PRODUCTS 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 present in selected fractions of the separated three basic components A, B, C of the postmicrosomal sediment (POMS) (Říman and Šulová, 1997a) were used as a source of nucleic acid synthesizing activities (NA-SAs) expressed in reactions *in vitro*. These were performed in the absence and presence of N^2-(p-butylphenyl) deoxyguanosine 5'-triphosphate (BuPdGTP), aphidicolin (Aph) and carbonyldiphosphonate (COMDP), inhibitors allowing differentiation between two DNA polymerases (pols) involved in a lagging DNA strand synthesis (LSS). Reaction products were isolated and analyzed by polyacrylamide gel electrophoresis (PAGE) in denaturing conditions. Products labelled with [alpha-^{32}P]dAMP or [alpha-^{32}P]AMP were represented by intermediates significant for LSS. Okazaki fragment precursors, whose synthesis was inhibited by BuPdGTP and resistant to Aph, and whose radioactive RNA label was DNase I-sensitive, represented products formed *in vitro* by NP complexes of POMS component C. Okazaki fragments 127 b in length, whose synthesis was insensitive to BuPdGTP but inhibited by Aph and COMDP, were characteristic of the reactions accomplished by NP complexes of POMS component B while products of NA-SAs of NP complexes of POMS component A were represented by Okazaki fragments up to 280 b in length, whose synthesis was most sensitive to Aph. In accord with previous data (Říman and Šulová, 1997b), COMDP strongly stimulated production of RNAs corresponding in length with initiator RNAs (iRNAs). However, in dependence on reaction conditions also ribodeoxyribonucleotide primers can be produced by NP complexes of POMS component C, suggesting the occurrence of two primase (Pr) catalytic modes influenced by dNTP/rNTP relation and thus, by COMDP, a strong competitor for dNTPs. These results represent the first direct evidence that an extrachromosomal DNA organized into special NP complexes can be replicated extrachromosomally by a mechanism of LSS.

Key words: extrachromosomal DNA; lagging DNA strand synthesis; primase; alpha DNA polymerase; epsilon DNA polymerase; carbonyldiphosphonate

Introduction

A 7 S DNA of avian myeloblastosis virus (AMV DNA) (Říman and Beaudreau, 1970) bound to the virus core (Deeney *et al.*, 1976; Dvořák and Říman, 1980a) and originating from an extrachromosomal small polydisperse DNA of chicken leukemic myeloblasts (CHLMs) (Dvořák and

Abbreviations: AMV = avian myeloblastosis virus; Aph = aphidicolin; As = activities; b = base; BSA = bovine serum albumin; BuPdGTP = N^2-(p-butylphenyl) deoxyguanosine 5'-triphosphate; ^{14}C-UR = [^{14}C]uridine; CHLM = chicken leukemic myeloblast; COMDP = carbonyldiphosphonate; DB = digestion buffer; dNTPs = deoxyribonucleotide triphosphates; DNase = deoxyribonuclease; DTT = dithiothreitol; EDTA = ethylenediamine tetraacetic acid; ^{3}H-mTdR = [methyl-^{3}H]thymidine; iRNA = initiator RNA; LSS = lagging DNA strand synthesis; NA = nucleic acid; NP = nucleoprotein; NP-40 = Nonidet P-40; nt = nucleotide; NTPs = ribonucleotide triphosphates; PAGE = polyacrylamide gel electrophoresis; PBS = phosphate-bufferred saline; PCNA = proliferating cell nuclear antigen; pol = polymerase; POMS = postmicrosomal sediment; Pr = primase; SAs = synthesizing activities; SDS = sodium dodecyl sulfate; ss = single-stranded

Říman, 1980b) is represented, actually, by minute replicative structures (Říman *et al.*, 1993a,b) highly bent up to circularization (Korb *et al.*, 1993). This DNA seems to be associated in the virus core with Pr and Pr-alpha DNA polymerase (pol) activities (As). The recent findings (Říman *et al.*, 1995) prompted our search centered on the cellular organization of this DNA into NP complexes associated, consequently, with enzymatic As relevant to an early DNA synthesis. To this end, we started to analyze systematically the material harboring this DNA and represented by the material forming POMS of CHLMs (Dvořák and Říman, 1980b). We have found (Říman and Šulová, 1997a) that the DNA present in the POMS material is organized into populations of NP complexes which can be intensely radioactively labelled for DNA and RNA and which can be separated by isopycnic sucrose density gradient centrifugation into three basic POMS components (A, B and C) differing in properties of labelling for DNA and RNA, density and sedimentation properties. Sedimentation and electrophoretic characteristics of the radioactively double-labelled NAs of the individual POMS components were found to be reminiscent of the products of three successive stages of a LSS taking place on short DNA pieces of three lengths cut out from lagging sites of strands of replicating chromosomal DNA. Our suggestions were strengthened by further findings showing that the POMS material is constantly associated with DNA- and RNA-SAs which correspond in nature with enzymatic As significant for LSS, as described previously (Říman and Šulová, 1997b). We have studied the influence of selective inhibitors and of proliferating cell nuclear antigen (PCNA) and found that two DNA pols are involved in LSS associated with POMS NP complexes, the alpha DNA pol and PCNA-insensitive delta DNA pol with a distribution characteristic of each of the POMS components. We have shown that the major portion of the RNA-SAs of the POMS material is represented by Pr-As, thus by RNA-SAs significant for LSS (Roth, 1987). Consequently, the NP complexes with such properties may represent special replication machineries that are able to accomplish extrachromosomally the synthesis of the DNA primers, Okazaki fragments and the mature Okazaki fragments in NP complexes of POMS components C, B and A, respectively. To confirm this hypothesis, it was necessary to analyze the products synthesized *in vitro* by NA-SAs typical for each of the individual POMS components. To this end, the relevant fractions of the POMS material were assayed *in vitro* for As of LSS comparatively, also in the presence of inhibitors selective for As of LSS. The isolated reaction products were then analyzed by PAGE in denaturing conditions. The results obtained support unambiguously the explanations and suggestions presented on this subject in both our precedent papers (Říman and Šulová, 1997a,b). Here, by a direct analysis of the reaction products, we confirm also the stimulatory effect of COMDP on Pr-As present in NP complexes of POMS component C (Říman and Šulová, 1997b).

Materials and Methods

Chemicals. Unlabelled rNTPs and dNTPs were from Calbiochem. Aph was from Sigma. BuPdGTP and COMDP were from Prof. E. Wright, University of Massachussets, Worcester, MA, USA (Říman and Šulová, 1997b). Acrylamide (N',N'-methylene-triacetylamide), TEMED (N, N, N',N'-tetramethylene diamide), ammonium persulfate, Bromophenol Blue (BPB) and Xylene Cyanol (XC) were from Serva. Nonidet P-40 (NP-40) was from LKB. All other chemicals were of highest analytical purity.

Radioisotopes. [alpha-^{32}P]deoxyadenosine-5'-triphosphate ([alpha-^{32}P]dATP) and [alpha-^{32}P]adenosine-5'-triphosphate ([alpha-^{32}P]ATP), 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 from ÚVVVR, Prague.

Deoxyribonuclease (DNase) I (ribonuclease-free) was from Worthington.

Buffers. Tris buffers, TBE and TNE, were according to Maniatis *et al.* (1982). Digestion buffer (DB) used for DNase I treatment was described previously (Říman *et al.*, 1993a).

CHLMs, their cultivation and radioactive labelling with ^{3}H-mTdR and ^{14}C-UR was described previously (Říman *et al.*, 1993a; Říman and Šulová, 1997a).

Source for studying the DNA- and RNA-SAs and their reaction products. The source was the NP complexes present in the portions of the radioactivity peak fractions of the individual POMS components (A, B and C) separated by isopycnic sucrose density gradient centrifugation as described previously (Říman and Šulová, 1997a).

Assay 1 for analyzing DNA-SAs reaction products synthesized in vitro by POMS material in the absence or presence of inhibitors. Reaction mixture (50 µl) contained 0.05 mol/l Tris-HCl pH 8.1, 0.005 mol/l $MgCl_2$, 0.04 mol/l KCl, 0.2 mmol/l dithiothreitol (DTT), 40 µmol/l unlabelled dGTP, dCTP and dTTP each, 4 µmol/l unlabelled dATP, 40 µmol/l unlabelled GTP, CTP, UTP and ATP each, 10 µCi [alpha-^{32}P]dATP, 0.05% Nonidet P-40 (NP-40), 1% glycerol, and 20 µl of gradient fractions (200 µl) of the relevant POMS components. Inhibitors, COMDP and BuPdGTP, were added in final concentrations of 50 µmol/l and 10 µmol/l, respectively. Aph was added in the amount of 0.5 µg per 50 µl of reaction mixture.

Assay 2 for analyzing RNA-SAs reaction products synthesized in vitro by POMS material in the absence or presence of COMDP. 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% bovine serum albumin (BSA), 0.05% NP-40, 40 µmol/l unlabelled UTP and CTP each, 4 µmol/l unlabelled GTP, 12 µmol/l unlabelled ATP, 40 µmol/l unlabelled dGTP, dCTP, dATP, and dTTP each, 24 µCi [alpha-^{32}P]ATP, 1% glycerol, and 20 µl of the gradient peak fraction (200 µl) of the relevant POMS components. The influence of COMDP was studied at final concentration of 50 µmol/l. In both assay types, the reactions procedeed for 20 mins at 37°C and were

stopped by adding ethylenediamine tetraacetic acid (EDTA) (Maniatis *et al.*, 1982) and sodium dodecyl sulfate (SDS) (to 0.2%).

Isolation of NAs. The reaction mixtures were extracted by phenol-chlorophorm, divided into equal portions, ethanol-precipitated at -20°C for 24 hrs (Chang *et al.*, 1984), sedimented and dried in vacuum.

DNase I treatment of NAs. For this purpose, one of the two sediments mentioned above was suspended in 5 µl of DB containing 10 µg/µl DNase I. The treatment was performed at 37°C for 6 hrs (Singh and Dumas, 1984). Then the reaction was stopped with EDTA and by heating for 2 mins at 100°C and cooling. Finally, both the native (untreated) and the DNase I-treated NA samples were prepared in a final volume of 12 µl in a formamide mixture (Maniatis *et al.*, 1982) for PAGE.

PAGE under denaturing conditions. Reaction products were electrophoresed in 12% polyacrylamide gels (17 x 12 x 0.04 cm) supplemented with urea (7 mol/l) in TBE at 300 V for 240 mins at 8°C. Series of compared samples were run in parallel with XC and BPB as internal size markers. ^{32}P-radioactivity was determined in 4 mm gel slices dried on Synpor 6 filter discs in a toluene-based scintillation fluid or on the basis of the Cerenkov effect in a Beckman spectrometer 6000 SE. The length of the reaction products in bases (b) was evaluated by comparing their gel positions with those of the internal markers and the marker DNAs analyzed identically (Nethanel *et al.*, 1988; Říman *et al.*, 1993b). Construction of a curve expressing the relation between the length and electrophoretic migration distances of the compared marker single-stranded (ss) DNA fragments turned out useful.

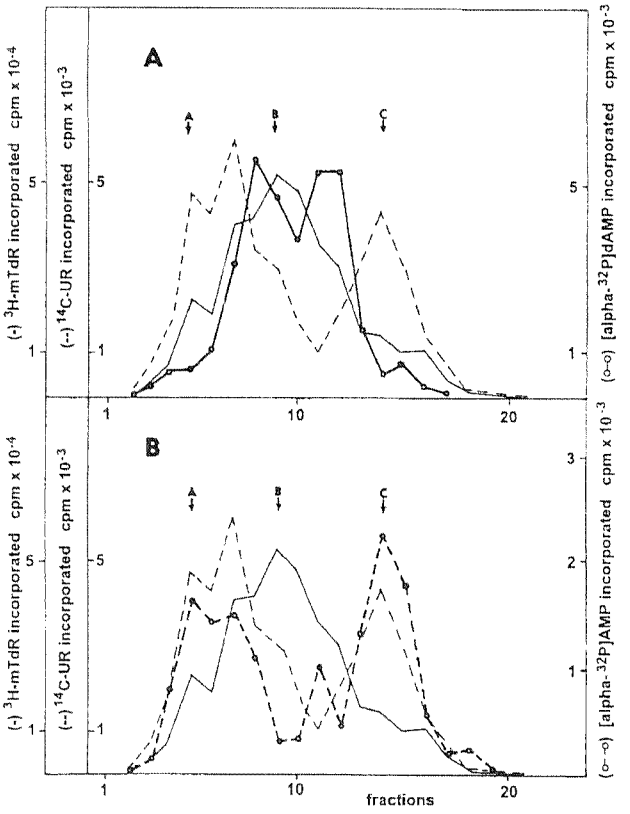

Fig. 1
Separation of POMS material by isopycnic sucrose density gradient centrifugation
Profiles of ^{3}H-mTdR- (continuous thin line) and ^{14}C-UR radioactivity (broken thin line). Vertical arrows indicate fractions of POMS components A, B and C, whose NP complexes served as a source of NA-SAs in reactions performed *in vitro*. A: DNA-SAs, radioactivity profile of the incorporated [alpha-^{32}P]dAMP ((o), continuous line). B: RNA-SAs, radioactivity profile incorporated [alpha-^{32}P]AMP ((o), broken line).

Results and Discussion

Reaction products of DNA-SAs and influence of BuPdGTP, Aph and COMDP

[Alpha-^{32}P]dAMP-labelled NA products were synthesized *in vitro* by NP complexes present in the peak fractions of POMS components A, B and C, separated by isopycnic sucrose density gradient centrifugation (Fig. 1). Reactions were done under conditions of Assay 1 and contained complete sets of dNTPs and rNTPs, the substrates needed for expression of DNA- and RNA-SAs, respectively, both using the common endogenous template DNA, i.e., the pieces of an extrachromosomal DNA harbored in the POMS NP complexes (Říman and Šulová, 1997a,b). The synthesis of the reaction products was accomplished either in the absence or presence of BuPdGTP, a selective inhibitor of the alpha DNA pol-Pr-As (Byrnes, 1985; Lee *et al.*, 1985; So and Downey, 1988), Aph, a general inhibitor of As of DNA pols of the alpha family (Wang, 1991), and COMDP, a selective inhibitor of PCNA-insensitive delta DNA pol- (or epsilon DNA pol) As (Syväoja and Linn, 1989; Talanian *et al.*, 1989), or delta DNA pol class type As (Wright *et al.*, 1994). The concentrations of BuPdGTP and Aph, 10 µmol/l and 10 µg/ml, respectively, corresponded with those used in *in vitro* studies of the SV40 replicating systems (Nethanel and Kaufmann, 1990). In the case of COMDP, the concentration of 50 µmol/l was used, as needed for the inhibition of epsilon DNA pol-As in some systems (Syväoja *et al.*, 1990). In addition, IC_{50} of 40 µmol/l of COMDP was found in the case of delta DNA pol using a natural DNA template (Talanian *et al.*, 1989). Analysis of the isolated reaction products performed by PAGE in denaturing conditions gave the following results. Reaction products synthesized *in vitro* by NA-SAs of NP complexes present in the peak fraction of POMS component A (Fig. 1) revealed the presence of several DNA components (Fig. 2). The most prominent of them were ssDNAs 90 – 280 b in length that corresponded with those of mature Okazaki fragments consisting of 250 (Nethanel and Kaufmann, 1990) to 300 nucleotides (nt) (Lee *et al.*, 1991). Synthesis of these products

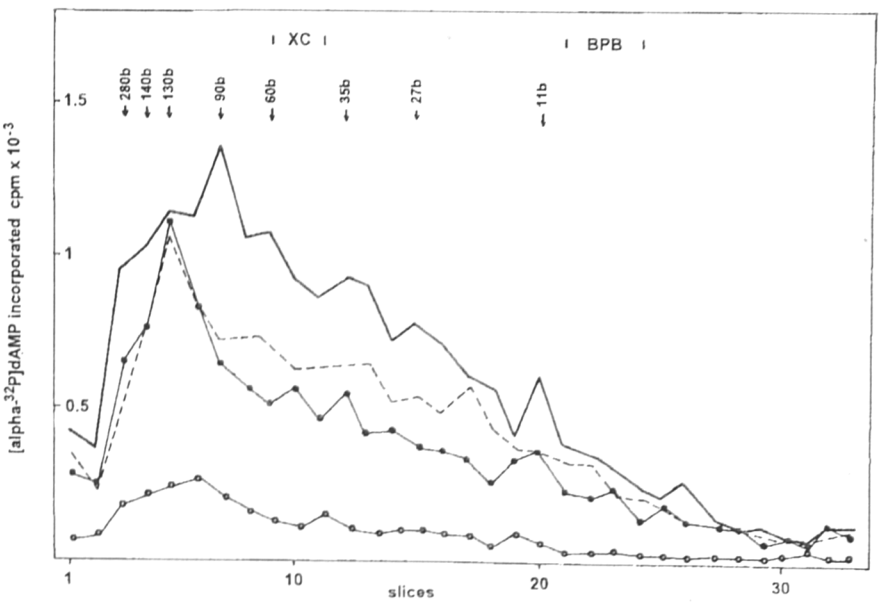

Fig. 2

PAGE in denaturing conditions of products of NA-SAs in NP complexes of POMS component A

Gel distribution of [alpha-^{32}P]dAMP radioactivity incorporated in NAs. Products synthesized in the absence of inhibitors (continuous thick line), in the presence of BuPdGTP (10 μmol/l) (●), COMDP (50 μmol/l) (broken line) and Aph (10 μg/ml) (o). XB and BPB, internal markers. Vertical arrows indicate gel positions of ssNAs of a length given in the number of b.

was most strongly inhibited by Aph (Fig. 2), which showed an about 80% inhibitory influence on DNA-SAs present in this gradient fraction. BuPdGTP and COMDP exhibited a much weaker but noticeable inhibitory effect on synthesis of products 27 – 90 b long (Fig. 2). A similar characteristic of the reaction products synthesized by NP complexes present in the gradient peak fraction of POMS component B (Fig. 3) revealed that the length of the most prominent 127 b product corresponded well with that of a classical Okazaki fragment in eukaryotes (DePamphilis and Wassarman, 1980) and, interestingly, with the length of majority of the DNA strands of AMV DNA (Říman *et al.*, 1993b; Korb *et al.*, 1993). As described previously (Říman and Šulová, 1997a), the ^{3}H-mTdR-labelled DNAs of POMS component B with their length of about 150 bp were most similar to the majority of the radioactively labelled (for DNA) molecules of AMV DNA isolates (Říman *et al.*, 1993b). Synthesis of the most prominent reaction product 127 b in length was not influenced by BuPdGTP (Fig. 3A) but, in contrast, it was markedly (by about 50%) inhibited by Aph (Fig. 3B) and even more by COMDP (Fig. 3C). These characteristics of an early DNA synthesis taking place *in vitro* in NP complexes of POMS component B correspond well with the concept that there exist two successive steps of LSS, accomplished by cooperation of two pols (Nethanel and Kaufmann, 1990; Podust and Hübscher, 1993; Wright *et al.*, 1994), a BuPdGTP-sensitive and Aph-resistant Pr-alpha DNA pol that synthesizes DNA primers (Nethanel *et al.*, 1988), and a DNA pol of delta class (delta or epsilon DNA pol) that is sensitive to Aph and COMDP (Syväoja *et al.*, 1990) and responsible for elongation of DNA primers into Okazaki fragments. Consequently, the synthesis of the 127 b long reaction product, resistant to BuPdGTP but sensitive to Aph and COMDP seems to start in NP complexes of POMS component B from preexisting DNA primers with the involvement of a DNA pol of delta pol class, which may be represented by an epsilon DNA pol because it is insensitive to PCNA (Říman and Šulová, 1997b). The reaction products of DNA-SAs of NP complexes present in the peak fraction of the POMS component C (Fig. 4) were represented, according to the incorporated [alpha-^{32}P]dAMP, by short strands 10 – 20 b in length. Their synthesis was sensitive to BuPdGTP and insensitive to Aph which had, conversely, rather slightly activating effect. These results complement our previous findings that DNA-SAs of POMS component C are BuPdGTP-sensitive and Aph-resistant (Říman and Šulová, 1997b). They are also in accord with the results obtained by Nethanel *et al.* (1988) showing in *in vitro* studies of the SV40 DNA replication system that the As of the Pr-alpha DNA pol complexes leading to the synthesis of short RNA-DNA strands are BuPdGTP-sensitive and highly Aph-resistant. This characteristic clearly signif-

icant for a Pr-alpha DNA pol synthesis is *eo ipso* typical of LSS performed, indeed, by the DNA-SAs in the NP complexes of POMS component C. In our precedent paper (Říman and Šulová, 1997b), these DNA-SAs were found to be markedly stimulated by COMDP which is known as a selective inhibitor of DNA pol-As of delta class (Talanian *et al.*, 1989; Syväoja *et al.*, 1990). In accord with this observation was the impact of the influence of COMDP on the synthesis of [alpha-^{32}P]dAMP-labelled products, the majority of which were represented, interestingly, by short strand pieces 7 – 8 b in length. Additonal data, which may help to explain this phenomenon, will be presented and discussed below.

Summarizing the results obtained, it is possible to conclude that the analysis of the reaction products synthesized by DNA-SAs of this POMS material brought their characteristics closely similar to those significant for LSS performed in cooperation of the two different DNA pols (Nethanel and Kaufmann, 1990). Accordingly, the products of BuPdGTP-sensitive and Aph-insensitive DNA-SAs in NP complexes of the selected fraction of POMS component C are strongly reminiscent of the Okazaki fragment precursors (DNA primers) synthesized by Pr-alpha DNA pol-As (Nethanel *et al.*, 1988). Similarly, the products of DNA-SAs less sensitive to BuPdGTP and distinctly more sensitive to Aph and COMDP (DNA-SAs of POMS components B and A) are reminiscent of the products, Okazaki fragments in nature, synthesized successively from DNA primers by involvement of As of a DNA pol of delta class (Nethanel and Kaufmann, 1990; Lee *et al.*, 1991). In addition, the characteristics obtained by PAGE of the reaction products in denaturing conditions are quite comparable with those performed with native ^{3}H-mTdR- and ^{14}C-UR-labelled NAs of POMS components A, B and C analyzed in 4% NuSieve agarose gels (Říman and Šulová, 1997a).

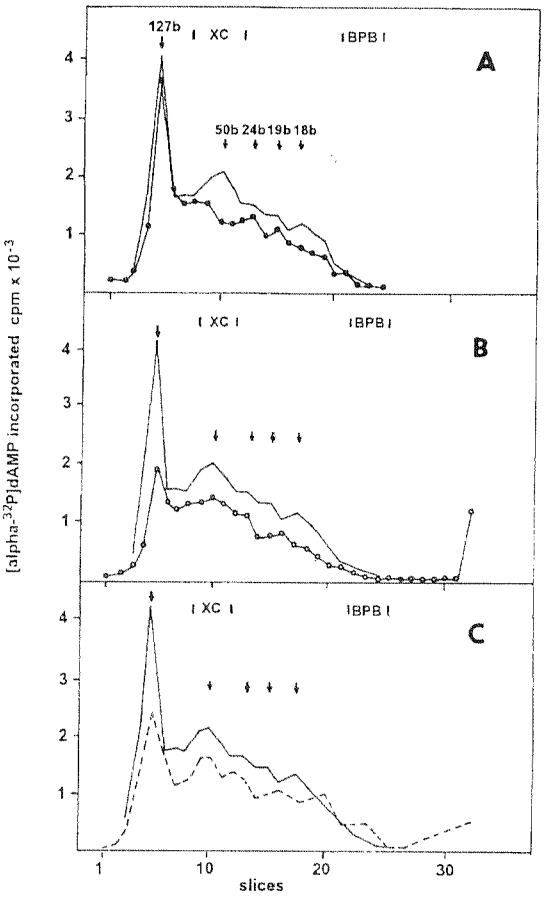

Fig. 3
PAGE in denaturing conditions of products of NA-SAs in NP complexes of POMS component B
Gel distribution of [alpha-^{32}P]dAMP radioactivity incorporated in NAs. XB and BPB, internal markers. A: Products synthesized in the absence (continuous line) and in the presence of BuPdGTP (10 µmol/l) (●). B: Products synthesized in the absence (continuous line) and in the presence of Aph (10 µg/ml) (○). C: Products synthesized in the absence (continuous line) and in the presence of COMDP (50 µmol/l) (broken line).

Reaction products of RNA-SAs and influence of COMDP

Previously, the POMS material was found to be efficiently labelled for DNA and RNA (Říman and Šulová, 1997a). Consequently, this implicated the presence of the relevant NA-SAs. Such activities were, indeed, constantly associated with NP complexes of all three individual POMS components (Říman and Šulová, 1997b). The RNA-SAs (Fig. 1B) were markedly associated with the material forming POMS components A and C. Component C was richest in these As which, in comparison with those of component A, were homogeneous in their nature (Říman and Šulová, 1997b). In contrast to RNA-SAs of component A, they were highly resistant to alpha-amanitin like the Pr-As (Gronostajski *et al.*, 1984) and similarly to Aph. Accordingly, in comparison to those of components B and A, the proteins separated from NP complexes of component C were most enriched in Pr-As (Říman and Šulová, 1997b). Finally, the RNA-SAs of component C even more than its DNA-SAs were stimulated by COMDP (Říman and Šulová, 1997b). For this reason, we analyzed by PAGE in denaturing conditions the reaction products of RNA-SAs of NP complexes present in the peak fraction of component C (Fig. 1B) expressed under conditions of Assay 2. Products synthesized in this way exhibited the presence of two distinct [alpha-^{32}P]AMP-labelled ssNA species of 54 and 23 b in length (Fig. 5). The radioactivity belonged in this case to very short pieces of RNA covalently bound to the newly synthesized but unlabelled DNAs because DNase I treatment of both these products led to a distinct decrease of the radioactive RNA label together with its shift into the electrophoretic mobility zone of oligoribonucleotides max-

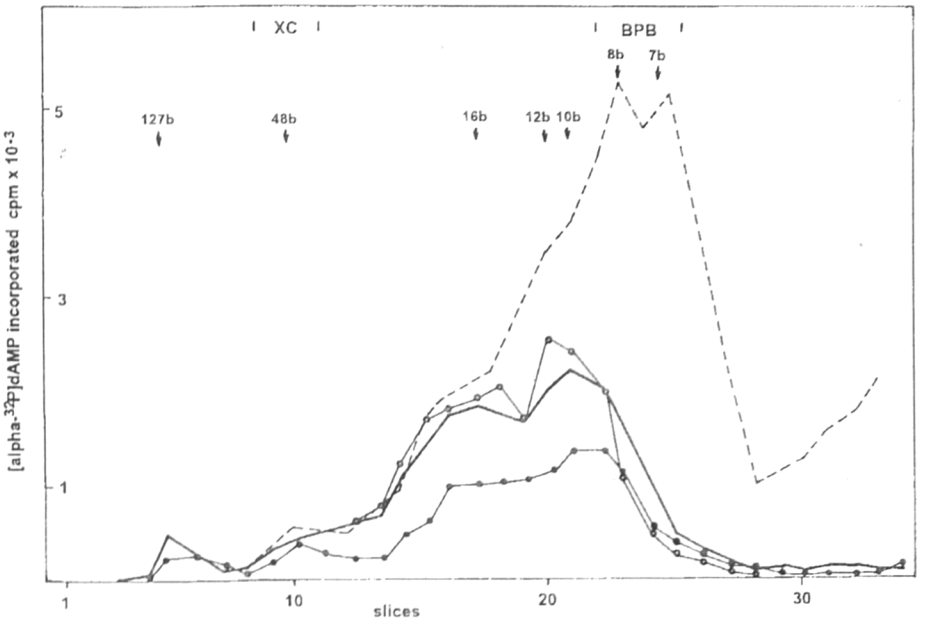

Fig. 4

PAGE in denaturing conditions of products of NA-SAs in NP complexes of POMS component C

Gel distribution of [alpha-^{32}P]dAMP radioactivity incorporated in NAs. Products synthesized in the absence of inhibitor (continuous line) and in the presence of BuPdGTP (10 μmol/l) (●) and Aph (10 μg/ml) (o). [Alpha-^{32}P]dAMP incorporation into oligonucleotides of the iRNA length in the presence of COMDP (50 μmol/l) (broken line). XB and BPB, internal markers.

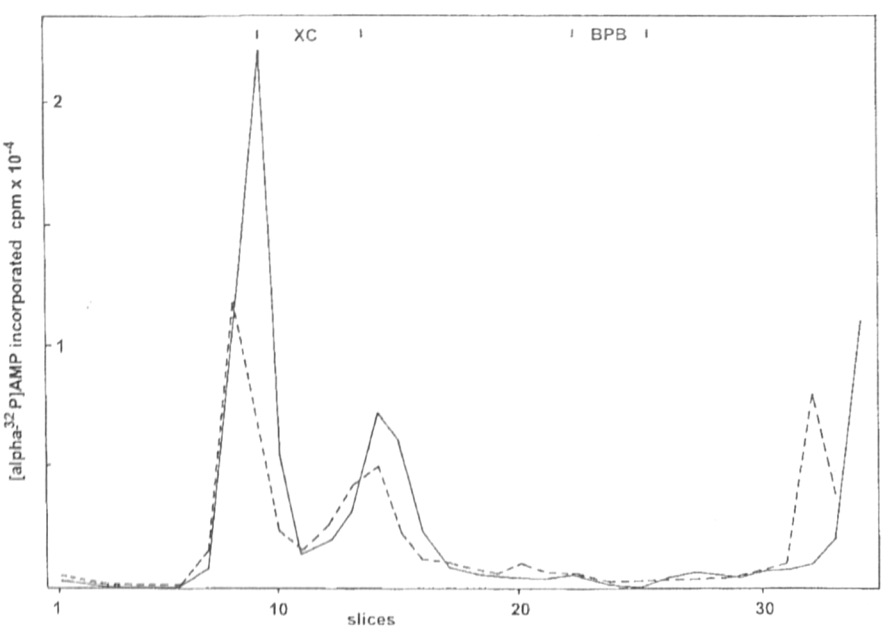

Fig. 5

PAGE in denaturing conditions of products of NA-SAs in NP complexes of POMS component C labelled for RNA

Gel distribution of [alpha-^{32}P]AMP radioactivity incorporated in NAs *in vitro* (continuous line). The same product treated prior to this analysis with DNase I (broken line). XB and BPB, internal markers.

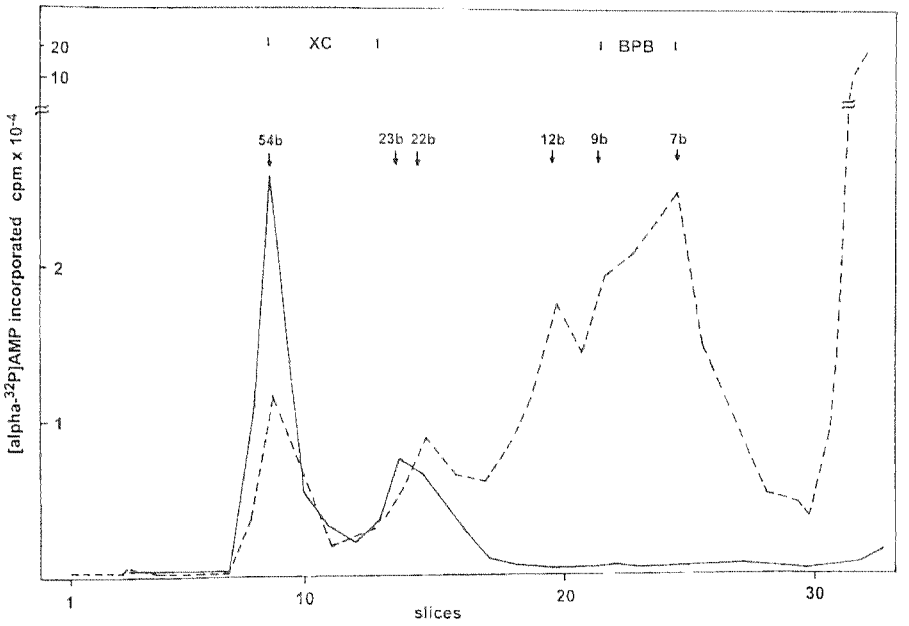

Fig. 6

PAGE in denaturing conditions of products of NA-SAs in NP complexes of POMS component C labelled for RNA

Gel distribution of [alpha-^{32}P]AMP radioactivity incorporated in NAs *in vitro* in the absence (continuous line) and in the presence of COMDP (50 µmol/l) (broken line). XB and BPB, internal markers.

imally 3 b in length. In such properties including the length of 23 and 54 b these RNA-DNA molecules are closely similar to Okazaki fragment precursors or immature Okazaki fragments (Nethanel *et al.*, 1988; Bullock *et al.*, 1991) which represent the products of a coupled reaction of Pr-As and alpha DNA pol-As performed by the enzyme complex of Pr-alpha DNA pol (Wang, 1991; Bambara and Huang, 1995). Similar ssRNA-DNA molecules were constantly found as a minor portion of isolates of radioactively double-labelled AMV DNA (Říman *et al.*, 1993b). Using the ssRNA-DNA molecules of AMV DNA separated by hydroxylapatite column chromatography, we have presented earlier in this case by phosphorylation reaction direct evidence of a covalent attachment of RNA to the 5'-end of DNA (Říman *et al.*, 1993a). Thus, the presence of Okazaki fragment-like molecules in AMV DNA (Říman *et al.*, 1995) originating from POMS components B and C (Říman and Šulová, 1997b) is evident. For comparison, we analyzed in this study in the same way the reaction products of RNA-SAs of NP complexes in the peak fractions of POMS components B and A. In these cases, the radioactive [^{32}P]RNA label, located and continuously increasing from the gel positions of strands from about 130 to 30 b in length, was also found DNase I-sensitive in accordance with the nature of Okazaki fragments (data not shown). Otherwise, complete DNase I digestion of the reaction products of the Pr-alpha DNA pol-As is always a problem (Říman *et al.*, 1995). Attempts were made to solve it by using vely long (up to 10 hrs) digestion times (Gronostajski *et al.*, 1984).

According to our precedent paper (Říman and Šulová, 1997b), RNA- and DNA-SAs of POMS component C belong to Pr- and alpha DNA pol-As, respectively (Říman and Šulová, 1997b), or are expressed by the Pr-alpha DNA pol enzyme complex As. Besides, COMDP was found to stimulate markedly the DNA-SAs, but, even more the RNA-SAs of this portion of POMS material. In the foregoing section of this paper (Fig. 4), we characterized by PAGE in denaturing conditions the reaction products of DNA-SAs influenced by COMDP. Here, we are complementing this characteristic by the same analysis of the COMDP influence on RNA-SAs of the same material of POMS component C. In contrast to the Okazaki fragment precursor nature of the two distinct NA species 54 and 23 b in length composing the reaction products of the RNA-SAs expressed *in vitro* in the absence of COMDP (Fig. 5), those synthesized *in vitro* in the presence of COMDP were represented minimally by four electrophoretic mobility NA species (Fig. 6). Two of them representing minor portions of the total [^{32}P]RNA label were by their length of 54 and 23 b reminiscent of the Okazaki fragment precursors (Fig. 5) depicted for comparison also in Fig. 6. A major portion of this [^{32}P]RNA label was located in the gel region of oligonucleotides 12 – 7 b in length. This range of length is that

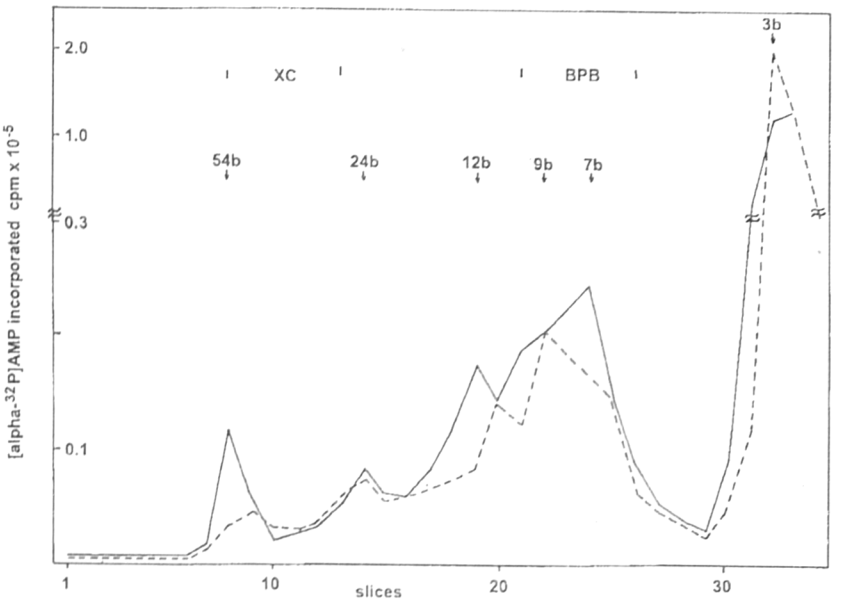

Fig. 7

PAGE in denaturing conditions of products of NA-SAs in NP complexes of POMS component C

Gel distribution of [alpha-^{32}P]AMP radioactivity incorporated in NAs *in vitro* in the presence of COMDP (50 µmol/l) (continuous line). The same product treated prior to this analysis with DNase I (broken line). XB and BPB, internal markers.

given for RNA primers (Roth, 1989), the products of DNA primase As, i.e., the initiator RNAs (iRNAs) (Reichard *et al.*, 1974), the average length of which is between 6 and 10 nt. Indeed, the peak of the radioactivity of this product was located at gel positions of 7 nt.

To distinguish which part of this product labelled for RNA was attached to DNA, we analyzed comparatively in the same way the product synthesized in the presence of COMDP and treated thereafter with DNase I (Fig. 7). From this comparison it was evident that the product representing an NA species 54 b in length was DNase I-sensitive like the product of the same length synthesized in the absence of COMDP (Figs. 5 and 6) and reminiscent of Okazaki fragment precursor. A slight DNase I sensitivity was also noted in the case of the 23 b NA species which represented the second of two NA products synthesized in the absence of COMDP (Figs. 5 and 6). The DNase I treatment of the major portion of the product labelled for RNA and synthesized in the presence of COMDP, which removed evidently very short DNA pieces covalently bound to RNA, confirmed that its prevalent portion was represented actually by iRNAs only, as indicated already by the electrophoretic characteristic of the same product but not treated with DNase I (Figs. 6 and 7). These findings demonstrate that, in the case of the DNA replicative systems represented by NP complexes of POMS component C, COMDP stimulated strongly the DNA Pr-As to synthesize under conditions used the RNA primers in the range of length given for iRNAs (Roth, 1987). Accordingly, they confirm our suggestions (Říman and Šulová, 1997b) that the RNA-SAs stimulated by COMDP in NP complexes of POMS component C belong actually to Pr-As stimulated directly by this strong inhibitor of DNA pol-As of delta class. According to Talanian *et al.* (1989), COMDP inhibits the delta DNA pol-As by mimicking a dNTP and by binding at a site within a dNTP binding domain of this enzyme. Similarly, such a powerful competitive effect of COMDP with dNTPs on Pr-As may be most probably responsible for a strong activating effect of COMDP on Pr-As and the mode of their expression. As shown by Hu *et al.* (1984), a Pr exhibits distinct catalytic properties that are able to catalyze the synthesis either of oligoribonucleotides or oligodeoxyribonucleotides or their hybrids. These properties are regulated by mechanisms exquisitely sensitive to concentrations of dNTPs reflecting, according to these authors, the occurrence of two Pr catalytic centers (or two conformers of a single center) which are coupled synchronously mutually exclusive and stringent for respective rNTP and dNTP substrates. In accord with these findings and views are observations that the RNA synthesis effected by a Pr is strongly depressed by all four dNTPs (Rowen and Kornberg, 1978). Consequently, in our experiments, addition of COMDP to the reaction mixture (Assay 2), containing besides the complete set of rNTPs all four dNTPs, stimulated the Pr RNA-SAs by eliminating the influence of dNTPs. Interestingly,

in contrast, the presence of COMDP led in this case to the decrease in formation of the NAs 54 and 23 b in length reminiscent of Okazaki fragment precursors which constituted the only products of these NA-SAs when expressed in the absence of COMDP (Fig. 5). This indicated an inhibitory influence of COMDP on alpha DNA pol-As which was 20 times lesser than that on delta DNA pol-As observed by Talanian *et al.* (1989). A strong stimulatory influence of COMDP on these RNA-SAs that was noted previously (Říman and Šulová, 1997b) in reactions containing only the rNTPs may indicate that COMDP eliminates the inhibitory influence of the residual dNTPs which may be present in POMS material and/or in trace amounts in the rNTPs used (Hu *et al.*, 1984). This was indicated by the characteristics of the products labelled for DNA in the presence of COMDP (Fig. 4). In this case, the [alpha-^{32}P]dAMP-labelled reaction products 12 – 7 b long were strikingly similar to iRNAs which may be represented by ribodeoxyribonucleotide primer molecules synthesized by Pr (Rowen and Kornberg, 1978) at a dNTP/rNTP relation resulting from the COMDP interference with ambient dNTPs. The incorporated radioactive DNA label may then occupy each position in the nt sequence of the primer with the exception of the first position of the nt initiating the primer synthesis. This is initiated *in vivo* always by ATP (Roth, 1987). A detailed analysis of this phenomenon is beoynd the scope of this characterization and deserves a separate study in which we could start from the disclosure of the strong stimulatory effect of COMDP on Pr-As described in our precedent paper (Říman and Šulová, 1997b) and confirmed in the present experiments.

Concluding remarks

The characteristics of the reaction products of NA-SAs of NP complexes of the individual POMS components performed in this way confirmed the conclusions presented in our precedent papers on this subject (Říman and Šulová, 1997a,b). We showed once more that in the case of NA-SAs of this material we have been dealing with As significant for LSS (Bambara and Huang, 1995) which led to the formation of Okazaki fragment precursors, Okazaki fragments and mature Okazaki fragments in NP complexes of POMS components C, B and A, respectively. The synthesis of these reaction products including the synthesis of iRNAs, accomplished *in vitro* by NP complexes of this POMS material, represents the first direct evidence that an extrachromosomal DNA organized into highly specialized NP complexes can be replicated extrachromosomally by the mechanism of LSS up to the stage of mature Okazaki fragments. Consequently, AMV DNA molecules assembled into NP complexes of POMS components B and C (Říman and Šulová, 1997a,b) enter the virus core as minute replicative active structures as suggested earlier (Říman *et al.*, 1993a,b). Possible consequences for reactions accomplished by the virus core reaction machinery (Grandgenett and Mumm, 1990) are evident. For example, an involvement of epsilon DNA pol-As and other cellular proteins of an early DNA synthesis, besides Pr- and Pr-alpha DNA pol-As (Říman *et al.*, 1995), in virus core reactions. Other questions are connected with the possible functional meaning of this extrachromosomal DNA and its NP complexes for the life cycle of a growing cell as suggested previously (Říman and Šulová, 1997a).

At last but not least, these and the precedent findings (Říman and Šulová, 1997a,b) are evidently correcting after years the interpretation of the functional meaning of the cytoplasmic NP particles strongly labelled for DNA and RNA, so-called "informosomes" (I-somes) harboring and "informational DNA" (I-DNA) (Bell, 1969). This discovery was disputed in 70-ties (see, e.g., the Editorial in Nature New Biol. **239**, 33, 1971). On the basis of our characterization of the POMS material of CHLMs, we believe that the Bell's NP particles originating from POMS of growing animal cells are closely similar to our NP complexes harboring a replicative active extrachromosomal DNA with all components necessary for LSS with the relevant DNA- and RNA-SAs responsible for their strong labelling for DNA and RNA, respectively.

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