Acta virol. **37**: 343–359, 1993

AVIAN MYELOBLASTOSIS VIRUS CORE-BOUND 7 S DNA, HIGHLY BENT MINUTE STRUCTURES WITH SEQUENCE-DIRECTED CURVATURE

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Received January 20, 1993; revised March 4, 1993

Summary. – Structural properties and length distribution profile of 7 S avian myeloblastosis virus (AMV) DNA were studied by means of electron microscopy using two different techniques. This DNA represents mostly double strands, the single strands being in minority. We have shown directly that this DNA forms a bent structure typical of the majority of molecules. These bends are sensitive to the distamycin treatment which stretches most of the bent molecules. Some amount (up to 30 %) of circular DNA molecules was detected also in DNA preparations, the nature and the size of which are reminiscent of electron microscopic data on microbubbles of replicating DNA. No specific AMV DNA structural features were found using osmium-tetroxide treatment. The basic size of AMV DNA was estimated to be approximately 150 bp, but its multimers were also detected. Their presence and significance is discussed.

Key words: AMV DNA; bent DNA structures; replicative DNA forms; electron microscopy

Introduction

The 7 S DNA species were discovered in retroviral particles as their constant component (Říman and Beaudreau, 1970; Levinson *et al.*, 1970; Biswal *et al.*, 1971). The study of 7 S DNA isolated from the core of AMV revealed its partly single-stranded nature and complementarity to the host genome (Dvořák and Říman, 1980*a*). This DNA revealed some peculiar features in its CsCl-buoyant density and velocity sedimentation properties, labelling kinetics and response to inhibitors of RNA and DNA synthesis (Říman and Beaudreau, 1970; Říman, 1971; Křemen *et al.*, 1971). A certain fraction of cytoplasmic nonmitochondrial DNA was considered as a possible origin of this DNA (Dvořák and Říman, 1980*b*).

Detailed biochemical and biophysical analysis has recently shown that more than 20 % of AMV DNA is of single-stranded nature and the rest is in double-stranded form. About 30 % of these molecules are composed of covalently

bound ribonucleotides, having properties of Okazaki fragments, the structures present in eukaryotic DNA replicative systems (Říman et al., 1993*a*). This AMV DNA possesses the properties of DNA replicative structures with possible functional consequences (Říman *et al.*, 1993*a*).

High degree od sequence homology between AMV DNA and DNA of different eukaryotic organisms carrying autonomously replicating sequence (ARS) motifs was found by sequence analysis. Presence of sequences identical with matrix-attached DNA regions (MAR) and scaffold-attached DNA region (SAR) sequential motifs resulting from sequence data analysis was confirmed also by hybridization experiments with scaffold-bound DNA isolated from chicken leukaemic myeloblasts (Říman *et al.*, 1993*b*). The 7 S AMV DNA undergoes some kind of maturation changes inside retroviral particles, as it was apparent from comparative electrophoretic mobilities of DNA isolated from cores of 7 hrs old virions and of virions isolated from different pools of chicken leukaemic plasma (Říman *et al.*, 1993*b*).

All these findings suggest a possible involvement of AMV DNA in the synthesis of the final viral DNA product in an endogenous reverse transcriptase reaction, or in the mechanism of integration of viral sequences into the cell genome, and generally in virus-cell interaction.

The findings that AMV DNA contains prevalently AT-rich molecules of high homology with ARS consensus sequences (Říman *et al.*, 1993*b*) have indicated that this DNA may represent structures surrounding the origins of replication and therefore these sequences may be involved in the control of replication and recombination, transcriptional regulation and DNA packing into nucleosomes (Bracco *et al.*, 1989; Travers, 1990; Galli *et al.*, 1992). It is known that AT-rich DNA is the preferred target of basic molecules (Travers, 1989). Moreover, it is well established that AT-tracts have an impact on DNA properties such as bendability and flexibility (Trifonov, 1985; Travers, 1989). The function of these AT-tracts is not entirely clear. However, the most probable, a very basic mechanism in the regulation of replication involves weak base pairing and bending potential of these AT-rich stretches (Deb *et al.*, 1986). On the other hand, they represent target sequences for a number of cellular factors (Malkas and Baril, 1989). AT-stretches are a characteristic feature of many viral and several prokaryotic and eukaryotic origins of replication (Kearsey, 1984; Zahn and Blattner, 1985).

An accurate study of viral AT-stretches may provide some useful insight into the mechanisms that underlie cellular replication.

The aim of this paper was to gather more information on the structure of 7 S AMV DNA and its length distribution. We made a first attempt to elucidate the AMV DNA structure by electron microscopy, a powerful approach to investigations of DNA bending (Griffith *et al.*, 1986). Our results are discussed with regard to the biochemical and biophysical properties of AMV DNA presented elsewhere (Říman *et al.*, 1993*a, b*).

Materials and Methods

Isolation and purification of AMV was made from large pools of blood plasma of chickens in terminal stages of AMV-induced acute myeloblastic leukaemia by isopycnic and velocity sucrose gradient centrifugation as described previously (Říman *et al.*, 1993*a*).

AMV cores were isolated from virus according to Stromberg and Litwack (1973) with minor modifications (Říman *et al.*, 1993*a*).

AMV DNA was isolated and purified by electrophoresis in agarose gels (Říman *et al.*, 1993*a*).

Electron microscopy. Two different electron microscopy techniques were used to exclude the influence of the visualization method on the structure and length distribution profile of AMV DNA. (a) Technique of activated carbon-coated grids (Coggins, 1987), where a thin carbon membrane was treated either with benzyldimethylalkylammonium chloride (BAC, 50 μg/ml for 3 hrs or 5 μg/ml overnight), or with ethidium bromide (50 μg/ml for 3 hrs). DNA was mounted directly onto a thin carbon film placing a 50 μl drop of DNA (0.01–0.5 μg/ml) in 0.01 mol/l Tris, 0.1 mmol/l EDTA, 0.15 mol/l NaCl and 2 mmol/l spermidine, pH 8.0. Spermidine is known to promote tight binding of DNA to the support membrane, whereas NaCl prevents aggregation of DNA (Griffith and Christiansen, 1978). (b) Modified BAC spreading technique (Štokrová *et al.*, 1989). Electron micrographs were recorded at magnifications of 10 000 or 15 000 in JEOL JEM 1200EX microscope operating at 60 kV. Length measurements of DNA molecules were performed with Olivetti PD3 digitizer linked to Olivetti M24 calculator system. Double-stranded DNAs of plasmids pBR322 and pUC18 were used as internal molecular length standards (Brack, 1981).

Distamycin treatment. DNA was incubated for 15 min on ice with antibiotic distamycin in ratios of 1 drug molecule to 6.7 and 4 nucleotides, respectively.

Osmium-tetroxide treatment. A typical reaction mixture contained AMV DNA (30 μg/ml) in 0.01 mol/l Tris, 1 mmol/l EDTA pH 7.8, 1 mmol/l OsO_4 and 2 % (v/v) pyridine. It was incubated under conditions described by Paleček (1992).

Results and Discussion

Structural analysis of 7 S AMV DNA

DNA for electron microscopic analysis was prepared from highly purified pools of AMV isolated from blood plasma of leukaemic chickens. To exclude any contamination by host cell DNA adsorbed on or occluded in the virus envelope, DNA isolated also from highly purified AMV cores was used as well. AMV cores appear mostly as intact structures encapsulated by core membrane (Fig. 1). No contamination with outer viral envelope was observed in our preparations.

The size of AMV DNA molecules ranges from 20 to 950 bp according to electrophoretic analysis, as shown elsewhere (Říman *et al.*, 1993*b*). Electron microscopic analysis of DNA, however, is limited to the molecules of higher size, whereas DNA of smaller size is beyond the scope of electron microscopic evaluation (Meyer, 1981). Therefore only three electrophoretic fractions of DNA isolated from AMV particles and/or AMV cores, which represented the main portion of the whole AMV DNA (Říman *et al.*, 1993*b*) and corresponded to 900–1000 bp (F1), 220–250 bp (F2) and 180 bp (F3), respectively, were analyzed by electron microscopy (Fig. 2).

The structure of 7 S AMV DNA prepared by the BAC spreading technique, which prevents the formation of artificial bends (Vollenweider *et al.*, 1975) is

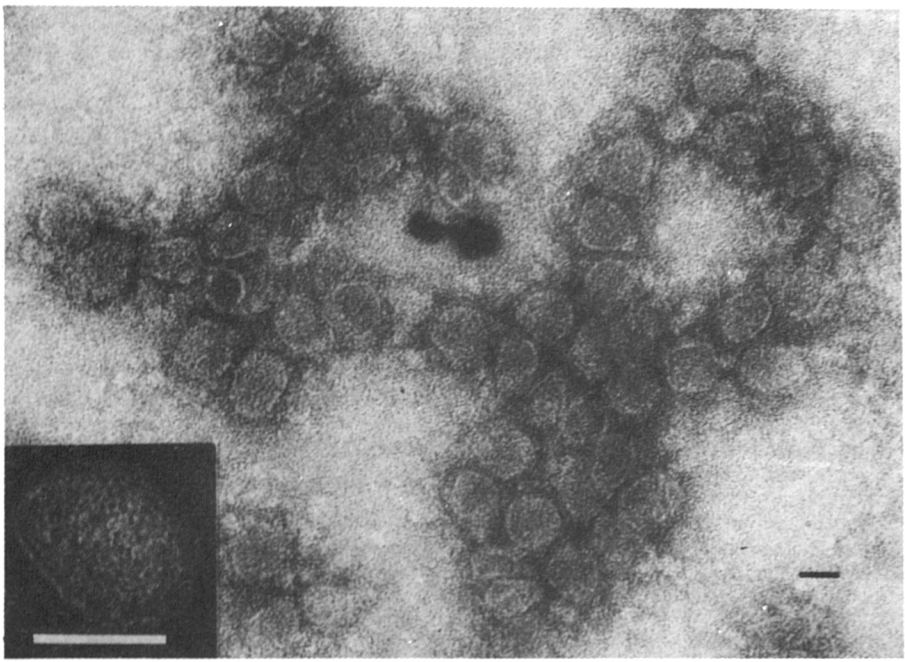


Fig. 1
Electron micrograph of an intact core AMV preparation made by negative stain technique Intact core particle void of the viral envelope is shown at higher magnification in the insert. Bar = 0.05 μm.

shown in Fig. 3. Most of the molecules are double-stranded in all three fractions, but some amount of single-stranded molecules is also apparent. This observation confirms the finding of Říman *et al.* (1993*b*).

Previous studies on the gel mobility properties and the nucleotide sequence of AMV DNA (Říman *et al.*, 1993*b*) led to a conslusion that this DNA might have sequence-directed bend. The first suggestions for this type of bending came from Selsing *et al.* (1979). Since also other unusual structures in DNA helix (e.g. cruciforms (Gough and Lilley, 1985), lariats (Grabowski *et al.*, 1985)) can also affect the electrophoretic mobility and the presence of such unusual structures in replicating DNA is probable, the direct evidence of bending by means of electron miccroscopy is a method of choice allowing an unequivocal interpretation. Indeed, the bent structure was a typical appearance of AMV DNA molecules in all three DNA fractions. About 80 % of DNA exhibited this feature, whereas the rest of DNA molecules (about 20 %) were straight. The bending was

localized usually at the ends of AMV DNA, but sometimes a bend in the inner part of the molecules was observed as well.

According to Trifonov and Sussman (1980) the periodic positioning of small bends could result in the observed unidirectional curvatures. Naturally occurring bends were first described in some kinetoplast DNA molecules (Marini *et al.*, 1982; Griffith *et al.*, 1986). The variable distribution of bent and straight AMV DNA forms probably reflects high flexibility of these DNA molecules.

Besides these bent structures, some small amount of circular molecules in AMV DNA preparations was also observed by this technical approach (less than 10 %).

In electron microscopy, however, artefacts formed during sample preparation should be eliminated as the amount of bent molecules depends on preparative conditions (Marini *et al.*, 1982; Diekmann and Wang, 1985; Griffith *et al.*, 1986). Transfer of DNA molecules from 3D to 2D support for visualization, formation of monomolecular BAC film during spreading, effect of drying, and temperature might play an important role and significantly affect the observed DNA structure. Therefore in paralell with the BAC spreading technique another method of DNA sample preparation was used, where naked DNA was adsorbed

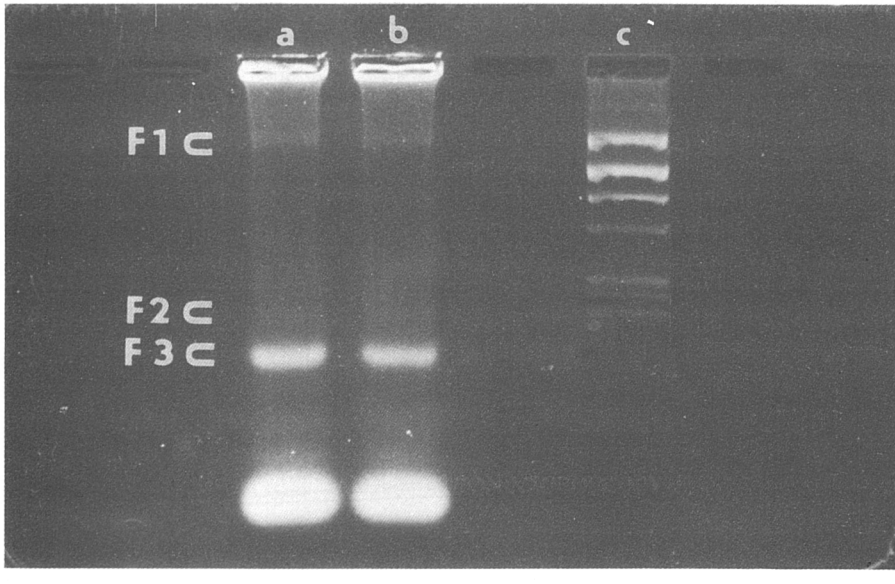
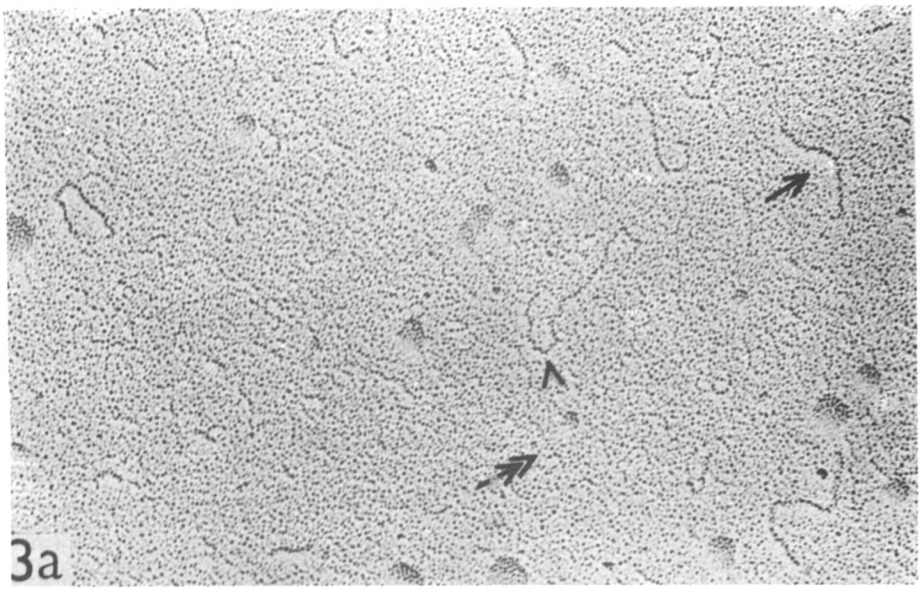
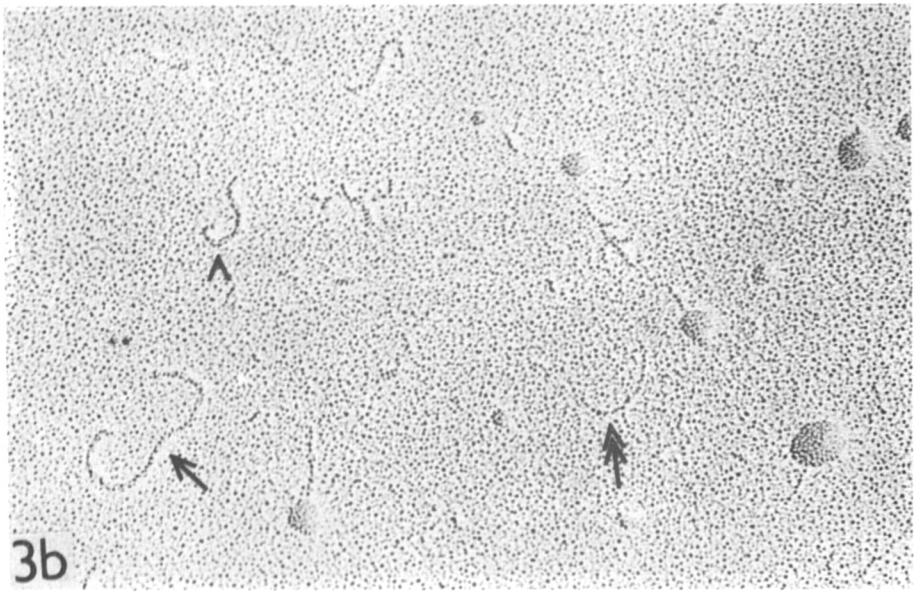


Fig. 2
Agarose gel electrophoresis of AMV DNA
AMV DNA isolated from pool No. 1 (lane a) and pool No. 2 (lane b) of chicken leukaemic plasma. *Alu*I pBR322 marker fragments in bp: 910, 657 (2 ×), 521, 403, 281, 257, 226.



3a



3b

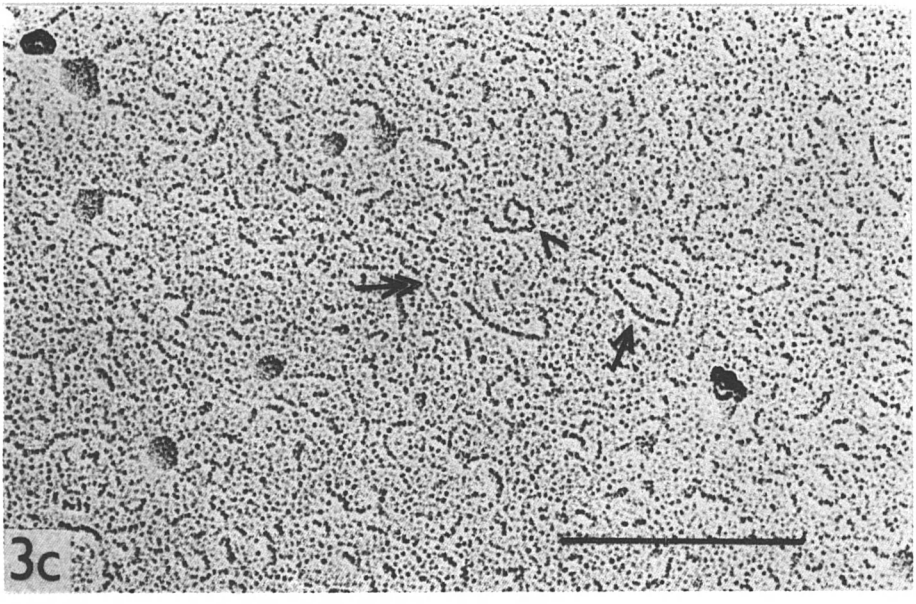


Fig. 3
Electron micrograph of typical AMV DNA double-stranded (arrows) and single-stranded (double arrows) structures
Fraction 1 (3a), Fraction 2 (3b) and Fraction 3 (3c) from Fig. 2 visualized by electron microscopy using BAC spreading technique. Note the presence of bent molecules (see arrowheads). Bar = 0.5 μm.

on BAC-activated carbon film. While the first technique could result in artificially stretched DNA molecules, using the second, more gentle technique, on the contrary, some amount of collapsed molecules could be observed.

AMV DNA adsorbed directly on carbon film activated either by BAC or ethidium bromide revealed structural features similar to those observed by the BAC spreading method, but a significantly higher proportion of bent DNA molecules was apparent. Also the bending curvature was more distinct and circular molecules were found in this type of visualization more frequently. The highest amount of circular DNA molecules (about 30 %) was observed in fraction 3 (Fig. 4). The size of these molecules (BAC-activated membrane) was quite homogeneous with the mean length of 0.1117 ± 0.0185 μm (Fig. 5). When DNA molecules were adsorbed on ethidium bromide-activated carbon membrane, the mean length of this DNA increased to 0.1349 ± 0.0243 μm. This is in accordance with the well known effect of the intercalating agent, ethidium bromide, on changes in linear densities of DNA (Koller *et al.*, 1974). The nature and the length of observed circular AMV DNA are reminiscent of electron

Fig. 4
Electron micrograph of circular molecules of AMV DNA
Fraction 3 from Fig. 2 analyzed by the technique of BAC-activated carbon-coated grids. Bar = 0.5 μm.

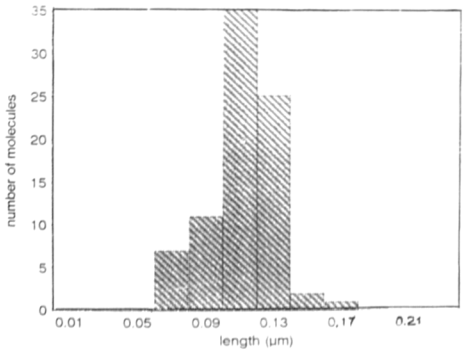


Fig. 5
Length distribution profile of circular AMV DNA molecules shown in Fig. 4

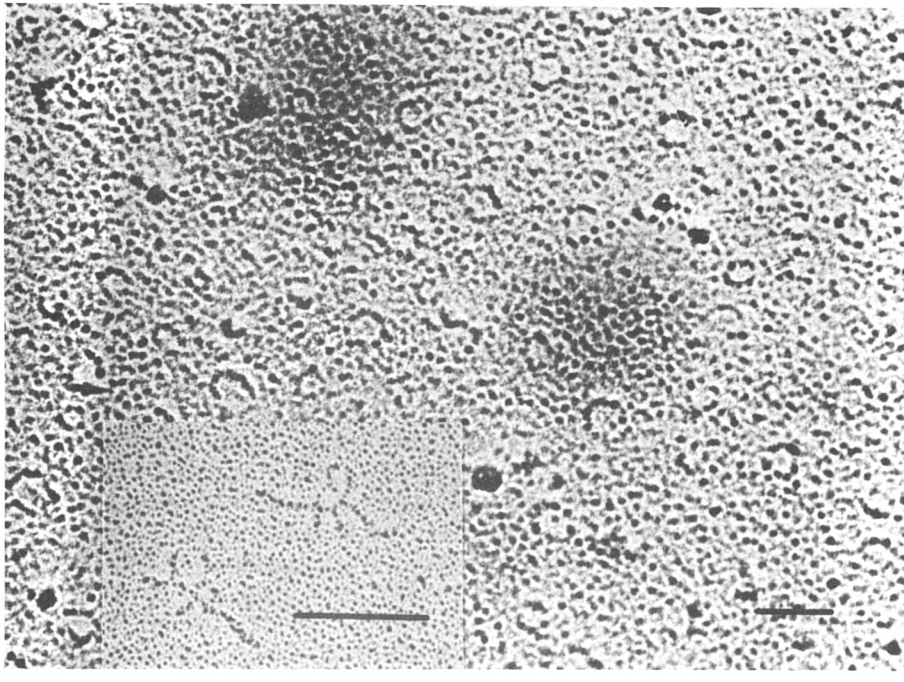


Fig. 6
Electron micrograph of bent molecules of DNA isolated from the postmicrosomal fraction of leukaemic myeloblast lysate
Unusual DNA structures also found in this fraction are shown in the insert. Bar = 0.1 μm.

microscopic data of minute replicative DNA structures, called microbubbles or small eyes present in DNA of sea urchin embryos in the third S-phase after fertilization (Baldari *et al.*, 1978).

Because of the small size of circular AMV DNA molecules it is difficult to decide whether the ends of DNA are covalently closed, forming a circle, or if there is a short, electron microscopically unidentified region, or a small indetectable gap. The small size circular molecules were also detected in the cytoplasmic postmicrosomal fraction of leukaemic myeloblast cell lysate (Fig. 6). Both the form and the size of these molecules were identical with those found in AMV DNA isolates from AMV cores. Besides circular DNA molecules, additional structures typical of replicating DNA molecules were also observed in the postmicrosomal fraction (Fig. 6, insert). The presence of circular DNA molecules in this fraction is strikingly reminiscent of the data published by Smith and Vinograd (1972) who found the "small polydisperse circular cytoplasmic DNA" in the postmicrosomal fraction of HeLa cells, the size of which ranged from 0.1 μm to 2.0 μm (Smith and Vinograd, 1972).

Chemical modification of AMV DNA

To prove that AMV DNA bending was directed by sequence properties of AMV DNA and did not reflect the interaction of these molecules with BAC and/or support film, the DNA was treated with distamycin prior to mounting for electron microscopy. Distamycin was shown by Wu and Crothers (1984) to abolish the anomalous electrophoretic behaviour of DNA containing a bent helix and to straighten these bent molecules (Griffith *et al.*, 1986). To examine the structural behaviour of 7 S AMV DNA in the presence of distamycin, DNA was incubated with the drug in a ratio of 1 molecule of distamycin to 6.7 and 4 nucleotides, respectively. Incubation of AMV DNA with distamycin at the 1:6.7 ratio resulted in straightening of almost 70 % of all observed molecules, while only 30 % remained slightly bent (Fig. 7). In control experiments without distamycin treatment, 65 % of the molecules were scored as highly bent. The amount of straight DNA molecules increased to about 80 % at drug/nucleotide ratio 1:4.

Distamycin specifically recognizes and binds oligo (dA)-oligo (dT) runs (Fox and Waring, 1984). It is known that the interaction of distamycin with SAR sequences results in complete suppression of binding to either scaffold or histone H1 and that these tracts play a direct role in mediating these specific interactions (Käs *et al.*, 1989). Moreover, distamycin is a potent inhibitor of transcription and replication. It is apparent that the specific structure of AT-rich stretches of DNA for these interactions is involved in the regulation of replication and transcription.

To characterize the structural properties of AMV in more detail, we analyzed the structural changes in the presence of osmium tetroxide. It is known that OsO_4, together with a suitable tertiary amine, forms stable complexes with pyrimidine bases and that it reacts specifically with single-stranded and distorted double-stranded regions in DNA (Paleček, 1992).

Incubation of DNA with different concentrations of OsO_4-pyridine under different conditions resulted in complete denaturation of double-stranded molecules, following the all-or-none rule. Either no changes in the double-stranded structure were apparent, or only single strands were found in DNA preparations. This phenomenon might be due to either the sequence properties of AMV DNA or its low molecular mass.

Length distribution of AMV DNA

Lengths of 7 S AMV DNA molecules were calculated from samples prepared by both adsorption and BAC spreading electron microscopy techniques using appropriate length standards.

AMV DNA prepared by direct adsorption on BAC-activated carbon film revealed rather heterogeneous length distribution profile in all three fractions, with preferential mean lengths of 0.209 ± 0.022 μm (F1), 0.051 ± 0.011 μm (F2) and 0.040 ± 0.008 μm (F3), respectively. These data are in good agreement with electrophoretic measurements (Říman *et al.*, 1993*b*). Besides the molecules of

the mean length, both shorter and longer DNA molecules were present in all three fractions. These molecules form special size classes which are multiples of the "monomer" length. This observation raises the question of possible degradation and/or association of AMV DNA molecules.

This possibility was tested in detail using the BAC spreading technique, which is known to act on DNA molecules by stretching forces. The length distribution profiles of DNA fractions F1, F2 and F3 prepared by this method are shown in Fig. 8. The same heterogeneity mentioned above at DNA analyzed by the adsorption method was also apparent when the BAC spreading technique was used. The presence of 6, 6 and 3 peaks was observed on the length distribution profiles of DNA fractions F1, F2 and F3, respectively. The quantitative evaluation of data obtained is presented in Table 1.

It is apparent that in fraction F3 83 % of the molecules corresponds to the length expected from electrophoretic data (137±47 bp). However, 11 and 3.5 % of the DNA molecules are apparently dimers and tetramers, respectively.

In fraction F2, the majority of DNA (44 %) represents the size which is in good agreement with electrophoretic measurements (363±70 bp), but both longer and shorter molecules are present in this fraction, 20 % of them being characterized by the length one half of size in the main peak (181±35 bp). On the other hand, longer molecules, which could be dimers and trimers (652±29 bp, 959±82 bp), are also present.

In fraction F1, only a minor part of DNA molecules falls in the length range expected from electrophoretic analysis (about 9 %). The majority of DNA is

Table 1. The quantitative evaluation of length distribution profiles shown in Fig. 8

Peak on the length distribution	F1		F2		F3	
	Mean length (bp)	% of molecules	Mean length (bp)	% of molecules	Mean length (bp)	% of molecules
1	178 ± 35	26.5	181 ± 35	20	137 ± 47	83
2	368 ± 79	42.5	363 ± 70	44	389 ± 64	11
3	587 ± 55	12	535 ± 41	7	625 ± 43	3.5
4	728 ± 47	10	652 ± 29	13.5		
5	912 ± 38	6	778 ± 20	6		
6	1155 ± 61	3	959 ± 82	9.5		
No. of molecules measured	162		118		195	

Per cent of molecules falling into individual size classes was calculated from the total number of molecules measured. pUC dsDNA (2688 bp) was included as a size standard.

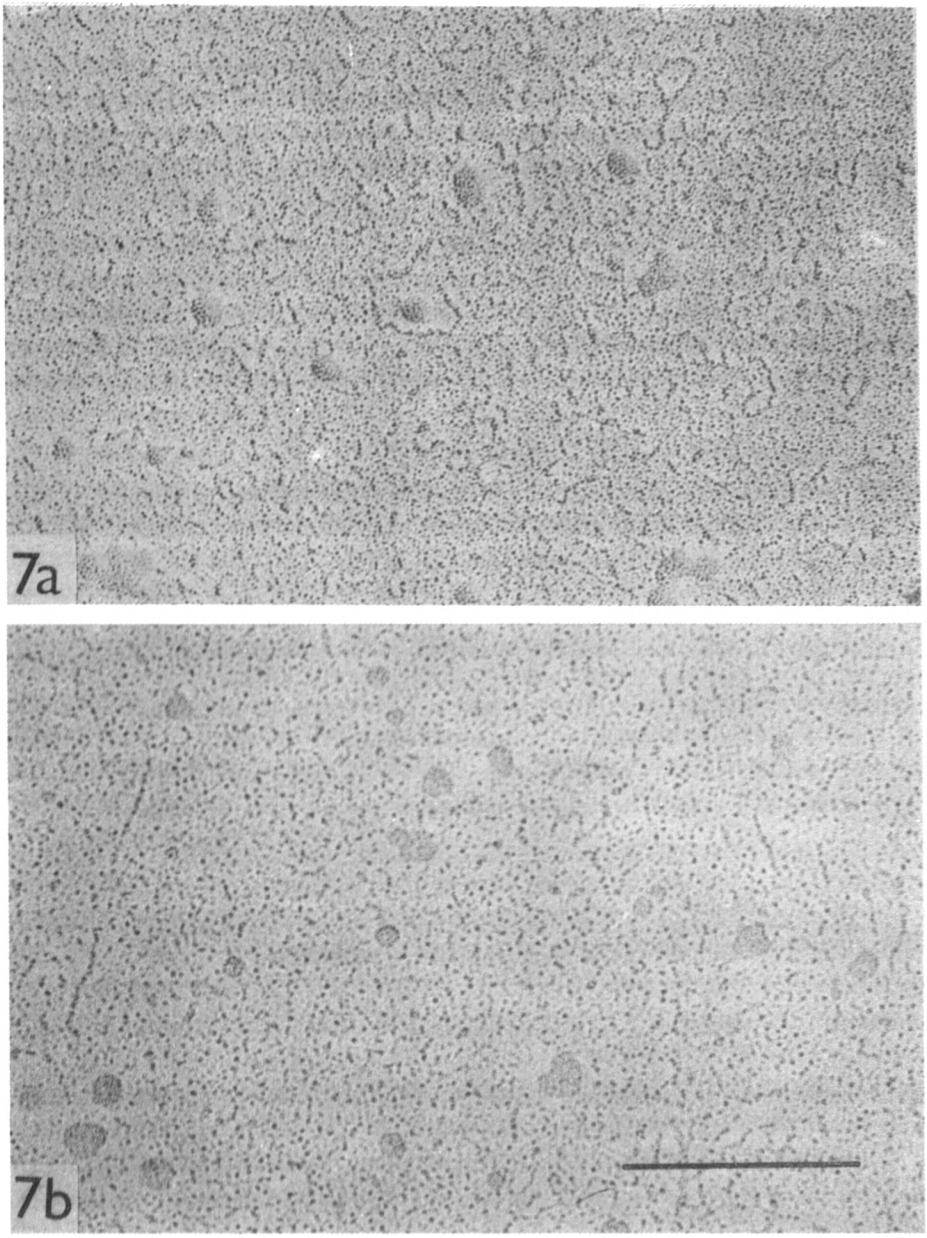
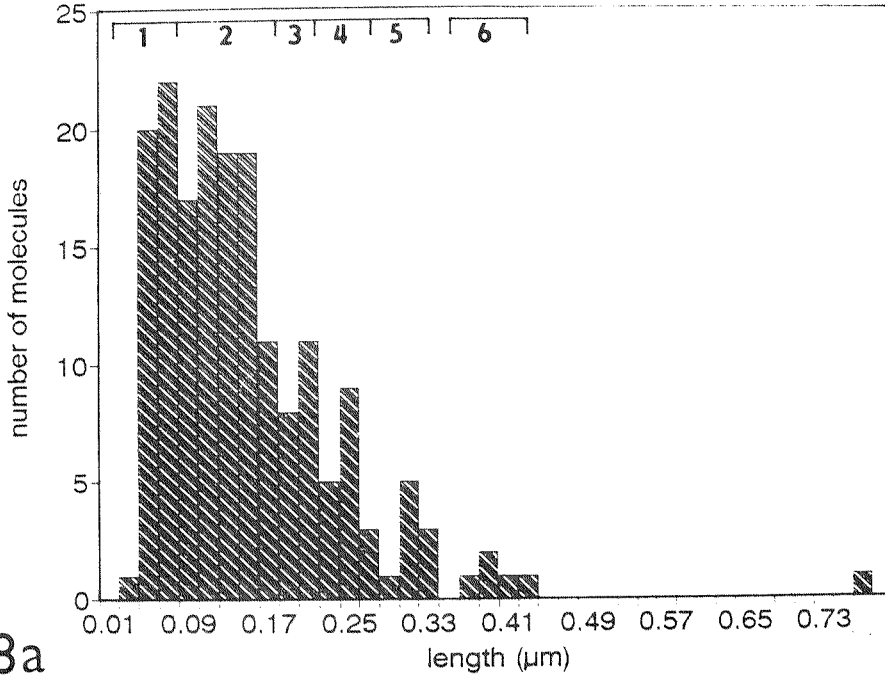


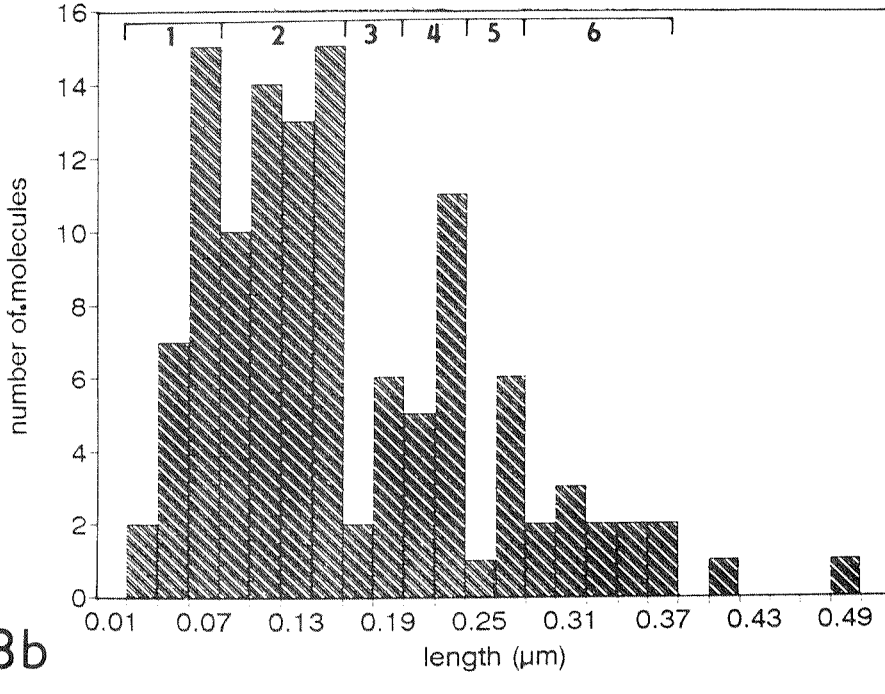
Fig. 7

Effect of distamycin treatment on the structure of bent AMV DNA

AMV DNA visualized by BAC spreading technique in the absence of distamycin (Fig. 7a) and in the presence of distamycin (ratio 1:6.7, Fig. 7b). Bar = 0.5 μm.


1
2
3
4
5
6
number of molecules
0
5
10
15
20
25
0.01
0.09
0.17
0.25
0.33
0.41
0.49
0.57
0.65
0.73
length (µm)

8a


1
2
3
4
5
6
number of molecules
0
2
4
6
8
10
12
14
16
0.01
0.07
0.13
0.19
0.25
0.31
0.37
0.43
0.49
length (µm)

8b

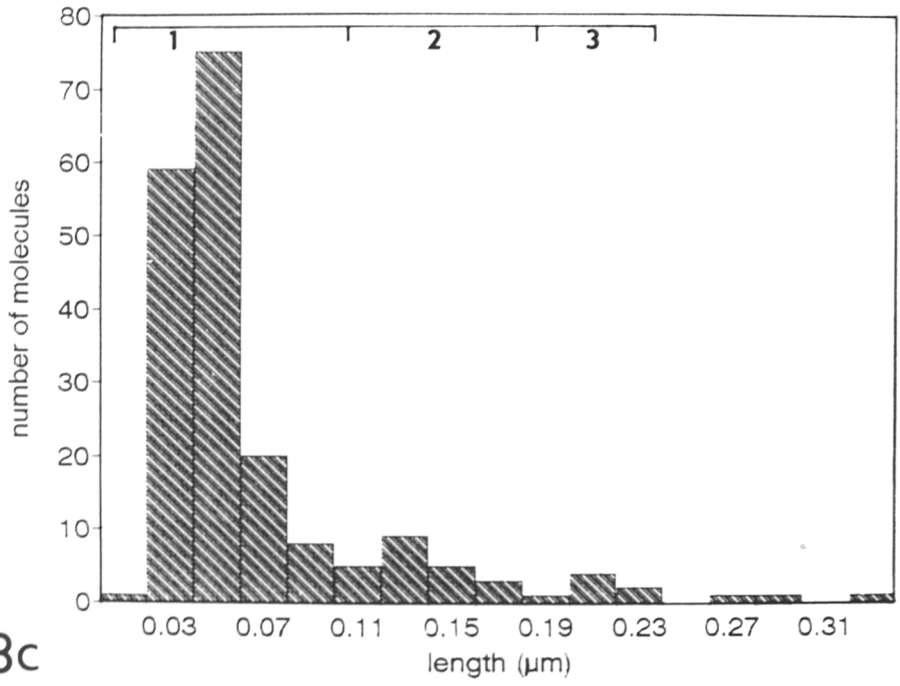


Fig. 8
Length distribution profile of AMV DNA visualized by BAC spreading technique
Fraction F1 (Fig. 8a), F2 (Fig. 8b) and F3 (Fig. 8c).

characterized by shorter molecules, the length of which is identical with those found in fractions F2 and F3.

From the results obtained we can conclude that in all three fractions F1 - F3 the DNA molecules represent multimers derived from the basic DNA size of about 150 bp (137±47 bp, 181±35 bp, 178±35 bp). One can imagine the formation of such multimers with DNA having cohesive ends. The existence of such DNA is quite possible. For example, in the case of AMV DNA, involvement of some enzymes such as viral endonuclease and topoisomerase I, both detected also in retroviruses (Grandgenett *et al.*, 1978; Weiss and Faras, 1981; Priel *et al.*, 1990), could result in the formation of such cohesive ends. Their existence was supported also by the observation of extremely effective labelling of AMV DNA using the Klenow fragment of DNA polymerase I (Říman *et al.*, 1993*b*).

Cohesive ends having sufficient complementarity could allow formation of observed multimers of AMV DNA. This might explain the presence of DNA molecules of higher size in fractions F2 and F3. On the other hand, sufficient but

not full complementarity of the ends could explain also the fragility and susceptibility of these regions to the preparative conditions, resulting in easy cleavage of observed multimers. This is in accord with the observed presence of shorter DNA molecules in fractions F1 and F2.

The data presented here are in accordance with the observation that the maximal length of denatured AMV DNA molecules is about 150 bp (Říman *et al.*, 1993*b*), the size of which falls in the range of single-stranded units of discontinuous DNA synthesis in eukaryotic cells (Huberman and Horovitz, 1973).

Electron microscopic analysis proved the bent structure of 7 S AMV DNA molecules which can form multimers, most probably due to the presence of cohesive ends.

In these studies the AMV DNA was isolated from virions produced by chicken leukaemic myeloblast *in vivo*, where virus pools are of a broader age range (up to 36 hrs) (Říman, 1965), with a minor portion of virions up to 7 hrs old. It has been previously shown that some specific virus age-dependent cleavage changes of AMV DNA occur *in vivo* inside the virus core structure (Říman *et al.*, 1993*b*). Analysis of structural changes of AMV DNA during the maturation of AMV virions is of interest and such structural studies are in progress.

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