A DNA polymerase ε inhibitor activates the ribo and deoxyribo modes of primase expression and induces a unique phenomenon of primer accumulation[1]

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Abstract Carbonyldiphosphonate (COMDP), a selective inhibitor of DNA polymerase (pol) ε, strongly stimulates expression of the ribo and deoxyribo modes of primase (Pr) activities of the Pr–DNA pol α enzyme complex associated with special cytoplasmic nucleoprotein complexes of chicken leukemic myeloblasts [J. Říman and A. Šulová, Acta Virol. 41 (1997) 181–214]. Besides stimulation, COMDP uncouples the Pr activities from those of DNA pol α, inducing in this way a unique phenomenon of accumulation of primers of basic length. In the presence of dNTPs, the COMDP effect is counteracted by excess of mimosine. The mutually exclusive effects of these agents are discussed.

Key words: Carbonyldiphosphonate; Mimosine; Primase; DNA polymerase α; Primer molecule

1. Introduction

In the course of our recent search for the origin of avian myeloblastosis virus core-bound 7S DNA [1,2], metabolically active [3,4], extrachromosomal in nature [5], and descending from origin (ori) regions of chromosomal DNA replication [6], we have found that this kind of DNA is organized in the cell into nucleoprotein (NP) complexes forming three basic components (A, B, C) of the postmicrosomal sediment (POMS) of chicken leukemic myeloblasts (CHLMs) [7]. In accord with the descent of their DNAs they are equipped with outstanding activities significant for early DNA synthesis [8]. Accordingly, these NP complexes are able to synthesize in vitro the relevant reaction products, using as endogenous templates their occluded DNAs [9]. To distinguish the various DNA polymerases (pols) associated with these NP complexes we used corresponding inhibitors, among them also carbonyldiphosphonate (COMDP) [8], a selective inhibitor [10] of proliferating cell nuclear antigen-insensitive DNA pol δ [11], later called DNA pol ε [12]. Analyzing in this way also POMS component C–NP complexes exhibiting minimally advanced early DNA synthesis [7,8], we have found that, in this case, COMDP strongly stimulates their labelling for DNA and even strikingly more for RNA [7,8]. Later, we have shown that this effect of COMDP is due to its strong stimulation of primase (Pr) activities of the Pr–DNA pol α enzyme complex associated with these NP complexes [9]. To investigate this event more accurately and in greater detail, we analyzed directly, as recommended [13], the reaction products radioactively labelled for RNA or DNA and synthesized in vitro by POMS component C–NP complexes in the absence or presence of COMDP by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions. The reaction conditions used were suitable for the ribo or deoxyribo modes [14] of expression of Pr activities or those of both pols of the Pr–DNA pol α enzyme complex [15]. Comparatively, we have used BuPdGTP, a selective inhibitor of DNA pol α [15] and mimosine (MIMO), which was recently found to preserve the coupling of Pr and DNA pol α activities [16].

Here, we show that COMDP, besides activating both modes of expression of Pr activities, uncouples them from those of DNA pol α, inducing in this way a unique phenomenon of accumulation of primers of basic length. BuPdGTP does not influence these COMDP effects while MIMO, under certain reaction conditions, counteracts the uncoupling potency of COMDP.

2. Materials and methods

2.1. Chemicals

COMDP and BuPdGTP were from Prof. G.D. Wright, University of Massachusetts, Worcester, MA, USA. MIMO (1-mimosine-(α-amino-β-[*N*-(3-hydroxy-4-pyridone)]propionic acid) was from Sigma. All other chemicals were of analytical purity.

2.2. Radioisotopes

[α-^{32}P]ATP) and [α-^{32}P]dATP, 110 TBq/nmol each, were from Amersham.

2.3. Source of Pr–DNA pol α activities

This was represented by NP complexes of POMS component C with a sucrose density of 1.108 g/cm^3, isolated [7] from isopycnic sucrose gradients of cytoplasm of CHLMs. These NP complexes descending from ori regions of chromosomal DNA replication [6] possess all components, including the occluded short template DNAs [8,17], necessary for synthesizing in vitro products significant for early lagging strand DNA synthesis [9].

2.4. Enzymatic reactions

They were accomplished at 37°C for 30 min with aliquots of the POMS component C material originating from $3–4\times10^7$ cells and residing in 20 μl of the relevant isopycnic sucrose gradient enzyme fraction (220 μl) [9]. The following assays were used.

Assay 1 (reaction conditions suitable for expression of the ribo

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[1] The author dedicates this piece of work in memory of his teacher, Professor F. Bruno Straub.

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mode 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, 0.05% Nonidet P-40, 1% glycerol, 40 μmol/l unlabelled UTP and CTP each, 4 μmol/l unlabelled GTP, 12 μmol/l unlabelled ATP, 24 μCi [α-^{32}P]ATP and 20 μl of the enzyme fraction.

Assay 2 (reaction conditions suitable for expression of Pr (ribo mode) and DNA pol α activities). The reaction mixture (50 μl) contained: 40 μmol/l unlabelled UTP and CTP each, 4 μmol/l unlabelled GTP, 12 μmol/l unlabelled ATP, 24 μCi [α-^{32}P]ATP, 40 μmol/l each of the four common unlabelled dNTPs. All other ingredients, including the enzyme, were as in assay 1.

Assay 3 (reaction 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 unlabelled dGTP, dCTP, dTTP each, 4 μmol/l unlabelled dATP, 48 μCi [α-^{32}P]dATP. All other ingredients, including the enzyme, were as in assay 1.

Assay 4 (reaction conditions suitable for expression of Pr and DNA pol α activities [15]). The reaction mixture (50 μl) contained: 40 μmol/l each of the four common unlabelled rNTPs, 40 μmol/l unlabelled dGTP, dCTP, dTTP each, 4 μmol/l unlabelled dATP, 48 μCi [α-^{32}P]dATP. All other ingredients, including the enzyme, were as in assay 1.

COMDP, BuPdGTP and MIMO were added at concentrations of 50 μmol/l [8], 10 μmol/l [15] and 400 μmol/l [18], respectively, as recommended.

2.5. DNase I treatment

DNase I treatment of reacted samples has already been described [16].

2.6. PAGE under denaturing conditions

Samples of reacted products isolated as described [9] were electrophoresed in 12% polyacrylamide gels (17×12×0.04 cm) supplemented with urea (7 mmol/l) at 300 V for 240 min at 8°C. The samples were run in parallel with xylene cyanol (XC) and bromophenol blue (BPB) markers [19]. The length of the reaction products expressed in the number of bases was estimated as described [4,9,15].

2.7. Radioactivity measurements

These were accomplished with 4-mm gel slices dried on filter disks, in toluene-based scintillation fluid in a Beckman spectrometer 6000 SE.

3. Results and discussion

3.1. The ribo mode of Pr expression, its influencing by COMDP

A comparative PAGE analysis of the reaction products radioactively labelled for RNA with [α-^{32}P]AMP and synthesized in vitro in the absence or presence of COMDP under reaction conditions suitable for expression of the ribo mode of Pr activities of the Pr–DNA pol α enzyme complex (assay 1) of NP complexes of POMS component C [9] led to the following characteristics: in the absence of COMDP, most of the radioactivity was associated with two reaction products by their length of 24 and 54 bases (Fig. 1), reminiscent of an Okazaki fragment precursor and an immature Okazaki fragment, respectively [10]. According to the relevant tests [16], these reaction products are RNA–DNA molecules in nature and their DNA portion is synthesized like in the case of Okazaki fragments by DNA pol α [10]. Thus, they will be further designated here early immature (EI) Okazaki fragments. A small but perceptible amount of radioactivity was noted, in addition, at gel positions of RNA primers or initiator (i) RNAs [20] of the basic length [13] constituted of 6–12 nucleotides. This characteristic suggests that even in the presence of rNTPs only in the reaction mixture, a great portion of newly synthesized iRNAs was used in a coupled reaction by DNA pol α activities for initiation of DNA synthesis and, consequently, for synthesis of the DNA portion of both EI Okazaki fragments. The necessary expression of DNA pol α activities was, in this case, evidently enabled by trace amounts of dNTPs (≤0.1% of total rNTPs) [14] always present in rNTP samples of high purity. In contrast, in the presence of COMDP, an outstanding accumulation was noted to occur at gel positions of the iRNAs of the basic length and, at the same time, a distinct decrease of the radioactivity was found to be associated with both EI Okazaki fragments (Fig. 1). This indicated clearly that COMDP not only strongly activates the ribo mode expression of Pr activities, but also uncouples them from those of DNA pol α. Consequently, COMDP seems to be also responsible for the appearance of the phenomenon of accumulation of iRNAs of the basic length, newly synthesized but not further used for initiation of DNA synthesis. A radioactivity increase at gel positions of the smallest reaction products indicates the presence of initiation [21] or degradation products, or both, significant for a precipitous primer synthesis.

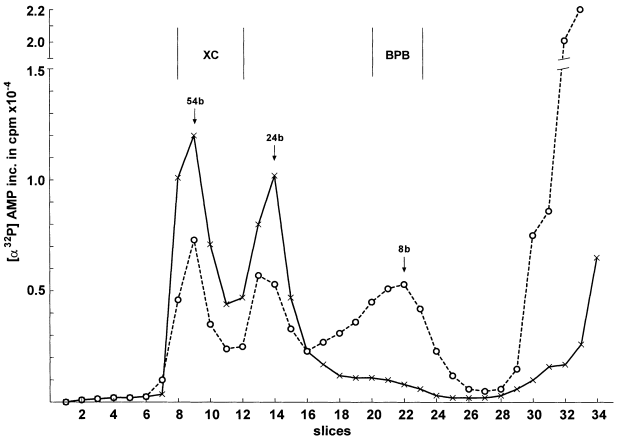

Fig. 1. Denaturing PAGE characteristics of the reaction products radioactively labelled with [α-^{32}P]AMP for RNA and synthesized in the absence (continuous line, ×) or presence of COMDP (50 μmol/l) (broken line, ○) under reaction conditions suitable for expression of the ribo mode of Pr activities of the Pr–DNA pol α enzyme complex (assay 1). The following comments apply to all figures: XC and BPB, internal markers. Vertical arrows indicate gel positions of single-stranded (ss) nucleotide acids of a length given in number of bases (b).

3.2. Pr–DNA pol α enzyme complex activities and COMDP

Closely similar, but even more pronounced characteristics were obtained by analyses of the reaction products similarly radioactively labelled for RNA, but synthesized with both types of four common NTPs (rNTPs+dNTPs) in the reaction medium (assay 2). In this case, in the absence of COMDP the radioactive RNA labelling of both EI Okazaki fragments was augmented (Fig. 2) and no perceptible radioactivity was noted at gel positions of iRNAs of the basic length. This suggested that the newly synthesized primers were immediately used, in this case, in a coupled reaction for DNA synthesis (Fig. 2). In accord with the increase of radioactive labelling in general was also the increase of radioactivity at gel positions of the smallest reaction products. In contrast, the presence of COMDP under these reaction conditions again led to the accumulation

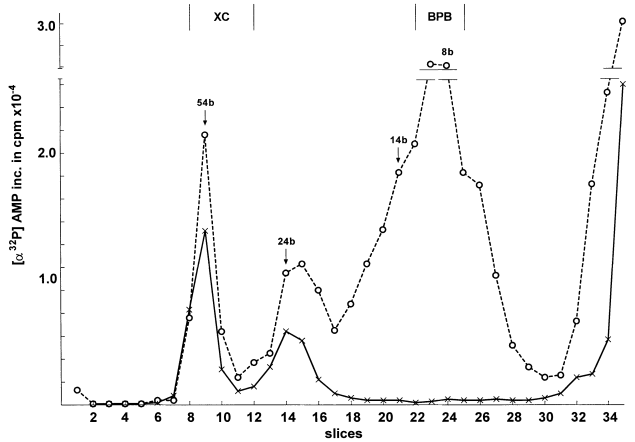

Fig. 2. Denaturing PAGE characteristics of the reaction products radioactively labelled with [α-^{32}P]AMP for RNA and synthesized in the absence (continuous line, ×) or presence of COMDP (50 μmol/l) (broken line, ○) under reaction conditions suitable for expression of RNA and DNA synthesizing activities of Pr–DNA pol α enzyme complex (assay 2).

of primers of the basic length, but this time the manifestation of this phenomenon was even more pronounced (Fig. 2). With respect to a multiple increase in accumulation of newly synthesized iRNAs, an about two times only increase of radioactivity in both EI Okazaki fragments in this case actually suggested that COMDP exhibits its uncoupling effect also under those reaction conditions. Interestingly, COMDP at the concentration used (50 μmol/l), which is optimal for Pr activation [8], is overcoming the inhibitory effect of concentration of ambient dNTPs on synthesis of RNA primers [21]. This should be fully exhibited [21] at the total concentration of 90 μmol/l of dNTPs significant for assay 2. The accumulated radioactive RNA label, in this case, like that induced by COMDP in the presence of rNTPs only, was found to be alkali-sensitive (not shown), suggesting the RNA nature of primers synthesized under those reaction conditions.

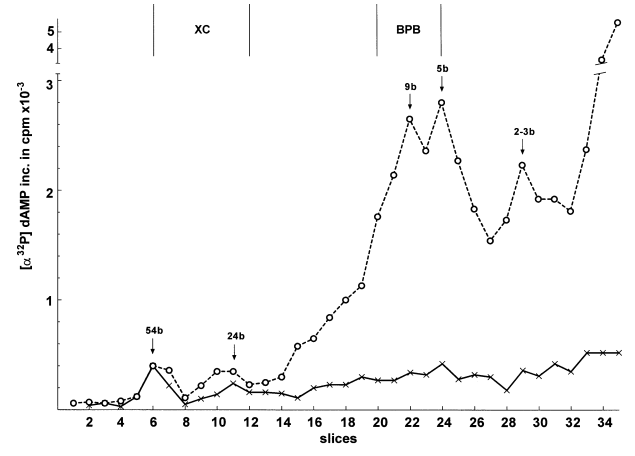

Fig. 3. Denaturing PAGE characteristics of the reaction products radioactively labelled with [α-^{32}P]dAMP for DNA and synthesized in the absence (continuous line, ×) or presence of COMDP (50 μmol/l) (broken line, ○) under reaction conditions suitable for the deoxyribo mode expression of Pr activities of the Pr–DNA pol α enzyme complex (assay 3).

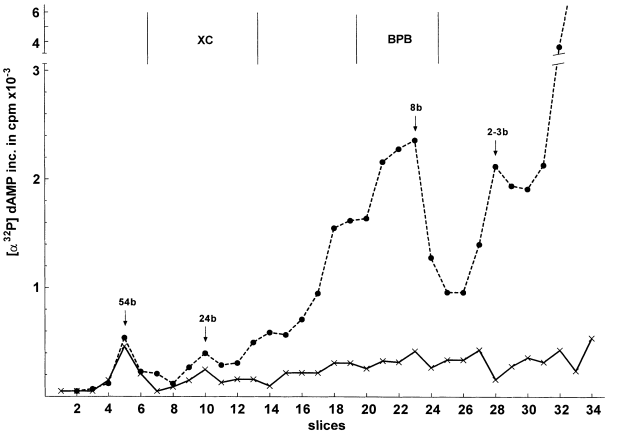

Fig. 4. Denaturing PAGE characteristics of the reaction products labelled with [α-^{32}P]dAMP for DNA and synthesized in the absence (continuous line, ×) of the drugs or presence of COMDP (50 μmol/l) and BuPdGTP (10 μmol/l) (broken line, ○) under reaction conditions as in Fig. 3 (assay 3).

3.3. Deoxyribo mode of Pr expression, its influencing by COMDP

It is known that besides rNTPs, each of the dNTPs can be incorporated by Pr into a ribo–deoxyribonucleotide hybrid primer, though the incorporation of dNTPs was found to be less effective [21]. Consequently, the Pr is able to add either a ribonucleotide or a deoxyribonucleotide to the 3′-OH of either a ribo or deoxyribo residue of the primer terminus [14,21]. This led to the suggestion that Pr may possess two catalytic centers or two conformers of one catalytic center, which are synchronously coupled, mutually exclusive for respective rNTPs or dNTPs [14] and, in this way, responsible for the ribo or deoxyribo modes of Pr expression. Thus, to answer the question whether COMDP also influences the deoxyribo mode of Pr expression, we comparatively analyzed the reaction products radioactively labelled for DNA with [α-^{32}P]dAMP and synthesized in the absence or presence of COMDP with all four common dNTPs in the reaction mixture only (assay 3), permitting, in general, the expression of DNA synthesizing activities. As evident from Fig. 3, COMDP at the same concentration (50 μmol/l) as used in the case of analysis of the ribo mode of Pr expression (Figs. 1 and 2) also strongly activated the deoxyribo mode of Pr expression, again inducing the accumulation of primers radioactively labelled for DNA, but this time the extent of the radioactive labelling was more than one order less than that for RNA in the presence of rNTPs only or both NTP types in the reaction mixture. Interestingly, the radioactivity profile of primer accumulation revealed, in this case, a bifurcation of the main peak into two at gel positions of oligonucleotides of about five and nine bases in length, reminiscent of mono- and dimers, respectively, of an assumed Pr 'synthesis unit' [14]. A broadening of this profile to the gel positions of the reaction products up to about 20 bases in length also suggested the presence of even longer multimers. Another radioactivity peak at the gel position of di- and trinucleotides (Fig. 3) may indicate the presence of initiation products significant for a precipitous primer synthesis. A small radioactive DNA label associated with both EI Okazaki fragments suggested, together with the characteristic obtained in the absence of COMDP (Fig. 3), that under these reaction conditions the DNA pol α activity seems to be

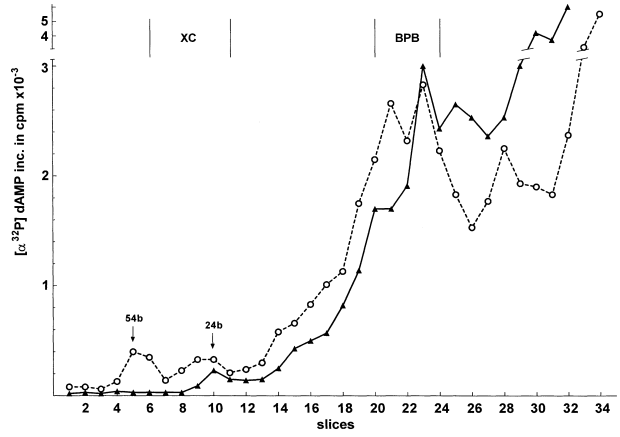

Fig. 5. Denaturing PAGE characteristics of the reaction products radioactively labelled with [α-^{32}P]dAMP for DNA and synthesized in the presence of COMDP (50 μmol/l) (broken line, ○) under reaction conditions as in Fig. 3 (assay 3) and thereafter treated with DNase I [16] (continuous line, ▲).

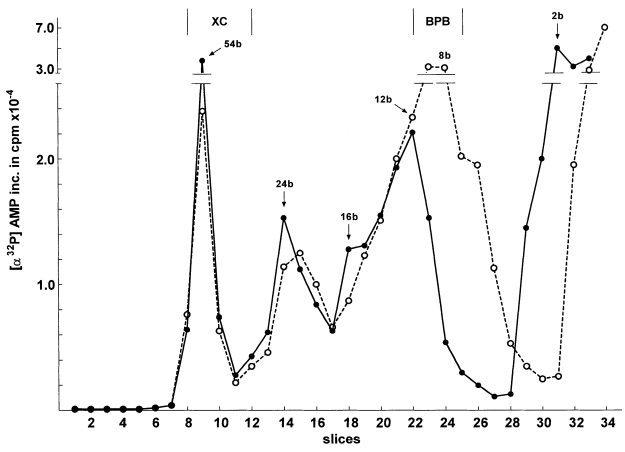

Fig. 7. Denaturing PAGE characteristics of the reaction products radioactively labelled with [α-^{32}P]AMP for RNA and synthesized in the presence of COMDP (50 μmol/l) (broken line, ○) or COMDP (50 μmol/l) and MIMO (400 μmol/l) (continuous line, ●) under reaction conditions suitable for expression of the RNA and DNA synthesizing activities of the Pr–DNA pol α enzyme complex (assay 2).

only weakly stimulated, due to its very low ability to use the DNA primers for initiation of 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 primer accumulation, is not inhibited by BuPdGTP, a selective inhibitor of DNA pol α (Fig. 4). As shown previously, the Pr activities of the ribo mode expression are also resistant to this inhibitor [16]. As regards the nature of the accumulated primers, they seem to be composed mostly of deoxynucleotides. Their material, labelled for DNA, composed of about seven nucleotides and more was found to be sensitive to DNase I (Fig. 5), while the shorter primers were evidently less suitable targets for this enzyme [14]. The material of these primers was alkali-resistant up to 90% according to the remainder of the radioactivity in its acid-precipitable portion [2]. These and the preceding data indicated that these primers are constituted mostly of deoxynucleotides. However, as assumed, the primer synthesis begins always [21,22] or most frequently [14] with rATP. The shortest RNA primer is believed to be the ribodinucleotide pppApGp [21]. If so, then this shortest RNA primer, the constituents of which may be recruited from trace amounts of rNTPs always contaminating the dNTPs in turn [21], could be used, in this case, for addition of deoxynucleotides by the deoxyribo mode of Pr expression. In such instances the radioactive labelling for DNA with [α-^{32}P]dAMP of the primer should reflect its internal labelling because of the presence of two respective non-labelled ribonucleotides at its 5′ end. Nevertheless, a striking radioactive labelling of the di- and trinucleotides apparent in Fig. 3 invites speculation about a possible role of dATP in initiation of primer synthesis under certain reaction conditions.

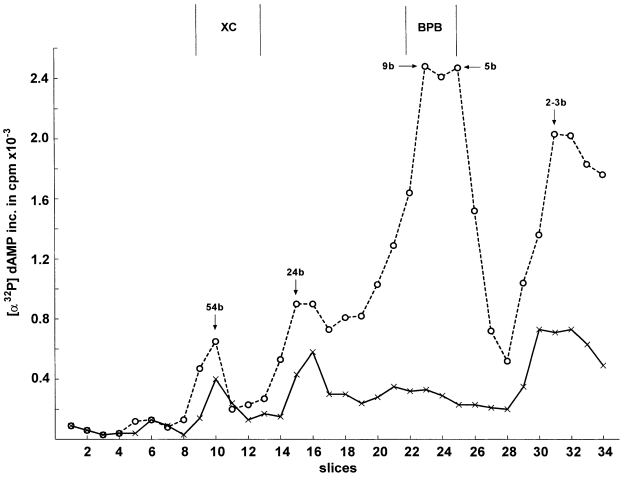

Fig. 6. Denaturing PAGE characteristics of the reaction products radioactively labelled with [α-^{32}P]dAMP for DNA and synthesized in the absence (continuous line, ×) or presence of COMDP (50 μmol/l) (broken line, ○) under reaction conditions suitable for expression of RNA and DNA synthesizing activities of the Pr–DNA pol α enzyme complex (assay 4).

3.4. Deoxyribo mode of Pr expression in the presence of dNTPs and rNTPs and its influencing by COMDP

Similarly, we have analyzed DNA radioactively labelled reaction products synthesized in the absence or presence of COMDP with all four common rNTPs and dNTPs in the reaction medium (assay 4). In this case (Fig. 6), in the absence of COMDP the radioactive labelling of both EI Okazaki fragments was more distinct. These DNA synthesizing events may reflect, this time, rather the use of unlabelled RNA primers for initiation of DNA synthesis, since perceptible amounts of radioactivity at gel positions of primers of the basic length suggest a low efficiency in using DNA primers by DNA pol α activity. The presence of COMDP again led, under those reaction conditions, to an outstanding accumulation of newly synthesized primers of the basic length, radioactively labelled for DNA. Interestingly, a bifurcation of the main radioactivity peak into two seems to be characteristic for accumulation of primers synthesized by the deoxyribo mode of Pr expression (see also Fig. 8). Besides this radioactivity, its increase,

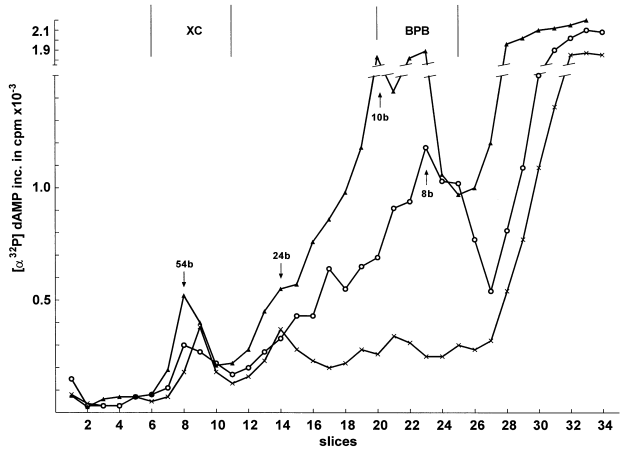

Fig. 8. Denaturing PAGE characteristics of the reaction products radioactively labelled with [α-^{32}P]dAMP for DNA and synthesized in the presence of COMDP (50 μmol/l) (continuous line, ▲) or COMDP (50 μmol/l) and MIMO (400 μmol/l) (continuous line, ○) under reaction conditions suitable for the deoxyribo mode expression of Pr activities of the Pr–DNA pol α enzyme complex (assay 3).

though not adequate to the extent of stimulation of Pr activity, was found to be associated with both EI Okazaki fragments. This characteristic may again indicate that COMDP also influences, in this case, the coupling of deoxyribo mode Pr activity with that of DNA pol α. An about three times increase of the radioactivity at gel positions of the di- and trinucleotides might again be challenging for speculation about the role of dATP in initiation of primer synthesis accomplished by the deoxyribo mode of Pr expression. Otherwise, the radioactive labelling was again, in general, one order less, regardless of the presence or absence of rNTPs besides dNTPs (compare with Fig. 2). 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 [14,21]. They were also found to be equally alkali-resistant.

3.5. Possible targets of COMDP intervention, its counteracting

The potency of COMDP to inhibit DNA pol ε was explained by its capacity to mimic dNTPs and bind within the dNTP binding domain [10]. However, the bound COMDP cannot be released by excess dNTPs, as also shown in this article by recording the COMDP effect at high concentrations of dNTPs as well (assays 2–4). Given the COMDP structure and its analogy with inorganic pyrophosphate, 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 [10]. COMDP might also be involved, like pyridoxal phosphate [23], 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 [24]. A 20 times lower inhibitory effect of COMDP on DNA pol α 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 [10]. Confronting these data on the COMDP inhibitory effect with those showing its activator and uncoupling potency on Pr activities presented in this article, we can point out, despite the complexity of the Pr functions [25], the following simplified deductions: the activation of Pr by COMDP may actually reflect stabilization of both (ribo and deoxyribo) modes of Pr expression. This effect is taking place at once, due, probably, to the binding of COMDP to an 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 [14]. As regards the potency of COMDP to uncouple the Pr and DNA pol α activities, that seems to be responsible for induction of the phenomenon of primer accumulation; it is possibly effected by a weak, but dNTP concentration-resistant inhibitory effect of COMDP on the dNTP binding site of DNA pol α. Consequently, this site should also be involved in the event of coupling that is not yet well understood [22]. This assumption was recently strengthened by finding the means to counteract the uncoupling effect of COMDP [16]. In accord with the suggested analogy of the inhibitory effects of COMDP and pyridoxal-5′-phosphate on DNA pols [10,23,24], we have found that MIMO [26], a pyridone compound [18] and a pyridoxal antagonist, counteracts the uncoupling potency of COMDP under reaction conditions suitable for expression of the whole Pr–DNA pol α enzyme complex (assay 2) (Fig. 7), or those suitable for expression of its DNA synthesizing activities (assay 3) (Fig. 8). In both these cases exhibiting the presence of dNTPs, addition of an excess of MIMO to COMDP led to a distinct decrease in primer accumulation. This effect was associated with an increase of synthesis of both EI Okazaki fragments, as clearly evident, consequently, under reaction conditions of assay 2 (Fig. 2). These findings suggest that COMDP and MIMO, though chemically quite different, compete for the active site responsible for coupling the Pr and DNA pol α activities and, consequently, that besides Pr active site(s), another target of COMDP intervention is represented by that responsible for maintenance of singularity of the Pr–DNA pol α reaction [27]. Otherwise, the findings presented here also possess their practical aspect in the proposed use of COMDP as an efficacious instrument for detection of Pr activities.

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