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STIMULATION OF CALF THYMUS DNA POLYMERASE α ACTIVITY BY NUCLEOLAR PROTEIN B23

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Protein B23 is a major RNA-associated nucleolar protein and putative ribosome assembly factor which exists in at least two isoforms designated B23.1 and B23.2. Recently, it has been reported that B23 is copurified with DNA polymerase α-primase complex. To examine its possible role in DNA replication, the effects of B23 on DNA polymerase activities were investigated. B23.1 purified from rat Novikoff hepatoma ascites cell nucleoli stimulated the activity of DNA polymerase α by as much as 3-to 4-fold in a dose-dependent manner, while it showed little effect on the activities of DNA polymerases β, γ, and primase. Rat recombinant B23.1 showed the same stimulation as that of B23.1 from Novikoff cells. In contrast, isoform B23.2 showed no effect on the activity of DNA polymerase α, suggesting that C-terminal region of B23.1 is important in its activity in the stimulation of DNA polymerase α.

Protein B23 is a major nucleolar protein (M_r = 37,000) which is more abundant in tumor cells and normal growing cells than in resting cells (1- 5), and is found to be significantly elevated in mitogen-stimulated normal lymphocytes (6, 7). Previous electrophoretic and cDNA analysis indicated the presence of at least two isoforms of the protein, differing in molecular weight (8- 10). They were designated B23.1 and B23.2 which are polypeptides of 292 and 257 amino acids, respectively (11). The untranslated 5' regions of the two cDNA's and N-terminal 255 residues are identical in the two isoforms. However, the 3' untranslated regions and sequences coding for the C-terminal ends of the two proteins are completely different. The two forms of the protein are generated from a single gene via alternative splicing of 3' exons at the mRNA level (12). Immunoelectron and immunofluorescence microscopic studies indicate that protein B23 is predominantly located in the granular region of the nucleolus (13), although a

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Abbreviations: HIV, human immunodeficiency virus; BSA, bovine serum albumin; DTT, dithiothreitol; IgG, immunoglobulin G.

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recent study suggests that some B23 is present in dense fibrillar component (14) and it also shown to be associated with preribosomal ribonucleoprotein particles (2, 15). In addition, protein B23 was shown to shuttle between the nucleolus and the cytoplasm (16) and it is able to form specific complexes with the other proteins such as the HIV-Rev protein (17). Finally, protein B23 (termed numatrin) has been found in the nuclear matrix (18), the apparent site of DNA synthesis.

It has been proposed that DNA replication is performed by several DNA polymerases and its accessory proteins in eukaryotic cells. Five different types of DNA polymerases have been found in eukaryotes. DNA polymerases α, δ and ε are probably involved in chromosomal DNA replication, whereas DNA polymerases β and γ are thought to take part in DNA repair synthesis and mitochondrial DNA replication, respectively (19). The regulatory mechanisms involved with DNA replication are poorly understood.

Recently, Feuerstein *et al.* (20) reported that B23 copurifies with the complex of DNA polymerase α-primase, which is essential for chromosomal DNA replication and is believed to function on the nuclear matrix (21). In the current study we have investigated the direct effects of protein B23 on eukaryotic DNA polymerases.

MATERIALS AND METHODS

Purification of B23.1 and nucleolin from Novikoff hepatoma cells - Novikoff hepatoma ascites cells were grown in male Sprague-Dawley rats. Six days after transplantation, rats were killed, then the cells were harvested. Nucleoli were prepared by the magnesium-sucrose sonication procedure as previously described (22). Protein B23 has been purified by the chromatography using heparin-Sepharose (Pharmacia) and Bio-Rad Q as previously described (22, 23). Nucleolin, another major nucleolar protein, was purified using heparin-Sepharose column chromatography as previously described (22).

Purification of recombinant B23.1 and B23.2 - The two B23 isoforms have been expressed in *E.coli* using the pKK223-3 vector and purified utilizing ammonium sulfate fractionation followed by chromatography using DEAE-cellulose (Whatman, DE-52), heparin-Sepharose and Bio-Rad Q as previously described (23).

Purification of DNA polymerase α-primase, β, and γ - DNA polymerase α-primase complex was purified from calf thymus extract by rapid purification on an immunoaffinity Sepharose 4B column conjugated with monoclonal antibody (MT17) directed against calf thymus DNA polymerase α as previously described (24). DNA polymerases β and γ were purified from calf thymus and calf liver, respectively, as previously described (25, 26).

DNA polymerase and primase assays - Three kinds of DNA polymerases were assayed as previously described (27) with several modifications. DNA polymerase α was assayed in 80 mM potassium phosphate (pH 7.2), 8 mM 2-mercaptoethanol, 200 μg/ml of activated calf thymus DNA, 80 μM each of dATP, dGTP, and dCTP, 40 μM [^{3}H]dTTP (250 cpm/pmol), 8 mM $MgCl_2$, and 5 μg of BSA. DNA polymerase β was assayed in 100 mM Tris-HCl (pH 8.8), 100 mM NaCl, 4 mM N-ethylmaleimide, 40 μg/ml of poly(dA), 8 μg/ml of oligo(dT)$_{12\text{-}18}$, 80 μM [^{3}H]dTTP (62.5 cpm/pmol), 0.5 mM $MnCl_2$, and 5 μg of BSA. DNA polymerase γ was assayed in 100 mM Tris-HCl (pH 7.5), 100 mM KCl, 40 μg/ml of poly(rA), 8 μg/ml of oligo(dT)$_{12\text{-}18}$, 10 mM DTT, 40 μM [^{3}H]dTTP (250 cpm/pmol), 0.5 mM $MnCl_2$, and 5 μg of BSA. DNA primase was assayed as previously described (28) in 50 mM Tris-HCl (pH 7.5), 40 μg/ml of poly(dT), 1.6 mM rATP, 2 mM DTT, 80 μM [^{3}H]dATP (100 cpm/pmol), 0.2 units *E.coli* DNA polymerase I (Klenow fragment), 2 mM $MgCl_2$, and 5 μg of BSA. After incubation for 60 min at 37°C, acid-insoluble radioactivity was measured.

Measurement of stimulatory effect of B23 on DNA polymerases and primase - Typically, 5 μl of enzyme solution were mixed with equal volume of B23 solution at 0- 4°C, then the DNA polymerase reaction mixture was added, in a total volume of 50 μl, and the activity of DNA polymerases and primase was measured as described above.

Immunoprecipitation with anti-B23 antibody - Anti-B23 antibody or control IgG (anti-α amylase inhibitor antibody (29)) were incubated with protein A-Sepharose beads (Pharmacia) at room temperature for 2 h. Subsequently, purified recombinant B23.1 was incubated with anti-B23 antibody or with control IgG-conjugated beads at 0- 4°C for 1 h, and centrifuged at 15,000 rpm for 15 min. Five-μl aliquots of the supernatants were then assayed for stimulatory effect on DNA polymerase α.

Quantitation of protein B23 - The protein content of purified B23 fractions was determined using the Bradford procedure (30), with γ-globulin as the protein standard.

RESULTS

Nucleolar protein B23.1 stimulates calf thymus DNA polymerase α

To test the effect of B23 on DNA polymerase α activity, calf thymus DNA polymerase α was mixed with highly purified B23.1 from Novikoff hepatoma ascites cells, and the DNA synthesis was carried out. In order to rule out the possibility that B23.1 might stabilize DNA polymerase α nonspecifically, a saturating amount of BSA (100 μg/ml) was added to the reaction system. As shown in Fig.1, addition of B23.1 stimulated DNA polymerase α activity in a dose-dependent manner. About 3-fold stimulation of DNA polymerase α activity was observed after 60 min incubation at 37°C. On the other hand, nucleolin, another major nucleolar protein, had no detectable effect (Fig.1A). Time course of the reaction with or without B23.1 was shown in Fig.1B.

Stimulation by B23.1 is specific for DNA polymerase α activity

As shown in Fig.2, the stimulatory effect of B23.1 was specific for DNA polymerase α, since B23.1 showed little effect on calf thymus DNA polymerase β or calf liver DNA polymerase γ when assayed under optimal conditions for these polymerases using poly(dA)-oligo(dT)$_{12-18}$ and poly(rA)-oligo(dT)$_{12-18}$ as templates, respectively. The activity of primase associated with the immunoaffinity-purified DNA polymerase α was also unaffected by addition of B23.1 when determined in the coupled primase/DNA polymerase I (Klenow fragment) assay, with poly(dT) as template.

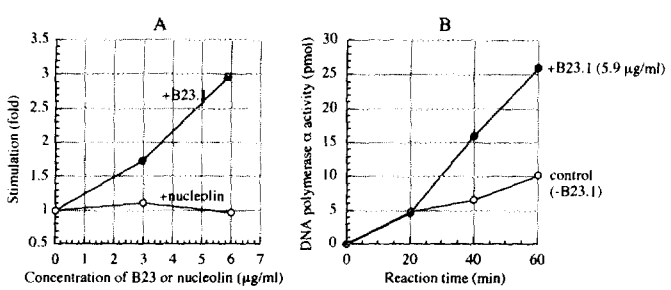

Fig. 1. **Stimulatory effect of B23.1 from Novikoff hepatoma cells on activity of calf thymus DNA polymerase α.** - A, calf thymus DNA polymerase α activity was assayed as described in "MATERIALS AND METHODS," after addition of different concentrations of B23.1 or nucleolin. Incubation was carried out for 60 min at 37°C. dNMP incorporation in the absence of B23.1 was 10 pmol for DNA polymerase α. B, the activity of DNA polymerase α, in the presence or absence of B23.1,was measured after incubation for 20, 40, and 60 min at 37°C. The data are means of triplicate samples.

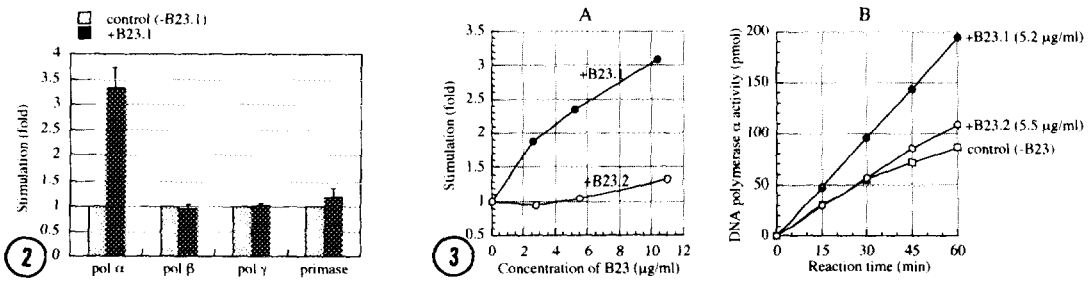

Fig. 2. **Specificity of stimulation by B23.1 from Novikoff cells.** - After addition of B23.1 (5.9 μg/ml), three kinds of DNA polymerases and primase were added with appropriate reaction mixtures, incubated for 60 min at 37°C, and assayed as described in "MATERIALS AND METHODS." dNMP incorporation in the absence of B23.1 was 10 pmol for DNA polymerase α (pol α), and 90, 45, and 15 pmol for DNA polymerase β (pol β), DNA polymerase γ (pol γ), and primase, respectively. The data represent the mean ± S.E. of quadruplicate determinations.

Fig. 3. **Comparison of stimulatory effects of recombinant B23.1 and B23.2 on activity of calf thymus DNA polymerase α.** - A, calf thymus DNA polymerase α activity was assayed as described in "MATERIALS AND METHODS," after addition of different concentrations of B23. Incubation was carried out for 60 min at 37°C. dNMP incorporation in the absence of B23 was 35 pmol for DNA polymerase α. B, the activity of DNA polymerase α, in the presence or absence of B23 was measured after incubation for 15, 30, 45, and 60 min at 37°C. The data are means of triplicate samples.

Activity of DNA polymerase α is stimulated by B23.1, but not by B23.2

As shown in Fig.3, highly purified rat recombinant B23.1 also stimulated DNA polymerase α activity about 3-fold after 60 min incubation at 37°C as well as B23.1 from Novikoff cell nucleoli. On the contrary, highly purified rat recombinant B23.2, which is 35 amino acid residues shorter than that of B23.1 in its C-terminal region, had very little stimulatory effect on DNA polymerase α activity.

Stimulation of DNA polymerase α activity is due to B23.1 itself

To confirm that the stimulation of DNA polymerase α is produced by B23.1 itself, an immunoprecipitation assay was done using anti-B23 antibody attached to protein A-Sepharose beads. Treatment with anti-B23 antibody decreased the stimulatory effect of B23.1 on DNA polymerase α activity (Fig.4). However, control IgG-conjugated beads did not abolish the

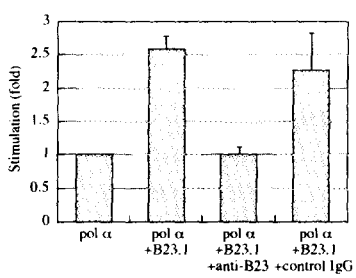

Fig. 4. **Immunoprecipitation with anti-B23 antibody.** - Purified rat recombinant B23.1 (1.8 μg) was incubated with anti-B23 antibody or control IgG conjugated with protein A-Sepharose beads. Aliquots (5 μl) of each supernatant were then assayed for DNA polymerase α activity as described in "MATERIALS AND METHODS." One-fold stimulation corresponds to 10 pmol of dNMP incorporated in 60 min. The data represent the mean ± S.E. of quadruplicate determinations.

stimulation. Therefore, the stimulation of DNA polymerase α activity appears to be due to B23.1 itself.

DISCUSSION

Our experiments reported here show that protein B23.1, one of the major nucleolar non-ribosomal proteins, stimulates the activity of DNA polymerase α, but does not stimulate DNA polymerase β or DNA polymerase γ. Recent investigation suggested that DNA polymerase α participates in the synthesis of lagging strand in the process of eukaryotic DNA replication (19). These observations suggest that B23.1 takes part in the DNA replication process, especially in lagging strand elongation by DNA polymerase α. These results also suggest that protein B23.1 is not involved in DNA repair synthesis or mitochondrial DNA replication, performed by DNA polymerase β or DNA polymerase γ respectively. Furthermore, this data is compatible with a previous report that B23 was found to copurify with DNA polymerase α throughout the conventional purification (20). We also found that the stimulatory ratio of B23.1 on immunopurified DNA polymerase α was significantly higher than that of conventionally purified (non-immunopurified) DNA polymerase α (data not shown). It is possible that the conventionally purified DNA polymerase α contain B23.1 as a minor component. Since B23.1 had little effect on primase activity, B23.1 might not participate in RNA priming process, but only in DNA chain elongation.

Protein B23 is expressed in two isoforms, B23.1 and B23.2 (11), the latter being 35 residues shorter than the former at the C-terminal end. Highly purified recombinant B23.1 produced in *E.coli* showed approximately the same level of stimulation of DNA polymerase α activity as B23.1 purified from Novikoff cells. However, the B23.2 isoform did not stimulate DNA polymerase α activity. Peculis and Gall (31) have shown that the C-terminal region of B23.1 is required for localization in nucleoli using C-terminal deletion mutant, and more recent work suggests that B23.1 is predominantly localized to the nucleolus, whereas B23.2 is only found in cytoplasm after cell fractionation (32). The stimulation of DNA polymerase α by B23.1 but not by B23.2, suggests that the C-terminal region of B23.1 is required not only for localization to nucleoli but also for the stimulatory effect of DNA polymerase α activity.

DNA replication factories are attached to a nuclear matrix (21) in nucleoplasm. If B23.1 is involved in DNA replication, therefore, B23.1 must also exist in the nucleoplasm. Yung *et al.* (33) have shown that B23 localized in nucleoli is capable of translocating from the nucleolus to the nucleoplasm, thereby supporting our hypothesis. Moreover, Feuerstein *et al.* (7) have observed that the numatrin (identical to protein B23) is synthesized synchronously with the progression of S phase, and its synthesis decreases rapidly at the end of S phase. The apparent association of B23 with the nuclear matrix further supports the possible requirement of B23 in DNA replication process.

The important role of B23 in ribosome biosynthesis and protein transport has been suggested by several lines of evidence (34), including its capability translocating from nucleolus to cytoplasm (16) and its high affinity for the HIV-Rev protein (17). The present results that B23, especially B23.1 had direct effect on DNA replication enzyme in *in vitro* raised another possibility that B23.1 may serve as one of the DNA replication factors.

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