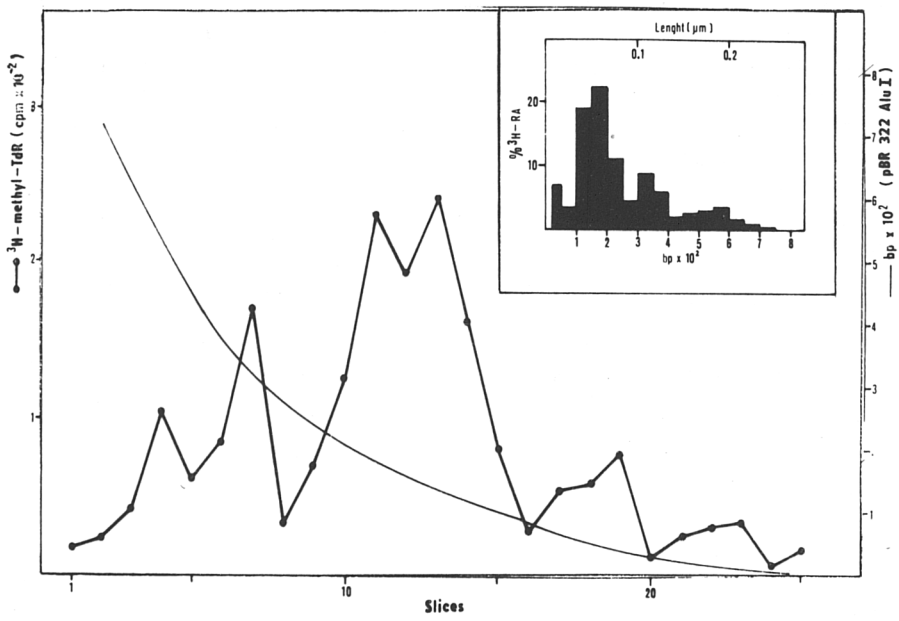

Fig. 2
Electrophoretic mobility patterns of native AMV DNA labelled with ^{3}H-mTdR, analyzed in 4 % NuSieve agarose gel
The insert shows a histogram of the size (bp) and length distribution of the molecules making up the 7 S AMV DNA. *Alu*I pBR322 marker fragments as in Fig. 1.

Okazaki fragments in this DNA (Říman *et al.*, 1993), as well as by other data to be presented further in this paper.

The basic mobility differences found by comparing both types of gel electrophoreses are just evident from comparison of migration curves of *Alu*I pBR322 marker fragments in PAG (Fig. 1) and in agarose (Fig. 2) gels. A little more rapid migration of the main as well as of the higher size class AMV DNA molecules in agarose gels reflects in this case, besides the above mentioned basic differences of electrophoretic mobilities in both types of gels, small differences in basic physicochemical properties of both analyzed AMV DNA samples obtained from different AMV-core preparations. Such differences may reflect especially in this case the minute differences in the core-bound DNA cleavage activities (the existence of which we shall demonstrate later) between the individual pools of AMV up to 7 hrs old.

Electrophoretic pattern of denatured AMV DNA

To evaluate the size of AMV ssDNA and dsDNA molecules and the RNA distribution among them, we analyzed the ss- and ds-portion of AMV DNA separated previously by hydroxylapatite column chromatography (Říman *et al.*, 1993) by PAG electrophoresis under denaturing conditions. The starting material was the same ^{3}H-mTdR- and ^{14}C-UR-labelled AMV DNA, the migration properties of which we defined above by electrophoresis in 8 % PAG (Fig. 1).

As shown in Fig. 3A, the denatured single-stranded portion of this AMV DNA separated in 8 % PAG in the presence of 7 M urea into four main components that migrate like DNA molecules consisting of about 100, 40–30, and 15–10 bases. According to the ^{3}H-mTdR-radioactivity distribution, the main portion (61 % of the total radioactivity) is represented by ssDNAs of smaller size. A non-negligible part of the AMV ssDNA portion (29.8 % of the total radioactivity) is represented by molecules of about 100 and even more (120–150) bases in length. This suggests that the DNA single-strands present in AMV DNA belong to the smaller as well as to the main size class AMV DNA molecules. The ^{14}C-UR label, representing in the ss-portion 80 % of the total ^{14}C-UR radioactivity of the unfractionated AMV DNA (Říman *et al.*, 1993), was associated prevalently with single strands of smaller size. However, the ^{14}C-UR label is present, even though in smaller proportions, also in DNA single strands 100 and more bases long. This indicates that the ssRNA-DNA molecules participating in AMV DNA are present in all AMV DNA size classes up to the main size class. On the basis of our previous data (Říman *et al.*, 1993), they are most probably represented in the case of single strands 100 and more bases long by Okazaki fragments and in the case of molecules 40–30 bases long by Okazaki fragment precursors, the so-called DNA primers. The occurrence of the latter in replicating eukaryotic DNAs has been recently demonstrated both *in vivo* (Nethanel *et al.*, 1989; Nethanel and Kaufmann, 1990), and *in vitro* (Bullock *et al.*, 1991). The presence of distinctly labelled molecules smallest in size (15–10 bases) may reflect in this case, like in other isolated replicative eukaryotic DNAs, an artificial or a natural

elimination of the initiator RNA from RNA-DNA molecules of various sizes (DePamphilis and Wassarman, 1980; Nethanel *et al.*, 1989).

In contrast, the dsAMV DNA portion (Fig. 3B) converted by denaturation to single strands and analyzed by PAG electrophoresis under denaturing conditions, consists mainly of molecules of about 100 and more (150–190) bases in size, representing 60.1 % and 12.9 % respectively, of the total ^{3}H-mTdR radioactivity of this AMV DNA portion and therefore belonging to the main size class of AMV DNA molecules. Besides these components, a minor part is represented by DNAs of about 80–20 bases in size, confirming the presence of shorter dsDNA molecules of this size found in native AMV DNA (Fig. 1). The ^{14}C-UR label, which is associated with the ds-portion and represents about 20 % of the total ^{14}C-UR radioactivity of the unfractionated AMV DNA (Říman *et al.*, 1993), is present in small amount in all ssDNA size classes produced by denaturation of the AMV dsDNA portion. However, an apparent prevalence of this label, when related to the relevant amount of ^{3}H-mTdR label, is associated with smaller molecules about 80–20 bases in size. Thus, these findings are again compatible with the expected distribution of Okazaki fragments (DePamphilis and Wassarman, 1980) and their precusors (Nethanel *et al.*, 1989) in eukaryotic replicating dsDNA structures. In addition, the characterization of AMV DNA obtained in this way helped to explain the occurrence and origin of DNAs of higher size classes which we have found to be constantly present in minor proportions in samples of AMV DNA preparations. In no instance did we find under denaturing conditions the AMV ssDNA molecules migrating like molecules of the

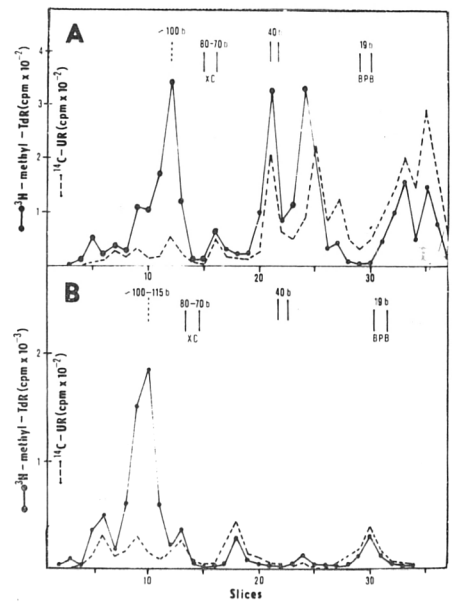

Fig. 3
Electrophoretic mobility patterns of ss-portion (A) and ds-portion (B) of AMV DNA double-labelled with ^{3}H-mTdR and ^{14}C-UR analyzed in 8 % PAG under denaturing conditions
XC and BPB, positions of the internal markers. Position of 40 b derived as described in Materials and Methods.

Table 1. Content of total AMV RNA and AMV DNA expressed per mg of protein, i. e. per 2.15×10^{12} virus particles

Total RNA μg/mg of protein	AMV DNA μg/mg of protein	AMV DNA % of total NA	No. of AMV DNA 220 bp-molecules per 2.15×10^{12} virus particles
51.99 ± 1.60	0.52 ± 0.05	0.99	2.17×10^{12}

The mean values of RNA and DNA in AMV with standard deviations are derived from four separate duplicite determinations of protein, RNA and DNA in samples (15 to 20 mg of protein) of the highly purified AMV from blood plasma of leukaemic chickens.

length expected after denaturation of DNAs of higher size classes, i.e. those of about 300–400, 500–600 and 700–800 bp in size. The maximal length of denatured AMV DNA molecules was always about 150–190 bases corresponding approximately to the length of the main size class portion of AMV DNA of about 150–250 bp in size. This size range corresponds by itself to the range of size of a ss-unit of discontinuous DNA synthesis in eukaryotes, which is supposed to be about 100–240 bases (Huberman and Horowitz, 1973; Kriegstein and Hogness, 1974). Thus, the higher size class AMV DNAs are most probably formed secondarily during AMV DNA isolation, due to the linking of 2–4 of those molecules of the main size class AMV DNAs which display sufficiently complementary ss-ends. Consequently, the main size class molecules of about 150–250 bp in size may represent the actual core-bound AMV DNAs in virions being up to 7 hrs old. This assumption is supported by other data on AMV DNA properties which will be presented further in this paper.

Basic evaluation of sequence characteristics of cloned AMV DNA

To confirm further that the 7 S DNA belongs to the category of minute early replicative structures of host DNA, we proceeded to its cloning and sequencing. We cloned total AMV DNA to get a survey of sequence properties of DNAs belonging to various molecular size classes.

After sequencing, we evaluated eight AMV DNA clones. Seven of them were AT-rich since their (A+T) content ranged from 54.8 to 85.6 8, with a mean value of 67.45 ± 3.02 %. AT-richness is one of the basic features of eukaryotic DNA derived from replication origins (Amati and Gasser, 1988). These findings fit well also with our conclusions about the (G+C) content of this DNA based on CsCl-buoyant density properties of denatured and RNase-treated 7 S AMV DNA ()Říman *et al.*, 1993), in which we did not exclude also the existence of a small portion (up to 10 %) of GT-rich AMV DNA molecules. After all, one of the eight basically evaluated cloned AMV DNAs had a (G+C) content of 71 %.

Its sequence (clone A-DNA) is depicted in Fig. 4. This table presents five examples of sequence properties of individual AMV DNA clones and illustrates a great sequence heterogeneity of DNA molecules, of which the 7 S AMV DNA is composed, thus explaining objectively the inability of this DNA to renature under standard conditions (Dvořák and Říman, 1980*a*).

Special evaluation of sequence characteristics of cloned AMV DNAs

The use of specific sequences as replication origins appears to be a norm for eukaryotic cells. Such sequences, which in general are AT-rich, are specifically recognized by initiation factors and provide a region of unwinding, in which the elongation complexes with accessory proteins can form (Diffley and Stillman, 1990). Accordingly, if AMV DNA represents a family of minute early replicative structures, the sequences of its individual AT-rich DNA molecules should contain the sequence motifs characteristic of the origins of replication. Such motifs are represented mainly by sequences of ARS type discovered in yeast (Stinchcombe *et al.*, 1979; Chan and Tye, 1980), which now belong to the best defined sequences of replication origins (Broach *et al.*, 1982; Snyder *et al.*, 1986; Umek *et al.*, 1989). Therefore we have focussed on searching for the presence of yeast ARS "core" elements in DNAs of AMV DNA clones. Since in the yeast the ARS "core" motifs coincide at a certain distance periodically with *Drosophila* topoisomerase II. (Topo II) binding sites (Sander and Hsieh, 1985; Amati and Gasser, 1988), we have also searched for Topo II consensuses using two approaches. First, the nucleotide sequences of cloned AMV DNAs I-2, I-18 and II-28 shown in Fig. 4 were scanned for homology with the yeast 11 bp ARS "core" motif and with the 15 bp Topo II consensus of *Drosophila*. Second, using the matrix method (Pustell and Kafatos, 1984), the sequence homology in the yeast ARS "core" consensus was compared also between these AMV DNA clones and the ARS-carrying sequences of DNAs of a variety of phylogenetically divergent eukaryotes from EMBL database (release 3, 1991). The AMV DNA clones I-2, I-18 and II-28 exhibited 77.0, 88.9 and 90.9 % homology, respectively, with the yeast ARS "core" consensus, as depicted in detail for clones I-18 and II-28 in Fig. 5 and Fig. 6, respectively. As shown further in these Figs, a high degree of homology (82 to 100 %) in ARS "core" consensuses between the above mentioned cloned AMV DNAs and ARS-carrying DNAs of various eukaryotes was found to reside in about 16 bp long nucleotide segments situated within, upstream as well as downstream of the 3'-flanking region of ARS "core" consensuses proposed in AMV DNAs. Taking into consideration a very low probability that such long homologous segments in eukaryotic DNAs would be a random event, the results obtained by this comparison support the previous indications that the examined AMV DNA clones carry the ARS-like motifs. This conclusion was confirmed further by examining the homology between complete sequences of clone I-2, I-18 and II-28 DNAs and various ARS-carrying DNAs, which reveals 46–57 % (mean 49.5 %) homology. In addition to this characterization, we could notice without a computerized search the presence of

a distinct ARS- like motif also in the sequence of the shortest (29 bp long) cloned AMV DNA which was 85 % AT-rich (Fig. 4, clone B).

In analyzing the homology between cloned AMV DNAs and the 15 bp Topo II consensus of *Drosophila,* a discontinuous alignment with a gap score of 1 to 3 revealed several Topo II consensus canditates. The best potential Topo II motifs were situated in clone I-18 and II-28 DNAs, as shown in Fig. 5 and Fig. 6, downstream 204 and 29 bp, respectively, from the proposed ARS consensuses of AMV DNAs (in the case of clone I-2 DNA, 29 bp upstream; data not shown). In addition, cloné II-28 DNA revealed other extensive long nucleotide homologies (with a gap of 1 to 2) with ARS-carrying DNAs of various eukaryotes situated within and downstream of our proposed partial Topo II consensuses. Moreover, besides the above mentioned homologies, we observed about 14 bp long homologous (85 %) segments which did not coincide either with ARS or Topo II consensuses in clones I-18 and II-28. Interestingly, the 14 bp segment of clone I-18 DNA (beginning at position 126, Fig. 4) showed 85–95 % homology with the gene segments coding for 18 S rRNA which are conserved in DNAs of nine phylogenetically divergent eukaryotes, suggesting a possible gene family relation in this case of cloned AMV DNA.

Finally, an examination of a special sequence characterization of AMV DNA AT-rich clones revealed a high incidence of segments composed of four to six A-motifs (see Fig. 4, clone II-28) which carry the DNA conformation requirement for DNA bending (Paleček, 1991). Accordingly, an electronoptical analysis of individual AMV DNA molecules revealed a high incidence of their bending up to circularization as well as other distinct properties (Korb *et al.*, 1993) associated with phenomena of a sequence-directed DNA bending (Koo *et al.*, 1986). Thus, the data reported above confirm that AMV DNA represents, also according to basic and special sequence properties of its individual AT-rich DNA molecules, a collection of minute early replicating host DNA structures.

Sequence similarity of AMV DNA with myeloblastic nuclear scaffold-bound DNA

The findings that AMV DNA contains prevalently AT-rich DNA molecules with sequence motifs of high degree of homology with yeast ARS "core" consensus sequences indicated that these DNA molecules might represent AT-rich DNA structures closely surrounding the replication origins which in eukaryotes are supposed to be associated with nuclear skeleton proteins (Razin *et al.*, 1986) of matrix (Berezney and Coffey, 1974) or scaffold (Lebkowski and Laemmli, 1982) nature. It is known that the nuclear skeleton structures are held as sites of origin of newly replicated DNA (Jackson and Cook, 1986; Razin *et al.*, 1986). Consequently, according to sequence properties of a majority of its molecules, the total AMV DNA, even if not cloned, should exhibit a higher degree of homology with scaffold-bound DNA than with total nuclear host-cell DNA. To test this assumption, we hybridized comparatively (using special conditions described in Materials and Methods) the ^{3}H-mTdR-labelled denaturated ds-portion of AMV DNA with two samples of myeloblastic nuclear

AMV DNA Clones

I-2

```
          10         20         30         40         50
GAGCTCGGTA CCCATTTTTT TTTCCCTCTG ATTTGTAACT TAAGAATGTC
          60         70         80         90        100
TTAAACTTTT TATTGAAAGA GAGAGAAGTG ATTAGGTGTG AAGATGGTGA
          10         20         30         40         50
GAAGATCTTT GCAGTAACAA AAAAGTTGTT TAGATTTCAG TTACTGTAAG
          60         70         80         90        200
GATTGGTTTT TATATCTGTT GGAAAAAAAG GGGGATCCTC TAGAGTCGAC
          10         20         30         40         50
CTGCAGGCAT GCAAGCTTGG CGTAATCATG GTCATAGCTG TTTCCTGTGT
          60         70         80         90        300
GAAATTGTTA TCCGCTCACA ATTCCACACA ACATACGAGC CGGAAGCATA
          10         20         30         40         50
AAGTGTAAAG CCTGGGGTGC CTAATGAGTG AGCTAACTCA CATTAATTGC

GTT
```

I-18

```
          10         20         30         40         50
TCGAGCTCGR TACCCAACCT CACTCAAAAA AATTTTTTCC TGAGTAAGCA
          60         70         80         90        100
TTCCTATTTC TTCCTCCACA AAAATTCAGA AAAATTTATG TTTCATTTAC
          10         20         30         40         50
CCAAAAAAGC ATTTCAGATC AAGAAAGTTG ATCAGATTTG TTAGCCTTTG
          60         70         80         90        200
CCTTTTTCCG TTCGTCTCGT TAGAAAGATA AACTAAACCC TCCTGACAGT
          10         20         30         40         50
TTCACATCTG CAGAAGCCCG TTAGACACAG AATTCCAGGA GAGGTGTTTG
          60         70         80         90        300
CATGCCTGTA AAATGGCCAG GTTTGTGGAG CAGACAATGA CATTCAGTTC
          10         20
TGGAGAGGTC AGATTTTTTT CTT
```

II-28

```
          10         20         30         40         50
GAGCTCGGTA CCCGTCATGT CTTTTTTGAA ATAGATTTCT GTCCATAGGC
          60         70         80         90        100
CTCTTTGATG TGTTCCTTAC TGCTTGGCAT TATTTAGACC TCACAACTTG
          10         20         30         40         50
CAACTGGTAA AATTTAATCT ATTATAACTT TAAACTTAGT ATGTAATTAC
          60         70         80         90        200
TTGGTAGCTT GTTAAAATAA ATTCAATTCT GTGGTAACAT ACATAGATTT
          10         20         30         40         50
ATATTAGAGC ATATTTCTAT GTAATGTCTA CACTTCTTAT TTCTTGAGAT
          60         70         80         90        300
GTTTTCCAAA TCACACTCAG ACTGACTGAG GTTGAAAGGC TGTCTCATCC
          10         20
AACCACTTCA ATGGAAAAAA ACAC
```

A

```
          10         20         30         40         50
GATCCCGACG ACACGCGCGC TGGCGATCCG CTCGGCACGG TCGCATTCGC
          60         70         80
GGATGGATGC TGGACCAACG ACCGCGCCCG
```

B

```
          10         20
TTTACTGTAT TAAATATAAT ACAGTAAA
```

Fig. 4
Examples of sequence properties of clones of AMV DNAs
The left end is the 5'-site. ARS-like sequences are underlined.

scaffold-bound DNA isolated from different pools of chicken leukaemic myeloblasts and with total myeloblastic and calf thymus DNA, which together with the renaturation characteristic of AMV DNA served as controls.

Fig. 7 shows in its segments 0–E (see the legend) the percent expresion of ^{3}H-mTdR radioactivity of the individual phosphate molarity elution fractions obtained by hydroxylapatite column chromatography of the non-hybridized (0) and hybridized (A–E) AMV DNA samples. The insert in this figure depicts a final evaluation showing that AMV DNA under the conditions used exhibited

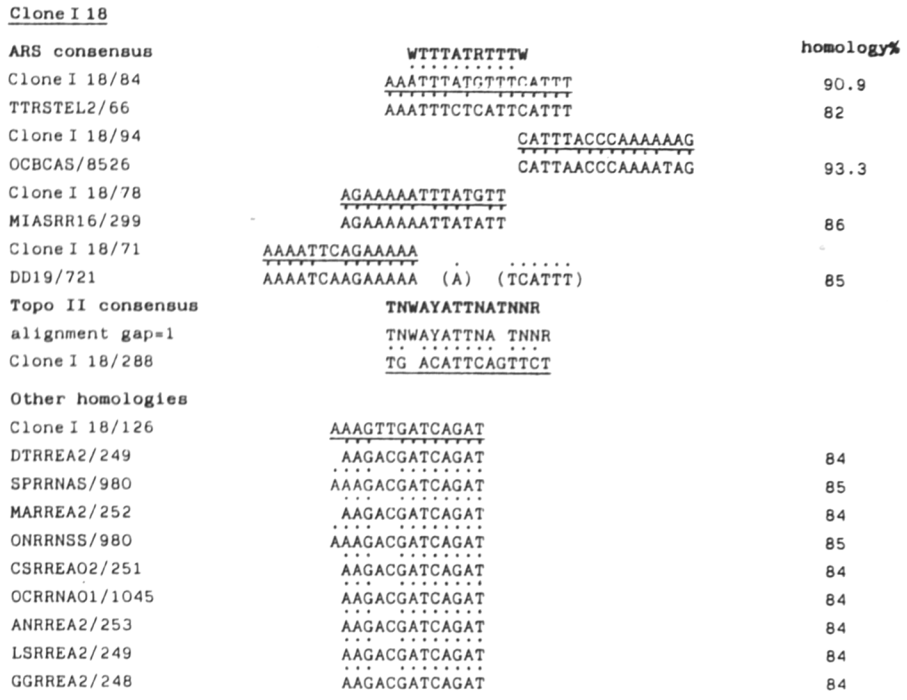

Fig. 5

Homologies between clone I-18 AMV DNA, the yeast ARS "core", *Drosophila* Topo II consensus sequences, and various eukaryotic ARS-carrying DNAs

Left column: List of segments of the clone I-18 (clone II-28 in Fig. 6) homologous to the yeast ARS, *Drosophila* Topo II consensus and to the ARS-carrying DNAs designated with entry names of the EMBL database. The numbers below slashes represent the first nucleotide of the corresponding sequence from the left (5'-site). Middle column: ARS "core"- and Topo II-consensus are in bold letters. Underlined sequences represent the homologous segments of the clone I-18 (clone II-28 in Fig. 6). The dots represent the corresponsing homologous nucleotides. Right column: per cent homology.

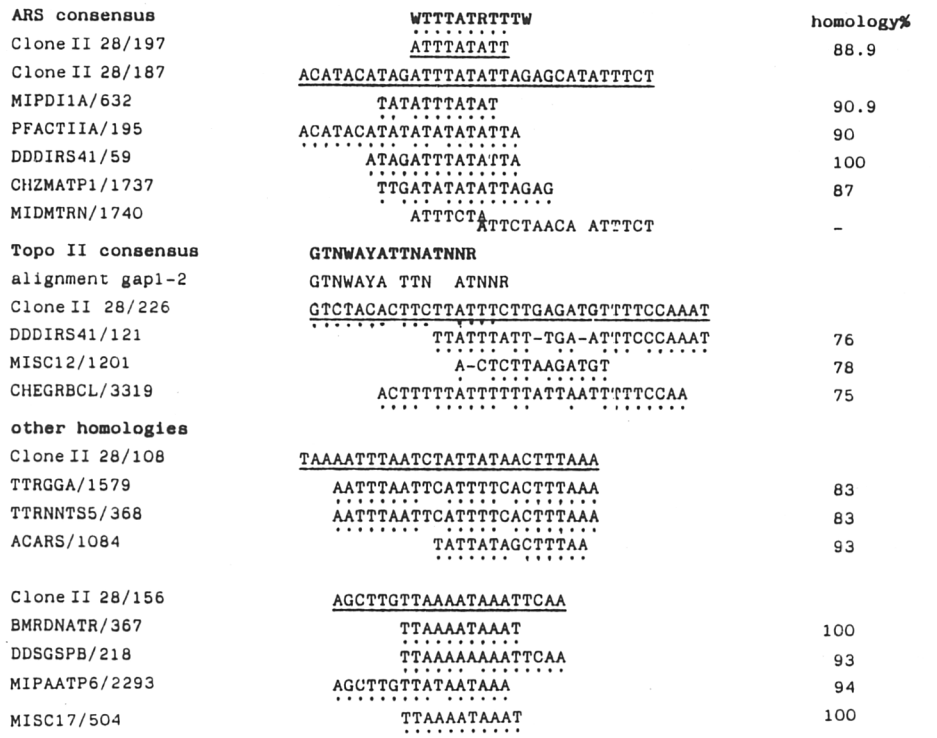

Fig. 6
Homologies between clone II-28 AMV DNA, yeast ARS "core", *Drosophila* Topo II consensus sequences and various ARS-carrying DNAs
For legend see Fig. 5.

little renaturation ability (A), did not hybridize with calf thymus DNA (reassociates in its presence) (B), and hybridized evidently with total myeloblastic DNA (C). However, a maximum of its hybridization exceeding two to three times its hybridization with total nuclear myeloblastic DNA was with both samples of myeloblastic nuclear scaffold-bound DNA (D, E)). Qualitatively a similar picture recorded by autoradiography was obtained using Southern transfer in order to follow hybridization of "*in vitro*" [alpha-^{32}P]dATP-labelled AMV DNA with *Eco*RI-treated chicken myeloblastic, chicken thymus, mouse thymus and two myeloblastic scaffold DNAs (data not shown). The highest (about 2–3-fold) radioactivity was recorded again in this case of comparatively tested DNAs by hybridization of ^{32}P-labelled AMV DNA with both myeloblastic nuclear scaffold-bound DNAs. Thus, we can conclude that the sequences

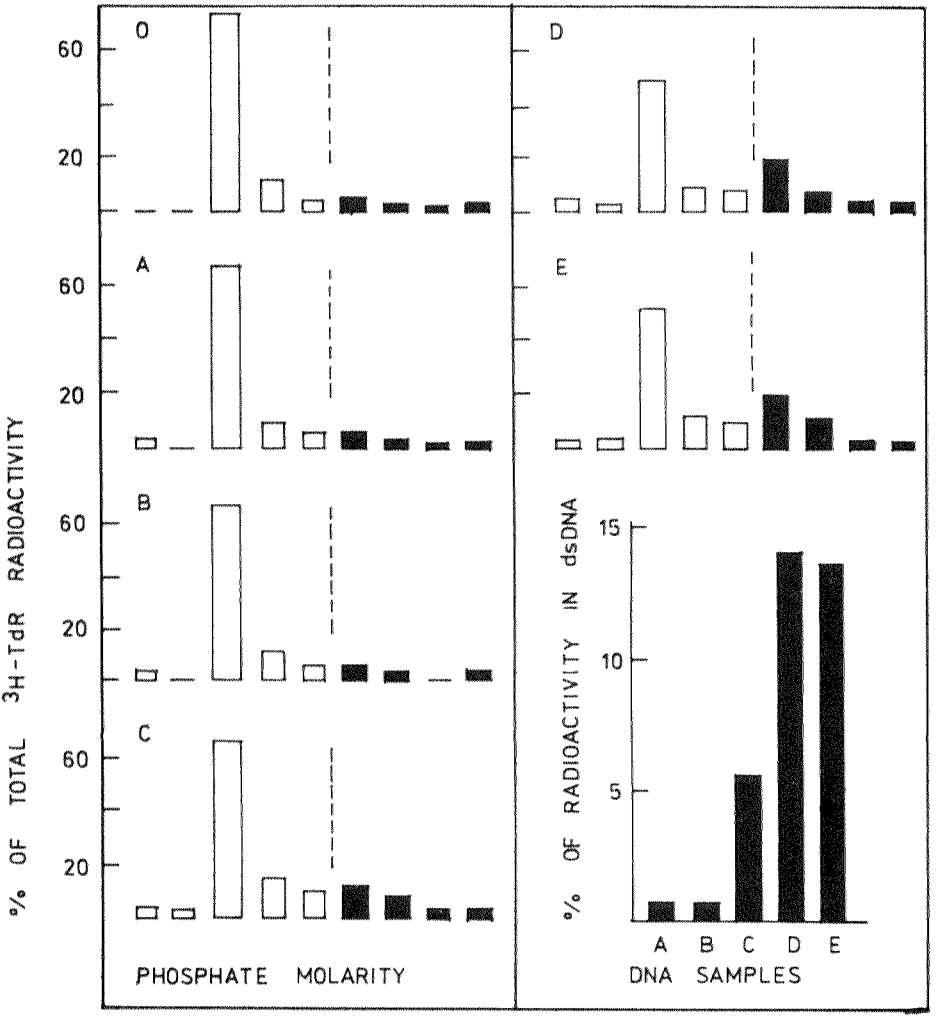

Fig. 7

Hydroxylapatite column chromatography of the double-stranded ^{3}H-mTdR-labelled AMV DNA Radioactivity elution profiles expressed in per cent of the total radioactivity. DNA probes exposed to the following conditions: 0 – denaturated, but not further treated; A – denaturated and PERT-treated; B – together with calf thymus DNA denatured and PERT-treated; C – together with myeloblastic DNA denatured and PERT-treated; D, E – together with myeloblastic scaffold DNAs (from two separate isolations) denatured and PERT-treated. The final evaluation of this search for AMV DNA homology shown in the insert was done by subtracting the radioactivity remaining bound to the ds-portion in experiment 0 from the radioactivity present in the same portion in all other individual experiments (A–E). Elution fractions were successively (from the left to the right) of 0.01, 0.01, 0.10, 0.18 (the ss-portion), 0.22, 0.30, 0.50 (the ds-portion), phosphate molarity.

present in AMV DNA molecules are enriched in scaffold-bound DNAs. Consequently, these findings implicate that our view of having to deal with minute replicative structures of host DNA in the case of AMV DNA can be extended by an indication that its DNA molecules are descendent from scaffold-bound DNAs bearing the origins of new DNA replication.

First indications of the functional significance of AMV DNA

(a) Constancy of AMV DNA virus content and the mode of segragation of this DNA

To the basic indications of some functional significance of a virus-occluded host component for the virus life cycle belongs, in general, the constancy of its virus content. In this respect a constancy of the virus content of the "small" host DNA present in all so far studied retroviruses, still remains doubtfull. This is due to its trace amounts (1–2 % of the total virus RNA according to Levinson *et al.* (1972)), as well as to the fluctuations of its amount in viruses produced by cells *in vitro*. Consequently, this makes the functional meaning of this DNA unlikely (Coffin, 1982). To contribute to this issue we analyzed four highly purified large pools of AMV isolated from blood plasma of leukaemic chickens for protein-, total RNA- and DNA-content. As shown in Table 1, it is evident that the estimated amount of AMV DNA expressed in μg per mg of AMV protein exhibits small variations and participates with 0.99 % in the total amount of virus-occluded nucleic acids (NAs). Expression of the amount of AMV DNA per mg of virus protein made it possible in the case of the well analyzed AMV to convert the estimated values of AMV DNA amount to its virus content values, taking 465 μg of AMV protein for 10^{12} virus particles (Deeney *et al.*, 1976). Consequently, assuming on the basis of the data presented in this paper that the main AMV DNA molecule is about 220 bp in size, the data obtained in this way on the AMV DNA content show that nearly each particle of the AMV complex contains one molecule of this DNA (Table 1). In addition, starting from the data obtained on the total RNA- and DNA-content of AMV and using other relevant data available (or tentative) (see the legend to Table 2), we estimated the base numbers of the individual NA species present in virions of AMV and thus, its tentative percent participation in the total NA virus content. In this way we found, as shown in Table 2, that besides the viral genome dimer RNA, all the host NA species also seem to be represented in virions of the AMV complex by certain non-fortuitous amounts. This opens again the question of a possible functional significance of the host NAs present in retroviruses, in general, and in the case of the special AMV DNA, in particular.

The constancy of the virus content in the occluded host components is closely related to their segregation selectivity, the determination of which is rather difficult (Coffin, 1982). Nevertheless, in the case of the core-bound AMV DNA we have shown earlier (Dvořák and Říman, 1980*a,b*) that its immediate segregation precursor is a DNA present in the postmicrosomal cytoplasmic fraction of chicken leukaemic myeloblasts since this DNA has physicochemical and hybridization properties identical with those of AMV DNA. We have now

Table 2. Tentative participation of the individual nucleic acid species in total nucleic acid content in virions of the AMV complex

Nucleic acid species	No. of molecules per virion		No. of bases per virion	% of total NA content
	A	B		
Viral dimer RNA	1	0.95	20 000	44.78
tRNA	125	120	8 750	19.59
8 S rRNA	2	1.91	4 000	8.95
28 S rRNA	2	1.91	10 290	23.27
5 S RNA	2	1.90	360	0.80
7 S RNA	2	1.91	820	1.83
AMV DNA	1*	1.01	440 (or 220 bp)	0.99

Instrumental for this estimation were: data obtained by direct analysis of RNA- and DNA-content in AMV, data available on viral RNA (Coffin, 1982), virus-occluded tRNAs (Erikson and Erikson, 1970; Coffin, 1982) and rRNAs (Říman *et al.*, 1972), data on molecular weights of RNAs (McMaster and Carmichael, 1977). Numbers of 5 S and 7 S RNA molecules were estimated tentatively, considering their relevance to ribosomes (Walker *et al.*, 1974; Coffin, 1982). In base number calculations the nucleotide conversion factors given by Coffin (1982) were used. *Taking into consideration a possible occurrence of basic AMV DNA structures 150–180 bp in size, their high (A+T) content and the theory of the DNA detection by diphenylamine (Burton, 1956), the presence of two molecules of the basic AMV DNA structure per virion is not excluded. A – tentative, B – calculated number of molecules.

to confirm this assumption by demonstrating (Table 3) that both the AMV DNA and the myeloblastic "cytoplasmic" DNA have the same range of specific radioactivity (10^{-5} cpm/μg of DNA) differing by two orders of magnitude from that of the total nuclear DNA (10^{-3} cpm/μg of DNA) when virus producers, the chicken leukaemic myeloblasts grown *in vitro*, were labelled continuously 14 hrs with ^{3}H-mTdR. The extent of the specific radioactivity of AMV DNA and that of the "cytoplasmic" DNA, reflect well the same replication activity of both these DNAs. The fact that the AMV DNA is core-bound implies that it segregates from cell cytoplasm into virions in association with the nucleoprotein core complex. Thus, some of the virus core components, especially those having the proper DNA-binding abilities, may be involved in the selective segregation of this DNA. In this respect, the specific DNA-binding property of the viral DNA endonuclease (Misra *et al.*, 1982) which represents an integral part of the beta-unit of the viral revertase, might be considered.

(b) Virus "age"-dependent cleavage changes of AMV DNA

More direct indications for a possible involvement of AMV DNA in the virus life cycle were provided by the findings suggesting that this DNA undergoes *in vivo*, inside the core structure, virus "age"-dependent cleavage changes accom-

Table 3. Specific ^{3}H-mTdR radioactivity of myeloblastic and AMV DNA after 14 hrs of the continuous labelling of chicken leukaemic myeloblasts grown in vitro

Kind of DNA	Specific radioactivity cpm/μg DNA
Nuclear DNA	$3.20 \pm 0.02 \times 10^{-3}$
"Cytoplasmic" DNA	$3.37 \pm 0.01 \times 10^{-5}$
AMV core-bound DNA	$3.72 \pm 0.04 \times 10^{-5}$

The values of specific radioactivities (cpm/μg of DNA) represent the mean values of four determinations. The cytoplasmic DNA represents DNA isolated, as described previously (Dvořák and Říman, 1980*a, b*), from the cytoplasmic fraction void of mitochondria and microsomes, sedimenting at 235 000 x g.
AMV DNA was isolated from the core fraction of virions produced by 14 hrs continuously labelled myeloblasts (Říman *et al.*, 1993).

plished evidently by the relevant virus core-bound enzymatic activities. This follows, as shown in Fig. 8, from a comparison of the electrophoretic mobility patterns in agarose gels of AMV DNA isolated from young (up to 7 hrs old) virions produced *in vitro* by chicken leukaemic myeloblasts with the same characterization of this DNA isolated from the virions produced by chicken leukaemic myeloblasts *in vivo* and collected from the blood plasma of chickens in the final stages of the viral acute myeloblastic leukaemia. In this case, in contrast to the former virus material, the virus pools are always represented by virus populations of much broader age range (up to 36 hrs, according to our evaluation; Říman, 1965)) with only a minor portion of virions up to 7 hrs old. In this case, the AMV DNA isolated either from highly purified virions or their core fraction exhibited, in contrast to the same characteristics of AMV DNA of young virions, the appearance of a distinct portion of DNA molecules of a small size-class, about 20 to 40 bp long accompanied with a marked decrease in the amount of the main (150–220 bp) and the higher size-class portion of AMV DNA molecules. We observed an analogous shift in size distribution of AMV DNA molecules by analyzing this DNA isolated from virions up to 24 hrs old produced *in vitro* (data not shown).

The observed virus "age"-dependent cleavage changes of AMV DNA taking place inside the retroviral nucleoprotein core complex implicate strongly an involvement of core-bound (Grandgenett *et al.*, 1978) dual (virus-DNA specific and host-DNA unspecific) endonuclease activities of the viral integrase (p32 or pp32 in avian retroviruses) present also in the alpha, beta-dimer of the revertase as a natural component of its beta-unit. These viral endonuclease activities are able to cleave site-specifically the ds- and ssDNA substrates (Duyk *et al.*, 1983; Grandgenett and Vora, 1985; Cobrinik, *et al.*, 1987; Grandgenett and Mumm, 1990). An involvement of viral DNA endonuclease in AMV DNA cleavage accomplished inside the virus core *in vivo*, rather than that of the virus-occluded

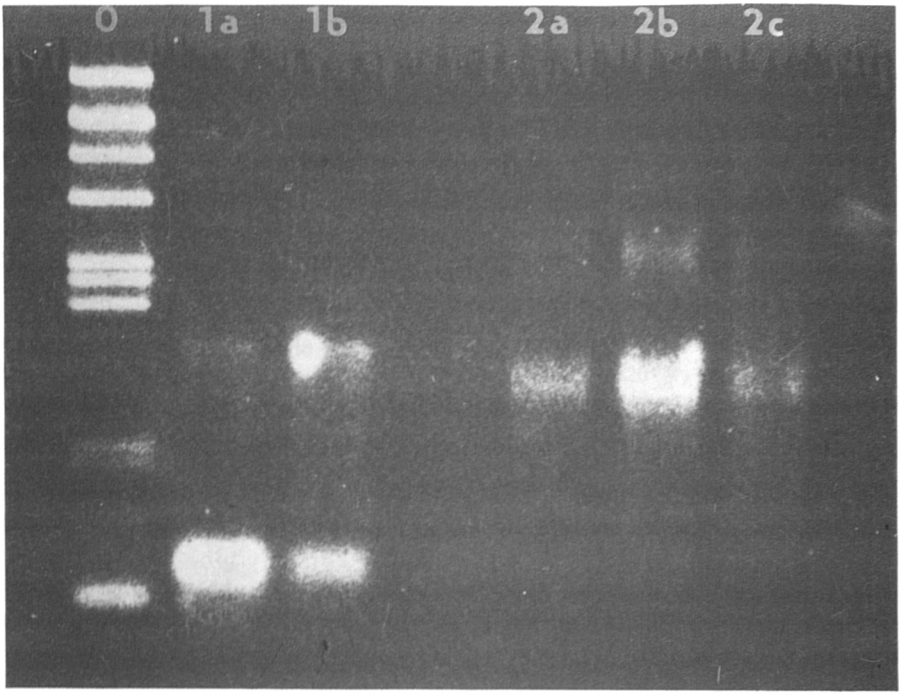

Fig. 8
Electrophoretic mobility patterns of AMV DNAs in 4 % NuSieve agarose gels
(1) - AMV DNA (8 μg/slot) of virus present in blood plasma of leukaemic chickens; (1a) - DNA isolated from virus; (1b) - DNA isolated from virus core. (2) - AMV DNA of virions up to 7 hrs old; (2a) - AMV DNA isolated from virus core (2 μg/slot); (2b) - DNA isolated from virus (4 μg/slot); (2c) - DNA isolated from virus core (2 μg/slot). (0) *Alu*I pBR322 markers.

topoisomerase I (Weiss and Faras, 1981; Priel *et al.*, 1990), is indicated by some characteristic properties of the AMV DNA nicking products such as (a) the occurrence of DNA molecules with 3'-recessed ends filled effectively in reactions with the Klenow fragment of the DNA polymerase I (Říman, unpublished data), which may explain that a minor part of native AMV DNA molecules tend to link into multimers, as mentioned already in this paper and confirmed by electron microscopical evaluation of AMV DNA molecules (Korb *et al.*, 1993); (b) the minimal size of 20–15 bp of the end products of cleavage of core-bound AMV DNA accomplished *in vivo* (see Fig. 8) is strongly reminiscent of the minimal substrate size requirement of AMV endonuclease observed in reactions performed *in vitro* with synthetic oligodeoxynucleotides (Katzman *et al.*, 1989). Besides possessing DNA nicking activity, viral DNA endonuclease exhibits

quite specific DNA-binding properties (Misra *et al.*, 1982; Knaus *et al.*, 1984) which might be responsible also for the selective segragation of AMV DNA into viral nucleoprotein core complex, most probably by binding of the AMV DNA to the beta-unit of the revertase. Further elucidation of these problems, toward which is directed our present work, may help to distinguish a possible functional significance of this special host DNA for the various activities of the biochemical machinery represented by the retroviral nucleoprotein core complex. Interestingly, this only contains all components necessary for synthesis of viral linear dsDNA as well as for its integration into host DNA (Brown *et al.*, 1987; Fujiwara and Mizuuchi, 1989). Besides other possibilities of involvement of this DNA in the life cycle of a retrovirus, one can imagine in the most simplest way that the AMV DNA may represent, by means of its cleavage products, primers suitable especially for accomplishment of certain steps in viral DNA synthesis, the detailed mechanism of which has not yet been fully elucidated. Indications for such an assumption may come from earlier observations (Kiessling *et al.*, 1972) that the AMV DNA contributes to the synthesis of the final viral DNA product in an endogenous reverse transcriptase reaction where both the AMV DNA and the viral RNA are present together. The issue of possible functional significance of 7 S host DNA constantly bound to the AMV nucleoprotein core complex is not restricted by far to the area of retrovirology only, since the cytoplasmic pool of this kind of DNA associated with the postmicrosomal cytoplasmic fractions seems to be a common property of proliferating vertebrate cells. As previously shown (Dvořák and Říman, 1980*b*), such DNA similar to AMV DNA and cytoplasmic DNA of chicken leukaemic myeloblasts in physicochemical and hybridization properties represents a constant cytoplasmic component of proliferating virus-free chick embryo fibroblasts. In addition, this DNA is strikingly similar also to the "small polydisperse circular DNA" which was found to be present in the postmicrosomal cytoplasmic fraction of HeLa cells (Smith and Vinograd, 1972). Thus, this kind of DNA, representing in principle according to its properties shown in the case of AMV DNA in this and our preceding paper (Říman *et al.*, 1993), a collection of basic units of cellular DNA replication, raises by itself a broad set of questions connected with its general meaning for the life cycle of proliferating vertebrate cells. In this respect, it remains to answer, e.g. (a) the mode of its transfer from cell nucleus into cytoplasm accomplished possibly in the phase of the cell cycle when the nuclear membrane ceases to exist (G_2-M phase); (b) the S phase time of its replication; (c) the motives of activation of the multiple origins of its replication; (d) the motives and modes of a premature break of its bidirectional synthesis leading to the release of minute replicative structures uniform in size derived from the immediate vicinity of replication origins; (e) its chromosomal regional location and its gene family relation (in proliferating normal and tumour cells); (f) the reason for its cytoplasmic cumulation which seems to be influenced by protein synthesis and cell growth conditions (Smith and Vinograd, 1972); (g) its possible involvement in various activities of the proliferating cell not excluding its possible participation by

means of its special sequence motifs in the formation of extrachromosomal autonomous DNA replicative structures. Finally, this DNA and its nucleoprotein complexes may represent a source suitable for studying the role of the various DNA polymerases and factors (Linn, 1991) engaged in the early events in DNA synthesis of vertebrate cells *in vivo*.

Dedication. This work is dedicated to Professor Dionýz Blaškovič at the occasion of his 80th birthday.

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