NUCLEOPROTEIN COMPLEXES HARBORING AN EXTRACHROMOSOMAL DNA CLOSELY RELATED TO 7 S DNA OF AVIAN MYELOBLASTOSIS VIRUS: PHYSICO-CHEMICAL PROPERTIES AND REPRESENTATION OF NUCLEIC ACIDS

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Summary. – The source of avian myeloblastosis virus (AMV) DNA, an extrachromosomal small polydisperse DNA, present in the material forming the postmicrosomal sediment (POMS) of lysed chicken leukemic myeloblasts (CHLMs) is organized into nucleoprotein (NP) complexes containing always RNA. This material, radioactively double-labelled for DNA and RNA, separated in isopycnic sucrose gradients into three POMS components (A,B,C) differing from one another in properties of labelling for DNA and RNA, sucrose densities (1.21, 1.18 and 1.08 g/cm^3 for components A, B and C, respectively) and sedimentation properties of NP complexes which constituted the individual POMS components. The NP complexes present in representative fractions of POMS components A, B and C sedimented at 37.3 and 27.3, at 15.3 and 7.4, and at 11.3 and 5.5, respectively. They differed also in the length of DNAs they were harboring. Radioactively double-labelled nucleic acids (NAs) of POMS components A, B and C sedimented at 9, 7 and 3.5 S, respectively, and the sedimentation characteristics of both labels corresponded with those significant for replication intermediates. Electrophoretic characteristics of these NAs indicated that we dealt with DNA and RNA products of a lagging DNA strand synthesis (LSS) taking place, evidently, on pieces of the lagging sites of replicating DNA strands that were cut out at predilected sites by nucleases. As regards the origin of AMV DNA, we show that the major and minor portions of this DNA might be descending from NAs harbored in NP complexes of POMS components B and C, respectively, pointing out selectivity of segregation of this DNA from the cell into AMV core.

Key words: avian myeloblastosis virus DNA; extrachromosomal DNA; small polydisperse circular DNA; lagging strand synthesis; replication intermediates; postmicrosomal sediment

Abbreviations: AMV = avian myeloblastosis virus; ^{14}C-UR = [^{14}C]uridine; DB = digestion buffer; DNase = deoxyribonuclease; ^{3}H-mTdR = [methyl-^{3}H]thymidine; CHLM = chicken leukemic myeloblast; iRNA = initiator RNA; LB = lysis buffer; LSS = lagging DNA strand synthesis; NA = nucleic acid; NP = nucleoprotein; NP-40 = Nonidet P-40; PBS = phosphate-bufferred saline; PCA = perchloric acid; PMSF = phenylmethyl sulfonyl fluoride; pol = polymerase; POMS = postmicrosomal sediment; Pr = primase; spc = small polydisperse circular; RNase = ribonuclease; SDS = sodium dodecyl sulfate; Tris = tris-(hydroxymethyl)-aminomethane

Introduction

AMV virions contain constantly a 7 S DNA (AMV DNA) (Říman and Beaudreau, 1970) which is of host origin and is virus core-bound (Deeney *et al.*, 1976; Dvořák and Říman, 1980a). The nature of this DNA began to be recognized recently by the findings demonstrating that isolates of AMV DNA actually represent a collection of minute replicative structures intensely labelled for DNA and RNA (Říman *et al.*, 1993a,b), the majority of which reveals

a sequence-dependent bending up to circularization (Korb *et al.*, 1993). It has been shown recently that molecules of AMV DNA serve as endogenous templates of primase (Pr) and Pr-alpha DNA polymerase (pol) activities (As) which were found to be constantly present in AMV core isolates (Říman *et al.*, 1995). These findings indicate clearly that AMV DNA comes from the cell into the virus complexed with highly specialized cell enzymes significant for initiation of DNA synthesis (Roth, 1987). This opens, consequently, a number of questions about a possible participation of this host DNA and/or of special proteins associated with it in reactions accomplished by the virus core NP complexes possessing all components that are necessary for replication and integration of the retroviral information (Grandgenett and Mumm, 1990). For elucidation of this problem, it was necessary to know more about the properties of NP complexes into which this DNA is organized prior to its entering the virus core NP complex. Studies in this direction were facilitated by the following data relevant to the occurrence of AMV DNA in chicken leukemic myeloblasts (CHLMs). It has been shown (Korb *et al.*, 1993) that AMV DNA resembles micromorhologically an extrachromosomal small polydisperse highly bent (up to circularization) DNA. Such DNA, a small polydisperse circular (spc) DNA, was shown to be present in all growing animal cells (Rush and Misra, 1985). Accordingly, an extrachromosomal highly bent small polydisperse DNA including a portion of the spc DNA was constantly present in POMS of CHLMs (Korb *et al.*, 1993). Indeed, this DNA was found to be identical with AMV DNA electron microscopically (Korb *et al.*, 1993), in physico-chemical and hybridization properties (Dvořák and Říman, 1980b) and specific radioactivity (Říman *et al.*, 1993b). These findings have indicated that AMV DNA, an extrachromosomal small polydisperse DNA in nature, possesses its cytoplasmic pool in CHLMs from which it enters the virus core NP complexes (Dvořák and Říman, 1980b). This suggests, consequently, that AMV DNA and POMS DNA have a common descent. This seems to be the nuclear scaffold-bound DNA of CHLMs since this DNA has a homology significantly greater to the AMV DNA than has AMV DNA to the total CHLMs DNA (Říman *et al.*, 1993b). Thus, the data showing the presence of AMV DNA-like DNA in POMS of CHLMs allowed us to use this material double-labelled with [methyl-^{3}H]thymidine (^{3}H-mTdR) and [^{14}C]uridine (^{14}C-UR) (Říman *et al.*, 1993a) for studying the properties of NP complexes into which the AMV DNA-like POMS DNA is organized. Here, we show that this DNA is organized in several populations of NP complexes containing always DNA and RNA and constituting three basic components (designated further as A, B and C) of POMS material which differ from one another in the properties of labelling for DNA and RNA, sucrose densities and sedimentation properties. DNAs and RNAs synthesized during the period of labelling and harbored in NP complexes of all three POMS components have sedimentation and electrophoretic characteristics strongly reminiscent of replicative intermediates of an early LSS most advanced, advanced and beginning in NP complexes of POMS components A, B and C, respectively.

Here, we show that NAs most intensively labelled for DNA and RNA residing in NP complexes of POMS components B and C, respectively, but not those of NP complexes of POMS component A, represent candidates for the NAs of isolates of AMV DNA double-labelled and characterized in the same way (Říman *et al.*, 1993a,b). These findings indicate a distinct selectivity of segregation of AMV DNA organized into relevant NP complexes from the cell into the virus core.

Materials and Methods

Chemicals. Phenylmethylsulfonyl fluoride (PMSF) was from Serva, NuSieve GTG agarose was from FMC BioProducts, Rockland, USA, Nonidet P-40 (NP-40) was from LKB. All other chemicals were of highest analytical purity.

Radioisotopes. ^{3}H-mTdR (1.5 – 2.0 TBq/mmole) and ^{14}C-UR (14 – 17 GBq/mmole) were from ÚVVVR, Prague.

Enzymes. Deoxyribonuclease (DNase) I (ribonuclease-free) and ribonuclease (RNase) A pretreated according to Hirose *et al.* (1973) were from Worthington. Proteinase K from Merck was thermally pretreated (Maniatis *et al.*, 1982).

Buffers. Lysis buffer (LB): 0.01 mol/l Tris-HCl, 0.14 mol/l NaCl, 0.0015 mol/l $MgCl_2$, 0.0001 mol/l spermidine, 0.5% NP-40 pH-7.5. Tris buffers TE, TBE, TNE and phosphate-buffered saline (PBS) were prepared according to Maniatis *et al.* (1982). Digestion buffer (DB) with $MgCl_2$ and $CaCl_2$ was described earlier (Říman *et al.*, 1993b).

Cells. Blood of white Leghorn chickens with acute myeloblastic leukemia induced by AMV (Říman, 1964) was a source of leukemic myeloblasts (CHLMs) cultivated *in vitro* (Říman and Beaudreau, 1970).

Radioactive labelling. Cells (5 – 8 x 10^7/ml) in 60 – 80 ml of medium were radioactively double-labelled with ^{3}H-mTdR (1.4 MBq/ml) and ^{14}C-UR (94.7 kBq/ml) for two successive 7-hr periods (Říman *et al.*, 1993a). After each labelling period, cells were sedimented and washed with PBS. In the same way were treated the unlabelled CHLMs originating from the same cell stock and cultivated in parallel. Final cell sediments were stored at -70°C.

POMS of CHLMs. This material represented a portion of the cytoplasm of CHLMs void of cell organelles and ribosomes which contained AMV DNA-like DNA (Dvořák and Říman, 1980b). This material remained in a homogenous suspension in the 138,000 x g (1 hr, 4°C) supernatant of the cell lysate fractionated by centrifugation. From this supernatant it sedimented in the next centrifugation step accomplished at 273,000 x g (4 hrs, 4°C) and leading to the formation of the POMS.

POMS of CHLMs was isolated as follows: Cells radioactivelly labelled or unlabelled (5 – 8 x 10^7/ml) were suspended and lysed for 10 mins at 0°C in LB, diluted two-fold with the same buffer

but without NP-40 and centrifuged at 800 x g for 15 mins at 4°C. The resulting supernatant was centrifuged at 138,000 x g (1 hr at 4°C) in a Beckman SW 50.1 rotor. The supernatant resulting from this centrifugation step served for separation of the POMS material in the form of a homogenous suspension required for characterization of this material. Therefore, we separated the POMS material by centrifuging directly the aliquots of the 138,000 x g supernatant of the lysed CHLMs either in the equilibrium or velocity sucrose density gradients. In the equilibrium density gradient centrifugation, the supernatant aliquots were layered on linear 20 – 60% sucrose gradients supplemented with TNE and centrifuged at 147,000 x g for 21 hrs at 4°C.

In the velocity density gradient centrifugation, the supernatant aliqouts were layered on linear 10 – 30% sucrose gradients piled up on a 0.5 ml cushion of 60% sucrose. The centrifugation was accomplished in a Beckman SW 50.1 rotor at 147,000 x g for 6 hrs at 4°C. In parallel, the myeloblastic 28, 18 and 4 S RNA were run as S markers (Říman *et al.*, 1972), and their positions were used for estimating (Martin and Ames, 1961) the sedimentation properties of the POMS material. Fractions (210 µl) of sucrose gradients of both types were collected from the bottom of the tubes into calibrated Eppendorf tubes. Aliquots of each fraction were used for estimating ^{3}H-mTdR and ^{14}C-UR radioactivity incorporated during the labelling period into the acid-insoluble portion of the POMS material (Říman *et al.*, 1993a). The rest of each fraction served for other analytical purposes.

Estimation of DNA, RNA, acid-soluble compounds and protein content. The three density peak fractions (150 µl each) of the individual POMS components (A, B and C) were diluted with precooled LB void of NP-40 and supplemented with PMSF (final concentration 0.1 mmol/l) to the final volume of 4 ml and layered on a cushion (1 ml) of 15% sucrose. The POMS material present in these samples was sedimented at 122,000 x g for 21 hrs at 4°C in a SW 50.1 Beckman rotor. Sediments of the POMS material were then quantitatively extracted with perchloric acid (PCA) in successive steps of the Procedure 1 described by Leslie (1955). In the relevant fractions of this extraction procedure the protein was estimated according to Bradford (1976) while the acid-soluble compounds, RNA and DNA were estimated spectrophotometrically (Ogur and Rosen, 1950). The content of DNA was estimated also in parallel and with identical results by the procedure of Burton (1968).

Isolation of NAs. The three joint density peak fractions of the individual POMS components were dialyzed overnight at 0°C against LB void of NP-40. The dialyzed samples supplemented with sodium dodecyl sulfate (SDS, 0.5% end concentration) were treated with proteinase K according to Maniatis *et al.* (1982). NAs were then subjected to phenol-chloroform extraction and ethanol precipitation under conditions precipitating very small NA molecules (Singh *et al.*, 1986). NA precipitates collected by centrifugation were dried before use in vacuum and then solubilized in relevant buffers. For analytical purposes, these NAs were used native (untreated) or treated with nucleases. The treatment with RNase A (50 µg/ml) was accomplished in $\frac{1}{5}$ TE buffer for 15 mins at 37°C while that with DNase I (400 µg/ml) was performed in DB for 1 hr at 37°C.

Sedimentation analysis of NAs is described in the legend to Fig. 5.

Agarose gel electrophoresis of NAs. Samples of NAs (2 µg) were run in 4% NuSieve agarose gels in TBE buffer supplemented with ethidium bromide (Maniatis *et al.*, 1982) for 60 mins at 15 V/cm and at room temperature. A set of *Alu*I-pBR322 marker fragments (960, 657(2x), 521, 403, 281, 257, 226, 100, 97, 57, 49, 46, 18, 15 and 11 bp) was run in parallel and their gel positions were detected by fluorescence in UV-light. To determine the gel distribution of the ^{3}H- and ^{14}C-radioactivity, the gels were cut into 2-mm slices which were solubilized at 65°C and extracted with a phenol-chloroform mixture. NAs separated into the water phase were then precipitated by conventional TCA technique and collected on Synpor No. 6 filters used for radioactivity determination (Říman *et al.*, 1993a).

Results and Discussion

Sucrose densities of the material forming the POMS

Separation of the POMS material double-labelled with ^{3}H-mTdR and ^{14}C-UR in equilibrium sucrose density gradients revealed three components (POMS components A, B and C) differing from one another by the extent of labelling for DNA or RNA. The most prominent labelling for DNA exhibited component B with ^{3}H-mTdR radioactivity peak at a sucrose density of 1.18 g/cm^{3} while components A and C showed distinct labelling for RNA with peaks of ^{14}C-UR radioactivity at sucrose densities of 1.21 and 1.108 g/cm^{3}, respectively (Fig. 1). Closely similar characteristics were obtained with POMS material isolated from different stocks of CHLMs grown in tissue culture, as will be presented elsewhere (Říman and Šulová, 1997b). In conclusion, the distinct differences in the radioactive labelling for DNA and RNA and the gradual decrease of the sucrose density from the POMS component A through B to C (with the sucrose densities of 1.21, 1.18 and 1.108 g/cm^{3}, respectively) indicate that the individual POMS components consist of populations of NP complexes differing accordingly in their NA and protein content as well as in DNA- and RNA-synthesizing activities. These activities are responsible for their properties of radioactive labelling, as will be shown elsewhere (Říman and Šulová, 1997b).

DNA, RNA and protein content of POMS components

To evaluate directly the NP nature of the POMS material, we estimated the content of DNA, RNA and protein in the radioactivity peak fractions of POMS components A, B and C. The mutual proportions of the DNA and RNA quantitatively extracted from the relevant gradient fractions of the individual components are depicted in Fig. 2. The components A and C were rich in RNA while the component B was richest in DNA. The figure also shows that NP complexes constituting the individual components possessed their constant pools of acid-soluble compounds absorbing at 260 nm. This property may reflect, besides nucleotides and nucleosides, very short oligoribonucleotides present in extracts

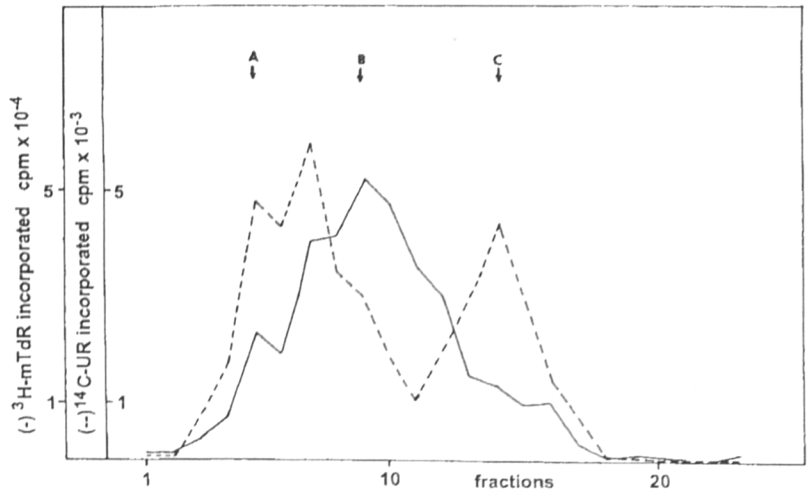

Fig. 1

Characteristics of POMS material of CHLMs double-labelled for DNA and RNA and separated by isopycnic sucrose density gradient centrifugation

Centrifugation was accomplished at 147,000 x g for 21 hrs at 4°C in a linear 60 – 20% sucrose density gradient. Vertical arrows A, B and C indicate the positions of sucrose densities of 1.21 (component A), 1.18 (component B) and 1.108 g/cm^3 (component C), respectively.

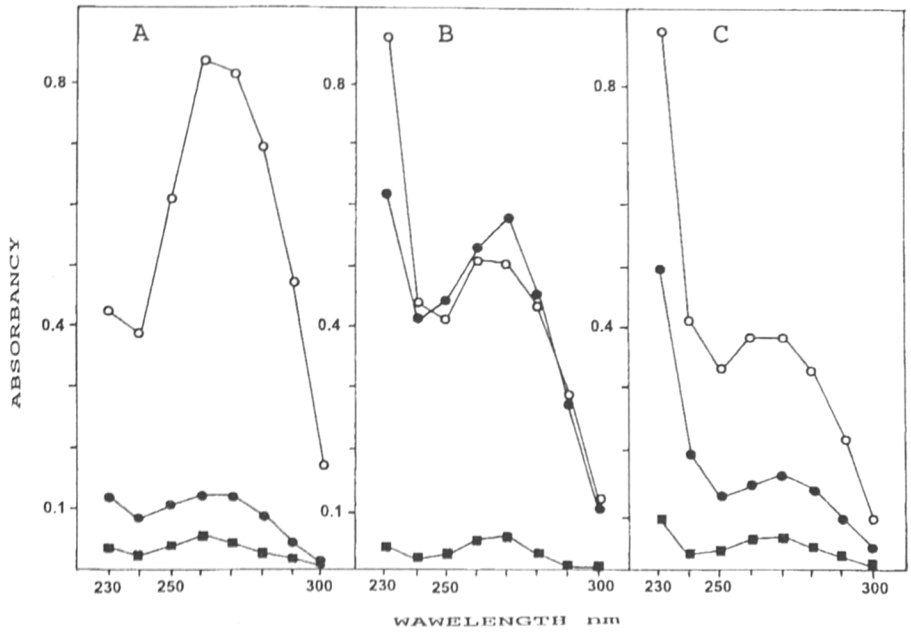

Fig. 2

Mutually comparable contents of DNA, RNA and acid-soluble compounds of NP complexes present in representative fractions of POMS components A, B and C

DNA (●), RNA (o) and acid-soluble compounds (■) absorbing at 260 nm were extracted quantitatively from NP complexes present in the sucrose density (radioactivity) peak fractions of POMS components A, B and C separated from 5.46 x 10^8 CHLMs by isopycnic sucrose density gradient centrifugation (Table 1).

of NAs of this material, as will be shown below. The actual content of DNA, RNA and protein in the relevant gradient fractions of all three POMS components is presented in Table 1. As evident, the NP complexes constituting the individual components differed in the content of NAs and protein. The differences were reflected in the different values of the NA/protein ratio signifi-

Table 1. Content of DNA, RNA, acid-soluble compounds and protein in the peak fractions of POMS components A, B and C

POMS components	Sucrose density g/cm³(%)	DNA (μg)	RNA (μg)	Acid-soluble compounds (μg)	Protein (μg)	NAs (μg)/protein (μg)
A	1.219 (49.5)	2.79 ± 0.21	16.2 ± 0.63	0.085 ± 0.002	16.21 ± 0.94	1.16
B	1.180 (42.0)	12.40 ± 0.56	9.08 ± 0.46	0.085 ± 0.002	45.50 ± 2.86	0.49
C	1.108 (26.5)	3.28 ± 0.69	7.40 ± 0.42	0.099 ± 0.003	84.76 ± 8.32	0.12

POMS components A, B and C were separated from 5.46 x 10^8 CHLMs on an equilibrium sucrose density gradient. Values of DNA, RNA, acid-soluble compounds and protein content in μg in NP complexes present in sucrose density peak fractions of the individual POMS components are mean values obtained from four estimations with relevant standard deviations.

cant for NP complexes of the individual components. Accordingly, the trend of the decrease of these values from component A through B to C was analogous to that reflected at the level of sucrose density.

Sedimentation properties of NP complexes of POMS components

The radioactivity peak fractions of sucrose density of 1.21, 1.18 and 1.108 g/cm³ of the double-labelled POMS components A, B and C, respectively, separated in equilibrium sucrose density gradients were dialyzed and analyzed in velocity sucrose density gradients. The characteristics obtained in this way and presented in Fig. 3 show that the material of the selected fractions of the components A, B and C was represented by the NP complexes sedimenting at 37.3 and 27.3 (Fig. 3A), at 15.3 and 7.4 (Fig. 3B), and at 11.3 and 5.5 S (Fig. 3C), respectively. As regards the labelling properties, a striking ^{3}H-mTdR radioactivity exceeding by one order of magnitude that of sedimentation species of NP complexes present in the selected fractions of the components A and C, was significant for NP complexes of the component B which sedimented at 15.3 S (Fig. 3B). These findings, in addition to the data previously obtained on a close similarity of specific ^{3}H-mTdR radioactivity of AMV DNA and POMS DNA (Říman *et al.*, 1993b), indicate that NP complexes of POMS component B that sediment at 15.3 S harbor the NAs with the properties of AMV DNA. The radioactive labelling for RNA, in general, was significant for NP complexes of the selected fractions of the components A and C. NP complexes most strongly labelled for RNA sedimented at 37.3 and 5.5 S and resided in the components A (Fig. 3A) and C (Fig. 3C), respectively.

Sedimentation characteristic of the total POMS material

To complement the data obtained on sedimentation properties of the NP complexes present in the selected fractions of POMS components separated in equilibrium sucrose density gradients, we analyzed directly in velocity sucrose density gradients the to-

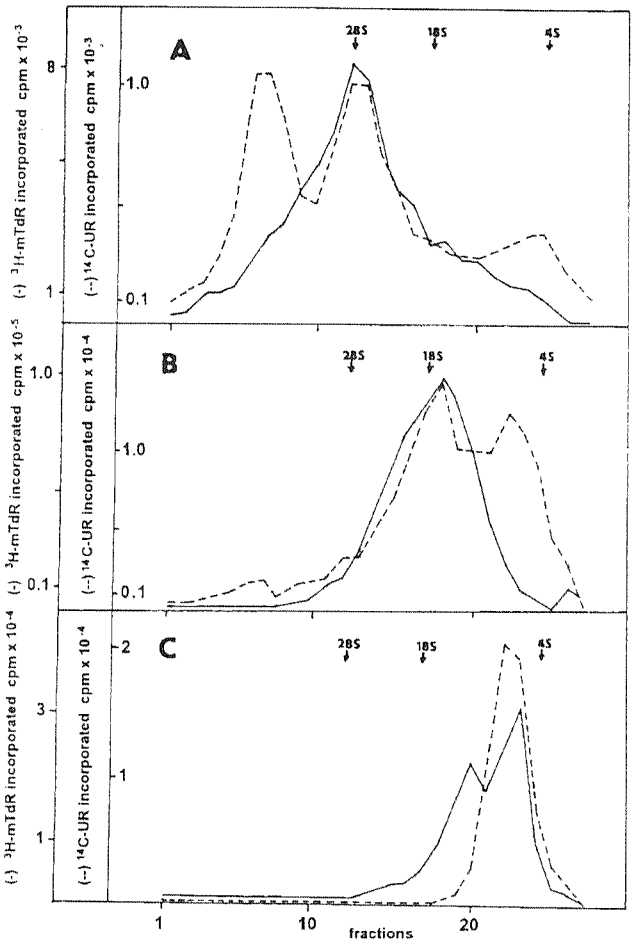

Fig. 3
Sedimentation characteristics of NP complexes present in representative fractions of individual POMS components radioactively double-labelled for DNA and RNA

Sedimentation profiles of ^{3}H-mTdR- and ^{14}C-UR-radioactivity of NP complexes present in the sucrose density (radioactivity) peak fractions of POMS components A, B and C. Velocity linear 30 – 10% sucrose density gradient centrifugation at 147,000 x g for 6 hrs at 4°C. Vertical arrows denote the S positions of the myeloblastic rRNA and tRNA run in parallel. The S values of NP complexes present in the representative fractions of individual POMS components are given in the text.

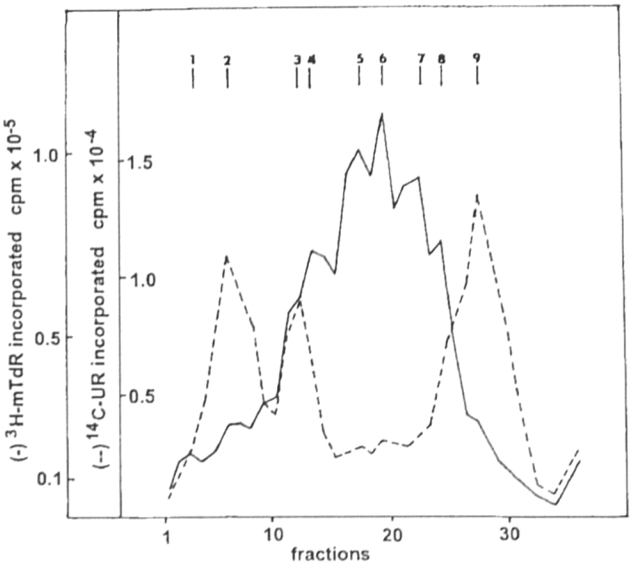

Fig. 4
Sedimentation characteristics of total POMS material double-labelled for DNA and RNA

Sedimentation profiles of ^{3}H-mTdR and ^{14}C-UR-radioactivity of total POMS material centrifuged at 147,000 x g for 6 hrs at 4°C in a linear 30 – 10% sucrose density gradient piled up on a cushion (0.5 ml) of 60% sucrose. Numbered vertical bars denote in parentheses the following S positions: 1 (48 S), 2 (37.8 S), 3 (28 S), 4 (25 S), 5 (19 S), 6 (15.3 S), 7 (13 S), 8 (11 S), 9 (7.4 S).

tal POMS material double-labelled with ^{3}H-mTdR and ^{14}C-UR as before. To detect a possible presence of components sedimenting at S values higher than 50, the linear (30 – 10%) sucrose gradient was piled up on a cushion of 60% sucrose.

Thus the material sedimenting at S values higher than those recorded previously for the material present in the selected fractions of the POMS components could be arrested at the cushion-gradient boundary. The sedimentation characteristics of the total POMS material obtained in this way (Fig. 4) show that this material contained several more populations of NP complexes sedimenting at 25, 19 and 13 S that were more distinctly labelled for DNA than for RNA. According to the labelling properties, these populations of NP complexes seem to belong to the family of NP complexes forming the POMS component B, the most prominent population of NP complexes, of which the maximally labelled one for DNA sedimented at 15.3 S. According to the lowest radioactivity located at the cushion-gradient boundary (Fig. 4), these sedimentation characteristics also show that the total POMS material was void of CHLMs ribosomes sedimenting at 80 S (Říman *et al.*, 1972) as well as of virus core NP complexes sedimenting at about 100 S (Grandgenett and Mumm, 1990). Retrovirus core structures also differ markedly from NP complexes of POMS material by much higher sucrose densities ranging from 1.23 to 1.25 g/cm^{3} (Grandgenett *et al.*, 1978). Finally, the sedimentation characteristics of the total POMS material (Fig. 4) compared with that obtained by equilibrium density gradient centrifugation (Fig. 1) pointed out a consonant correlation between sedimentation and density properties of these NP complexes. This phenomenon reflected in a close similarity of the distribution of both radioactivities of the POMS material in velocity and equilibrium sucrose density gradient centrifugations cannot be ex-

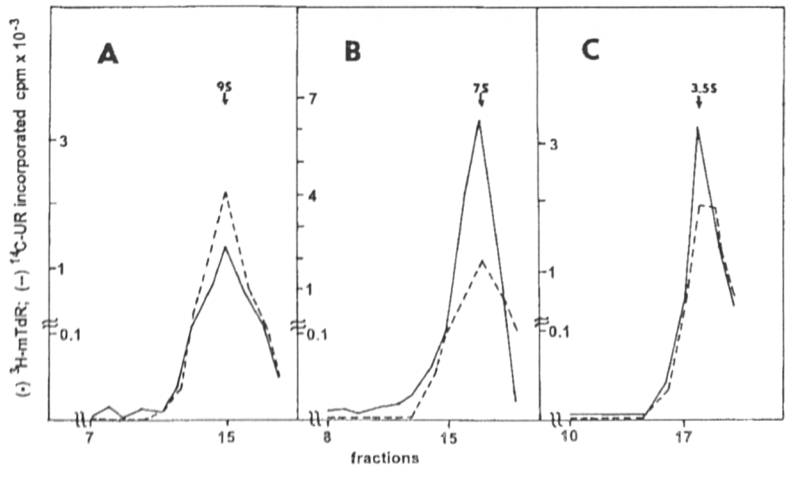

Fig. 5
Sedimentation characteristics of NAs of NP complexes radioactively double-labelled for DNA and RNA present in representative fractions of POMS components A, B and C

Sedimentation profiles of ^{3}H-mTdR and ^{14}C-UR-radioactivity of NAs isolated from NP complexes present in the sucrose density (radioactivity) peak fractions of POMS components A, B and C. NA samples were centrifuged in linear 40 – 10% glycerol density gradients at 147,000 x g for 280 mins at 4°C.

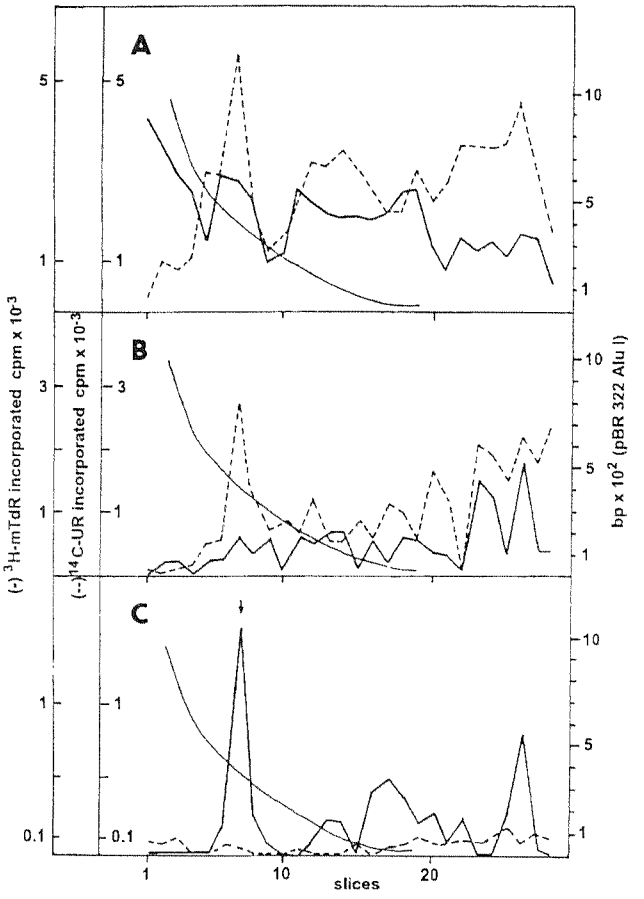

Fig. 6
Electrophoretic characteristics of NAs radioactively double-labelled with ^{3}H-mTdR and ^{14}C-UR isolated from a representative fraction of POMS component A

Gel distribution of ^{3}H-mTdR and ^{14}C-UR-radioactivity of NAs isolated from the sucrose density (radioactivity) peak fraction of POMS component A: native (A), DNase I- (B) and RNase A-treated (C) NAs. The vertical arrow in the compartment C indicates the position of the most prominent labelled DNA species which was in this case 250 bp in length. Continuous line represents the characteristics of the gel distribution of DNAs from 960 to 10 bp in length constructed using the positions of DNA fragments of the *Alu*I-pBR322 marker set.

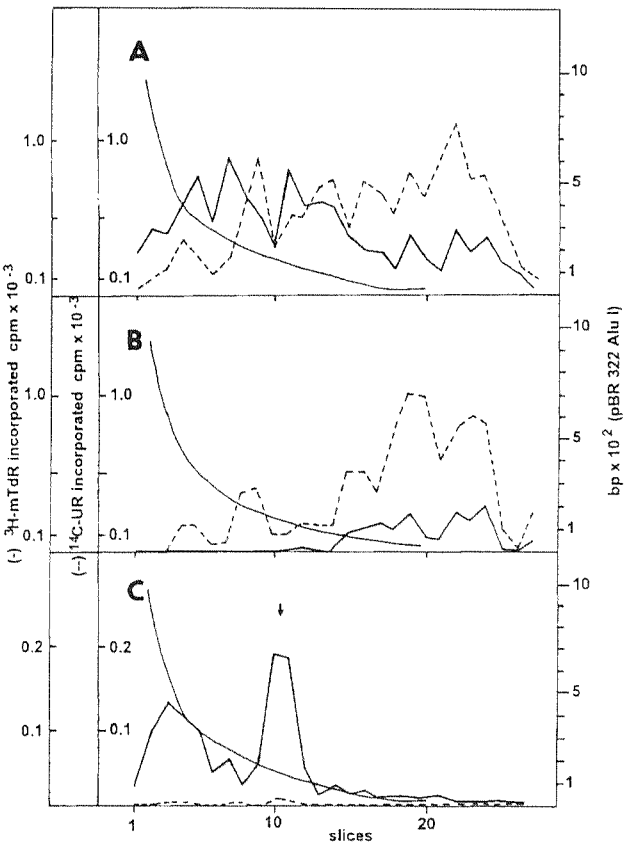

Fig. 7
Electrophoretic characteristics of NAs radioactively double-labelled with ^{3}H-mTdR and ^{14}C-UR isolated from a representative fraction of POMS component B

Gel distribution of ^{3}H-mTdR and ^{14}C-UR-radioactivity of NAs isolated from the sucrose density (radioactivity) peak fraction of POMS component B: native (A), DNase I- (B) and RNase A-treated (C) NAs. The vertical arrow in the compartment C indicates the position of the most prominent labelled DNA species which was in this case 150 bp in length. Continuous line – see the legend to Fig. 6.

plained simply by gradual changes in the NA/protein ratio recorded from POMS component A through B to C (see Table 1) since, in general, the sedimentation and density properties of the NP complexes were not directly mutually dependent or related. Consequently, in addition to the NA/protein ratio, other factors must contribute to this interesting property of NP complexes of POMS material. As will be shown below, the individual POMS components differed gradually (from A through B to C) in the length of DNAs they harbor and, accordingly, also in the extent of the equipment with enzymes significant for an early LSS (Říman and Šulová, 1997b). Thus, the DNAs harbored in the individual POMS components may reveal (from the component A through B to C) gradual changes in their organization into NP complexes reflecting changes in the multiple DNA-protein and protein-protein interactions significant for NP complexes endowed with early replicative events (Echols, 1986). Indeed, the NP complexes of all three POMS components are micromorphologically strongly reminiscent of NP complexes endowed with early replicative events (J. Korb and J. Štokrová, personal communications).

Double-labelled NAs of POMS components: sedimentation properties

Differences in radioactive labelling for DNA and RNA of the individual POMS components led us to characterize their

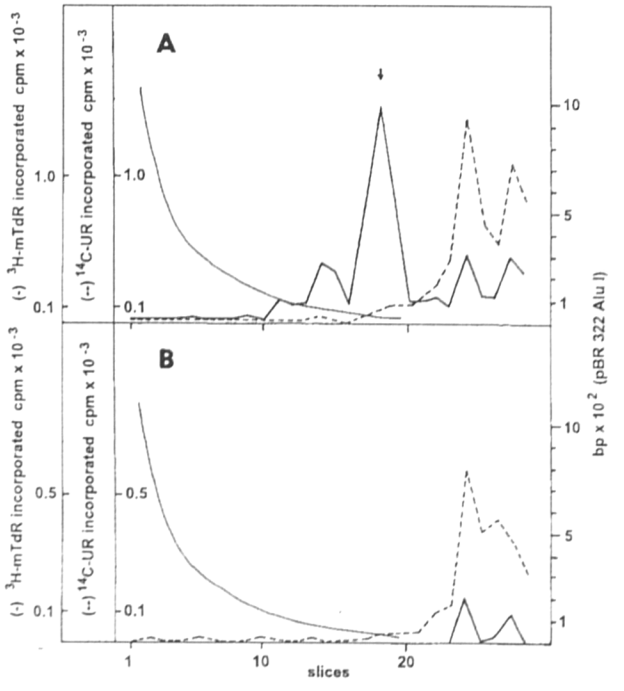

Fig. 8
Electrophoretic characteristics of NAs radioactively double-labelled with ^{3}H-mTdR and ^{14}C-UR isolated from a representative fraction of POMS component C
Gel distribution of ^{3}H-mTdR and ^{14}C-UR-radioactivity of NAs isolated from the sucrose density (radioactivity) peak fraction of POMS component C: native (A) and DNase I-treated (B) NAs. The vertical arrow in the compartment A indicates the most prominent labelled DNA species which was in this case 40 bp in length. Continuous line – see the legend to Fig. 6.

NAs synthesized during the labelling period. For these purposes, we analyzed by velocity glycerol density gradient centrifugation the NAs double-labelled for DNA and RNA and isolated from radioactivity peak fractions of the individual POMS components. Sedimentation characteristics of the NAs of the components A, B and C obtained in this way are presented in Fig. 5. In general, the labelled NAs of all three components were small in size. The gradient distribution of both their radioactive labels exhibited closely parallel sedimentation profiles, indicating a firm association of RNA with DNA with common peaks of ^{14}C-UR and ^{3}H-mTdR radioactivity at 9, 7 and 3.5 S in the NAs of the components A, B and C, respectively. The sedimentation characteristics of the NAs of all three components were strongly reminiscent of those of the double-labelled intermediates of DNA synthesis accomplished by the subnuclear systems of HeLa cells (Brun and Weissbach, 1978). A gradual decrease in the size of the NAs from the component A through B to C reflected the descent of these NAs harbored in NP complexes of different S values present in the individual POMS components (Fig. 3). The highest ^{3}H-mTdR radioactivity was found in the NAs of the component B containing NP complexes that sedimented at 15.3 S and were maximally labelled for DNA. Consequently, the double-labelled NAs originating from the 15.3 S NP complexes of the POMS material sedimented at 7 S like the double-labelled AMV DNA (Říman *et al.*, 1993a).

Double-labelled NAs of POMS components: electrophoretic properties

The double-labelled NAs of the individual POMS components were characterized in more detail by gel electrophoresis of the portions of the same samples of NAs used previously for determining the sedimentation characteristics. Electrophoresis in NuSieve agarose gels in contrast to polyacrylamide gels made it possible to extract easily (Maniatis *et al.*, 1982) and to determine without substantial quenching the gel distribution of both radioactivities. Moreover, this type of gel analysis was suitable especially for DNAs which were in the center of our interest. The electrophoretic mobilities of DNAs in agarose gels are not influenced by sequence properties significant for early replicative DNA structures (Amati and Gasser, 1988; Caddle *et al.*, 1990) and for the DNAs similar in nature like the AMV DNA (Říman *et al.*, 1993b) and, consequently, as may be expected for the DNAs harbored in NP complexes forming the POMS material. To make these characteristics oriented in the first place on DNA clearly legible, we analyzed comparatively always native (untreated), RNase A- and DNase I-treated NA samples isolated from all three individual components. The length of the radioactively labelled POMS DNAs was evaluated comparatively from the gel positions of the incorporated ^{3}H-mTdR radioactivity and from those of DNA length markers run in parallel. The positions of the radioactive RNA label in agarose gels cannot be converted into length of the respective RNAs unless analyzed under denaturing conditions. In order to determine these characteristics, we evaluated in this case the size of the labelled RNAs approximately only, using the terms "very short" (up to about 15 b), "short" (up to about 80 b) and "medium" (up to about 600 b) size RNAs. For such an evaluation we used the data from analysis of radioactively double-labelled AMV DNA (Říman *et al.*, 1993b) and ^{32}P-labelled products of the NA-synthesizing activities of POMS components analyzed under denaturing conditions (Říman and Šulová, 1997c), as well as the data on sedimentation properties of ^{14}C-UR radioactivity of these NAs presented above.

The characteristics of NAs of the components A, B and C obtained by this analysis are presented in Figs. 6, 7 and 8. Compartments A and B of these figures represent the characteristics of the NAs native (untreated) and treated with DNase I, respectively. Compartments C in Figs. 6 and 7 represent the characteristics of RNase A-treated NAs of the

components A and B, respectively. Compartment C in Fig. 8, depicting the characteristics of NAs of POMS component C, is omitted, because the treatment with RNase A did not change the basic characteristics of DNAs. They remained closely similar to those of native NAs of the component C, with only "very short" RNAs. Electrophoretic characteristics obtained in this way led to the following findings. A comparison of the gel distribution of ^{3}H-mTdR and ^{14}C-UR radioactivity of native NAs of the components A, B and C (Figs. 6A, 7A and 8A) revealed a gradual shift of both radioactive labels to positions of NAs smaller in size, taking place from the component A to B and, finally, to C. Its labelled DNAs and RNAs were found to be shortest in length. A more accurate evaluation of these changes was made possible by electrophoretic characterization accomplished with the same NA samples but treated before analysis with RNase A or DNase I. These treatments, besides direct testing the RNA or DNA nature of the respective radioactive labels, led to the detection of DNA and RNA species, which constituted the NAs of the individual POMS components. The treatment of NAs of the component A with RNase A disclosed the presence of about six DNA species (Fig. 6C) of 40, 60 – 80, 100 – 150, 250 and maximally to about 500 bp in length. But the most prominent of these DNAs was a DNA of 250 bp. The treatment of NAs of the component B with RNase A disclosed about five DNA species (Fig. 7C) ranging from about 40 to maximally 300 bp. The most prominent was a DNA of 150 bp. By the length and magnitude of its ^{3}H-mTdR radioactivity this DNA was closely similar to AMV DNA synthesized during the same labelling period (Říman *et al.*, 1993b). The NAs of the component C contained about six radioactively labelled DNA species ranging up to about 100 bp with the most prominent DNA species of about 40 bp. These unique characteristics of the representation of radioactively labelled DNAs were already clearly visible in the electrophoretogram of native NAs of the component C (Fig. 8A) and the RNase A treatment did not change this picture (data not shown). By its length the most prominent labelled DNA of this component was reminiscent of the DNAs of 40 – 50 bp which represented a constant minor portion of the molecules of AMV DNA radioactively labelled under the same conditions (Říman *et al.*, 1993b). Accordingly, the DNAs of the components B and C residing in NP complexes sedimenting at 15.3 and 5.5 S, respectively, may participate in the constitution of the virus core-bound AMV DNA. The absence of DNAs significant by length for the component A in virions of AMV suggests that segregation of the AMV DNA from the POMS material into virions is a selective process.

In the case of the native NAs of all three components, the gel distribution of the radioactive RNA label always revealed the presence of "very short" RNAs. In the native NAs of the components A and B, these RNAs represented about one third of the total radioactive RNA label (Figs. 6A and 7A) while in the native NAs of the component C the radioactive RNA label was localized mainly in the zone of "very short" RNAs only (Fig. 8A). A more instructive characteristic of RNAs in NAs of POMS components synthesized together with DNAs during the same labelling period was obtained by analysis of these NAs treated with DNase I. The treatment led, in general, to a decrease of ^{14}C-UR radioactivity in the electrophoretic mobility zone of DNAs 40 – 200 bp in length and simultaneously to an increase of this radioactivity in the zone of "very short" RNAs (compare Figs. 6A, 6B with Figs. 7A, 7B). This phenomenon may suggest the presence of RNA-DNA molecules of Okazaki fragment type from which the "very short" RNAs are liberated by DNase I treatment. This suggestion is supported by the occurrence of Okazaki fragments in isolates of AMV DNA (Říman *et al.*, 1993a) which originates from DNA of POMS DNA as indicated previously (Dvořák and Říman, 1980b) and in this paper. Moreover, the DNase I treatment of NAs of POMS components A and B disclosed, in addition to the "very short" RNAs, the presence of several RNA species with distinct electrophoretic mobilities. In both these cases (Fig. 6B and 7B), there were present several species of "short" RNAs (up to about 80 b). Their gel assortment makes together with the "very short" RNAs a staircase-like picture reminiscent of a ladder-like gel assortment of initiator RNAs (iRNAs) (Reichard *et al.*, 1974) and their multimers (Chang *et al.*, 1984) produced under certain reaction conditions by activities of a Pr-alpha DNA pol complex (Singh *et al.*, 1986). Besides the RNAs common to NAs of the components A and B, the NAs of the component A contained an RNA species localized in the electrophoretograms of native (Fig. 6A) or DNase I-treated (Fig. 6B) NAs at gel position of an RNA approximately 600 b in length (position of a DNA of about 300 bp). This RNA evidently not associated with DNA seems to be produced by enzymatic activities different from those responsible for synthesis of the "very short" and "short" RNAs. Interestingly, as will be shown elsewhere (Říman and Šulová, 1997b), in contrast to the components B and C, the component A contained besides PrAs also other types of RNA synthesizing activities. The latter are inhibited by alpha-amanitin at a concentration inhibiting RNA polymerase II (Manley, 1987). Consequently, such activities might be responsible for the occurrence of this RNA species in the component A. The results presented in this Section complement well the sedimentation characteristics of the NAs shown in Fig. 5. Here, an electrophoretic analysis revealed that the double-labelled NAs of all three components actually represented intermediates of three successive stages of an early DNA synthesis. Moreover, the data on the electrophoretic properties of the radioactively labelled

DNAs and RNAs of all three components suggest that the DNA synthesis takes place on pieces of the lagging sites of DNA strands and that the pieces of DNA strands harbored in NP complexes of the relevant POMS components are of three length classes, each of which is significant for one of the three individual POMS components. The length of the pieces of strands serving as templates for synthesis of these NAs seems to limit the extent and progress of the DNA synthesis. The most prominent DNA products were DNAs 40, 150 and 250 bp in length in NAs of NP complexes forming the POMS components C, B and A, respectively. In addition, the most prominent DNA species in the labelled NAs of NP complexes forming the POMS component C is by its length of 40 bp strongly reminiscent of the "DNA primer", the Okazaki fragment precursor (Nethanel *et al.*, 1988) that is synthesized on and associated with a piece of the lagging site of DNA strand of an appropriate length of about 100 b minimally. In this case (Fig. 8A), DNA synthesis advanced minimally in comparison to that of NAs in NP complexes of the components B and A, the most prominent DNA species of which were 150 and 250 bp, respectively. By the length these DNAs are reminiscent of the growing and mature Okazaki fragments formed from the building blocks of "DNA primers" by epsilon DNA polymerase activities (Nethanel and Kaufmann, 1990; Bambara and Huang, 1995). In accord with the suggestion, that in the case of the radioactively labelled NAs of the POMS material we deal with products of LSS, seems to be also the nature of the radioactively labelled RNAs. These RNAs, especially the "very short" RNAs reminiscent of iRNAs, indicate the presence of Pr- and Pr-alpha DNA pol As significant for initiation of LSS (Nethanel *et al.*, 1988). A direct confirmation of this suggestion will be presented elsewhere (Říman and Šulová, 1977b).

Concluding remarks

The findings presented in this paper indicate for the first time that an extrachromosomal DNA of growing animal cells may represent pieces of strands of replicating chromosomal DNA associated with RNA and DNA products of LSS and that these special NAs are harbored in NP complexes with distinct physico-chemical properties. The length classes of extrachromosomal DNA have been previously found also in other growing animal cells (DeLap and Rush, 1978; Bertelsen *et al.*, 1982; Tsuda *et al.*, 1983). A question remains whether these DNAs are similar in nature to the extrachromosomal DNAs of POMS material of CHLMs including the organization of these DNAs into similar NP complexes. Several mechanisms of the formation of extrachromosomal DNAs, especially spe DNAs, have been proposed: homologous or non-homologous recombination (Kiyama *et al.*, 1987), products of reverse transcription (Krolewski and Rush, 1984), products of reconstruction of immunoglobulin (Toda *et al.*, 1989) and T-cell receptor genes (Iwassato *et al.*, 1990), or processes splitting out the repetitive sequences (Kim and Wang, 1989). But all these modes of the formatin of an extrachromosomal DNA seem to represent special cases only, not the regular appearance of the small polydisperse DNAs in growing animal cells evidently dependent on growth conditions as demonstrated for the first time by Smith and Vinograd (1972). We believe that the occurrence of extrachromosomal small polydisperse DNA of CHLMs harbored in NP complexes of POMS material is due to nuclease activities cuting out pieces of three length classes at predilected nuclease-sensitive loci of the lagging sites of DNA strands of some early replicating chromosomal DNAs. A gradual increase from about 40 to 150 and 250 bp in length of the radioactively labelled DNAs from POMS component C to B and A, respectively, suggests that the nuclease activities are cutting out the pieces of lagging sites of the DNA strands associated with the relevant proteins at two sites: in the vicinity of the replication forks on one hand and, on the other hand, at three different distances upstream from them. Electron-microscopically estimated lengths of non-labelled DNAs of NP complexes of POMS components C, B and A were found to be maximally about 100, 200 and 400 b, respectively (J. Korb and J. Štokrová, personal communications). Interestingly, these distances are reminiscent of the length of one and two (maximally three) nucleosomal repeat units (Alberts *et al.*, 1983).

Accordingly, the rationale for this mode of occurrence of an extrachromosomal DNA in growing animal cells directly dependent on proteosynthesis (Smith and Vinograd, 1972) may be an asymmetrical segregation of parental nucleosomes from the lagging to the leading strand of replicating chromosomal DNA (Seidman *et al.*, 1979) associated with an increase in nuclease sensitivity of the nucleosome-free lagging sites of replicating DNA strands (Roufa and Marchionni, 1982). Otherwise, the mode of the formation of the small polydisperse extrachromosomal DNA of CHLMs, as indicated in this paper, may represent a general mechanism by which the growing animal cell is able to reduce its genetic material that is unnecessary for the cell life.

At last, there are a few data available that an extrachromosomal DNA of animal cells represents active replication structures (Kunisda *et al.*, 1983; Kiyama and Oishi, 1987). Our data presented in this paper contribute to this important possibility. They indicate that the small polydisperse extrachromosomal DNA organized into NP complexes forming the POMS material of CHLMS is a DNA still actively enganged in reactions of LSS and that the molecules of AMV DNA are replicative, most distinct representatives of this DNA.

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