# THE DEOXYRIBO-MODE EXPRESSION OF PRIMASE ACTIVITIES OF THE PRIMASE-α DNA POLYMERASE ENZYME COMPLEX ASSOCIATED WITH 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.** – The deoxyribo-mode expression of primase (Pr) activities of the Pr-α DNA polymerase (pol) enzyme complex belonging to the naturally occurring nucleoprotein (NP) complexes harboring an extrachromosomal DNA identical with avian myeloblastosis virus (AMV) core-bound DNA (Říman and Šulová, *Acta Virol.* **41**, 181–192 (1997)) is similarly influenced by carbonyldiphosphonate (COMDP) as the ribo-mode expression of Pr activities (Říman, *Acta Virol.* **45**, 109–124 (2001)). In the presence of all four common dNTPs only and dNTPs and rNTPs in the reaction medium, COMDP strongly activates the deoxyribo-mode of Pr activities and again induces a unique phenomenon of primer accumulation. These primers labeled for DNA are up to 90% alkali-resistant and sensitive to DNase I treatment. This suggests that they are constituted mostly of deoxynucleotides (dnts). In contrast to the stimulation of the ribo-mode expression of Pr activities by COMDP, the incorporated radioactivity is in this case more than one order lesser. 1-Mimosine-(α-amino-β-[N-(3-hydroxy-4-pyridone)]-propionic acid (MIMO) is again able to substantially eliminate the phenomenon of primer accumulation, suggesting that also in this case the effects of COMDP and MIMO are, at certain reaction conditions, mutually exclusive and that both agents compete for the same active site responsible for mutual coupling of Pr and α DNA pol activities.

**Key words:** α DNA polymerase; butylphenyl deoxyguanosine-5′-triphosphate; carbonyldiphosphonate; mimosine; Okazaki fragments; primase

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**Abbreviations:** AMV = avian myeloblastosis virus; b = base; BPB = bromphenol blue; BSA = bovine serum albumin; BuPdGTP = (p-butylphenyl)-deoxyguanosine-5′-triphosphate; CHLM = chicken leukemic myeloblast; COMDP = carbonyldiphosphonate; dnt = deoxyribonucleotide; EI = early immature; DNase = deoxyribonuclease; dNTPs = deoxyribonucleoside triphosphates; DTT = dithiothreitol; EDTA = ethylenediamine tetraacetate; iRNA = initiator RNA; MIMO = 1-mimosine-(α-amino-β-[N-(3-hydroxy-4-pyridone)]-propionic acid; NA = nucleic acid; NP = nucleoprotein; NP-40 = Nonidet P-40; nt = nucleotide; rnt = ribonucleotide; rNTPs = ribonucleoside-5′-triphosphates; ori = replication origin; PAGE = polyacrylamide gel electrophoresis; PCNA = proliferating cell nuclear antigen; pol = polymerase; POMS = postmicrosomal sediment; Pr = primase; ss = single-stranded; XC = xylene cyanol

## Introduction

Our recent search for the cell origin and NP organization of the avian myeloblastosis virus (AMV) core-bound 7S DNA (Říman and Beaudreau, 1970) brought the following new findings: this metabolically highly active (Říman *et al.*, 1972, 1993a) DNA, actually representing minute early replicative DNA structures (Říman *et al.*, 1993b) and inside of the virus core firmly associated with the Pr-α DNA pol enzyme complex (Říman *et al.*, 1995), descends from the origin (ori) regions of chromosomal DNA replication (Pajer *et al.*, 1999). In the cell this DNA was shown to be organized into NP complexes forming the three basic components (A, B, C) of the postmicrosomal sediment (POMS) of the chicken leukemic myeloblasts (CHLMs) (Říman and Šulová, 1997a). In accord with the descent of their short DNAs (Korb *et al.*, 1997), these NP complexes are equipped with outstanding enzymatic activities significant for early DNA synthesis, minimally, medium and maximally advanced in the NP complexes of POMS components C, B and A, respectively (Říman and Šulová, 1997b). These structures actually represent unique reaction machineries (Korb *et al.*, 1997) possessing all components, including the DNA templates, necessary for *in vitro* synthesizing the reaction products relevant to initiation of DNA synthesis (Říman and Šulová, 1997c). To distinguish the individual DNA pols associated with these NP complexes we used various inhibitors (Říman and Šulová, 1997b,c), among them also COMDP, a selective inhibitor (Talanian *et al.*, 1989) of proliferating cell nuclear antigen (PCNA)-independent δ DNA pol (Syväoja and Linn, 1989), now designated as ε DNA pol (Wright *et al.*, 1994). Using this drug we have found that besides its mentioned inhibitory effect, it stimulates labeling for DNA and even more that for RNA of NP complexes of POMS component C (Říman and Šulová, 1997b). Later, we have shown that this COMDP effect is due to a strong stimulation of Pr activities of the Pr-α DNA pol enzyme complex associated with the POMS component C-NP complexes (Říman and Šulová, 1997b) and that the Pr activation is accompanied by a unique phenomenon of the accumulation of RNA primers of basic length (Říman and Šulová, 1997c). Studying in detail this phenomenon of primer accumulation and the Pr-α DNA pol reaction accomplished by these NP complexes *in vitro*, we have shown only recently that COMDP, besides Pr activation, uncouples its activities from those of α DNA pol and that in this way this drug is also responsible for induction of the phenomenon of primer accumulation (Říman, 2001). In this study (Říman, 2001) we have also shown that the uncoupling potency of COMDP can be counteracted at certain reaction conditions by the excess of MIMO, a drug that stimulates both pol activities of the Pr-α DNA pol enzyme complex and preserves their mutual coupling. Since Pr of the Pr-α DNA pol enzyme complex is able to express, besides the ribo-, also the deoxyribo-mode of its activities (Hu *et al.*, 1984), the question arose whether and how COMDP can also influence the deoxyribo-mode of Pr expression. Analyzing the reaction products labeled for DNA and synthesized at two different reaction conditions, here we show that COMDP influences in the same way also the deoxyribo-mode expression of the Pr activities. Also in this case the primer accumulation is counteracted by MIMO.

## Material and Methods

*Chemicals.* COMDP and BuPdGTP were from Prof. G.D. Wright, University of Massachusetts, Worcester, MA, USA. MIMO was from Sigma. All other chemicals were of analytical purity.

*Radioisotope.* [alpha$^{32}$P]-deoxyadenosine-5′-triphosphate ([alpha$^{32}$P]dATP), 110 TBq/nmol, was from Amersham.

*The source of Pr-α DNA pol activities* was represented by NP complexes of the POMS component C of a sucrose density of 1.108 g/cm$^3$, isolated (Říman and Šulová, 1997a) from isopycnic sucrose gradients of the cytoplasm of CHLMs. These NP complexes descending from the ori regions of chromosomal DNA replication (Pajer *et al.*, 1999) possess all components, including the relevant short template DNAs (Říman and Šulová, 1997b; Korb *et al.*, 1997) necessary for synthesizing the *in vitro* products significant for early lagging strand DNA synthesis (LSS) (Říman and Šulová, 1997c).

*Enzymatic reactions* were accomplished at 37°C for 30 mins with aliquots of the 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) (Říman and Šulová, 1997c). The following assays were used:

Assay 1 (conditions suitable for expression of the deoxy-mode of Pr activities, DNA synthesizing activities, in general). The reaction mixture (50 µl) contained 0.05 mol/l Tris-HCl pH 8.1, 0.05 mol/l $MgCl_2$, 0.04 mol/l KCl, 0.2 mmol/l DTT, 40 µmol/l unlabeled dGTP, dCTP, dTTP each, 4 µmol/l unlabeled dATP, 48 µCi [alpha$^{32}$P]dATP, 0.05% NP-40, 1% glycerol and 20 µl of enzyme gradient fraction.

Assay 2 (reaction conditions suitable for expression of Pr and α DNA pol activities (Nethanel *et al.*, 1988)). The reaction mixture (50 µl) contained 40 µmol/l each of the four common unlabeled rNTPs, 40 µmol/l unlabeled dGTP, dCTP, dTTP each, 4 µmol/l unlabeled dATP, 48 µCi [alpha$^{32}$P]dATP. All other ingredients including the enzyme were added as in Assay 1.

*Polyacrylamide gel electrophoresis (PAGE) at denaturing conditions* was performed with samples of reaction products isolated as described (Říman and Šulová, 1997c). Samples were electrophoresed in 12% polyacrylamide gels supplemented with urea (7 mmol/l) at 300 V for 4 hrs at 8°C. Estimation of the length of the electrophoresed single-stranded NAs (ssNAs) of the reaction products was already described (Nethanel *et al.*, 1988; Říman *et al.*, 1993b; Říman, 2001).

All other methodical accomplished steps, including radioactivity measurements, were described previously (Říman, 2001).

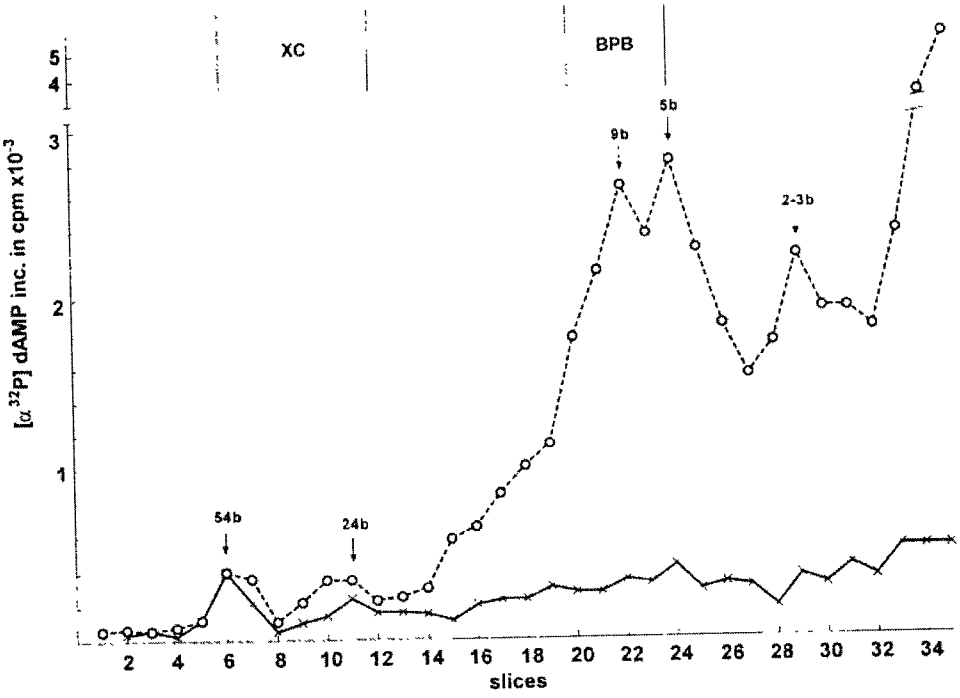


Fig. 1

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

Gel distribution of radioactivity of [alpha$^{32}$P]dAMP 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 ssNAs of a length given in the number of bases (b).

## Results and Discussion

### *Deoxyribo-mode of Pr expression and its influencing by COMDP*

A prerequisite for initiation of DNA synthesis *de novo* is synthesis of the RNA primer (Roth, 1987; Wang, 1991), required both for initiation of DNA synthesis at oris and for synthesis of Okazaki fragments on lagging DNA strands (Harrington and Perrino, 1995). The synthesis of the RNA primers, the initiator RNAs (iRNAs) (Reichard *et al.*, 1974), is accomplished by a highly conserved DNA-dependent RNA pol, the primase (Roth, 1987; Griep, 1995). The 3′-OH ends of its products, the iRNAs, are used in a coupled reaction by the α DNA pol activity for initiation of the DNA synthesis accomplished, consequently, by a single reaction of the Pr-α DNA pol enzyme complex (Coverley and Laskey, 1994). Thus, to the singularity of the reaction of this enzyme complex, the primase contributes by its RNA pol activities. In our precedent article (Říman, 2001) it was shown that COMDP strikingly activates these activities and uncouples them from those of α DNA pol, inducing in this way a unique phenomenon of accumulation of primers of the basic length. These effects of COMDP, even more strikingly evident at reaction conditions suitable for expression of both (RNA and DNA) pol activities of the Pr-α DNA pol enzyme complex, suggested that COMDP is able to overcome the inhibitory effect exerted by concentration of ambient dNTPs on synthesis of RNA primers (Rowen and Kornberg, 1978). In the same article of ours (Říman, 2001) it was also shown that this COMDP effect can be counteracted in the presence of dNTPs by excess of MIMO, which stimulates the Pr and α DNA pol activities and preserves their mutual coupling. These findings together with the knowledge that Pr is able, besides rnts, to incorporate also the dnts into a ribo-deoxyribonucleotide hybrid primer (Rowen and Kornberg, 1978) and that Pr is also able to add either a ribonucleotide (rnt) or deoxyribonucleotide (dnt) to the 3′-OH of either a ribo- or deoxyribo-residue of the primer terminus (Hu *et al.*, 1984), rose the question whether and how COMDP can

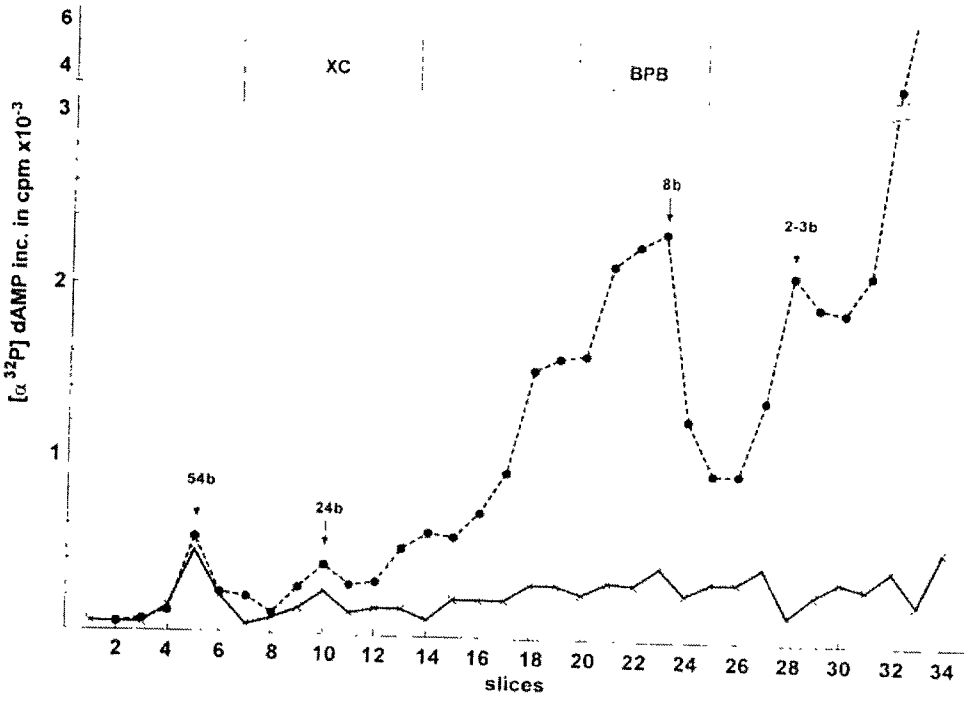


Fig. 2

**Denaturing PAGE characteristics of the reaction products labeled for DNA and synthesized in the absence of drugs or presence of COMDP and BuPdGTP at reaction conditions suitable for the deoxyribo-mode expression of Pr activities of the Pr-α DNA pol enzyme complex (Assay 1)**

Gel distribution of radioactivity of [alpha$^{32}$P]dAMP incorporated into NAs. Products synthesized in the absence of drugs (continuous line, x) or presence of COMDP (50 μmol/l) and BuPdGTP (10 μmol/l) (broken line, •). XC and BPB, internal markers. Vertical arrows indicate gel positions of ssNAs of a length given in the number of bases (b).

also affect the deoxyribo-mode of Pr expression. The importance of such a question was strengthened by the assumption that the Pr of the Pr-α DNA pol enzyme complex possesses two catalytic centers or two conformers of the one catalytic center, which are synchronously coupled, mutually exclusive for respective rNTPs or dNTPs (Hu *et al.*, 1984) and in this way responsible for the ribo- or deoxyribo-mode of Pr expression. Thus, to answer the question whether COMDP also influences the deoxyribo-mode of Pr expression, we have comparatively analyzed by denaturing PAGE the reaction products radioactively labeled for DNA with [alpha$^{32}$P]dAMP and synthesized in the presence of COMDP with all common four dNTPs in the reaction mixture only (Assay 1), permitting expression of DNA synthesizing activities, in general. As evident from Fig. 1, COMDP at the same concentration (50 μmol/l) as used in the case of analysis of the ribo-mode of Pr expression (Říman, 2001) strongly activated also the deoxyribo-mode of Pr expression, again inducing the accumulation of primers of basic length radioactively labeled for DNA, but this time the extent of the radioactive labeling was more than one order lesser, in general, than that noted for RNA in the presence of rNTPs only or both NTP types in the reaction mixture (Říman, 2001). Interestingly, the radioactivity profile of primer accumulation revealed, in this case, a bifurcation of the main peak at two at gel positions of oligonucleotides of about 5 and 9 nt in length, reminiscent of monomers and dimers of an assumed Pr "synthesis unit" (Hu *et al.*, 1984). A broadening of this profile to gel positions of oligonucleotides up to about 20 nt in length also suggested the presence of even longer multimers. Another radioactivity peak at gel positions of di- and trinucleotides (Fig. 1) indicated the presence of the initiation or degradation products, or both, significant for a precipitous reaction (Mendelman and Richardson, 1991). A small radioactive DNA label associated with reaction products constituted of 24 and 54 nts and identified in our precedent article as early immature (EI) Okazaki fragments (Říman, 2001) suggested, together with the characteristic obtained in the absence of COMDP (Fig. 1), that under these reaction conditions the

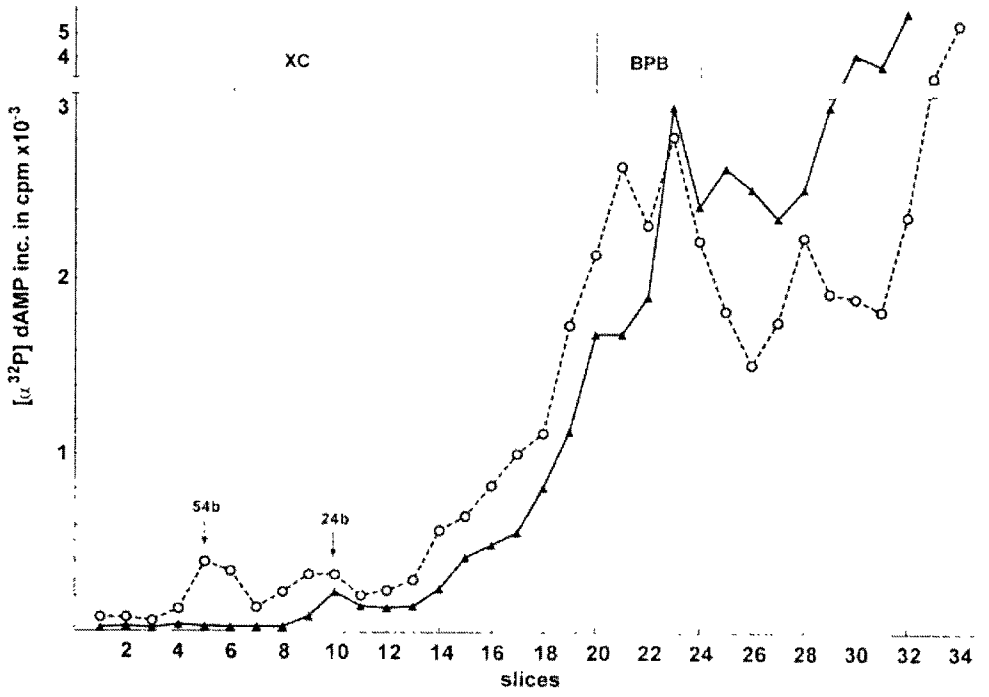


Fig. 3

**Denaturing PAGE characteristics of the reaction products radioactively labeled for DNA and synthesized in the presence of COMDP at reaction conditions suitable for expression of the deoxyribo-mode of Pr activities of the Pr-α DNA pol enzyme complex (Assay 1) and thereafter treated with DNase I (Říman, 2001)**

Gel distribution of radioactivity of [alpha$^{32}$P]dAMP incorporated into NAs. Products synthesized in the presence of COMDP (50 μmol/l) (broken line, o) and thereafter treated with DNase I (continuous line, ▲). XC and BPB, internal markers. Vertical arrows indicate gel positions of ssNAs of a length given in the number of bases (b).

α DNA pol activity seems to be only weakly stimulated, due to its very low ability to use the DNA primers for initiation of the DNA synthesis. Under such circumstances the uncoupling potency of COMDP may display itself even more strikingly, with relevant consequences for primer accumulation. In line with this explanation was also the observation that the activation of the deoxyribo-mode of Pr expression including the primer accumulation, is not influenced by BuPdGTP, a selective inhibitor of α DNA pol (Byrnes, 1985; Nethanel *et al.*, 1988) (Fig. 2). As shown previously, the Pr activities of the ribo-mode expression are also resistant to BuPdGTP (Říman, 2001). As regards the nature of the accumulated primers, they seem to be composed mostly of dnts. Their material labeled for DNA, composed of 7 and more nts, was found to be sensitive to DNase I treatment (Fig. 3), while the shorter primers were evidently less suitable targets for this enzyme (Hu *et al.*, 1984). The material of these primers was alkali-resistant up to 90% according to the remainder of the radioactivity in its acid-precipitable portion (Říman *et al.*, 1993a). These and the precedent data on this subject indicated that these primers are constituted mostly of dnts. However, as assumed, the primer synthesis begins always (Rowen and Kornberg, 1978; Kaguni and Lehman, 1988) or most frequently (Hu *et al.*, 1984) with rATP. The shortest RNA primer is believed to be the ribonucleotide pppApGp (Rowen and Kornberg, 1978). If so, then this shortest RNA primer, the constituents of which may be recruited from trace amounts of rNTPs always contaminating the samples of dNTPs (Rowen and Kornberg, 1978), could be used, in this case, for addition of dnts by the deoxyribo-mode of Pr expression. In such instance the radioactive labeling for DNA with [alpha$^{32}$P]dAMP of the primers should reflect their internal labeling because of the presence of two respective non-labeled ribonucleotides at their 5′ end. Nevertheless, a striking radioactive labeling of di- and trinucleotides apparent in Fig. 1 invite to speculate about a possible role of dATP in initiation of primer synthesis accomplished by the deoxyribo-mode of Pr expression under certain reaction conditions.

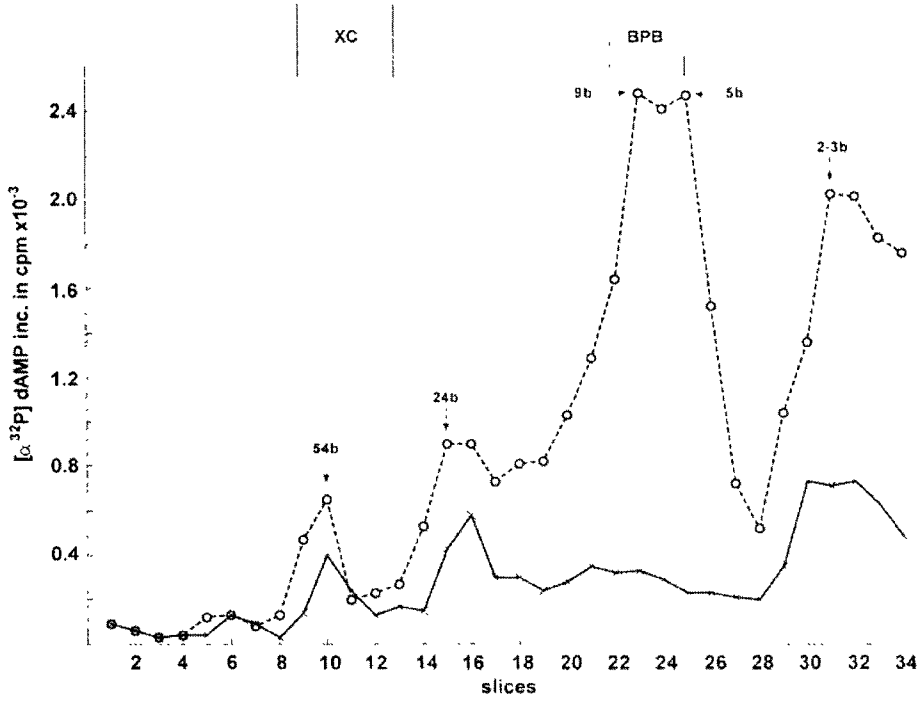


Fig. 4

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

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

*Deoxyribo-mode of Pr expression in the presence of rNTPs and dNTPs and its influencing by COMDP*

Similarly, we have analyzed for DNA the reaction products radioactively labeled with [alpha$^{32}$P]dAMP and synthesized in the absence or presence of COMDP with all four common rNTPs and dNTPs in the reaction medium (Assay 2). In this case (Fig. 4), in the absence of COMDP the radioactive labeling of both EI Okazaki fragments was more distinct. These DNA synthesizing events may reflect, this time, rather the use of the unlabeled RNA primers for initiation of DNA synthesis, since perceptible amounts of radioactivity at gel positions of primers of basic length suggest a low efficiency in using DNA primers by α DNA pol activity. The presence of COMDP again led, under these reaction conditions, to an outstanding accumulation of newly synthesized primers of basic length, radioactively labeled for DNA (Fig. 4). Interestingly, a bifurcation of the main radioactivity peak in two seems to be characteristic for accumulation of primers synthesized by the deoxyribo-mode of Pr expression (see also Fig. 5). Besides this radioactivity, its increase, though not adequate to the extent of stimulation of Pr activity, was found to be associated with both EI Okazaki fragments. Such a characteristic may again indicate that COMDP also influences, in this case, the coupling of the deoxyribo-mode of Pr activity with that of α DNA pol. An about threefold increase of the radioactivity at gel positions of di- and trinucleotides might again be challenging for speculation about the role of dATP in initiation of primer synthesis by the deoxyribo-mode of Pr expression. Otherwise, the radioactive labeling for DNA was again, in general, one order lesser than that for RNA (Říman, 2001), regardless of the presence or absence of the rNTPs besides dNTPs. As regards the nature of the primers, it is most probable that it is, in this case, similar to that of primers synthesized in the presence of dNTPs only, due to the high concentration of dNTPs in the reaction medium (Assay 2) (Rowen and Kornberg, 1978; Hu *et al.*, 1984). They were also found to be equally alkali-resistant.

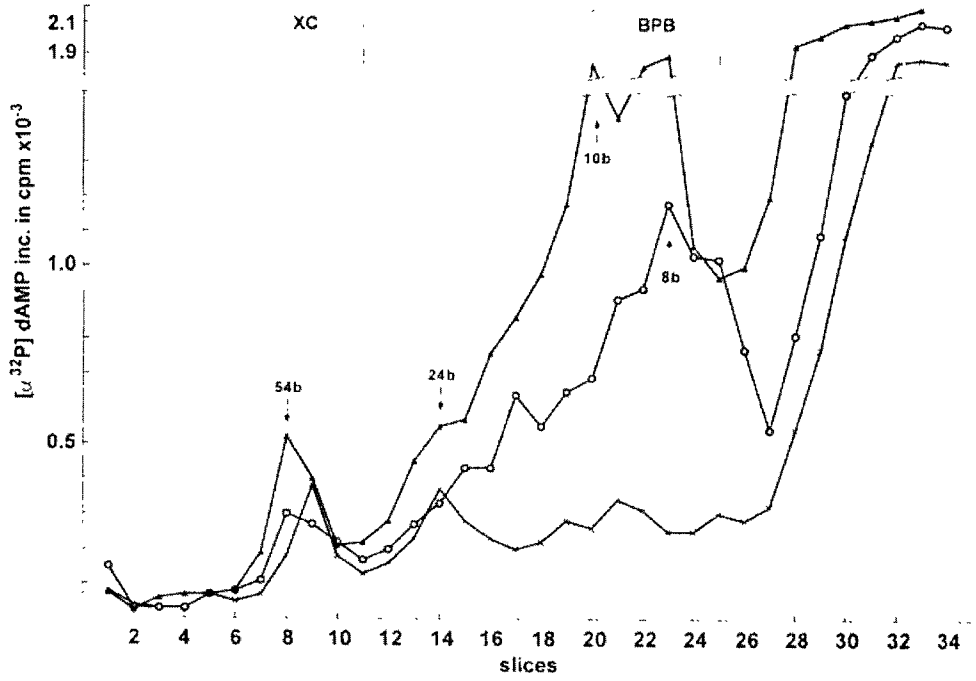


Fig. 5

**Denaturing PAGE characteristics of the reaction products radioactively labeled for DNA and synthesized in the presence of COMDP or COMDP and MIMO at reaction conditions suitable for the deoxyribo-mode expression of Pr activities of the Pr-α DNA pol enzyme complex (Assay 1)**

Gel distribution of [alpha$^{32}$P]dAMP radioactivity incorporated into NAs. Products synthesized in the absence of drugs (continous line, x), in the presence of COMDP (50 μmol/l) (continuous line, ▲) or COMDP (50 μmol/l) and MIMO (400 μmol/l) (continuous line, o). XC and BPB, internal markers. Vertical arrows indicate gel positions of ssNAs of a length given in the number of bases (b).

*Possible targets for COMDP intervention, its counteracting*

The potency of COMDP to inhibit α DNA pol was explained by its capacity of mimicking dNTPs and binding within the dNTP-binding domain (Talanian *et al.*, 1989). However, the bound COMDP cannot be released by an excess of dNTPs, as also shown in this and precedent (Říman, 2001) articles by recording the COMDP effects at high concentrations of dNTPs as well. Given the COMDP structure and its analogy with $PP_i$, it was posited that it binds at a part of the active site of the enzyme that reacts with the 5′-moiety of the incoming dNTP (Talanian *et al.*, 1989). COMDP might also be involved, like pyridoxal phosphate (Modak, 1976) in the case of inhibition of *Escherichia coli* DNA pol, in formation of a stable Schiff base with an amino acid residue critical to the functioning of the dNTP-binding domain (Basu and Modak, 1987). A 20-fold lesser inhibitory effect of COMDP on α DNA pol was ascribed to its lesser capacity to mimic dNTP, in this case, due to a non-specific drug-protein interaction independent of the active site (Talanian *et al.*, 1989). Confronting these data on COMDP inhibitory effect with those showing its activating and uncoupling potency on Pr activities presented in this and the precedent (Říman, 2001) articles, we can point out, despite the complexity of the primase functions (Griep, 1995), the following simplified deductions: the activation of Pr by COMDP may actually reflect stabilization of both (ribo- and deoxyribo-) modes of Pr expression, which is taking place at once. This is most probably due to the binding of COMDP to the amino acid residue critical to the functioning of the dNTP-binding domain involved in coupling of both Pr catalytic activities in dependence on dNTP concentration (Hu *et al.*, 1984). As regards the potency of COMDP to uncouple the Pr and α DNA pol activities, which seems to be responsible for induction of the phenomenon of primer accumulation, it is possibly effected by a weak but resistant to dNTP inhibitory effect of COMDP on the dNTP-binding site of the α DNA pol. Consequently, this site should also be involved in the event of coupling,

which is not yet well understood (Kaguni and Lehman, 1988). This assumption was recently strengthened by finding the means to overcome the uncoupling effect of COMDP. In accord with the suggested analogy of the inhibitory effect of COMDP and pyridoxal-5′-phosphate on DNA pols (Modak, 1976; Basu and Modak, 1987; Talanian *et al.*, 1989), it was shown that MIMO (Matsumoto *et al.*, 1951), a pyridone compound (Levenson and Hamlin, 1993) and a pyridoxal antagonist, counteracts the uncoupling potency of COMDP in the presence of dNTPs besides rNTPs in the reaction medium (Říman, 2001). At these reaction conditions MIMO strikingly decreases accumulation of the RNA primers of the basic length with a simultaneous increase of the RNA labelling of both EI Okazaki fragments. In contrast, this effect of MIMO was not recorded in the presence of rNTPs only in the reaction mixture (Říman, 2001). Here, it is shown that MIMO affects the accumulation of primers labeled for DNA, induced by COMDP in the case of the deoxyribo-mode of Pr expression, in a similar way (Fig. 5). This suggests that MIMO competes with COMDP for the same active site of α DNA pol that is responsible for coupling together the ribo- and deoxyribo-modes of Pr expression with the α DNA pol activities, though the use of the DNA primers for initiation of DNA synthesis seems to exhibit a very low efficiency. Consequently, these new data together with those published recently on this subject (Říman, 2001) suggest that as regards COMDP, besides Pr active site(s), another target of its intervention is represented by that responsible for the maintenance of singularity of the Pr-α DNA pol reaction (Wang, 1991). Otherwise, unraveling the potency of COMDP to strongly activate the Pr expression also possesses its practical aspect in proposing to use COMDP as an efficacious instrument for detection of Pr activities.

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