ANATOMY OF THE PRIMASE-ALPHA DNA POLYMERASE REACTION ACCOMPLISHED BY NUCLEOPROTEIN COMPLEXES HARBORING AN EXTRACHROMOSOMAL DNA IDENTICAL WITH AVIAN MYELOBLASTOSIS VIRUS CORE-BOUND DNA: INFLUENCING BY CARBONYLDIPHOSPHONATE, MIMOSINE AND BUTYLPHENYL DEOXYGUANOSINE-5′-TRIPHOSPHATE

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Summary. – Activities of the primase (Pr)-α DNA polymerase (pol) enzyme complex belonging to the naturally occurring reaction systems represented by special NP complexes harboring an extrachromosomal DNA identical with avian myeloblastosis virus (AMV) core-bound DNA (J. Říman, A. Šulová and K. Horská, *Acta virol.* **39**, 149–159 (1995); J. Říman and A. Šulová, *Acta virol.* **41**, 181–192 (1997)) were studied in the absence and presence of carbonyldiphosphonate (COMDP), mimosine (MIMO), to it related ciclopirox olamine (CPX) and butylphenyl deoxyguanosine-5′-triphosphate (BuPdGTP). Reaction products radioactively labeled for RNA and DNA and synthesized with the common four ribonucleoside triphosphates (rNTPs) or rNTPs and deoxyribonucleoside triphosphates (dNTPs) in the reaction medium, were analyzed by polyacrylamide gel electrophoresis (PAGE) at denaturing conditions. It was shown that COMDP strongly activates the Pr and uncouples its activity from that of α DNA pol with accumulation of initiator (i) RNAs of the basic length. This phenomenon is not affected by BuPdGTP. MIMO, in contrast, stimulates both pol activities of this enzyme complex and preserves their mutual coupling. The effects of COMDP, MIMO and CPX seem to be modulated by concentration of the ambient dNTPs. Addition of dNTPs to rNTPs makes the effects of COMDP and MIMO mutually exclusive, suggesting that both these agents, though chemically quite different, are competing for one active site responsible for coupling these both pol activities into the one Pr-α DNA pol reaction.

Key words: primase; α DNA polymerase; enzyme complex; carbonyldiphosphonate, mimosine; ciclopirox olamine; butylphenyl deoxyguanosine-5′-triphosphate; Okazaki fragments; initiator RNA; lagging DNA strand synthesis.

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Abbreviations: AMV = avian myeloblastosis virus; BPB = bromphenol blue; BSA = bovine serum albumin; BuPdGTP = p-butylphenyl-deoxyguanosine-5′-triphosphate; CPX = ciclopirox olamine (6-cyclohexyl-1-hydroxy-4-methyl-2[1H]-pyridone ethanolamine); ^{14}C-UR = [^{14}C]uridine; CHLMs = chicken leukemic myeloblasts; COMDP = carbonyldiphosphonate; DB = digestion buffer; EI = early immature; DNase = deoxyribonuclease; dNTPs = deoxyribonucleoside-5′-triphosphates (dATP, dGTP, dCTP, dTTP); DTT = dithiothreitol; EDTA = ethylenediamine tetraacetic acid; ^{3}H-mTdR = [methyl-^{3}H]thymidine; iRNA = initiator RNA; LSS = lagging DNA strand synthesis; MIMO = 1-mimosine-(α-amino-β-[N-(3-hydroxy-4-pyridone)] propionic acid); NA = nucleic acid, NP = nucleoprotein; NP-40 = Nonidet P-40, rNTPs = ribonucleoside-5′-triphosphates (ATP, GTP, CTP, UTP); ori = origin of replication; PAGE = polyacrylamide gel electrophoresis; PCNA = proliferating cell nuclear antigen; pol = polymerase; POMS = postmicrosomal sediment; Pr = primase; RNase = ribonuclease; ss = single-stranded; XC = xylene cyanol

Introduction

AMV like all other retroviruses so far studied contains a 7 S DNA (Říman and Beaudreau, 1970; Říman *et al.*, 1993a). This DNA actually represents minute early replicative structures (Říman *et al.*, 1993a,b) descending from origin regions of chromosomal DNA replication (ori) of the host cell (Pajer *et al.*, 1999). It enters the virus core in firm association with enzymes of initiation of DNA synthesis (Říman *et al.*, 1995). These findings prompted us to search for NP complexes into which this DNA is organized in the cell. Recently, we succeeded in showing that this DNA is present in NP complexes forming the three basic components (A, B, C) of the postmicrosomal sediment (POMS) of the cytoplasm of chicken leukemic myeloblasts (CHLMs) (Říman and Šulová, 1997a). Later, these complexes were proved to be highly specialized reaction machineries containing all components including the relevant DNA templates essential for early DNA synthesis, minimally, medium and maximally advanced in NP complexes of POMS components C, B and A, respectively (Říman and Šulová, 1997a,b). The NP complexes of POMS component C with a sucrose density of 1.108 g/cm^3 were found to be associated with outstanding Pr-α DNA pol activities (Říman and Šulová, 1997b). It turned out that these NP complexes synthesizing *in vitro* reaction products significant for early lagging DNA strand synthesis (LSS) (Říman and Šulová, 1997c) represent excellent naturally occurring reaction systems convenient for studying the properties of the Pr-α DNA pol reaction and, in this way, the principles of its singularity (Kaguni and Lehman, 1988). For separate influencing of α DNA pol and Pr activities we used BuPdGTP (Byrnes, 1985; Lee *et al.*, 1985) and COMDP, respectively. As regards COMDP, this agent was originally described as a selective inhibitor (Talanian *et al.*, 1989) of proliferating cell nuclear antigen- (PCNA) (Prelich *et al.*, 1987) -independent δ DNA pol (Syvaoja and Linn, 1989) activity, identical with that of the ε DNA pol (Syvaoja *et al.*, 1990; Lee *et al.*, 1991; Wright *et al.*, 1994). In our previous experiments (Říman and Šulová, 1997b) we have found that COMDP represents, in addition, a strong activator of Pr, which induces an outstanding accumulation of newly synthesized iRNAs of basic unit length (Říman and Šulová, 1997c). The suggested importance of the Pr-α DNA pol complex for functioning of the initiation complex (Dornreiter *et al.*, 1992) prompted us to analyze in this study also the influence of MIMO and a related substance, CPX (Levenson and Hamlin, 1993) on Pr-α DNA pol enzyme complex activities. MIMO, a rare toxic amino acid from mimosa and leucaena plants (Matsumoto *et al.*, 1951), has been recently rediscovered as a unique inhibitor of initiation of DNA synthesis in mammalian cells (Laland, 1990; Levenson and Hamlin, 1993; Tsvetkov *et al.*, 1997). Accordingly, an inhibitory effect of MIMO on the Pr-α DNA pol enzyme complex activities was expected.

To increase the accuracy of this study, we analyzed directly, as recommended by Roth (1987), the reaction products by PAGE at denaturing conditions. These products, radioactively labeled for RNA and DNA, were synthesized in the absence or presence of the agents mentioned above at reaction conditions suitable for expression of Pr or both pol activities (Nethanel *et al.*, 1988) of this enzyme complex.

Here, we show for the first time specific effects of COMDP and MIMO on Pr and α DNA pol activities and on their mutual coupling. In this way, this study contributes to a better understanding of the singularity of the Pr-α DNA pol reaction as well as to the modes by which COMDP and MIMO influence this reaction system.

Materials and Methods

Chemicals. Unlabeled rNTPs and dNTPs were from Calbiochem. COMDP and BuPdGTP were from Prof. G.S. Wright, Univ. of Massachusetts, Worcester, MA, USA. MIMO and CPX were from Sigma, acrylamide and TEMED, bromphenol blue (BPB) and xylene cyanol (XC) were from Serva. Nonidet P-40 (NP-40) was from LKB. All other chemicals were of analytical purity.

Radioisotopes. [α^{32}P]-adenosine-5′-triphosphate ([α^{32}P]ATP) and [α^{32}P]-deoxyadenosine-5′-triphosphate ([α^{32}P]dATP), 110 TBq/nmol each, were from Amersham.

Buffers. TRIS buffers containing EDTA (TNE) or borate (TNB) were prepared according to Maniatis *et al.* (1982). Digestion buffer (DB) for DNase I treatment was prepared as described earlier (Říman *et al.*, 1993a).

Enzyme. RNase-free DNase I was from Worthington.

Cells. CHLMs, their cultivation and radioactive labeling for DNA and RNA with ^{3}H-mTdR and ^{14}C-UR, respectively, were described in detail earlier (Říman *et al.*, 1993a).

Source of Pr-α DNA pol enzyme complex was represented by NP complexes of POMS component C with a sucrose density of 1.108 g/cm^3, isolated from the cytoplasm of CHLMs as described earlier (Říman and Šulová, 1997a). These NP complexes descend from ori regions of chromosomal DNA replication (Pajer *et al.*, 1999) and possess all components, including the short pieces of endogenous DNA template molecules (Korb *et al.*, 1997), necessary for the *in vitro* synthesis of reaction products significant for the early LSS (Říman and Šulová, 1997c).

Enzymatic reactions were performed with aliquots of POMS component C material originating from 3–4 x 10^7 cells and residing in 20 µl of the relevant isopycnic sucrose gradient fraction (220 µl). Individual reactions were carried out for 30 mins at 37°C at the following reaction conditions.

Assay I (suitable for expression of Pr activity). The reaction mixture (50 µl) contained: 0.05 mol/l Tris-HCl pH 7.2, 0.01 mol/l $MgSO_4$, 0.1 mmol/l dithiothreitol (DTT), 0.05% bovine serum albumin (BSA), 0.05% NP-40, 40 µmol/l unlabeled UTP and CTP each, 4 µmol/l unlabeled GTP, 12 µmol/l unlabeled ATP, 24 µCi [α^{32}P]ATP, 1% glycerol and 20 µl of the relevant isopycnic sucrose gradient fraction

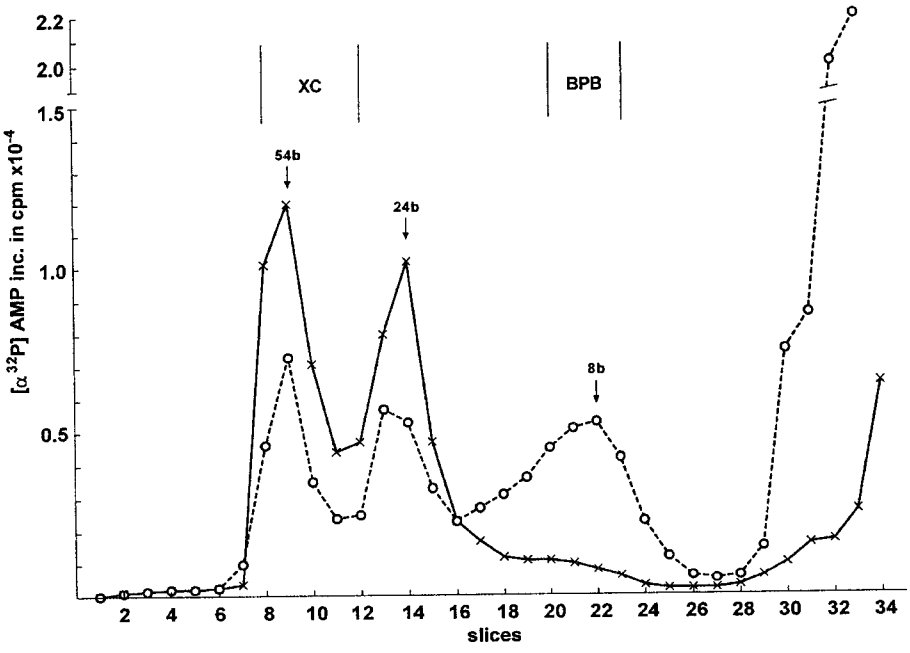

Fig. 1

Denaturing PAGE characteristics of the reaction products radioactively labeled for RNA and synthesized in the absence or presence of COMDP at reaction conditions suitable for expression of Pr activity (Assay 1) of the Pr-α DNA pol complex

Gel distribution of radioactivity of [α^{32}P]AMP incorporated into NAs. Products synthesized in the absence (continuous line, x) or presence (broken line, o) of COMDP (50 μmol/l). XC and BPB, internal markers Vertical arrows indicate gel positions of ss NAs of a length given in the number of bases.

Assay 2 (suitable for expression of both Pr and α DNA pol activities). The reaction mixture (50 μl) contained: 0.05 mol/l Tris-HCl pH 7.2, 0.01 mol/l MgSO4, 0.1 mmol/l DTT, 0.05% BSA, 0.05% NP-40, 40 μmol/l unlabeled UTP and CTP each, 4 μmol/l unlabeled GTP, 12 μmol/l unlabeled ATP, 24 μCi [α^{32}P]ATP, 40 μmol/l each of the common unlabeled dNTPs, 1% glycerol, and 20 μl of the relevant isopycnic sucrose gradient fraction.

Assay 3 (suitable for expression of both Pr and α DNA pol activities). The reaction mixture (50 μl) contained: 0.05 mol/l Tris-HCl pH 8.1, 0.05 mol/l MgCl2, 0.04 mol/l KCl, 0.2 mmol/l DTT, 40 μmol/l each of the unlabeled common rNTPs, 40 μmol/l unlabeled dGTP, dCTP, dTTP each, 4 μmol/l unlabeled dATP, 48 μCi [α^{32}P]dATP, 0.05% NP-40, 1% glycerol, and 20 μl of the relevant isopycnic sucrose gradient fraction.

Agents and their concentrations. The influence of COMDP was studied at the final concentration of 50 μmol/l (Říman and Šulová, 1997b), that of MIMO and CPX at 400 and 200 μmol/l, respectively (Levenson and Hamlin, 1993), and that of BuPdGTP at 10 μmol/l (Nethanel *et al.*, 1988).

Isolation of the reaction products and their preparation for analyses were described previously (Říman and Šulová, 1997c).

DNase I treatment. One of the two equal portions of a vacuum-dried reaction product sample was suspended in 5 μl of digestion buffer (DB) containing 10 μg/μl of DNase I and incubated for 6 hrs (Singh and Dumas, 1984) at 37°C. The reaction was stopped with EDTA (Maniatis *et al.*, 1982), and the sample was further incubated at 100°C for 2 mins. Another portion remained untreated. Then, both the treated and the untreated samples were dissolved in 12 μl of a formamide mixture, thermally denatured (Maniatis *et al.*, 1982) and subjected to PAGE.

PAGE at denaturing conditions. Samples of isolated reaction products were electrophoresed in 12% polyacrylamide gels (17 x 12 x 0.04 cm) supplemented with urea (7 mmol/l) in TBE at 300 V for 4 hrs at 8°C. Samples were run with xylene cyanol (XC) and bromphenol blue (BPB) as internal markers (Maniatis *et al.*, 1982). The length of reaction products was estimated by use of internal markers and DNA fragment markers run in parallel (Nethanel *et al.*, 1988; Říman *et al.*, 1993b) and was expressed in the number of bases (b). For this purpose, construction of a calibration curve expressing the relation of the length of the markers versus their electrophoretic migration distance turned out to be useful.

Radioactivity measurements were accomplished with 4-mm gel slices dried on Synpor 6 filter discs, in a toluene-based scintillation fluid or on the basis of Cerenkov effect in a Beckman spectrometer 6000 SE.

Results

Expression of the Pr activity of the Pr-α DNA pol enzyme complex and the influence of COMDP

A comparative analysis of the reaction products, radioactively labeled for RNA with [α^{32}P]AMP and

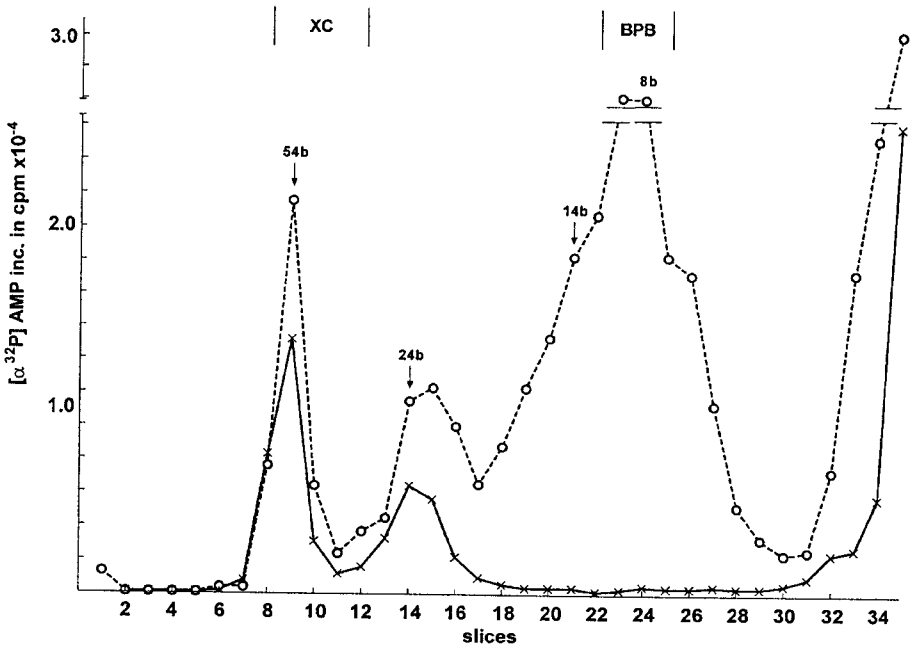

Fig. 2

Denaturing PAGE characteristics of the reaction products radioactively labeled for RNA and synthesized in the absence or presence of COMDP at reaction conditions suitable for expression of both pol activities (Assay 2) of the Pr-α DNA pol complex

Gel distribution of the radioactivity of [α^{32}P]AMP incorporated into NAs. Products synthesized in the absence (continuous line, x) or presence (broken line, o) of COMDP (50 μmol/l). XC and BPB, internal markers. Vertical arrows indicate gel positions of ss NAs of a length given in the number of bases.

synthesized *in vitro* in the absence or presence of COMDP at reaction conditions enabling expression of Pr activity (Assay 1) of the Pr-α DNA pol enzyme complex, associated with POMS component C NP complexes (Říman and Šulová, 1997b,c), led to the following characteristics of the electrophoretic gel distribution of the radioactive RNA label of the reaction products.

In the absence of COMDP (Fig. 1), most of the radioactive RNA label was found to be associated with two reaction products reminiscent, by their length of 24 and 54 nts, of an Okazaki fragment precursor and an immature Okazaki fragment, respectively. Both these reaction products will be further designated as early immature (EI) Okazaki fragments. Besides this radioactivity, its small but perceptible amount was noted at gel positions of the RNA primers (iRNAs) of a basic unit length consisting of 7 to 12 nts (Roth, 1987).

This characteristic was dramatically changed in the presence of COMDP in the reaction medium. In this case (Fig. 1), an outstanding accumulation of the radioactive RNA label was noted at gel positions of the iRNAs of the basic unit length and, simultaneously, a distinct decrease of radioactivity was found to be associated with both EI Okazaki fragments. These changes indicated that COMDP strikingly stimulated the Pr activity and uncoupled it from that of α DNA pol. Thus, in this way, COMDP is also directly responsible for accumulation of the newly synthesized iRNAs, which cannot be further used by α DNA pol for initiation of DNA synthesis and, consequently, for synthesis of the DNA portion of both EI Okazaki fragments. The uncoupling potency of COMDP seems to be responsible also for high selectivity of stimulation of Pr activity of the Pr-α DNA pol enzyme complex. The strong activation of RNA primer synthesis by COMDP may also be in direct relation to the distinct increase of radioactivity at gel positions of the smallest reaction products consisting of 2–3 nts (Fig. 1). This radioactivity may represent the occurrence of early initiation or degradation products or both (Singh and Dumas, 1984; Mendelmann and Richardson, 1991) accompanying a precipitous RNA primer synthesis. This interpretation of the influence of COMDP in the presence of rNTPs only in the reaction medium may help, in turn, to explain the characteristic obtained at the same reaction conditions but in the absence of COMDP. In this case, the increase of radioactivity in both types of EI Okazaki fragments and the presence of small but perceptible radioactivity at gel positions of iRNAs of the basic unit length indicated that a major portion of iRNAs, newly synthesized by Pr activity expressed in the presence of the

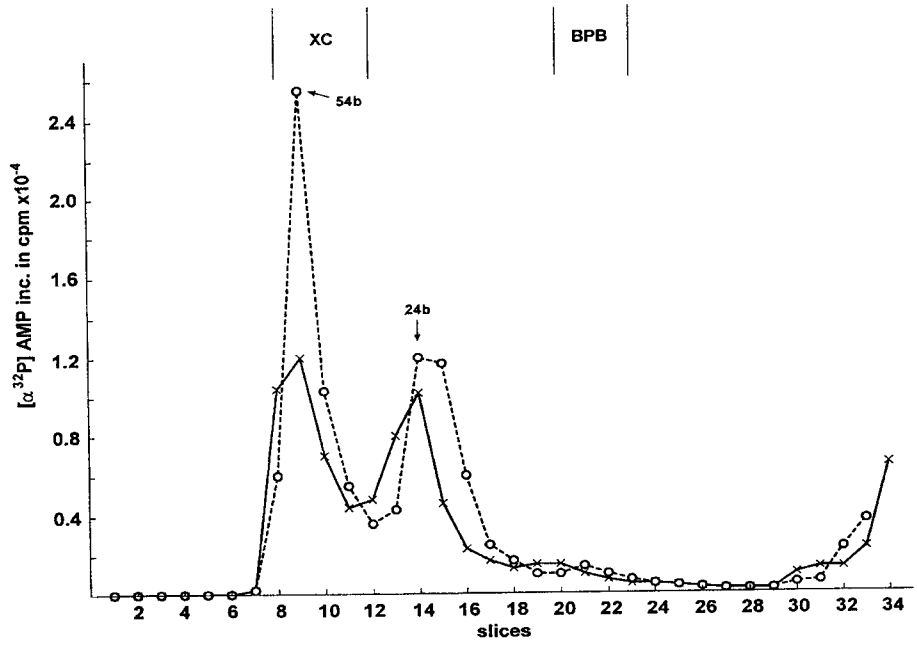

Fig. 3

Denaturing PAGE characteristics of the reaction products radioactively labeled for RNA and synthesized in the absence or presence of MIMO at reaction conditions suitable for expression of the Pr activity (Assay 1) of the Pr-α DNA pol complex

Gel distribution of the radioactivity of [α^{32}P]AMP incorporated into NAs. Products synthesized in the absence (continuous line, x) or presence (broken line, o) of MIMO (400 μmol/l). XC and BPB, internal markers. Vertical arrows indicate gel positions of ss NAs of a length given in the number of bases.

common four rNTPs only, were used in a coupled reaction by that of α DNA pol for synthesis of the DNA portion of both types of EI Okazaki fragments. The necessary expression of the α DNA pol activity was, in this case, evidently enabled by trace amounts of dNTPs, which are always present in rNTP samples, though they are of analytical purity (Chang *et al.*, 1984). In this way expressed α DNA pol activity was sufficient for using the greater but not the entire portion of the newly synthesized iRNAs for initiation of synthesis of the DNA component of both types of EI Okazaki fragments.

Expression of both pol activities of the Pr-α DNA pol enzyme complex and the influence of COMDP

A comparative analysis of reaction products, radioactively labeled for RNA and synthesized in the absence or presence of COMDP with the common rNTPs and dNTPs in the reaction medium (Assay 2), led to the characteristics presented in Fig. 2.

In the absence of COMDP no perceptible amounts of radioactivity were noted at gel positions of iRNAs of the basic length unit, but the radioactive labeling of both EI Okazaki fragments was strikingly augmented. Simultaneously, at gel positions of the smallest reaction products the radioactivity distinctly increased. Accordingly, at these reaction conditions the expression of both Pr and α DNA pol activities was stimulated in a mutually coupled manner. Thus, an increased amount of iRNAs was, in this case, immediately used for synthesis of the DNA component of both types of EI Okazaki fragments. A logical consequence of all these events is, in this case, a striking increase of radioactive labeling in general.

In contrast, the presence of COMDP again led to the accumulation of iRNAs of the basic unit length, but the presence of dNTPs besides rNTPs made the phenomenon of accumulation of iRNAs even more striking. This outstanding change in the gel distribution of the radioactivity was again accompanied by its decrease in both types of EI Okazaki fragments. This suggested that COMDP uncouples both pol activities of the Pr-α DNA pol enzyme complex even at conditions enhancing a mutually coupled expression of both pol activities of this enzyme complex. This implicated that COMDP may somehow interfere with the influence of ambient dNTPs on this enzyme complex (Kaguni and Lehman, 1988).

In conclusion, these experiments confirmed that COMDP uncouples Pr and α DNA pol activities and strongly

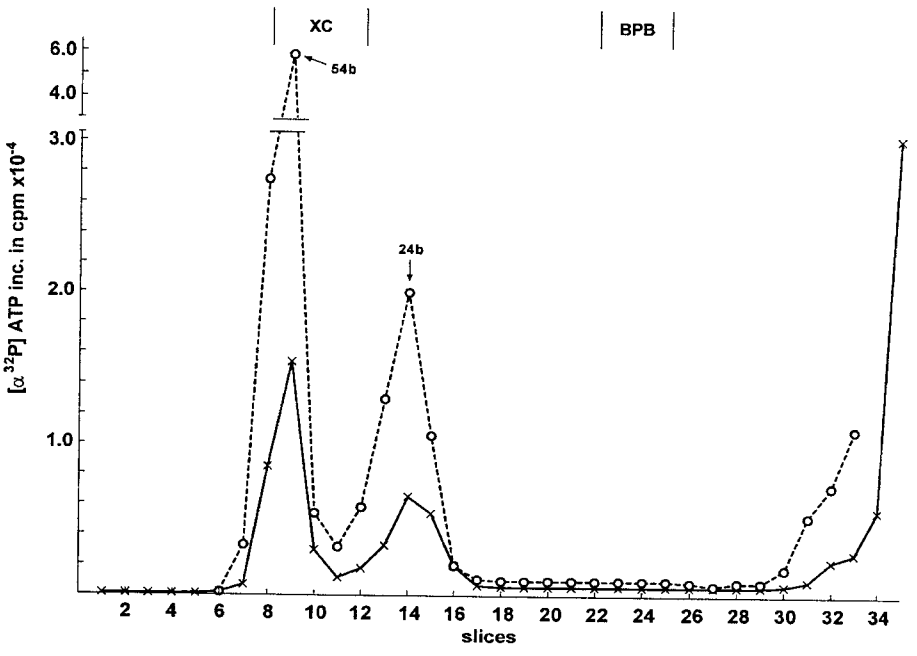

Fig. 4

Denaturing PAGE characteristics of the reaction products radioactively labeled for RNA and synthesized in the absence or presence of MIMO at reaction conditions suitable for expression of both pol activities (Assay 2) of the Pr-α DNA pol complex

Gel distribution of the radioactivity of [α^{32}P]AMP incorporated into NAs. Products synthesized in the absence (continuous line, x) or presence (broken line, o) of MIMO (400 μmol/l). XC and BPB, internal markers. Vertical arrows indicate gel positions of ss NAs of a length given in the number of bases.

stimulates the Pr activity, with the manifestation of the unique phenomenon of accumulation of iRNAs of the basic unit length.

Expression of Pr activity of the Pr-α DNA pol enzyme complex and the influence of MIMO

Detection of striking and different effects of COMDP on both pol activities of the Pr-α DNA pol enzyme complex, which represents an integral part of the initiation complex (Dornreiter *et al.*, 1992), led us to compare in the same way the effect of MIMO on Pr-α DNA pol complex activities. In this case, one could expect an inhibitory influence of MIMO on functioning of the initiation complex and, consequently, on Pr-α DNA pol activities, since this agent was recently suggested to inhibit chromosomal DNA synthesis at its very beginning (Laland, 1990). Despite the existence of different modes of explanation of the inhibitory effect of MIMO on DNA synthesis (see Discussion), we examined comparatively, in the same way, the influence of MIMO on Pr-α DNA pol activities.

As shown in Fig. 3, the presence of MIMO in reactions accomplished with the common rNTPs only (Assay 1) led, unexpectedly, to an increase of the radioactive labeling for RNA in both types of EI Okazaki fragments. As regards the radioactivity at the gel positions of very small reaction products, this was rather low. Such characteristic suggested that at these reaction conditions MIMO stimulates both pol activities of the enzyme complex with preservation of their mutual coupling. A decrease of radioactivity of the very small reaction products may indicate the proper functioning of the whole Pr-α DNA pol complex, including a more efficient use of newly synthesized RNA primers. The expression of α DNA pol activity occurring at these reaction conditions can be again explained by trace amounts of dNTPs present in samples of rNTPs as already suggested above.

Expression of both pol activities of the Pr-α DNA pol enzyme complex and the influence of MIMO

The presence of MIMO led, at reaction conditions suitable for expression of both pol activities (Assay 2), to a further outstanding increase in the radioactive labeling for RNA in both EI Okazaki fragments (Fig. 4). This suggested that this agent stimulated expression of both pol activities of the complex. Accordingly, an increase in the radioactive labeling for DNA in both types of EI Okazaki fragments was noted in the presence of MIMO with the common rNTPs and dNTPs

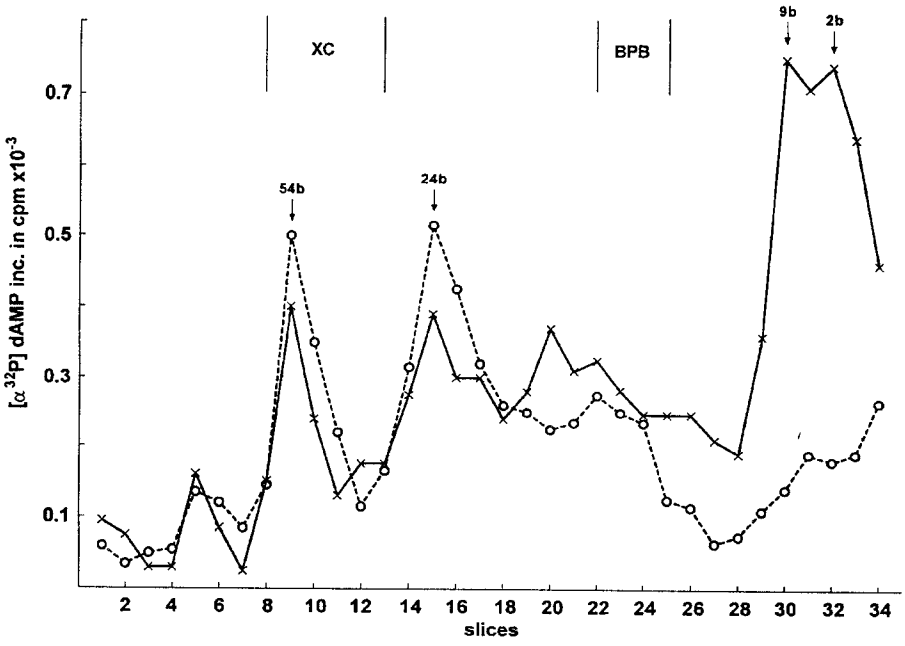

Fig. 5

Denaturing PAGE characteristics of the reaction products radioactively labeled for DNA and synthesized in the absence or presence of MIMO at reaction conditions suitable for expression of both pol activities (Assay 3) of the Pr-α DNA pol complex

Gel distribution of the radioactivity of [α^{32}P]dAMP incorporated into NAs. Products synthesized in the absence (continuous line, x) or presence (broken line, o) of MIMO (400 μmol/l). XC and BPB, internal markers. Vertical arrows indicate gel positions of ss NAs of a length given in the number of bases.

(Assay 3) in the reaction medium (Fig. 5). A simultaneous decrease of radioactivity at gel positions of very small reaction products may reflect an activation of DNA synthesis resulting in synthesis of DNA pieces of greater size (Fig. 5). In conclusion, MIMO, in contrast to COMDP, seems to activate both pol activities of the Pr-α DNA pol complex with preservation of their mutual coupling.

Influencing of the Pr-α DNA pol enzyme complex activities by CPX

Besides MIMO, another pyridone agent, CPX was recently described as a potent inhibitor of the initiation of chromosomal DNA synthesis (Levenson and Hamlin, 1993). This prompted us to use also this agent in our study. As shown in Fig. 6, in the presence of the common rNTPs only (Assay 1), CPX, interestingly, in contrast to MIMO, inhibited strongly but incompletely the radioactive labeling for RNA of the shorter EI Okazaki fragment (24 b) but completely that of the longer one (54 b). This change in the labeling characteristic together with a moderate increase of the radioactivity at gel positions of the smallest reaction products suggested that at these conditions CPX impaired the functioning of the whole Pr-α DNA pol complex. However, this inhibitory effect of CPX was abolished when the common dNTPs as well as rNTPs were present (Assay 3) in the reaction medium (Fig. 7). At these reaction conditions CPX stimulated even more than MIMO the αDNA pol activities of the enzyme complex. As regards the radioactive RNA labeling of both types of EI Okazaki fragments, this was at these reaction conditions reduced to a half only (data not shown). In conclusion, the end effects of both MIMO and CPX seem to be influenced by the rNTPs/dNTPs relation.

Testing the nature of the presumed EI Okazaki fragments

The most important data obtained in this study are those dealing with the properties of the chief reaction products. Consequently, their correct definition represents a prerequisite for a correct interpretation of the results achieved. In this respect, in contrast to the satisfactorily defined iRNAs of the basic unit length, both reaction products, 24 b and 54 b in length, radioactively labeled for RNA as well as for DNA, which we designated as EI Okazaki fragments, deserved an additional confirmation of their actual nature.

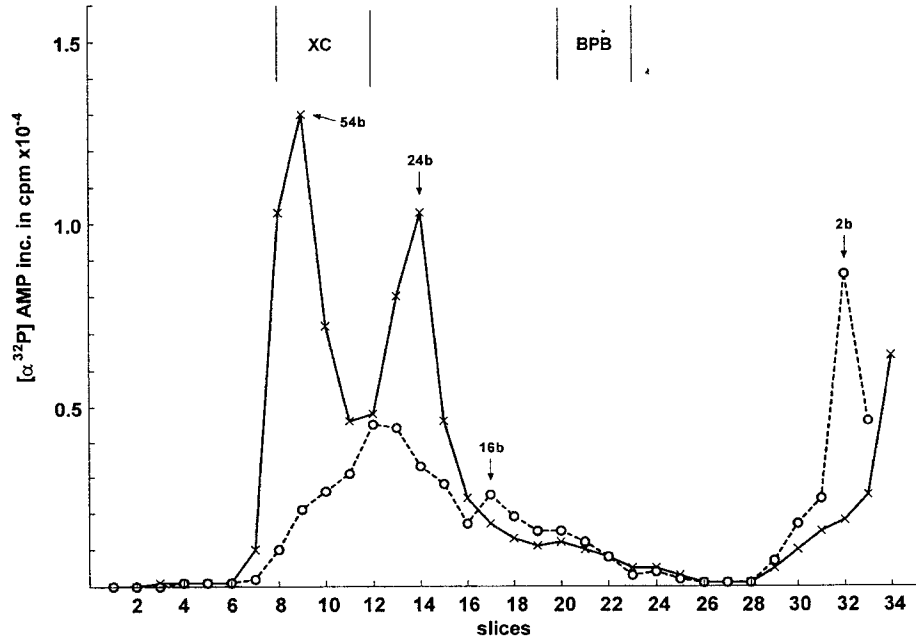

Fig. 6

Denaturing PAGE characteristics of the reaction products radioactively labeled for RNA and synthesized in the absence or presence of CPX at reaction conditions suitable for expression of the Pr activity (Assay 1) of the Pr-α DNA pol complex

Gel distribution of the radioactivity of [α^{32}P]AMP incorporated into NAs. Products synthesized in the absence (continuous line, x) or presence (broken line, o) of CPX (200 μmol/l). XC and BPB, internal markers Vertical arrows indicate gel positions of ss NAs of a length given in the number of bases

Testing the RNA-DNA nature of the presumed EI Okazaki fragments

For this purpose we used reaction products radioactively labeled for RNA and synthesized with the common rNTPs only (Assay 1) in the reaction medium. In order to increase the radioactive RNA labeling of both presumed EI Okazaki fragments, the reactions were also performed in the presence of MIMO. The reacted samples were divided into two equal portions and one of them was further treated with DNase I. Then, both the treated and untreated portions were subjected to PAGE at denaturing conditions. Fig. 8 shows that digestion with DNase I led to a decrease of radioactivity in both types of EI Okazaki fragments as compared to the undigested portion. This decrease in radioactivity was accompanied simultaneously by its increase at the gel positions of the smallest reaction products. Both these interrelated events suggested liberation of the radioactively labeled RNA primers from the unlabeled DNA portion of EI Okazaki fragments digested by DNase I. Such a property of NAs is held as confirmative (Chang *et al.*, 1984; Singh *et al.*, 1986) that, in this case, we have to deal with compounds that are, like the Okazaki fragments, RNA-DNA molecules in nature (DePamphilis and Wassarman, 1980).

Testing the liability of α DNA pol activity for the synthesis of the DNA portion of the presumed EI Okazaki fragments

An RNA primer serves for initiation of DNA synthesis of both, the leading and lagging DNA strands (Harrington and Perrino, 1995). In the case of a LSS (Podust and Hübscher, 1993), also accomplished by the reaction system used in this study (Říman and Šulová, 1997c), the RNA primers synthesized by the Pr activity are used in a coupled reaction by α DNA pol activity of the same enzyme complex for initiation of DNA synthesis and, consequently, for synthesis of the DNA portion of EI Okazaki fragments (Nethanel *et al*, 1988; Nethanel and Kaufmann, 1990). Accordingly, a selective inhibition of α DNA pol activity of the Pr-α DNA pol complex should lead to the inhibition of DNA synthesis and, consequently, to a drop in the use of the newly synthesized RNA primers for starting the DNA synthesis and thus to a decrease in the radioactive labeling for RNA of both types of EI Okazaki fragments. Therefore, we used to this end BuPdGTP, a selective inhibitor of the α DNA pol As (Byrnes, 1985; Lee *et al.*, 1985; Nethanel *et al.*, 1988).

We added this agent alone or with MIMO to the reaction medium containing the common rNTPs and dNTPs (Assay

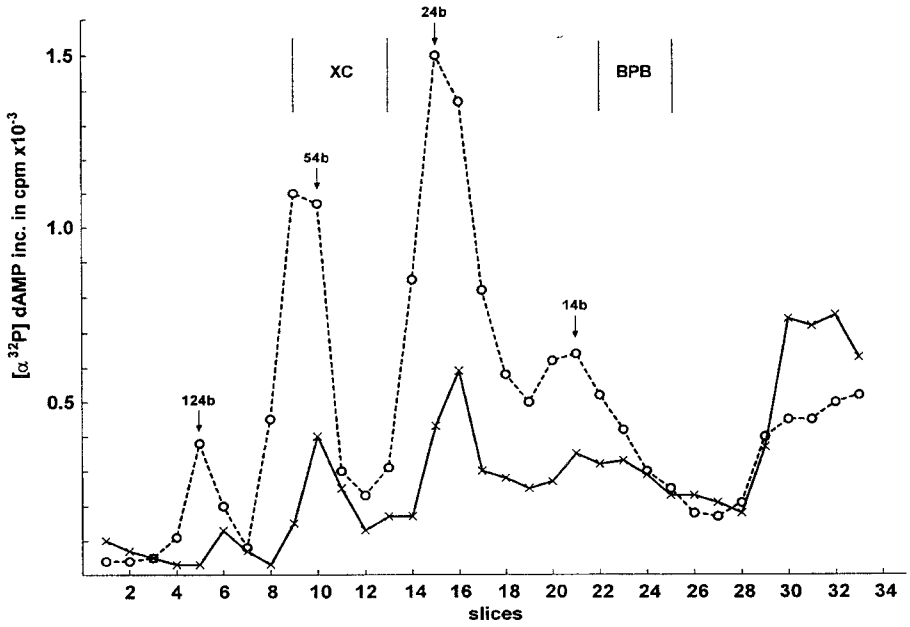

Fig. 7

Denaturing PAGE characteristics of the reaction products radioactively labeled for DNA and synthesized in the absence or presence of CPX at reaction conditions suitable for expression of both pol activities (Assay 3) of the Pr-α DNA pol complex

Gel distribution of the radioactivity of [α^{32}P]dAMP incorporated into NAs. Products synthesized in the absence (continuous line, x) or presence (broken line, o) of CPX (200 μmol/l). XC and BPB, internal markers. Vertical arrows indicate gel positions of ss NAs of a length given in the number of bases.

2). The reaction products synthesized and analyzed in the same way as before gave characteristics shown in Fig. 9. The presence of BuPdGTP alone or with MIMO led to a decrease in the radioactive RNA labeling of both types of presumed EI Okazaki fragments. Simultaneously, an increase in radioactivity was noted at gel positions of the smallest reaction products, actually representing the degradation products of the RNA primers newly synthesized by Pr activity but not further used by α DNA pol activity inhibited by BuPdGTP. Very similar characteristics were obtained when BuPdGTP was present in the reaction medium alone, without MIMO (data not shown).

In conclusion, the changes in the radioactive labeling for RNA of both types of presumed EI Okazaki fragments occurring in the presence of BuPdGTP clearly indicate that the DNA portion of both reaction products, 24 b and 54 b in length, is synthesized by α DNA pol activity as it is in the case of immature Okazaki fragments (Nethanel *et al.*, 1988; Nethanel and Kaufmann, 1990). Thus, the outcome of both tests performed in this and the precedent sections substantiated the Okazaki fragment nature of both reaction products consisting of 26 and 56 nts. In addition, an invariable inhibitory effect of BuPdGTP on the α DNA pol activity, noted also in the presence of MIMO, contributes to the selectivity of this inhibitor.

Selectivity of the effects of COMDP and BuPdGTP

To demonstrate directly the selectivity of the activating and inhibitory effects of COMDP and BuPdGTP, respectively, on the pol activity of the Pr-α DNA pol enzyme complex, we analyzed comparatively the reaction products radioactively labeled for RNA and synthesized in the presence of COMDP or COMDP together with BuPdGTP in the reaction medium containing the common rNTPs and dNTPs (Assay 2). As shown in Fig. 10, BuPdGTP did not influence the activating effect of COMDP on Pr activity, including the accumulation of newly synthesized iRNAs of the basic unit length. Nevertheless, the presence of BuPdGTP together with COMDP made the decrease of radioactivity of both types of EI Okazaki fragments, caused by COMDP, even more pronounced. This implicates that the decline of use of the newly synthesized RNA primers for initiation of DNA synthesis, affected by uncoupling both pol activities by COMDP, was additionally deepened by direct inhibition of the α DNA pol activity by BuPdGTP. In conclusion, BuPdGTP inhibits the α DNA pol activity more deeply than COMDP, which impairs the proper functioning of both pol activities in the coupled Pr-α DNA pol reaction.

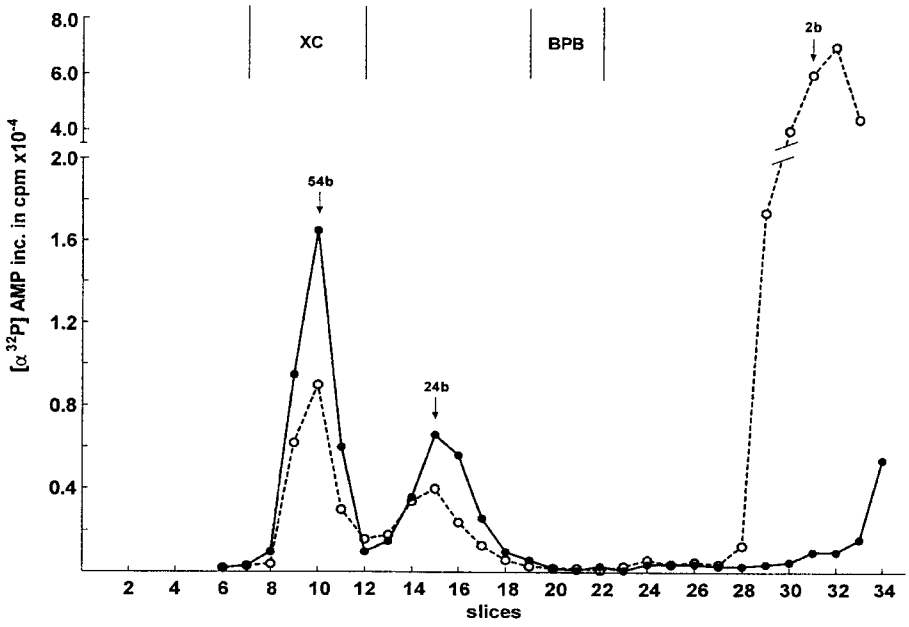

Fig. 8

Denaturing PAGE characteristics of native and DNase I-treated reaction products labeled for RNA and synthesized in the absence or presence of MIMO at reaction conditions suitable for expression of the Pr activity (Assay 1) of the Pr-α DNA pol complex

Gel distribution of the radioactivity of [α^{32}P]AMP incorporated into NAs. Products synthesized in the absence or presence of MIMO (400 μmol/l) and further non-treated (continuous line, ●) or treated (broken line, o) with DNase I. XC and BPB, internal markers. Vertical arrows indicate gel positions of ss NAs of a length given in the number of bases.

Simultanenous influencing of the Pr-a DNA pol activities by COMDP and MIMO

As described above, COMDP and MIMO influence the coupling of both pol activities of the Pr-α DNA pol enzyme complex in an opposite manner. This rose a question whether the influencing of the enzyme complex by COMDP and MIMO is mutually exclusive and thus competitive. Answering this question seemed to be important for recognition whether both these agents do not influence the same active site that might be responsible for coupling of both pol activities of the enzyme complex. To this end we analyzed comparatively the reaction products radioactively labeled for RNA and synthesized in the presence of COMDP or COMDP and MIMO in the reaction medium containing the common rNTPs only (Assay 1). As shown in Fig. 11, MIMO did not influence the activating effect of COMDP on Pr activity including of the accumulation of newly synthesized iRNAs of the basic unit length. Nevertheless, a perceptible increase in the radioactivity in both types of EI Okazaki fragments suggested that even at those reaction conditions, characteristic by the occurrence of trace amounts of dNTPs (Chang *et al.*, 1984), MIMO begins to counteract the uncoupling effect of COMDP. This suggestion was confirmed by the characteristic of reaction products radioactively labeled for RNA and synthesized in the presence of COMDP and MIMO with the common rNTPs and dNTPs in the reaction medium (Assay 2). In this case (Fig. 12), a dramatic decrease in accumulation of radioactivity at gel positions of iRNAs of the basic unit length, accompanied by an increase in radioactivity of both types of EI Okazaki fragments, was recorded. This indicated clearly that MIMO is able, in dependence on dNTP concentration, to counteract the uncoupling effect of COMDP. This phenomenon implicates that COMDP and MIMO, two agents chemically profoundly different, compete for the site on the enzyme complex that is responsible for mutual coupling of both pol activities into the one Pr-α DNA pol enzyme complex reaction.

Discussion

Pr-α DNA pol enzyme complex represents the most conspicuous member of the family of replication enzymes. It is the only eukaryotic DNA pol that can initiate DNA synthesis *de novo* (Wang, 1991). It represents an integral part of the initiation complex (Dornreiter *et al.*, 1992), the

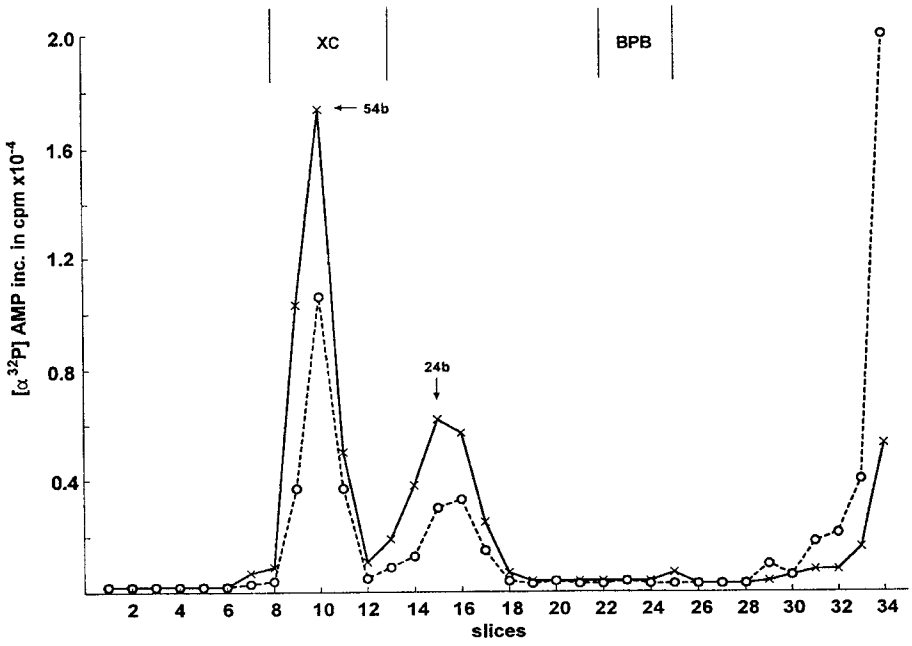

Fig. 9

Denaturing PAGE characteristics of the reaction products radioactively labeled for RNA and synthesized in the absence or presence of MIMO plus BuPdGTP at reaction conditions suitable for expression of both pol activities (Assay 2) of the Pr-α DNA pol complex

Gel distribution of the radioactivity of [α^{32}P]AMP incorporated into NAs. Products synthesized in the absence (continuous line, x) or presence (broken line, o) of MIMO (400 μmol/l) plus BuPdGTP (10 μmol/l). XC and BPB, internal markers. Vertical arrows indicate gel positions of ss NAs of a length given in the number of bases.

formation of which is a prerequisite for initiation of DNA synthesis. It is required both for initiation of DNA synthesis at replication oris and for synthesis of Okazaki fragments on lagging DNA strands of replication forks (Harrington and Perrino, 1995). This dual role of this enzyme complex makes it a likely target for mechanisms that control the cell cycle S phase entry and progression (Foiani *et al.*, 1997). The functional singularity of this enzyme complex resides in its two pols, which use for their mutually coupled expression (Kaguni and Lehman, 1988) the same DNA template. A highly conserved RNA pol, the Pr (Roth, 1987; Griep, 1995), synthesizes RNA primers, iRNAs (Reichard *et al.*, 1974), the 3′-OH ends of which are used by α DNA pol for initiation of DNA synthesis (Coverley and Laskey, 1994). The problem of the functional singularity of this enzyme complex, intimately related to the still not well understood phenomenon of coupling of Pr and α DNA pol activities into one Pr-α DNA pol complex reaction, deserves permanent attention. The results presented in this study contribute right to this problem.

Convenient for studying this problem more deeply turned out to be a naturally occurring reaction system for early DNA synthesis represented by NP complexes forming the POMS component C of CHLMs (Říman and Šulová, 1997a). They possess outstanding Pr-α DNA pol activities (Říman and Šulová, 1997b), which use as endogenous templates short pieces of DNA (Korb *et al.*, 1997) descending from ori regions of chromosomal DNA replication (Pajer *et al.*, 1999). These reaction complexes are able to synthesize *in vitro* reaction products significant for early LSS (Říman and Šulová, 1997c), as confirmed also by this study. Thus, using this reaction system we synthesized reaction products at various reaction conditions and analyzed them by PAGE at denaturing conditions. In this way we confirmed our precedent findings that COMDP, a selective inhibitor of δ DNA pol activity independent of PCNA (Talanian *et al.*, 1989), strongly stimulates the Pr activity of the Pr-α DNA pol enzyme complex (Říman and Šulová, 1997b), and that this stimulation is accompanied by a striking accumulation of iRNAs of basic unit length (Říman and Šulová, 1997c). In addition, we showed that this unique phenomenon is due to the potency of COMDP to uncouple the Pr activity from the α DNA pol activity of this enzyme complex. These new findings also brought evidence that the effect of COMDP on the Pr activity is not changed by BuPdGTP, a selective inhibitor of the α DNA pol activity (Nethanel *et al.*, 1988). In contrast, BuPdGTP deepened the inhibition of synthesis of the DNA portion of the EI Okazaki fragments affected

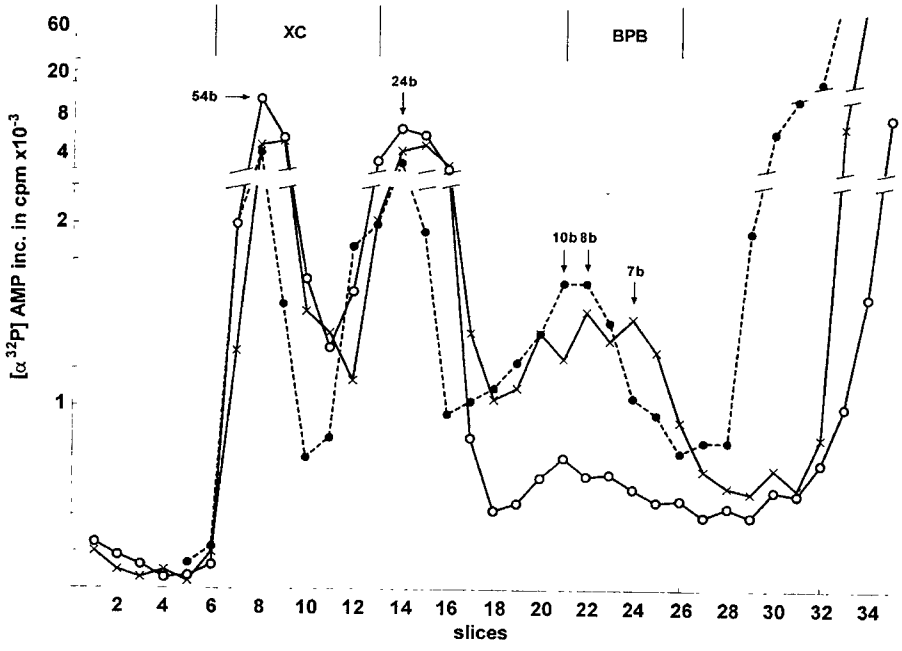

Fig. 10

Denaturing PAGE characteristics of the reaction products radioactively labeled for RNA in the presence COMDP or COMPD plus BuPdGTP or in the absence of these agents at reaction conditions suitable for expression of both pol activities (Assay 2) of the Pr-α DNA pol complex

Gel distribution of the radioactivity of [α^{32}P]AMP incorporated into NAs. Products synthesized in the presence of COMDP (50 μmol/l) (continuous line, x) or COMDP (50 μmol/l) plus BuPdGTP (10 μmol/l) (broken line, •) or without any agent (continuous line, o). XC and BPB, internal markers. Vertical arrows indicate gel positions of ss NAs of a length given in the number of bases.

by the uncoupling effect of COMDP. Both the uncoupling effect and that of stimulation of the Pr activity, accomplished by COMDP, seem to have a common denominator. This might be represented by the capacity of COMDP to interfere strongly with dNTPs on the relevant active sites of the Pr-α DNA pol complex. One of them belongs to that of the α DNA pol subunit, the other to that of the Pr subunit of the enzyme complex. As supposed by Hu *et al.* (1984), the Pr dNTP site (catalytic center or conformer) is synchronously coupled, mutually exclusive and stringent for respective dNTP or rNTP substrates, with the Pr rNTP active site. Transitions between two catalytic modes (deoxy- and ribo-) of this enzyme are regulated by concentration of ambient dNTPs, as suggested earlier by Rowe and Kornberg (1978). Accordingly, by blocking the dNTP binding active sites, COMDP may simultaneously impair initiation of DNA synthesis by uncoupling both pol activities and activate the synthesis of RNA primers by stabilizing the ribo-mode of expression of the Pr activity. A simultaneous locking up of the relevant dNTP binding sites on both pols of the enzyme complex, accomplished by COMDP, seems to be a prerequisite for accumulation of newly synthesized iRNAs further not used for initiation of DNA synthesis. This indicated that the COMDP effects on the Pr-α DNA pol activities, modulated by the dNTP/rNTP relation, may correspond with that assumed by Talanian *et al.* (1989) for the selective inhibitory effect of this agent on the δ DNA pol independent on PCNA (Syväoja and Linn, 1989). In this case, it was postulated that COMDP mimics a dNTP at the enzyme active site. In contrast to these authors, we were able to demonstrate by the analytical approach used, that COMDP affects also the α DNA pol activity, though its capacity to inhibit this enzyme was lower than that of BuPdGTP.

For comparison, we analyzed in the same way the influence of MIMO, an agent that has been recently described as a unique inhibitor of the cell-cycle traverse in the late G_1 prior to the onset of DNA synthesis (Laland, 1990). Consequently, it was suggested that MIMO inhibits, in contrast to the inhibitors of DNA chain elongation, DNA synthesis at replication oris (Mosca *et al.*, 1992; Levenson and Hamlin, 1993).

However, also a dual effect of MIMO on chain initiation and elongation (Kalejta and Hamlin, 1997; Tsvetkov *et al.*, 1997) or on chain elongation only (Hughes and Cook, 1996) has been suggested. Though the mechanism of inhibition of DNA synthesis by MIMO needs further elucidation, it has been clearly shown by Gilbert *et al.* (1995) that MIMO

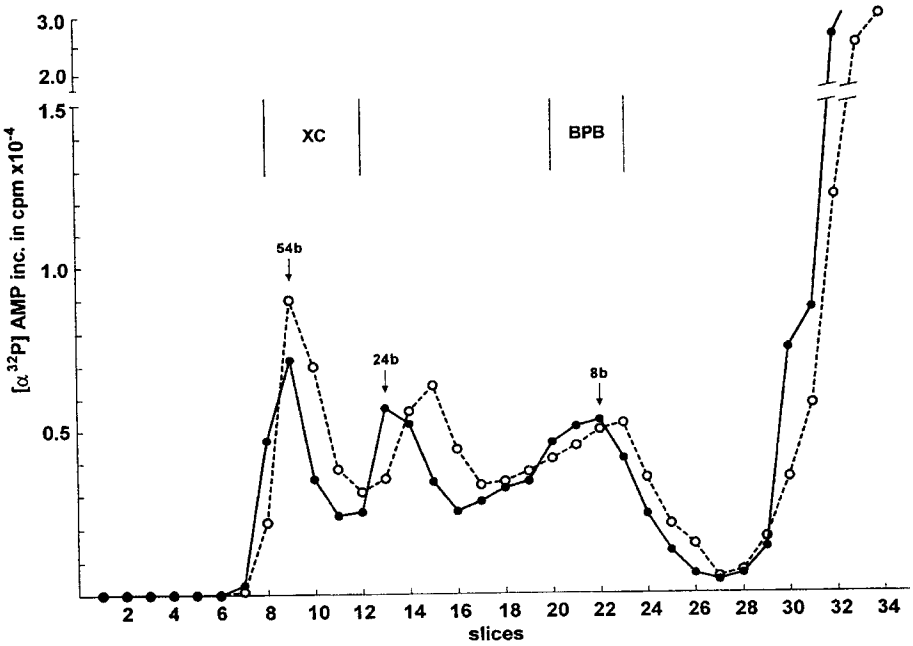

Fig. 11

Denaturing PAGE characteristics of the reaction products radioactively labeled for RNA in the presence of COMDP or COMDP plus MIMO at reaction conditions suitable for expression of the Pr activity (Assay 1) of the Pr-α DNA pol complex

Gel distribution of the radioactivity of [α^{32}P]AMP incorporated into NAs. Products synthesized in the presence of COMDP (50 μmol/l) (continuous line, •) or COMDP (50 μmol/l) plus MIMO (400 μmol/l) (broken line, o). XC and BPB, internal markers. Vertical arrows indicate gel positions of ss NAs of a length given in the number of bases.

arrests DNA synthesis at replication forks by inhibiting the deoxyribonucleotide metabolism in a manner reminiscent of the effect of hydroxyurea, which inhibits ribonucleotide reductase. The same authors have shown that MIMO, in contrast to aphidicolin, does not inhibit DNA synthesis in cell lysates supplied with exogenous dNTPs or in cells with a high content of endogenous dNTPs. MIMO is a strong iron chelator (Kulp and Villiet, 1995), as indicated long ago (Matsumoto *et al.*, 1951). In this capacity it inhibits ribonucleotide reductase by chelating its iron (Mosca *et al.*, 1995). MIMO is believed to be also an inhibitor of serine hydroxymethyl transferase (Lin *et al.*, 1996), involved in the penultimate step of thymidylate synthesis.

Nevertheless, all these and other data on this subject missed until now those related to the direct effect of MIMO on the activities significant for initiation of DNA synthesis. This problem also became a target of this study. Here, we demonstrated that MIMO does not inhibit but stimulates both pol activities of the Pr-α DNA pol enzyme complex. In contrast to COMDP, it preserves their mutual coupling. Accordingly, the newly synthesized iRNAs are, in this case, immediately used for synthesis of the DNA portion of both types of EI Okazaki fragments. These findings implicate that the inhibitory effect of MIMO on early DNA synthesis occurring at the cellular level is accomplished evidently by a mechanism suggested by Gilbert *et al.* (1995).

As regards the activating effect of MIMO on the Pr-α DNA pol complex activities, it deserves at least an attempt at explanation. In this respect, some relevant points can be mentioned. MIMO, a pyridone compound, is considered an antagonist of pyridoxal (Yoshida, 1944). Pyridoxal compounds are able of "pyridoxylation" of the relevant amino groups of cationic amino acid residues located at the active sites of DNA and RNA pols (Basu and Modak, 1987). The inhibitory effect of pyridoxal-5′-phosphate on DNA (and RNA) pols is reversible by dNTPs (Modak, 1976). As regards the MIMO effect on both pol activities of the Pr-α DNA pol complex, it is also strongly influenced by ambient dNTPs and it is competing with that of COMDP, which mimics them at active pol sites, as shown by Talanian *et al.* (1989) and in this study. Thus, the relevant conformation changes, induced most probably by the pyridone component of MIMO affecting the active sites of both pols of the Pr-α DNA pol complex, might be responsible for the activating MIMO effect. Such conformation changes induced in this subject should be, consequently, opposite to those evoked by pyridoxal compounds. Accordingly, CPX, a substance chemically quite different from but related to MIMO

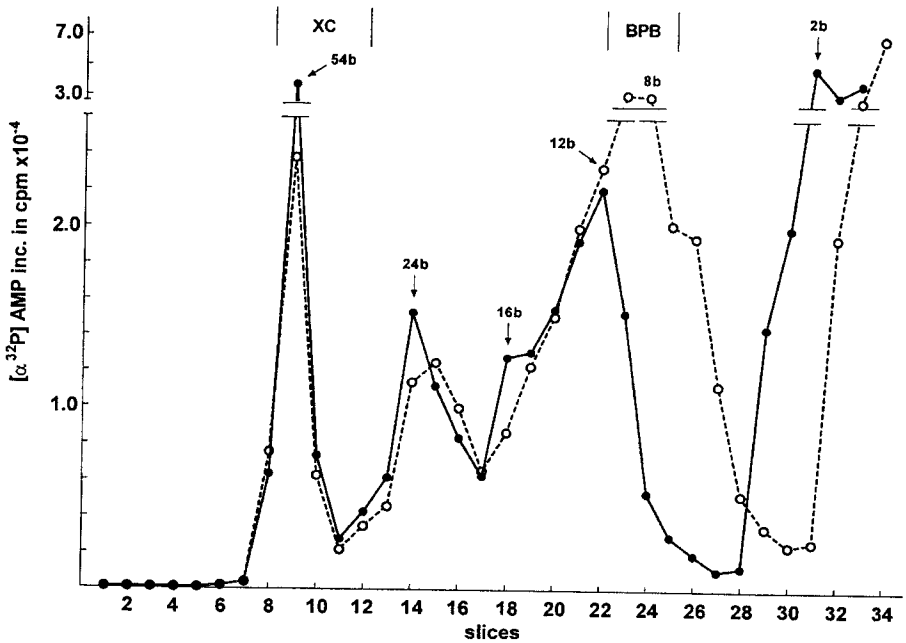

Fig. 12

Denaturing PAGE characteristics of the reaction products radioactively labeled for RNA in the presence of COMDP or COMDP plus MIMO at reaction conditions suitable for expression of both pol activities (Assay 2) of the Pr-α DNA pol complex

Gel distribution of the radioactivity of [α^{32}P]AMP incorporated into NAs. Products synthesized in the presence of COMDP (50 μmol/l) (broken line, o) or COMDP (50 μmol/l) plus MIMO (400 μmol/l) (continuous line, •). XC and BPB, internal markers. Vertical arrows indicate gel positions of ss NAs of a length given in the number of bases.

(Levenson and Hamlin, 1993) by its pyridone component, exhibited, like MIMO, an activating effect on both pol activities of the complex in dependence on dNTPs.

Finally, this study showed that, at certain reaction conditions, the effects of COMDP and MIMO are mutually exclusive and that both these agents, though chemically profoundly different, compete for the same active site that binds dNTPs. This active site belonging evidently to that located on the α DNA pol subunit seems to be responsible for mutual coupling of both pol activitiess into one reaction significant for functioning of the whole Pr-α DNA pol enzyme complex. In this way, the findings of this study contribute to the still not well understood problem of coupling the Pr and the α DNA pol activities and, consequently, to the functional singularity of this enzyme complex.

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