ACTIVITIES OF A LAGGING DNA STRAND SYNTHESIS OF NUCLEOPROTEIN COMPLEXES HARBORING AN EXTRACHROMOSOMAL DNA CLOSELY RELATED TO AVIAN MYELOBLASTOSIS VIRUS CORE-BOUND DNA

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Summary. – Nucleoprotein (NP) complexes constituting the material of the postmicrosomal sediment (POMS) and its three basic components (A, B, C) (Říman and Šulová, 1997a), harboring an extrachromosomal DNA closely related to AMV DNA were found to possess DNA- and RNA-synthesizing activities (SAs) reflecting the ability of this material to be intensely labelled for DNA and RNA, respectively. The types of these NA-SAs were compatible with those significant for a lagging DNA strand synthesis (LSS). The use of selective inhibitors and of the proliferating cell nuclear antigen (PCNA) disclosed a successive involvement of alpha DNA polymerase (pol) and PCNA-insensitive delta DNA pol in LSS. In this respect, we show gradual changes in the representation of activities (As) of both mentioned DNA pols in the NP complexes of the individual POMS components. Those of POMS component C contained alpha DNA pol As only, while a distinct portion of DNA SAs of POMS component B was represented on expense of alpha DNA pol As by PCNA-insensitive delta DNA pol (epsilon DNA pol), As which represented practically all the DNA SAs of POMS component A. The type of RNA SAs of this material represented mostly by primase (Pr) As corresponded well with the nature of LSS. An exception was represented by a minor portion of RNA-SAs of POMS component A which was alpha amanitin-sensitive like RNA pol II. Moreover, analyzing this natural model replication system, we found that the carbonyldiphosphonate (COMDP), a selective inhibitor of the PCNA-insensitive delta DNA pol, was a strong activator of Pr-As and/or Pr-alpha DNA pol As of NP complexes of POMS component C.

Key words: avian myeloblastosis virus DNA; extrachromosomal DNA; lagging DNA strand synthesis; alpha DNA polymerase; PCNA-insensitive delta DNA polymerase; carbonyldiphosphonate

Abbreviations: AMV = avian myeloblastosis virus; Aph = aphidicolin; As = activities; BuPdGTP = N^2-(p-butylphenyl) deoxyguanosine 5'-triphosphate; COMDP = carbonyldiphosphonate; ^{14}C-UR = [^{14}C]uridine; DB = digestion buffer; DNase = deoxyribonuclease; DTT = dithiothreitol; ^{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; dNTPs = deoxyribonucleotide triphosphates; NTPs = ribonucleotide triphosphates; PBS = phosphate-bufferred saline; PCA = perchloric acid; PCNA = proliferating cell nuclear antigen; PMSF = phenylmethyl sulfonyl fluoride; pol = polymerase; POMS = postmicrosomal sediment; Pr = primase; SAs = synthesizing activities; SB = solubilizing buffer; spc = small polydisperse circular; RNase = ribonuclease; SDS = sodium dodecyl sulfate; Tris = tris-(hydroxymethyl)-aminomethane

Introduction

In our precedent paper (Říman and Šulová, 1997a) we have shown that the extrachromosomal small polydisperse DNA present in POMS of lysed CHLMs (Dvořák and Říman, 1980b; Říman *et al.*, 1993b; Korb *et al.*, 1993) and closely related to AMV DNA (Říman and Beaudreau, 1970) is organized into NP complexes. The latter form three basic POMS components (A, B and C) differing not only in sucrose densities, but also in their properties of labelling for DNA and RNA. Their DNAs and RNAs, synthesized during the labelling period, are by sedimentation and electrophoretic properties strongly reminiscent of the products of LSS of three successive stages. The synthesis takes place evidently on pieces

of lagging sites of DNA strands of three length classes that are cut out in the vicinity of replication forks on one hand and on the other hand in three length distances upstream from them. By these properties the radioactively double-labelled NAs of POMS components B and C are closely similar to the major and minor portion, respectively, of the molecules forming the identically labelled AMV DNA (Říman *et al.*, 1993b). This implicates that NP complexes harboring such NAs should be associated with enzymatic As relevant to LSS. This suggestion is strengthened by our recent findings (Říman *et al.*, 1995) that AMV DNA bound to AMV core is associated with Pr- and Pr-alpha DNA pol As significant for LSS (Roth, 1987). Here, we show that the NP complexes harboring the extrachromosomal DNA closely related to AMV DNA possess RNA- and DNA-SAs reflecting quite well the capacity of the radioactive labelling of this material in the cell with ^{3}H-mTdR and ^{14}C-UR. Differential inhibition of these As associated with NP complexes in fractions of the three individual POMS components with N^2-(p-butylphenyl) deoxyguanosine 5'-triphosphate (BuPdGTP), aphidicolin (Aph) and COMDP as well as no effect of PCNA correspond well to the suggestion that the NP complexes forming the POMS components C, B and A contain As significant for LSS occurring in the earliest stage in POMS component C and in successively more advanced stages in POMS components B and A. Unexpectedly, it was found in this model system that COMDP, a potent inhibitor of PCNA-independent delta DNA pol (epsilon DNA pol), is a strong activator of DNA- and RNA-SAs associated with the NP complexes of POMS component C.

Materials and Methods

Chemicals. Unlabelled ribonucleoside triphosphates (NTPs), deoxyribonucleoside triphosphates (dNTPs) and calf thymus DNA were from Calbiochem. Aphidicolin (Aph) was from Sigma. Alpha-amanitin was from Serva. BuPdGTP and COMDP were generous gifts from Dr. E. Wright, Department of Pharmacology, University of Massachussetts, Medical School, Worcester, MA, USA. All other chemicals were of the highest analytical purity.

Radioisotopes. [alpha-^{32}P]adenosine-5'-triphosphate ([alpha-^{32}P]ATP) and [alpha-^{32}P]deoxyadenosine-5'-triphosphate ([alpha-^{32}P]dATP), 110 TBq/mmole each, were from Amersham. [Methyl-^{3}H]thymidine (^{3}H-mTdR, 1.5 – 2.0 TBq/mmole) and [^{14}C]uridine (^{14}C-UR, 14 – 17 GBq/mmole) were both from ÚVVVR, Prague.

Proteins. Deoxyribonuclease (DNase) I (ribonuclease-free) and ribonuclease (RNase) A were from Worthington. Proliferating cell nuclear antigen (PCNA) was purified (Kramata *et al.*, 1996) from the bacterial strain (*Escherichia coli* BL 21/DE R) harboring a plasmid encoding a human PCNA cDNA sequence and kindly provided by Dr. Stillman, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA.

Buffers. Tris buffers, TNE and TBE, were according to Maniatis *et al.* (1982). Solubilizing buffer (SB) prepared according to Weber *et al.* (1973) was enriched in 0.1 mmol/l phenylmethylsulfonyl fluoride (PMSF). Hepes buffer was used according to Talanian *et al.* (1989).

Cells. Chicken leukemic myeloblasts (CHLMs) were cultivated in a cell suspension as described earlier (Říman and Beaudreau, 1970; Říman and Šulová, 1997a).

Radioactive labelling. Cells (5 – 8 x 10^7/ml) in 60 – 80 ml of medium were 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).

Source for studying enzymatic As of NP complexes forming POMS material. The source was the POMS material present in the individual fractions of an equilibrium sucrose density gradient in which the POMS material of lysed CHLMs was separated as described previously (Říman and Šulová, 1997a).

Separation of proteins from NP complexes of the POMS material. After dilution with SB without detergent the peak fractions of the individual POMS components were centrifuged at 180,000 x g in a Beckman SW 41.0 rotor for 22 hrs at 2°C. The individual pellets were gently mixed at 0°C for 30 mins with solubilizing buffer (SB) (Říman *et al.*, 1995). Then, aliquots of this suspension (1 – 2 mg of protein) were run according to Weber *et al.* (1973) in isopycnic CsCl gradients (starting density of 1.40 g/cm^3) at 2°C (see the legend to Fig. 9).

Assay 1 for DNA-SAs. Reaction mixture (100 µl) contained 0.005 mol/l Tris-HCl pH 8.1, 0.005 mol/l $MgCl_2$, 0.040 mol/l KCl, 0.2 mmol/l dithiothreitol (DTT), 40 µmol/l unlabelled dGTP, dCTP, dTTP each, 2 µmol/l ATP, 1.5 µCi [alpha-^{32}P]dATP, 0.05% Nonidet P-40 (NP-40), 1% glycerol, and 30 µl of gradient fraction used for reaction.

Assay 2 for RNA-SAs. Reaction mixture (100 µl) contained 0.05 mol/l Tris-HCl pH 7.2, 0.01 mol/l $MgSO_4$, 0.01 mmol/l DTT, 0.05% bovine serum albumin (BSA), 0.05% NP-40, 40 µmol/l unlabelled GTP, CTP and UTP each, 2 µmol/l ATP, 1.5 µCi [alpha-^{32}P]ATP, 1% glycerol, and 30 µl of gradient fraction.

Assay 3 for studying the influence of COMDP on DNA-Sas. Reaction mixture (100 µl) contained 0.075 mol/l Hepes, 0.01 mol/l $MgCl_2$, 0.01 mol/l KCl pH 7.5 (adjusted with KOH), 40 µmol/l unlabelled dGTP, dCTP and dTTP each, 6 µmol/l unlabelled dATP, 1.5 µCi [alpha-^{32}P] dATP, 0.5% NP-40, 1% glycerol, and 15 µl of gradient fraction (200 µl).

Assay 4 for detection of Pr-As in portions of the CsCl isopycnic gradient fractions containing proteins separated from POMS components. Reaction mixture (50 µl) contained 0.05 mol/l Tris-HCl pH 7.2, 0.01 mol/l $MgSO_4$, 0.1 mmol/l DTT, 0.05% BSA, 0.1% NP-40, 1.5 µCi [alpha-^{32}P]ATP, 0.22 µg poly(dT), and an aliquot (7.5 µl) of CsCl gradient fraction.

Inactivation of reactions using the endogenous template DNA was accomplished by DNase I (40 µg per 100 µl of the reaction mixture). For activation of the enzymatic As, calf thymus DNA activated (Maniatis *et al.*, 1982) and thermally denatured was added in an amount of 10 µg per 100 µl reaction mixture. Concentrations of 10 µmol/l and 10 µg/ml of BuPdGTP and Aph, respectively, were used according to Nethanel *et al.* (1988). COMDP (50 µmol/l) and PCNA (330 ng/50 µl) were used (Kramata *et al.*, 1996).

Estimation of enzymatic As. The reaction mixtures incubated for 30 mins at 37°C were processed for estimation of the radioac-

tivity as described previously (Řiman *et al.*, 1993a). ^{3}H-mTdR and ^{14}C-UR radioactivity incorporated into acid-insoluble material that was precipitated and collected on Millipore filters was measured in toluene-based scintillation fluid, while that of [alpha-^{32}P]dAMP or [alpha-^{32}P]AMP incorporated during the reaction period was processed similarly and estimated on the basis of the Cerenkov effect, using a Beckman spectrometer LS 6000 SE.

Results and Discussion

DNA-SAs associated with POMS material

The DNA and RNA double-labelled POMS material separated from lysed CHLMs by isopycnic sucrose density gradient centrifugation into three POMS components (A, B and C) (Říman and Šulová, 1997a) was tested for DNA-SAs. Portions of each gradient fraction were subjected to Assay 1. The gradient distribution of [alpha-^{32}P]dAMP radioactivity incorporated during the reaction into the acid-insoluble portion of POMS material is depicted in Fig. 1 on the background of the gradient distribution of ^{3}H-mTdR and ^{14}C-UR radioactivity incorporated during the period of labelling of the cells for DNA and RNA. As evident, the POMS material possessed DNA-SAs most expressed in the NP complexes forming the POMS component B which were also found to be most labelled for DNA during the double-labelling of the cells. This implicates, consequently, that a DNA synthesis took place inside the NP complexes of POMS material and, using its natural endogenous DNA templates, was accomplished by the relevant associated enzymes. In contrast to ^{3}H-mTdR, the profile of [alpha-^{32}P]dAMP radioactivity revealed two distinct peaks indicating the occurrence of two populations of NP complexes with pronounced DNA-SAs. Such a characteristic of the distribution of these As was quite frequent, but not constant, and seems to reflect differences in POMS material separated from different stocks of cells (data not shown).

Natural endogenous templates of DNA-SAs of POMS material

DNA-SAs associated with NP complexes forming the POMS material used as natural templates the DNAs harbored in NP complexes, i.e., the small polydisperse extrachromosomal DNAs. This was indicated by a marked decrease in DNA-SAs when they were tested in the presence of DNase I in the reaction mixture of Assay 1. Fig. 2 gives

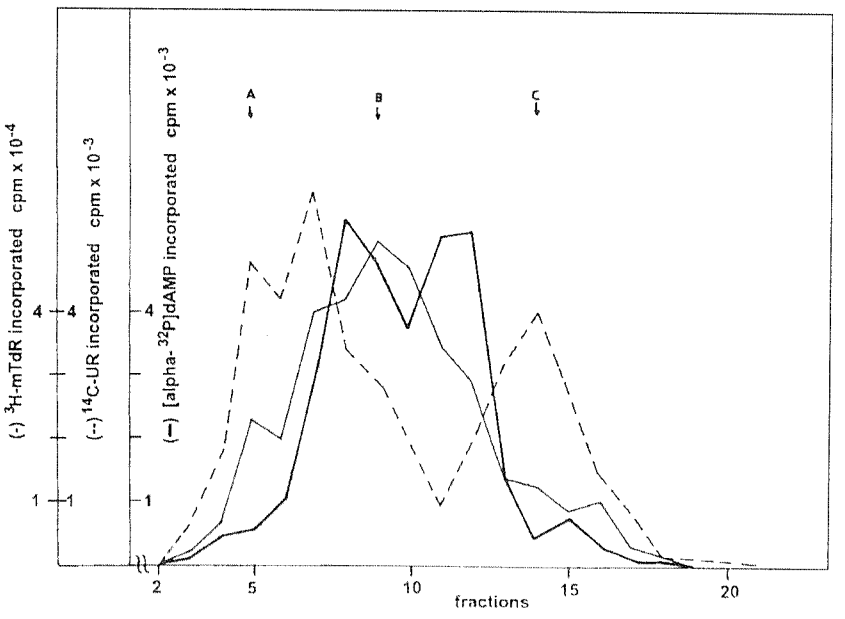

Fig. 1
Comparison of the distribution of ^{3}H-mTdR and ^{14}C-UR-radioactivity of the double-labelled POMS material and its DNA-SAs (^{32}P-radioactivity) in an isopycnic sucrose density gradient

Arrows A, B and C indicate the positions of the peak fractions of POMS components A, B and C, respectively. Separation of the radioactively double-labelled POMS material was accomplished by isopycnic centrifugation in a linear 60 – 20% sucrose density gradient at 147,000 x g for 21 hrs at 4°C. DNA-SAs (thick continuous line) in the individual gradient fractions were estimated in the absence of the exogeneous DNA template by the Assay 1.

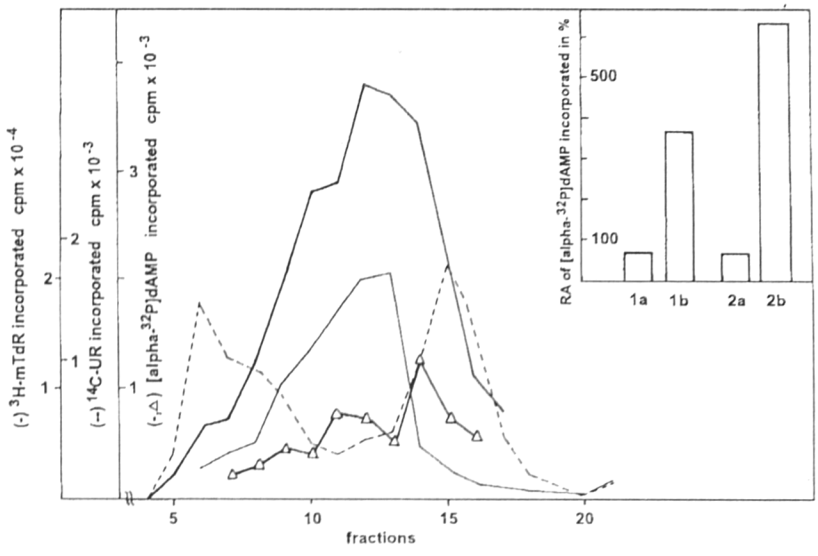

Fig. 2

Basic characteristics of DNA-SAs of POMS material

Gradient distribution of DNA-SAs of the POMS material tested in Assay 1 in the absence of an exogeneous DNA template (continuous thick line) and in the same way but with inclusion of DNase I into the reaction mixture (Δ). The thin continuous and broken lines denote the gradient distribution of the radioactive DNA and RNA label, respectively, of the POMS material separated as described in Fig. 1. Insert: behaviour of DNA-SAs present in their gradient peak fraction under the following reaction conditions of Assay 1: columns 1a and 1b show % of the radioactivity (RA) of DNA-SAs expressed in the presence of dATP only and with the complete set of dNTPs, respectively. Columns 2a and 2b show DNA-SAs under conditions of Assay 1 and after addition of the activated and denatured template DNA.

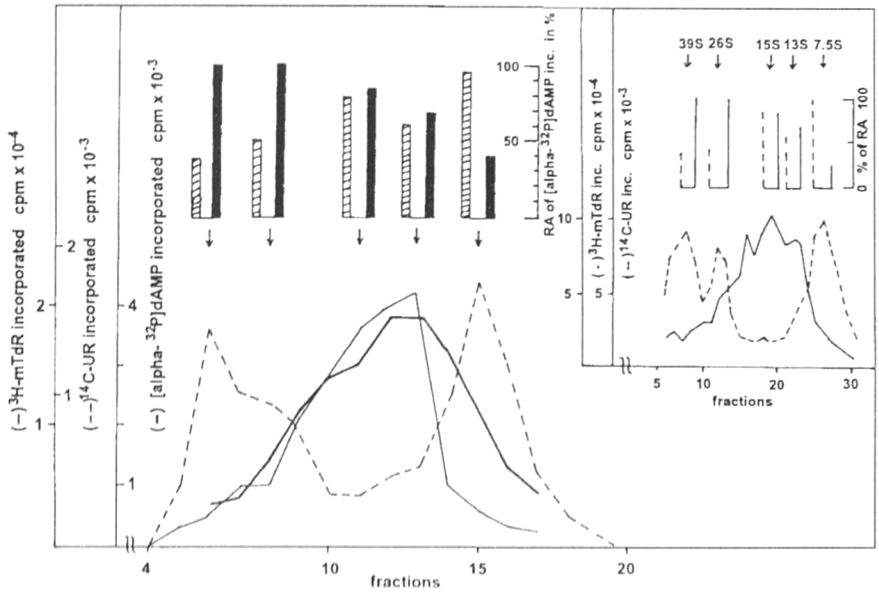

Fig. 3

Influence of BuPdGTP and Aph on DNA-SAs present in selected fractions of the POMS material double-labelled with ^{3}H-mTdR and ^{14}C-UR and separated in isopycnic sucrose density gradients

Gradient fractions the DNA-SAs of which were tested with inhibitors are denoted with vertical arrows. The inhibitory influence expressed in % of the radioactivity (RA) incorporated in the absence of the inhibitor in Asaay 1 (no addition of an exogenous DNA template) is depicted by black and hatched columns in the case of BuPdGTP (10 μmol/l) and Aph (10 μg/ml), respectively. Distribution of ^{3}H-mTdR and ^{14}C-UR-radioactivity of the double-labelled POMS material is indicated by thin continuous and broken lines, respectively. Insert: influence of BuPdGTP (10 μmol/l) (full vertical lines) and Aph (10 μg/ml) (broken vertical lines) located at positions of selected fractions of the radioactively double-labelled POMS material separated by velocity centrifugation in linear 30 – 10% sucrose density gradients (147,000 x g, 6 hrs at 4°C). The vertical arrows indicate the S positions of the tested fractions of POMS material.

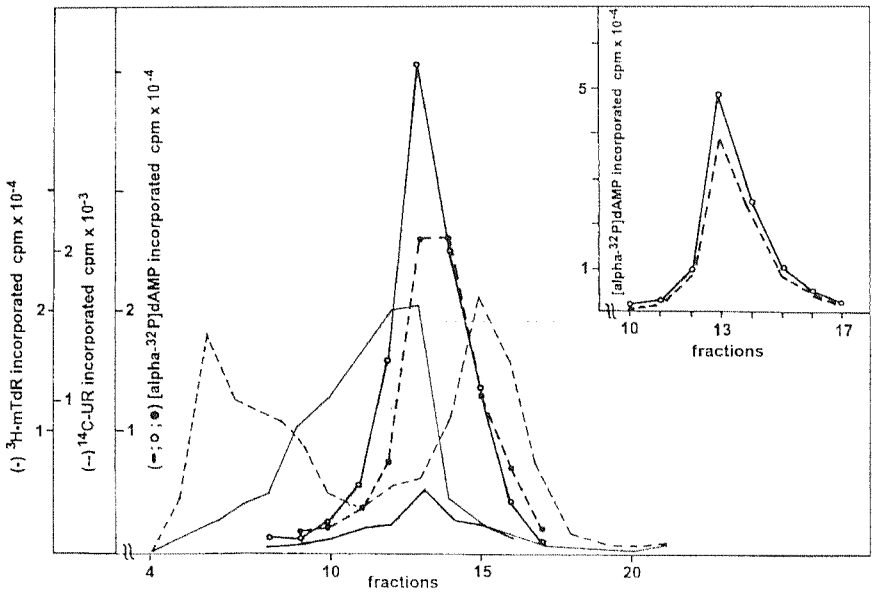

Fig. 4
Search for As of delta DNA pol class among DNA-SAs of the POMS material
Fractions of the same gradient of the separated POMS material (see Figs. 2 and 3) were tested in Assay 3 in the absence of the exogenous DNA template (continuous thick line), in the presence of the activated and denatured DNA template (o) and after addition of COMDP (50 μmol/l) to the reaction accomplished with activated and denature DNA template (●). For comparison, this figure depicts also the gradient distribution of ^{3}H-mTdR- (continuous thin line) and ^{14}C-UR-radioactivity (broken thin line). Insert: the distribution and extent of DNA-SAs expressed in reactions (Assay 3) with addition of the activated and denatured template DNA (o) and in the presence of activated and denatured template DNA and the PCNA added in the amount of 330 ng per reaction (broken line).

a comparison of the gradient distribution of [alpha-^{32}P]dAMP radioactivity resulting from the tested untreated and DNase I-treated samples of POMS material present in the individual gradient fractions. The DNA-SAs increased in the presence of the complete set of dNTPs and after addition of the activated denatured template DNA to the reaction mixture (Fig. 2, insert).

Influence of BuPdGTP and Aph on DNA-SAs of POMS material

Inhibitors recognizing, first of all, the As of a Pr-alpha DNA pol enzyme complex (Gronostajski *et al.*, 1984) significant for the early stages of LSS (Bambara and Huang, 1995) were used to identify the DNA pol types that are responsible for DNA-SAs associated with NP complexes forming the POMS material and to distinguish them from the other types of DNA pol belonging to the alpha family that are responsible for DNA replication in animals (Wang, 1991). To this end, we tested the DNA-SAs present in selected fractions of the individual POMS components separated by isopycnic sucrose density gradient centrifugation with BuPdGTP, a selective inhibitor of alpha DNA pol As (Lee *et al.*, 1985; So and Downey, 1988) and Aph, which acts in various concentrations differently on alpha DNA pol As on one hand and on those of delta (or epsilon) DNA pol on other hand (Basnakian *et al.*, 1989). The results obtained are depicted graphically in columns indicated by vertical arrows above the relevant gradient fractions in Fig. 3. As evident, maximally inhibited with BuPdGTP (by about 60%) were the DNA-SAs present in POMS component C (see the peak fraction of ^{14}C-UR radioactivity). In contrast, the DNA-SAs present in this fraction were practically not inhibited by Aph in concentrations used. This suggests that in NP complexes forming POMS component C we deal with enzymatic As significant for very early stages of LSS represented by the As of a Pr-alpha DNA pol complex that are relatively highly resistant to Aph (Nethanel *et al.*, 1988). These As are considered responsible for synthesis of the Okazaki fragment precursors, the DNA primers (Nethanel and Kaufmann, 1990). Consequently, these findings strengthen also our suggestion that the sedimentation and electrophoretic properties of the radioactively double-labelled NAs of POMS component C are strongly reminiscent of the products of very early LSS (Říman and Šulová, 1997a). In comparison to DNA-SAs of POMS component C, those of components B and A revealed a gradual decrease and increase in sensitivity towards BudGTP and Aph, respectively. The Aph inhibition of DNA-SAs present in the peak

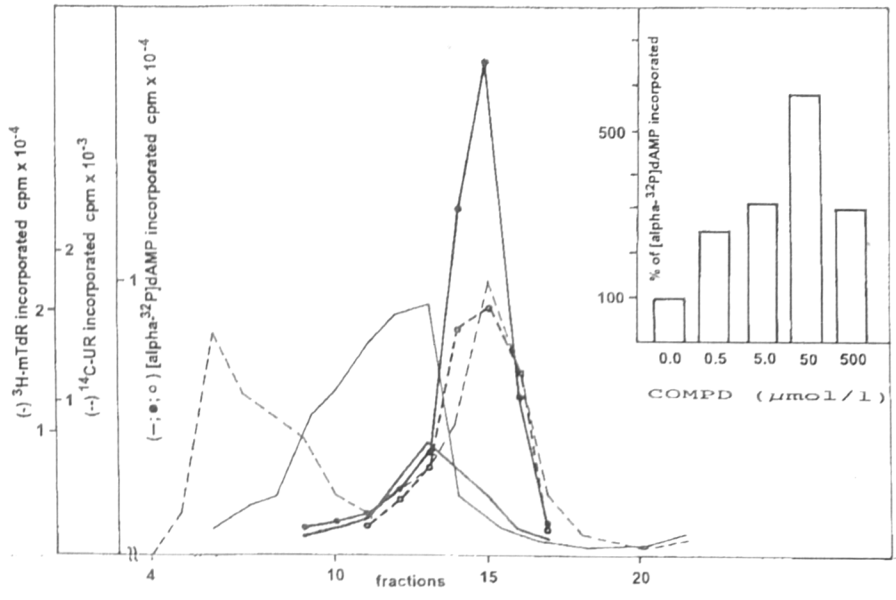

Fig. 5

Activating influence of COMDP on DNA-SAs of NP complexes of POMS component C

Gradient fractions were tested in Assay 3 in the absence of exogenous template DNA (thick continuous line) after addition of COMDP in final concentration of 50 µmol/l (●) and 500 µmol/l (o). For comparison and recording the shift of DNA-SAs to the region of POMS component C , the gradient distribution of the ^{3}H-mTdR- (continuous thin line) and ^{14}C-UR-radioactivity (broken thin line) of POMS material, is added. The insert depicts the activating influence of various concentrations of COMDP on DNA-SAs present in the gradient enzyme activity and ^{14}C-UR-radioactivity peak fraction.

fraction (^{14}C-UR radioactivity and the sucrose density 1.21 g/cm^{3}) of POMS component A reached about 60% while these As were practically resistant towards BuPdGTP. This phenomenon suggests that the NP complexes forming POMS components B and A become gradually enriched in As of a delta or epsilon DNA pol type that are considered responsible for elongation of the DNA primers into Okazaki fragments 150 and 250 b in length (Nethanel and Kaufman, 1990). Indeed, the most prominent radioactively labelled DNA species present in POMS components B and A were by length strongly reminiscent of the abovementioned Okazaki fragments (Říman and Šulová, 1977a). Interestingly, in our precedent paper (Říman and Šulová, 1997a), we have noted a similar distribution of the radioactively double-labelled POMS material in velocity and equilibrium sucrose density gradients permitting evaluation of the sedimentation properties of POMS NP complexes on the basis of their sucrose density. Consequently, testing the influence of BuPdGTP and Aph on DNA-SAs present in the selected fractions of the velocity sucrose density gradient of the total POMS material enabled us to characterize the properties of the DNA-SAs associated with POMS NP complexes of various sedimentation features (Fig. 3, insert). The characteristics obtained in this way were closely similar to those obtained with this material defined by its density properties.

Influence of COMDP and PCNA on DNA-SAs of POMS material

The nucleus of eukaryotic cells contains three DNA polymerases, alpha, delta and epsilon (for review see Bambara and Huang, 1995), which are jointly engaged in the replication of DNA. According to the contemporary views, the delta DNA pol activated with PCNA (Prelich *et al.*, 1987) elongates the leading DNA strand whereas the alpha DNA pol participates in LSS (Coverley and Laskey, 1994). By substitution of delta DNA pol with epsilon DNA pol in the dipolymerase SV40 DNA replication system it was shown that the epsilon DNA pol, which is PCNA-insensitive, cannot substitute the delta DNA pol in the leading DNA strand synthesis. Consequently, it was indicated in these experiments that the PCNA-insensitive or epsilon DNA pol may participate in the course of LSS in the formation of complete Okazaki fragments (Lee *et al.*, 1991). In keeping with this proposal are the findings reinforcing the idea of two distinct steps in LSS. One step, a BuPdGTP-sensitive and Aph-insensitive, accomplished by Pr-alpha DNA pol As, ends with the formation of RNA-DNA molecules about 40 b in length, the so-called DNA primers. The other step, a BuPdGTP-insensitive and Aph-sensitive, accomplished by delta or epsilon DNA pol, is engaged in

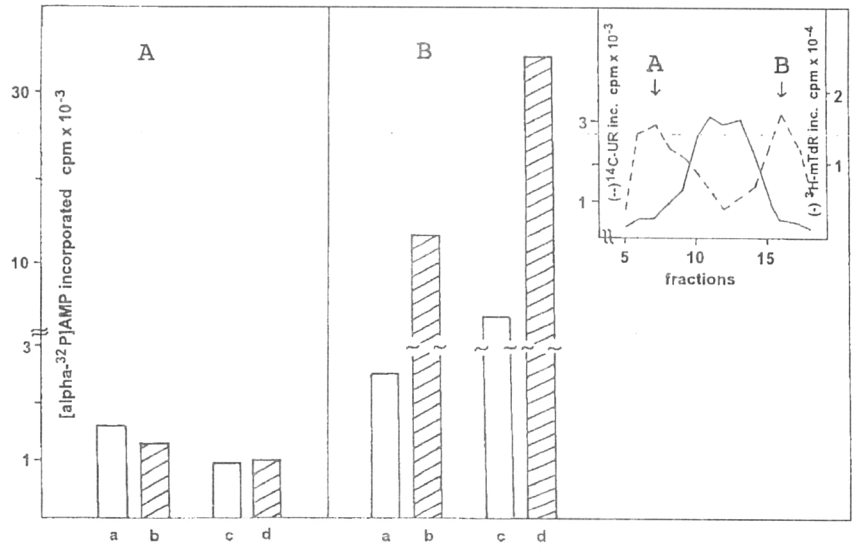

Fig. 6

Activating influence of COMDP on RNAs of NP complexes of POMS component C

Compartment A depicts the RNA-SAs present in the ^{14}C-UR-radioactivity peak of the POMS component A tested in Assay 2 in the presence of ATP only (column a) and after addition of COMDP (50 µmol/l) (column b), in the presence of the complete set of NTPs (column c) and after addition of COMDP (50 µmol/l) (column d). Compartment B depicts RNA-SAs present in ^{14}C-UR-radioactivity peak fraction of POMS component C tested in Assay 2 with ATP only (column a), after addition of COMDP (50 µmol/l) (column b), in the presence of the complete set of NTPs (column c) and after addition of COMDP (50 µmol/l) (column d). Insert: arrows A and B indicate the gradient fractions used for testing. Gradient distribution of the radioactive label of the POMS material is designated as in the precedent figures.

the elongation of DNA primers into mature Okazaki fragments (Nethanel *et al.*, 1988; Nethanel and Kaufmann, 1990). This concept is supported by Bullock *et al.* (1991). To contribute to the recognition of the nature of DNA-SAs of the POMS material which were found Aph-sensitive, these As were tested in the presence of COMDP, a potent and selective inhibitor of delta DNA pol As independent of PCNA (Talanian *et al.*, 1989). The influence of COMDP was tested under reaction conditions of Talanian *et al.* (1989) (Assay 3) in the presence of activated and denatured DNA template, addition of which increased the DNA-SAs expressed with endogenous template DNA more than four-fold. Fig. 4 shows that COMDP in concentration used inhibited accordingly the Aph-sensitive DNA-SAs of POMS material located in the gradient region of POMS component B from about 30 to 60%. Similarly, an about 50% inhibition by COMDP revealed the much less pronounced DNA-SAs tested separately in the peak fraction (sucrose density 1.21 g/cm^3) of POMS component A (data not shown). This inhibitory effect of COMDP indicating the presence of PCNA-insensitive delta DNA pol As in the relevant fractions of POMS material prompted us to test the DNA-SAs of the same gradient fractions in the presence of PCNA. As evident from the insert in Fig. 4, an addition of PCNA to the reaction mixture had no stimulatory effect and its rather low inhibitory effect is strongly reminiscent of that of PCNA on activities of the PCNA-independent delta DNA pol isolated from HeLa cells (Syväoja and Linn, 1989). Thus, these findings correspond well with arguments of Lee *et al.* (1991) that epsilon DNA pol participates in LSS, which is accomplished in two successive steps by two different DNA pols (Nethanel and Kaufmann, 1991).

Activating effect of COMDP on Pr-As associated with Pr-alpha DNA pol complex

Studying the influence of COMDP on DNA-SAs of POMS material, we tested also the influence of this inhibitor on DNA-SAs not activated by the exogenous DNA template. Testing in this way the portions of the same gradient fractions we found quite unexpectedly that an addition of 50 µmol/l COMDP (concentration used in our precedent tests of DNA-SAs expressed in the presence of added DNA template) strongly stimulated the incorporation of [alpha-^{32}P]dAMP into NP complexes of the POMS component C associated with DNA-SAs sensitive to BuPdGTP like the As of a Pr-alpha DNA pol complex. This phenomenon together with the shift of the gradient profile of DNA-SAs to the location of the ^{14}C-UR radioactivity peak of POMS com-

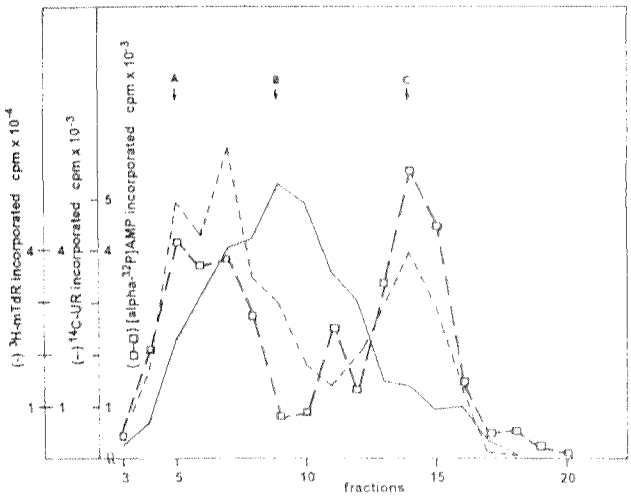

Fig. 7

Distribution of RNA-SAs in POMS material radioactively double-labelled for DNA and RNA and separated by isopycnic sucrose density gradient centrifugation

The gradient profile of RNA-SAs (□) tested in Assay 2 parallels closely the gradient distribution of ^{14}C-UR-radioactivity (broken thin line) of the POMS material. A continuous thin line designates as previously the distribution of ^{3}H-mTdR-radioactivity of the POMS material.

ponent C (sucrose density 1.108 g/cm^3) is depicted in Fig. 5. It also shows that even a ten-fold higher concentration of COMDP (500 μmol/l) still exerted an activation effect even though less by half. The influence of various concentrations of COMDP on stimulation of DNA-SAs present in the peak fraction of POMS component C are shown in the insert in Fig. 5. The stimulation of POMS DNA-SAs with COMDP seems to reflect an activation of alpha DNA pol As. Since the latter are in animals cooperatively tightly associated with DNA Pr-As (Roth, 1987), we decided to test directly also the influence of COMDP on RNA-SAs of the Pr nature present, as suggested (Říman and Šulová, 1997a), in the POMS material. To this end, we tested comparatively in Assay 2 the occurrence of RNA-SAs and the influence of COMDP on them in portions of the ^{14}C-UR radioactivity peak fractions of POMS components A and C (vertical arrows A and B, respectively, in the insert in Fig. 6). Fig. 6A shows the level of RNA-SAs present in the peak fraction of POMS component A. In general, these As were low, regardless of whether they were expressed in the presence of [alpha-^{32}P]ATP only (a) and with added COMDP (b), or in the presence of [alpha-^{32}P]ATP and the other three common unlabelled NTPs (c), and with added COMDP (d). In contrast, the RNA-SAs present in the peak fraction of POMS component C (Fig. 6B) were pronounced already in the presence of [alpha-^{32}P]ATP only (a). These As (reflecting initiation of iRNA synthesis) rose about five-fold after addition of COMDP (b). They increased more than twice after addition of the three common unlabelled NTPs (c) and further about five-fold after addition of COMDP (d). These results show clearly that COMDP activated even more strongly the RNA-SAs, present in POMS component C and belonging to Pr-As, than DNA-SAs as will be demonstrated below as well as elsewhere (Říman and Šulová, 1997c). An explanation of this phenomenon seems to be important for better understanding of the cooperative functioning of both DNA pols suggested as engaged in LSS (Nethanel and Kaufmann, 1990). The highly selective inhibition of a PCNA-insensitive delta DNA pol As by COMDP was explained (Talanian *et al.*, 1989) by competition of COMDP with dNTPs for the active enzyme site(s). In contrast, As of the Pr-alpha DNA pol complex were found twenty times less sensitive to the inhibitory influence of COMDP (Talanian *et al.*, 1989), and no direct activation of one of the components forming this enzyme complex was recorded. However, the authors cited above have studied the influence of COMDP on isolated enzymes using mostly synthetic templates. Thus, the behaviour of the isolated DNA pols may differ from that of these enzymes naturally organized into NP complexes (Basnakian *et al.*, 1989). Such a situation may occur in POMS NP complexes that are micromorphologically strongly reminiscent of highly specialized NP structures (J. Korb and J. Štokrová, personal communications) descending from origins of replication as shown in the case of bacterial replicating NP complexes (Dodson *et al.*, 1986), the micromorphology of which reflects multiple DNA-protein and protein-protein interactions (Echols, 1986). An analysis of the products of NA-SAs expressed in the presence of COMDP could contribute to the elucidation of the mode of stimulation of DNA- and RNA-SAs of POMS component C replicating systems.

Characteristics of RNA-SAs of POMS material

In precedent sections of this paper we have shown that the POMS material was associated with DNA-SAs, the gradient distribution of which paralleled that of the radioactive ^{3}H-mTdR DNA label. In experiments shown in Fig. 7 the POMS material was also associated with RNA-SAs, the gradient distribution of which paralleled accordingly for a change the gradient distribution of the radioactive ^{14}C-UR RNA label of this material with common peaks of RNA-SAs and ^{14}C-UR radioactivity at a sucrose density of 1.21 (component A) and 1.108 g/cm^3 (component C), respectively. In contrast to the extent of ^{14}C-UR radioactivity associated with components A and C, the RNA-SAs associated with component C were always higher than those associated with component A. Characteristics of RNA-SAs of POMS material were obtained in the following way. Portions of the gradient fractions of radio-

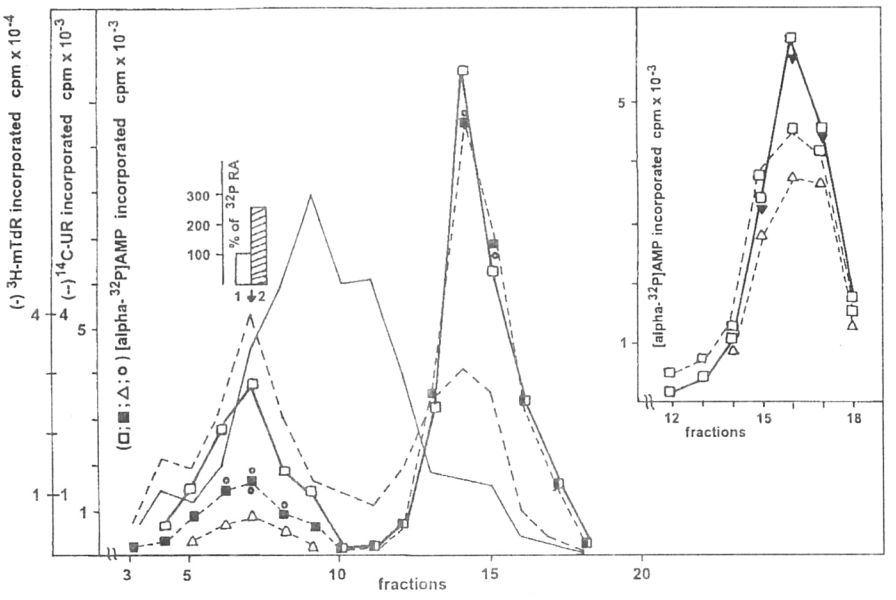

Fig. 8

Basic characteristics of RNAs-SAs of NP complexes of POMS components A and C

RNA-SAs present in fractions obtained by isopycnic sucrose density gradient centrifugation of radioactively double-labelled POMS material were estimated in Assay 2 in the absence of added activated and denatured template DNA (□), after incubation with DNase I included into the reaction mixture (D) after incubation with alpha-amanitin in concentrations of 1 μg/ml (■) and 100 μg/ml (o). Columns above RNA-SAs peak fraction of POMS component A depict RNA-SAs of this fraction tested without (1) and with addition of the activated and denatured DNA template (2). Insert: complementary data on RNA-SAs of POMS component C: RNA-SAs tested in Assay 2 (□, continuous line), in the presence of Aph (2 μg/ml) (▼), after addition of activated and denatured DNA template (□, discontinuous line), and after inclusion of DNase I into the reaction mixture (Δ).

actively double-labelled POMS material separated (Říman and Šulová, 1997a) from a new batch of CHLMs were assayed for RNA-SAs. Other portions of the same gradient fractions were used for testing the properties of these RNA-SAs important for basic definition of their nature. In this analysis we concentrated on properties of RNA-SAs present in fractions of components A and C most labelled for RNA and associated with most pronounced RNA-SAs. As evident from Fig. 8, the RNA-SAs present in the gradient fractions of component A were expressed in the absence of added DNA template. They were DNase I-sensitive and were stimulated by added activated and denatured template DNA (Fig. 8, columns 1 and 2 above the peak fractions tested). Accordingly, these RNA-SAs were DNA-dependent and their natural templates were pieces of DNA strands harbored in NP complexes forming the POMS material. The minor part of these RNA-SAs of component A was sensitive to alpha-amanitin concentration (1 μg/ml) inhibiting the RNA pol II, whereas the major part of these As was resistant to this inhibitor even in concentrations inhibiting RNA pol III (Manley, 1987). Interestingly, the alpha-amanitin-sensitive part of RNA-SAs present in fractions forming component A might be responsible for the appearance of the new prominent RNA species detected by analysis of radioactively double-labelled NAs of this component (Říman and Šulová, 1997a). In contrast, the RNA-SAs present in gradient fractions forming component C were resistant to alpha-amanitin similarly to the case of DNA Pr-As (Gronostajski *et al.*, 1984). As evident from the insert in Fig. 8 depicting in more detail the features of RNA-SAs present in this gradient region, these As were, as in the case of DNA Pr-As, resistant to Aph (Gronostajski *et al.*, 1984). They were DNase I-sensitive but, in contrast to those present in gradient fractions of component A, they were not stimulated by added activated and denatured DNA which, in this case, had a rather inhibitory effect, indicating quite special demands of these RNA-SAs on the template structure. The results obtained by this analysis of RNA-SAs of POMS material indicate that prevalent part of these As was represented by DNA Pr-As, as suggested already previously on the basis of electrophoretic properties of the radioactively labelled RNAs of the individual POMS components (Říman and Šulová, 1997a). However, this suggestion needs direct proof significant for detection of DNA Pr-As.

Detection of Pr-As in proteins of individual POMS components

For detection of Pr-As in AMV core isolates (Říman *et al.*, 1995) we used, comparatively, a set of assays including

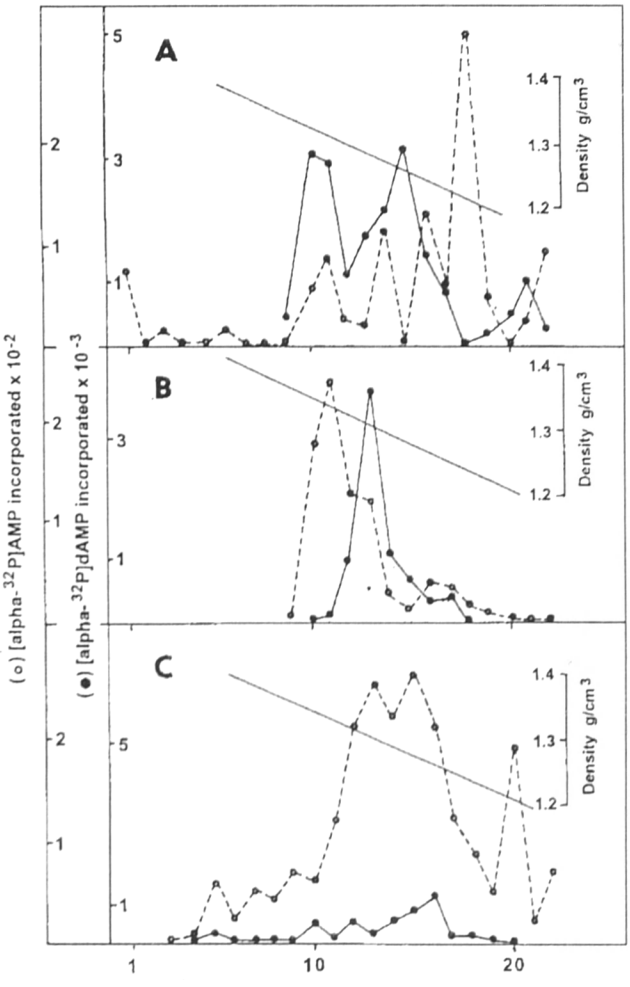

Fig. 9
Characteristics of Pr-As and DNA-SAs present in proteins separated from NP complexes present in representative fraction of the individual POMS components

NP complexes were isolated from the density (radioactivity) peak fraction of the POMS material double-labelled for DNA and RNA and separated by isopycnic sucrose density gradient centrifugation. Separation of proteins from these NP complexes was performed in isopycnic CsCl gradients of a starting density of 1.40 g/cm^3 run for 44.5 hrs at 165,00 x g at 2°C. DNA-SAs (●) were tested in Assay 1 and Pr-As (o) in Assay 4. Figs. 9A, B and C depict characteristics of representation of DNA-SAs and Pr-As in proteins separated from NP complexes of POMS components A, B and C, respectively.

the test of the ability of Pr-As to be expressed, in contrast to other RNA pols, with poly(dT) and ATP as template and substrate, respectively (Roth, 1987). This test should be performed with proteins separated from the relevant biological material. If it occurs in scarce amounts, a convenient method for separation of proteins is needed. One, especially convenient for separation of proteins from NP complexes, is centrifugation of the detergent-treated material in isopycnic CsCl density gradients (Weber *et al.,* 1973). It permits separation of NAs from proteins but, moreover, the bulk of proteins having a buoyant density of 1.23 g/cm^3, from template-primer-free replicating enzymes, viral (Weber *et al.,* 1973) or cellular (Říman *et al.,* 1995), the CsCl buoyant density of which is always markedly higher than 1.23 g/cm^3. Thus, we separated the proteins of NP complexes forming the individual POMS components in this way and used the portions of the individual gradient fractions for estimation of Pr-As using reaction mixture with poly(dT) and [alpha-^{32}P]ATP (Assay 4). We tested comparatively another portion of each gradient fraction for DNA-SAs using Assay 1 complemented with activated and denatured DNA template. This assay estimates by itself the DNA-SAs without differentiating their individual DNA pol types. The characteristics of the representation of Pr-As in proteins of the individual POMS components accomplished in this way (Fig. 9) corresponded well with the nature of RNA-SAs suggested above (Fig. 8) and mentioned earlier (Říman and Šulová, 1997a). The NP complexes forming POMS component C were indeed, maximally equipped with proteins exhibiting Pr-As (Fig. 9C). In contrast, these NP complexes possessed the smallest proportion of DNA-SAs (compare Fig. 9C with Figs. 9B and 9A) which were represented by molecules of the Pr-alpha DNA pol enzyme complex only, according to the sensitivity to BuPdGTP and insensitivity to Aph (Nethanel *et al.,* 1988) as shown in Fig. 3. Consequently, the mutual proportions of Pr-As and DNA-SAs in proteins separated from NP complexes of POMS component C (Fig. 9C) may indicate that a greater part of Pr-As belonged to "free" Pr molecules, not associated with Pr-alpha DNA pol enzyme complexes (Roth, 1987; Říman *et al.,* 1995). This suggestion is supported by the characteristics of the CsCl isopycnic density gradient distribution of Pr-As and DNA-SAs of proteins separated from NP complexes of POMS component B. In this case, a distinst portion of Pr-As was clearly separated from their other portion which remained in association with DNA-SAs (Fig. 9B). These DNA-SAs should be represented, according to their response to BuPdGTP, Aph, COMDP and PCNA (Figs. 3 and 4), by As of Pr-alpha DNA pol complex and PCNA-insensitive delta (or epsilon) DNA pols. Finally, the representation of Pr-As in proteins of NP complexes of POMS component A (Fig. 9A) indicates the presence of two Pr-As species not associated and of two Pr-As species associated with DNA-SAs, the prevalent portion of which should be represented, according to their response to the inhibitors and effectors used, by As of PCNA-insensitive delta DNA pol type. Interestingly, an association of Pr-As with a PCNA-insensitive delta DNA pol (an epsilon DNA pol) has been noted (Focher *et al.,* 1988). In conclusion, these findings confirm that the NP complexes of the POMS material are equipped with enzymes typical of LSS, the natural template

of which are short pieces of an extrachromosomal DNA cut out evidently from lagging sites of replicating chromosomal DNA strands as suggested earlier (Říman and Šulová, 1997a). In connection with the minute replicative structures representing AMV DNA (Říman *et al.*, 1993a,b), originating from NP complexes of POMS components B and C (Říman and Šulová, 1997a), the findings presented in this paper implicate that the AMV core NP complexes should contain besides the Pr- and Pr-alpha DNA pol As (Říman *et al.*, 1995) also epsilon DNA pol As as well as other specialized proteins significant for initiation of DNA replication.

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