A Single Stranded DNA Binding Protein Isolated from HeLa Cells Facilitates Ni^{2+} Activation of DNA Polymerases *in Vitro*†

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ABSTRACT: The divalent nickel ion (Ni^{2+}) is one of several metal ions that can substitute for Mg^{2+} in the activation of DNA polymerases *in vitro*, but usually with very low efficiency. We have purified and partially characterized a Ni^{2+}-binding protein (p40) from HeLa cell extracts that can specifically enhance the polymerase activity of DNA polymerase α (pol α) and other DNA polymerases in response to Ni^{2+}. This protein, with a molecular mass of 40 kDa, is a single stranded DNA binding protein that binds to a M13 DNA template-primer with an optimum stoichiometry of approximately 90 equiv of protein per equiv of DNA template and enhances the affinity of pol α for the primer-template. In the presence of Ni^{2+}, p40 exhibits an increased affinity for DNA. The p40 increased by 3- to 6-fold the rates at which pol α and the Klenow fragment of *Escherichia coli* DNA polymerase I (KF) replicate different DNA templates in response to Ni^{2+}. The low processivity of Ni^{2+}-activated pol α on primed M13 ssDNA was also enhanced by the presence of p40. The rates of Ni^{2+}-dependent replication by inherently more processive enzymes, DNA polymerase δ and T4 DNA polymerase, were not significantly increased by p40 when M13 ssDNA was used as a template; however, p40 did increase the activity of T4 polymerase on an activated calf thymus DNA template. The protein did not stimulate Mg^{2+}-activated DNA replication.

Nickel has been widely studied for its genotoxic effects and for its effects on DNA replication (Christie & Katsifis, 1990; Coogan et al., 1989; Costa, 1991). Divalent metal cations are well-known activators of the catalytic functions of DNA polymerases, the primary function being DNA polymerization (Bessman et al., 1958). Polymerase activation by Mg^{2+} is the most extensively studied since Mg^{2+} is the presumed physiological activator of all known polymerases. However, some nonphysiological metal ions, e.g., Mn^{2+} and Co^{2+}, can effectively replace Mg^{2+} while others, e.g., Ni^{2+}, Zn^{2+} and Fe^{2+}, are only weak activators of DNA replication (Sirover & Loeb, 1977; Burgers & Eckstein, 1979; Sirover et al., 1979; Esteban et al., 1992). Compared to Mg^{2+}, the activation of polymerization by these metal ions occurs at much narrower optimal concentrations and is immediately followed by inhibition at higher concentrations. The fidelity of DNA replication is also decreased in the presence of metal ions other then Mg^{2+} (Sirover & Loeb, 1976; Chin et al., 1994b).

The polymerizing activity of purified DNA polymerases catalyzed by Ni^{2+} usually yields no more than 10% of the maximal levels obtained by Mg^{2+} (Burgers & Eckstein, 1979; Sirover et al., 1979). DNA replication studies using purified enzymes showed that, in the absence of Mg^{2+}, the direct effects of Ni^{2+} on polymerases included both activation at low doses (up to 250 μM Ni^{2+}) and significant inhibition at higher doses, whereas in the presence of Mg^{2+}, Ni^{2+} produced a dose-dependent inhibition of polymerase activity (Chin et al., 1994b). Although nickel has been largely reported to inhibit DNA replication in many cell types or animal organs (Nishimura & Umeda, 1979; Hui & Sunderman, 1980; Conway et al., 1986), several studies showed that Ni^{2+} at relatively nontoxic doses stimulated DNA replication in murine splenocytes, rat tracheal epithelial cells, human thymocytes, and peripheral blood lymphocytes (Nordlind & Henze, 1984; Warner & Lawrence, 1986; Patierno et al., 1993). Nickel also increased cell proliferation of EBV-positive human lymphoblastoid cell lines (Wu et al., 1986). In addition to these affects on DNA replication in general, Patierno et al. (1987) also found that Ni^{2+} pretreatment of Chinese hamster ovary cells significantly increases the amount of [^{14}C]thymidine incorporation into the Mg^{2+}-insoluble fraction of the isolated chromatin (heterochromatin). Reports of effects of nickel on DNA replication indicate that nickel both interferes with and stimulates DNA synthesis, either by interaction with polymerases or by interaction with other proteins critical for the regulation of DNA replication.

Using a partially purified sea uchin nuclear DNA polymerase, Sirover and Loeb (1976) observed that DNA replication in response to Ni^{2+} can reach 30% of the rate of replication catalyzed by Mg^{2+}. In previous studies, we (Chin et al., 1994a) reported that, with crude HeLa cell extracts as the source of DNA polymerases, the rate of Ni^{2+}-catalyzed replication can reach about 34% of the rate catalyzed by Mg^{2+} and that Ni^{2+}-binding proteins isolated from the HeLa extracts can enhance Ni^{2+}-dependent replication. In this study, a 40 kDa Ni^{2+}-binding protein was purified from HeLa cell extracts by sequential column chromatography and its effects on Ni^{2+}-dependent DNA replication were analyzed. It was found that the p40 is a single strand DNA binding protein with increased affinity for DNA in the presence of Ni^{2+}. Binding of the p40 to a primed M13 DNA template increased the affinity of pol α for the primer-template and increased Ni^{2+}-dependent polymerase activity and processivity by up to 6-fold. This effect is specific for Ni^{2+} and, to a lesser extent, Zn^{2+}.

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MATERIALS AND METHODS

Materials. Radioactive substrates used were as follows: [α-^{32}P]dCTP (3000 Ci/mmol), [α-^{32}P]dATP (3000 Ci/mmol), and [γ-^{32}P]ATP (4500 Ci/mmol) from New England Nuclear. T4 polynucleotide kinase was purchased from New England Biolabs. Sephadex G-50 spin columns were obtained from Boehringer Mannheim. Unlabeled rNTPs, dNTPs, activated calf thymus DNA (ctDNA), M13mp18 ssDNA, phosphocellulose, and all metal salts were purchased from Sigma. The ssDNA−cellulose (made with denatured calf thymus DNA), poly(dT), and poly(dA·dT) were from Pharmacia. Ni^{2+}−nitrilotriacetic acid (NTA) resin was purchased from Qiagen. Hydroxylapatite, protein assay kit, and silver stain were from Bio-Rad. DNA polymerase I Klenow fragment (KF) and T4 DNA polymerase were from United States Biochemical. A primer of 15 nucleotides (oligomer-15) was synthesized by the Howard Hughes Medical Institute at the University of Washington. Mouse IgG column was from Hyclone. Antibodies to DNA polymerase α (SJK 237) were obtained from American Type Culture Collection (ATCC). DNA polymerase δ from calf thymus was kindly contributed by Dr. A. G. So at the University of Miami. All chemicals were reagent grade or better, and deionized water was used. All metal salt solutions were freshly prepared.

Oligomer-15 or poly(dT) labeled at the 5′ terminus with [γ-^{32}P]ATP was prepared by incubating the nucleotide with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP according to the method of Sambrook et al. (1989). Poly(dA·dT) labeled at the 3′ terminus with [α-^{32}P]dTMP was prepared by incubating poly(dA·dT) with KF in the presence of [α-^{32}P]dTTP. After incubation for 30 min at 30 °C, the reaction mixture was phenol-extracted and filtered through Sephadex G-50 spin columns.

Preparation of HeLa Cell Extracts and Pol α. HeLa cells were grown in spinner flasks in S-MEM medium (Gibco) containing 5% fetal bovine serum. Mid-log phase cultures (50 L, 6 × 10^5 cells/mL) were harvested by centrifugation, and the cell pellets were washed twice with phosphate buffered saline and once with hypotonic buffer [20 mM Hepes, pH 7.5, 5 mM KCl, and 0.5 mM dithiothreitol (DTT)]. The cell pellets were then pooled and resuspended in the same hypotonic buffer for swelling on ice for 10 min. The cells were disrupted by Dounce homogenization (30 strokes, B pestle). The lysate was adjusted to 0.2 M NaCl and immediately centrifuged at 50000*g* for 30 min at 4 °C. The supernatant fraction was then collected, pooled, and dialyzed for 12 h against two changes of phosphate buffer (20 mM $NaPO_4$, pH 8.0, 0.1 mM EDTA, 20% glycerol). The dialysate was clarified by centrifugation at 30000*g* for 30 min.

Preparation of monoclonal antibody-purified pol α from the above prepared HeLa cell extracts was conducted via the protocol of Murakami et al. (1986) which was slightly modified as described in detail previously (Chin et al., 1994b).

Purification of the 40 kDa Stimulatory Protein. All steps were performed at 4 °C:

(1) Ni^{2+}−NTA Chromatography. Before loading the dialyzed HeLa cell extracts, the Ni^{2+}−NTA column (10 mL) was charged with Ni^{2+} (freshly prepared 100 mM $NiSO_4$) and then extensively washed and equilibrated with phosphate buffer A [20 mM sodium phosphate, pH 8.0, and 1 mM phenylmethanesulfonyl fluoride (PMSF)]. After loading the cell extracts, the column was washed with 5 column volumes of the same phosphate buffer (pH 8) followed by sequential step elution with 5 volumes of the same phosphate buffer at pH 6, and then 5 volumes of phosphate buffer at pH 5, each at a flow rate of 1 mL/min. Fractions that contained the stimulatory activity were pooled and dialyzed overnight against 100 volumes of phosphate buffer B (25 mM potassium phosphate, pH 7.5, 0.25 mM DTT, 1 mM EDTA, 1 mM PMSF, and 20% glycerol). The column was regenerated by extensively washing with 20 mM phosphate buffer A, pH 3.5, according to the manufacturer's protocol.

(2) Phosphocellulose Chromatography. After dialysis the stimulatory activity from the Ni^{2+}−NTA column was loaded onto a phosphocellulose column and was washed with the balance buffer (25 mM potassium phosphate, pH 7.5, 1 mM EDTA, 0.25 mM DTT). A stepwise elution was conducted with 2 column volumes each of 0.15, 0.2, 0.3, 0.4, and 0.5 M KCl in the above phosphate buffer (pH 7.5). The fractions of stimulatory activity were eluted from the column at 0.4∼0.5 M KCl, pooled, and dialyzed overnight against 200 volumes of Tris buffer (15 mM Tris·HCl, pH 7.5, 500 mM NaCl, 0.5 mM DTT, 1 mM EDTA, 1 mM PMSF, and 20% glycerol) with three changes.

(3) Denatured DNA−Cellulose Chromatography. The fractions containing the enhancing factor from the phosphocellulose column were pooled and applied at a ratio of 2−5 μg of protein/mL of packed column volume in the presence of 0.5 mM Ni^{2+} to a column of denatured DNA−cellulose which had been pre-equilibrated with Tris buffer (50 mM Tris·HCl, pH 7.5, 50 mM KCl, 0.5 mM $NiSO_4$, and 20% glycerol). The loaded column was washed with 4 volumes of the same buffer, followed by stepwise elution with 2 column volumes each of 0.15, 0.25, 0.5, and 1 M KCl in Tris buffer (20 mM Tris·HCl, pH 7.5, 0.25 mM DTT, 1 mM EDTA, and 20% glycerol). The stimulatory fractions, which eluted with approximately 0.5 M KCl, were pooled.

(4) Hydroxylapatite Chromatography. The enhancing factor from the denatured DNA−cellulose column was dialyzed overnight against 200 volumes of phosphate buffer (20 mM potassium phosphate, pH 7.5, 0.5 mM DTT, 1 mM EDTA, and 20% glycerol) and was then loaded onto a hydroxylapatite column (1 × 5 cm). The loaded column was washed with phosphate buffer (10 mM potassium phosphate, pH 8.0, 0.25 mM DTT, 10% glycerol, 1 mM PMSF, and 0.5% Triton X-100) followed by stepwise elution with 10 column volumes each of phosphate buffer containing, respectively, 0.01, 0.05, 0.1, 0.2, and 0.3 M potassium phosphate. The peak of stimulatory activity was eluted from the column with approximately 0.1 M phosphate. The purified activity was dialyzed overnight against Tris buffer (20 mM Tris·HCl, pH 7.2, 2 mM EDTA, and 20% glycerol) and stored at −80 °C.

Gel Electrophoresis. Peptides present in the purified fractions were analyzed on SDS−polyacrylamide gels prepared as described by Laemmli (1970) using a 6% stacking gel and a 10% separating gel, and the gels were stained with Coomassie Blue or silver stain.

Standard Replication Assay for the Activity of the Stimulatory Protein. Throughout this work, the stimulatory protein was assayed by its ability to enhance DNA polymerase activity in the presence of Ni^{2+} and the absence of Mg^{2+}. The reaction mixtures contained 20 mM Tris·HCl, pH 7.2,

10% glycerol, oligomer-15 primed M13 ssDNA (3−20 μg/mL), 25 μM [^{32}P]dCTP, 50 μM each of dATP, dTTP, and dGTP, DNA polymerase, either pol α or KF, at a concentration of 0.05−0.1 unit per reaction, and various amounts of p40. The nickel concentration used was between 0.125 and 0.25 mM. After 30 min at 37 °C, the reaction was stopped by the addition of trichloroacetic acid (TCA) to a final concentration of 5%. Acid-insoluble radioactivity was counted as described previously (Christie & Tummolo, 1989). One unit of stimulatory activity was defined as the additional incorporation of 1 nmol of dCMP in 1 h at 37 °C using an oligomer-15 primed M13 ssDNA template and 1 unit of KF in the presence (versus the absence) of p40 plus optimum Ni^{2+}.

Processivity Assay. The rate of DNA synthesis and the processivity of pol α were determined under conditions where the primer-template was in excess as described by Sabatino et al. (1988). Assays were run in 20 μL total volume and contained 20 mM Tris·HCl (pH 7.2), 50 μM each of dCTP, dGTP, dATP, and dTTP, 5% glycerol, 20 μg/mL [^{32}P]-end-labeled oligomer-15 primed M13 ssDNA (1 μCi), varying amounts of p40, and the indicated metal (either $MgCl_2$ or $NiCl_2$) as described for each experiment. Reactions were initiated with 0.1 unit of pol α and were incubated at 37 °C for 15 min followed by the addition of a stop solution which contained EDTA to a final concentration of 20 mM. The product size was then determined by electrophoretic separation of aliquots containing approximately equal amounts of radioactivity on 8% denaturing polyacrylamide gels.

DNA Binding Assay. The nitrocellulose filter binding assay was carried out as described by Nagata et al. (1983). The standard assay (10 μL) contained 20 mM Tris·HCl (pH 7.2), $NiCl_2$ at a concentration described for each experiment, 5% glycerol, 0.1 mg/mL BSA, p40 (≤80 μg/mL), and 20 μg/mL M13 ssDNA primed with [5′-^{32}P]-labeled oligomer-15. After 5 min at 37 °C, the mixture was passed through a nitrocellulose filter which was then washed three times with 5 mL of the same buffer. After drying, the radioactivity adsorbed to the filters was measured by liquid scintillation counting.

Assay Conditions for Activities Associated with p40. Primase activity was assayed in the presence of the KF as described by Fry et al. (1985). M13 ssDNA served as an unprimed template, and all four ribonucleotide triphosphates were added at 50 μM. Incorporation of [^{32}P]dCMP beyond the primer into acid-insoluble products was measured as described for the assay of DNA polymerase activity. *3′−5′ Exonuclease activity of the stimulatory factor* was assayed by measuring the release of [^{32}P]dTMP from poly(dA·dT)·[^{32}P]dTMP. The reaction mixture contained (in 10 μL) 20 mM Tris·HCl, pH 7.5, 1 mM DTT, 0.1 mg/mL BSA, 2 mM $MgCl_2$, 32 μg/mL p40, and 0.22 μg of 3′-labeled poly(dA·dT). After incubation for 10 min at 37 °C, the reaction was stopped by the addition of EDTA, and the samples were precipitated, washed, and counted as described above. *5′−3′ exonuclease activity of the 40 kDa Ni^{2+}-binding protein* was assayed under the same conditions as described above for 3′−5′ exonuclease, only using [5′-^{32}P]poly(dT) as substrate in a final volume of 30 μL. *Endonuclease activity* was assayed by measuring the conversion of covalently closed M13mp 18 dsDNA either to the nicked circular form or to linear duplex DNA according to Moor et al. (1977). Samples were incubated in the presence of 1 μg of M13mp18 dsDNA for up to 1 h in the standard replication assay buffer without polymerase or primed single stranded DNA.

Table 1: Purification of a Stimulatory Factor for Ni^{2+}-Dependent DNA Polymerase Activity[a]

purification step	protein (mg)	total act. (units)[b]	sp act. (units/mg)	purifn (x-fold)	yield (%)
I. crude extracts	1820	270	0.15	1.0	100
II. Ni^{2+}−NTA	90.5	164	1.81	12.1	60.7
III. phosphocellulose	10.9	95	9.05	60.3	35.2
IV. ssDNA−cellulose	0.9	32	35.5	236.7	11.9
V. hydroxylapatite	0.07	9.8	140	933.3	3.6

[a] In this representative purification scheme, cells were harvested from 50 L of suspended HeLa cells with a cell number of 5 × 10^6/mL. [b] One unit of stimulatory activity is defined as the incorporation of an additional 1 nmol of dCMP into oligomer-15 primed M13 ssDNA by 1 unit of KF in the presence of 0.25 mM $NiCl_2$ per hour under standard replication conditions at 37 °C.

RESULTS

Purification of the Stimulatory Activity. In earlier experiments using HeLa cell extracts, we found that optimum Ni^{2+} (approximately 250 μM) catalyzes up to 34% as much dTMP incorporation as Mg^{2+} (at a concentration of 2 mM). In contrast, when purified DNA polymerase (pol α, pol δ, or KF) was used, Ni^{2+} was much less able to substitute for Mg^{2+}, i.e., less than 10% as much dTMP incorporation was seen as in the presence of Mg^{2+} (Chin et al., 1994a). These results indicated that Ni^{2+} may affect DNA replication not only through direct interaction with DNA polymerases, but possibly also through its interaction with other protein factors.

We hypothesized that a stimulatory factor affecting Ni^{2+}-dependent DNA replication was likely to be a nickel-binding protein. We therefore investigated whether Ni^{2+}-binding proteins from HeLa cell extracts could stimulate Ni^{2+}-dependent DNA replication *in vitro*. The crude HeLa cell extracts were chromatographed consecutively on columns of Ni^{2+}−NTA, phosphocellulose, denatured DNA−cellulose, and hydroxylapatite. DNA polymerase activity was eluted from the Ni^{2+}−NTA column by 20 mM phosphate buffer at pH 6, whereas the stimulatory factor eluted at pH 5. Elution of the polymerase activity not in the void volume but at a higher pH than the stimulatory factor indicates that DNA polymerases bind to Ni^{2+} ions, but more weakly than the stimulatory factor. Following further purification, the enhancing activity was eluted from the phosphocellulose column at 0.4−0.5 M KCl, and the pooled sample displayed multiple polypeptide bands on an SDS−polyacrylamide gel. The stimulatory activity also bound to denatured DNA−cellulose and could be eluted off with 0.5 M KCl. The stimulatory activity was further purified on a hydroxylapatite column and exhibited a single silver stained band with a molecular mass of approximately 40 kDa, as determined by comparison with the molecular mass markers ovalbumin (42.7 kDa) and carbonic anhydrase (31 kDa).

A summary of the purification of the p40 stimulatory protein with yield and relative specific activity is given in Table 1. Throughout purification the stimulatory activity was measured by the ability of the protein fractions to increase DNA polymerase activity in response to Ni^{2+} ions using either singly primed M13 ssDNA or activated calf thymus DNA as a template. The pooled fractions which

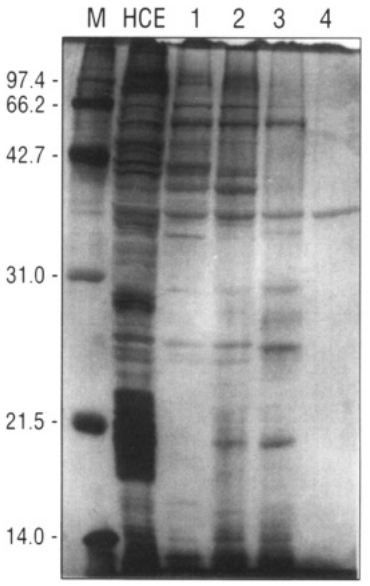

FIGURE 1: SDS gel electrophoresis of samples from the purification protocol. The gel was run as given under Materials and Methods and stained with silver. The amount of protein loaded varied between 4 and 50 μg. Lane M, molecular mass markers; lane HCE, HeLa cell extracts; lane 1, Ni^{2+}–NTA; lane 2, phosphocellulose; lane 3, denatured DNA–cellulose; lane 4, hydroxylapatite.

showed Ni^{2+}-dependent DNA replication stimulatory activity from each column were electrophoresed on an SDS–polyacrylamide gel and are shown in Figure 1.

Properties of the Purified 40 kDa Ni^{2+}-Binding Protein. The stimulatory activity of p40 was 94% inactivated by trypsin digestion and by heating at, or above, 60 °C for 10 min. The purified protein had no detectable DNA polymerase activity [<0.7 pmol of dNMP incorporated/(h·μg of p40)]; no primase activity [<0.4 pmol of dCMP incorporated on an unprimed template/h·μg of p40 in the presence of 1 unit of KF]; no 3′–5′ exonuclease activity [<0.08 pmol of [^{32}P]dTMP released/(h·μg of p40)]; no 5′–3′ exonuclease activity [<0.05 pmol ^{32}P released/(h·μg of p40)]; and no endonuclease activity, as determined by the assays described under Materials and Methods.

DNA Binding Effects. Since the stimulatory protein was seen to bind to DNA during purification, we used a filter-binding assay to measure DNA binding in a low salt buffer over a wide range of protein/DNA ratios. The p40 was mixed with M13 ssDNA primed with [^{32}P]-end-labeled oligomer-15 and filtered through nitrocellulose filters. Under the conditions used, both the protein and the protein–DNA complex were trapped on the filters, whereas the free DNA readily passed through. As can be seen in Figure 2, primed M13 ssDNA was bound to p40 in the presence of either Mg^{2+} or Ni^{2+}; however, the relative rate and maximum amount of binding were significantly higher in the presence of Ni^{2+}. In the presence of Ni^{2+} (0.25 mM), the rate of DNA binding to p40 is 2.7-fold greater than in the presence of Mg^{2+} (2 mM) and the maximum binding of 55 fmol of DNA in the presence of Ni^{2+} is 5.5-fold higher than the maximum binding in the presence of Mg^{2+}. However, the optimum stoichiometry of p40 binding to the primed M13 ssDNA [as determined by the intersection between the slope of the initial binding curve and the binding plateau (Fersht, 1985)] is approximately the same in the presence of either metal., i.e., 85 equiv of protein/mol of M13 DNA in the presence of Mg^{2+} and 93 in the presence of Ni^{2+}. The approximate

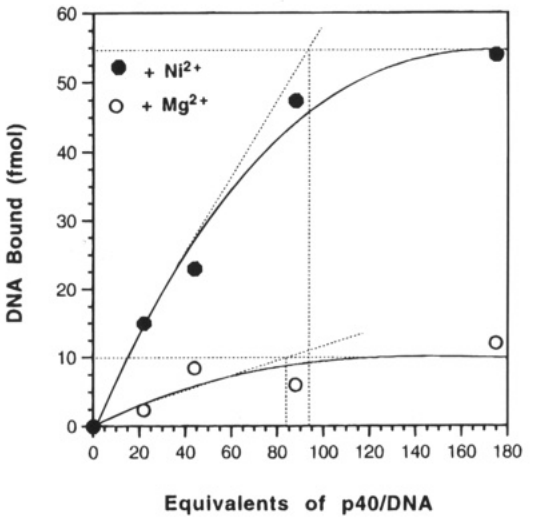

FIGURE 2: DNA binding activity of p40. The p40 protein was examined for its ability to bind to DNA primer-template using the nitrocellulose filter assay as described under Materials and Methods. The p40 protein was incubated with M13 ssDNA primed with [^{32}P]-end-labeled oligomer-15 in the absence or presence Ni^{2+} for 30 min at 25 °C. The reaction mixtures were then filtered through nitrocellulose filter membranes and washed thoroughly, and the amount of bound [^{32}P] was counted.

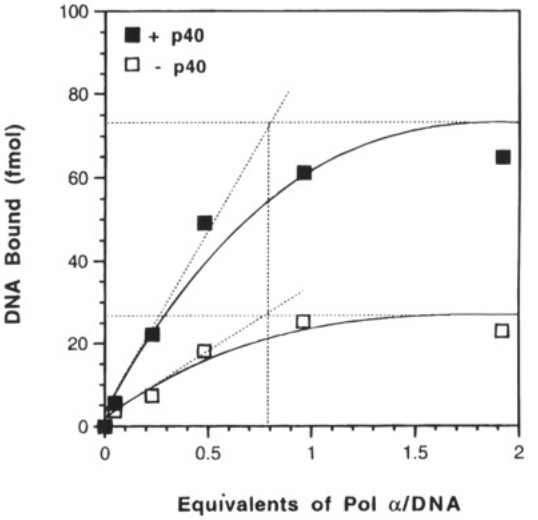

FIGURE 3: Effect of p40 on the binding of pol α to primed M13 ssDNA. Binding was determined as described under Materials and Methods. Each reaction mixture contained [^{32}P]oligomer-15 primed M13 ssDNA and from 0 to 4 μg/mL pol α in the presence of 0.1 mM Ni^{2+} and in the presence or absence of 10 μg/mL Ni^{2+}-binding protein.

binding constant (K_D), calculated by replotting the data as a double reciprocal plot (not shown), was determined to be approximately 0.56 μM in the presence of Ni^{2+} and 1.57 μM in the presence of Mg^{2+}, respectively.

We also examined the effect of p40 on the binding of pol α to primed M13 ssDNA in the presence of Ni^{2+}. As shown in Figure 3, incubation of increasing amounts of pol α in the presence of a fixed amount of p40 and 0.1 mM Ni^{2+} resulted in the retention of increased amounts of DNA in contrast to the low binding of pol α to the primed DNA in the presence of Ni^{2+} alone. The binding of pol α to the primed template was stimulated approximately 3-fold by the presence of p40, indicating that p40 increases the affinity of the DNA polymerase for the DNA primer-template. Moreover, the optimum stoichiometry of binding of pol α to the

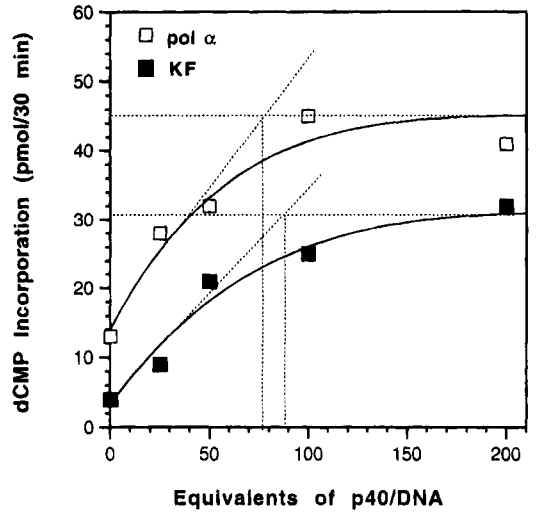

FIGURE 4: Stimulatory effect of p40 on Ni^{2+}-dependent DNA polymerase activity. The reactions (0.1 mL) contained oligomer-15 primed M13 ssDNA (0.015 mg/mL), 50 μM each of dATP, dGTP, and dTTP, 25 μM [^{32}P]dCTP, 5% glycerol, 0.25 mM $NiCl_2$, various amounts of p40, and either pol α (0.075 unit) or KF (0.05 unit). The reactions were incubated at 37 °C for 30 min, filtered, and counted as described in Materials and Methods.

primed M13 ssDNA is approximately 0.78 equiv of pol α/mol of M13 DNA and is not affected by the presence of p40. It should be noted that the DNA was primed with a 1:1 molar ratio of primer to template, and as a consequence the fraction of primed M13 DNA is expected to be less than 100% of the total DNA. Therefore, the observed stoichometry of pol α binding to the primed DNA is approximately 1:1, as would be predicted.

DNA Polymerase, DNA Template, and Metal Specificities. Figure 4 shows the effect of increasing concentrations of the 40 kDa protein on the rate of dCMP incorporation using primed M13 ssDNA with two different enzymes, pol α and KF. The nucleotide incorporation in response to increasing concentrations of p40 reached a plateau of 45 pmol/30 min for pol α and about 30 pmol/30 min for KF. The optimum stoichiometry of binding of p40 to the template is again about 80 equiv of protein/equiv of DNA molecule for both pol α and KF to reach their maximum rate of incorporation. No inhibition of polymerase activity was observed even in the presence of a large excess of p40 for either enzyme. Enzyme and template specificities for the stimulatory activity of p40, shown in Table 2, indicate that the Ni^{2+}-binding protein stimulates several different DNA polymerases from diverse sources. The rates of DNA synthesis using either activated calf thymus DNA or singly-primed M13 ssDNA by KF and HeLa cell pol α were increased approximately 3- to 6-fold over the controls by the p40 Ni^{2+}-binding protein. Replication of an activated calf thymus DNA template by T4 DNA polymerase (Jarvis et al., 1991) was also increased by 3-fold. However, replication of the singly-primed M13 template by T4 polymerase was increased by only 20% over the control. In contrast to these enzymes, the efficiency of DNA replication of either template by the highly processive calf thymus pol δ was increased only 20−40% over the control values. The effect of p40 on the rate of replication using a denatured DNA template and each of these polymerases was similar to that seen with the other two templates (not shown). It is notable that the Ni^{2+}-dependent synthesis by those polymerases with lower intrinsic processivity (pol α and KF) is most affected by the presence of p40, while the more processive polymerases (T4 and pol δ) are only weakly affected by the presence of p40, especially on a single stranded template.

Table 2: Polymerase and DNA Template Specificities of the Enhancing Factor[a]

	DNA template					
	activated ctDNA			primed M13 ssDNA		
enzyme	(−)	(+)	x-fold increase	(−)	(+)	x-fold increase
KF	10.5	48.3	(4.6)	5.2	31.0	(6.0)
HeLa pol α	20.5	65.4	(3.2)	10.6	45.2	(4.3)
thymus pol δ	23.7	29.1	(1.2)	13.2	18.6	(1.4)
T4 pol	1.2	3.5	(2.9)	3.9	5.4	(1.2)

[a] Rates of Ni^{2+}-dependent replication of different DNA templates by different DNA polymerases were determined in the presence (+) or absence (−) of p40 (35 μg/mL). The Ni^{2+} concentration was optimal for each polymerase and ranged from 0.125 to 0.25 mM. The rate is given as pmol of dCMP incorporated per 30 min reaction and is an average of duplicate assays. The numbers in parentheses are the x-fold enhancement of the replication in the presence of p40.

Table 3: Effect of p40 on Polymerase Activity Initiated by Different Metals

metal	−p40	+p40	x-fold increase
Mg	349.4	321.5	0.9
Mn	369.3	384.0	1.0
Co	356.5	358.6	1.0
Zn	44.9	98.8	2.2
Ni	34.2	147.1	4.3
Cu	9.2	7.5	0.8

[a] Reactions contained primed M13 ssDNA (0.02 mg/mL), KF (0.25 unit), and chloride salts of metal ions, in the presence (+) or absence (−) of p40 (35 μg/mL), and were incubated at 37 °C, 60 min. Metal ions of optimal concentration were used: 2 mM Mg^{2+}, 0.2 mM Mn^{2+}, 1 mM Co^{2+}, 2 mM Zn^{2+}, 0.25 mM Ni^{2+}, and 0.5 mM Cu^{2+}. The rate is given as pmol of dCMP incorporated per 60 min and is an average of duplicate assays.

In addition to its affect on Ni^{2+}-dependent polymerase activation, the p40 stimulatory protein increased the optimum concentration of Ni^{2+} required for DNA synthesis by KF (data not shown). In the presence of p40, the optimum Ni^{2+} concentration required for DNA replication with primed M13 ssDNA is 0.25 mM, whereas in the absence of p40, the optimum Ni^{2+} concentration is approximately 0.125 mM. In the absence of any added divalent metal ions, no polymerase activity was seen, even in the presence of purified p40. Although p40 does not affect the optimal concentration of Ni^{2+} required for the Ni^{2+}-dependent activation of pol α (not shown), it increased the rate of Ni^{2+}-dependent DNA synthesis by pol α by a factor of 4 (Table 2). The effect of p40 on DNA replication catalyzed by other metal ions was also tested. Other than Ni^{2+}, only Zn^{2+}-dependent replication showed a 2-fold enhancement of DNA polymerase activity in the presence of p40. Replication catalyzed by other metal ions such as Mg^{2+}, Mn^{2+}, Co^{2+}, and Cu^{2+} was not enhanced by the presence of p40 (Table 3). These results indicate that the stimulatory effect of this protein is specific to nickel and, to a lesser extent, zinc.

Effects on DNA Polymerase Processivity. To examine whether the stimulatory effect of p40 on the replication of heteropolymeric DNA was due to a change in DNA polymerase processivity, we analyzed the size distribution of the products of M13 ssDNA replication under conditions

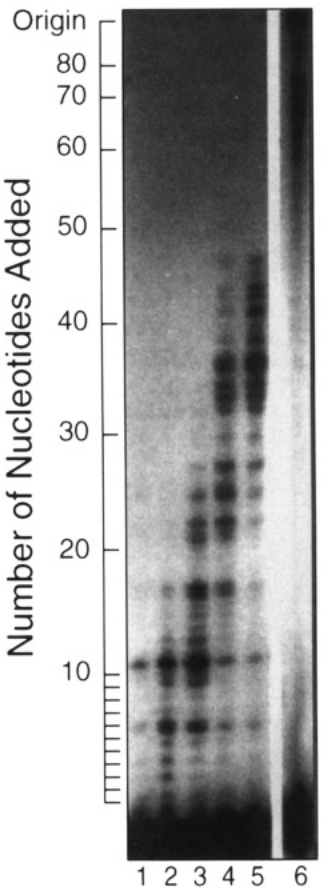

FIGURE 5: Effect of p40 on the size of the products formed by a limiting concentration of pol α with singly primed M13 ssDNA template. Lanes 1−5 show the products of DNA replication obtained using 0.25 mM Ni^{2+} as the activating cation in the presence of 0, 5, 10, 20, and 40 μg/mL p40. Lane 6 shows the products of DNA replication obtained using 2 mM Mg^{2+} as the activating cation in the absence of Ni^{2+} and the p40 protein. The approximate length number of nucleotides added to the extended primers as determined by densitometric scanning of the gel is shown on the left. The reaction conditions were as described under Materials and Methods.

whereby the polymerase concentration is limiting. Circular single stranded M13 DNA was primed with [^{32}P]oligomer-15 and was copied for 15 min by a low concentration of pol α either in the absence or in the presence of p40. Products of the DNA primer extension reactions were resolved by denaturing polyacrylamide gel electrophoresis. As shown in Figure 5, the extent of Ni^{2+}-dependent processive DNA synthesis by pol α on the single stranded template is strongly retarded in the absence of p40 but is increased by approximately 4-fold in response to saturating amounts of p40. Thus, the low intrinsic processivity observed for the Ni^{2+}-activated polymerase (as opposed to the relatively high processivity of Mg^{2+}-activated pol α, Figure 5, lane 6) was dramatically increased by the presence of p40. In contrast, the intrinsically higher processivity of pol α in the presence of Mg^{2+} is much less affected by the presence of the p40 (not shown).

DISCUSSION

DNA replication in intact cells is regulated by divalent metal ions as well as by numerous protein factors. Divalent nickel, although an essential trace element in animals, is also a carcinogen both in animals and in humans (Nielsen & Ollerich, 1974; Anke et al., 1984; Sunderman, 1984; Costa, 1991). In order to understand some of the diverse functions of nickel, we have been studying the effects of Ni^{2+} on DNA replication *in vitro*. We previously observed that crude HeLa cell extracts exhibit a significantly greater rate of Ni^{2+}-dependent DNA replication than do purified polymerases (Chin et al., 1994a). It has also been shown that nickel ions have a much stronger affinity for proteins than for DNA, and intracellular distribution studies indicate that up to 90% of the nickel that enters intact cells is bound to proteins rather than to DNA (Martell, 1971; Coogan et al., 1989).

On the basis of the assumption that proteins that could enhance Ni^{2+}-dependent DNA replication are likely to bind nickel, we carried out large scale purification of Ni^{2+}-binding proteins from HeLa cell extracts using Ni^{2+}−NTA chromatography followed by sequential purification on phosphocellulose, DNA cellulose, and hydroxylapatite. This process yielded a 40 kDa nickel-binding protein (p40) which can enhance the response of purified DNA polymerases to Ni^{2+}. To our knowledge this is the first protein that specifically enhances polymerase activity in response to a metal other than Mg^{2+}. Although p40 has some characteristics that are similar to other polymerase accessory proteins, it is in many ways unique. The protein we isolated is a single strand DNA binding protein which appears to bind noncooperatively to DNA (i.e., although binding cooperativity was not explicitly tested, the initial binding of the protein to the DNA appeared to exhibit linear kinetics). The optimum stoichiometry of binding was found to be approximately 1 protein per 80 nucleotides and was unaffected by the presence of Ni^{2+} or Mg^{2+}. However, the relative binding affinity of p40 for single stranded DNA is increased more than 3-fold in the presence of Ni^{2+}.

The p40 also increased the affinity of pol α for the DNA template by greater than 3-fold without altering the (~1:1) stoichiometry of binding of pol α to the primed DNA template. In the absence of p40, Ni^{2+}-activated pol α exhibits weakly processive DNA polymerase activity. This may be the result of weaker binding of the polymerase to the template in the presence of Ni^{2+} relative to Mg^{2+}. This would be consistent with the increased binding and increased processivity seen in the presence of p40. As the result of the increased binding of the polymerase to the primer-template, the p40 enhanced the activity of pol α by a factor of 4 with an approximately equal increase in processivity as seen by the accumulation of longer DNA product molecules (Figure 5). The fact that the stoichiometry of the protein binding to the DNA template was the same as the stoichiometry of the p40−DNA interaction which gave rise to increased DNA polymerase activity indicates that the DNA binding activity of p40 is critical for its stimulatory effect on replication. These data indicate that p40 binding to single stranded DNA promotes the increased ability of the DNA polymerase to utilize Ni^{2+} as an activating cation. It is unknown whether p40 can also interact directly with the polymerase molecule to promote Ni^{2+} binding to the active site of the enzyme or to limit access of the Ni^{2+} ions to the inhibitory binding sites hypothesized to be on the enzyme molecule (Chin et al., 1994b). However, the evidence that the p40 increases the optimum concentration of Ni^{2+} required for activation of KF (not shown) indicates that it may slightly reduce the concentration of available Ni^{2+} in solution.

Unlike other polymerase-specific accessory proteins, Ni^{2+}-dependent polymerase activation by p40 is not enzyme

specific. The Ni^{2+}-dependent activation of several other weakly processive enzymes is greatly enhanced by the protein (Table 2). Significantly, p40 increases the activity of T4 DNA polymerase on an activated calf thymus DNA template, but not on a single stranded M13 DNA template. T4 polymerase is a relatively processive enzyme on a single stranded DNA template but does not easily bypass regions of DNA secondary structure or displace double stranded DNA during replication in the absence of the homologous accessory proteins (Jarvis et al., 1991). Thus p40 can, at least partially, substitute for the homologous single stranded binding protein, gene 32 protein, and increase the rate of Ni^{2+}-dependent DNA synthesis by T4 polymerase on a partially double stranded template.

No replicative DNA polymerases act alone. All function optimally in the presence of multiple replication factors, including various DNA binding proteins, processivity enhancing factors, exonucleases, and other fidelity enhancing factors (So & Downey, 1992). Mammalian DNA polymerase α, in particular, has repeatedly been isolated in a variety of protein complexes containing accessory factors such as human single strand binding protein, primer recognition proteins, and other processivity factors (Sharf et al., 1988; Asna et al., 1989; Shioda et al., 1991). Other HeLa cell proteins of similar molecular mass to the p40 described here have been reported to enhance polymerase binding to the primer and to increase polymerase processivity. For example, two multifunctional proteins, annexin II (36 kDa) and phosphoglycerate kinase (PGK, 41 kDa), have been shown to enhance polymerase binding to DNA templates (Vishwanatha et al., 1983). These two proteins, collectively known as primer recognition proteins, enable DNA polymerase α to efficiently utilize substrates with low primer-template ratios (Prichard et al., 1983; Vishwantha et al., 1992). Other replication factors of similar molecular mass have been described (Lee et al., 1989; Kenny et al., 1989; Prelich et al., 1987). A protein with similar activity, but larger molecular mass (150 kDa), the α accessory factor (AAF) from mouse cell extracts, also acts as a template affinity factor for mammalian DNA polymerase α and increases its processivity (Goulian & Heard, 1990). Unlike p40 AAF protein acts with a stoichiometry of 1:1 with the DNA polymerase. There is no evidence that any of these factors are the same as the p40 described here; however, further clarification of this point awaits detailed characterization of p40.

Most polymerase accessory factors are presumed to be nuclear or nuclear matrix associated. The cellular localization of p40 has not yet been studied. However, p40 may be a nuclear protein since a 40 kDa protein similar to that described here was observed by SDS−polyacrylamide gel electrophoresis in the nickel-binding fraction obtained after separation of a HeLa nuclear extract on a Ni^{2+}−NTA column (data not shown). The p40 differs from other replication factors characterized in mammalian cells in being specific for a metal activator other than Mg^{2+}. Unlike other replication proteins, p40 enhances only Ni^{2+}-dependent DNA polymerase activity (pol α or KF) and only 3- to 6-fold. The rate of Ni^{2+}-dependent DNA replication, even in the presence of p40, is still lower than the rate of replication initiated by optimum Mg^{2+}. All previously reported studies on replication factors have looked exclusively at Mg^{2+}-dependent DNA replication, which this protein does not affect. Further studies of p40 and other Ni^{2+}-binding proteins and their role in the DNA replication process or their role in nickel toxicity or the induction of nickel resistance (Wang & Costa, 1989) may contribute to a better understanding of metal effects on DNA replication as well as to elucidate the process of Ni^{2+}-induced carcinogesis *in vivo*.

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