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The Second-Largest Subunit of the Mouse DNA Polymerase α-Primase Complex Facilitates Both Production and Nuclear Translocation of the Catalytic Subunit of DNA Polymerase α

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DNA polymerase α-primase is a replication enzyme necessary for DNA replication in all eukaryotes examined so far. Mouse DNA polymerase α is made up of four subunits, the largest of which is the catalytic subunit with a molecular mass of 180 kDa (p180). This subunit exists as a tight complex with the second-largest subunit (p68), whose physiological role has remained unclear up until now. We set out to characterize these subunits individually or in combination by using a cDNA expression system in cultured mammalian cells. Coexpression of p68 markedly increased the protein level of p180, with the result that ectopically generated DNA polymerase activity was dramatically increased. Immunofluorescence analysis showed that while either singly expressed p180 or p68 was localized in the cytoplasm, cotransfection of both subunits resulted in colocalization in the nucleus. We identified a putative nuclear localization signal for p180 (residues 1419 to 1437) and found that interaction with p68 is essential for p180 to translocate into the nucleus. These results indicate that association of p180 with p68 is important for both protein synthesis of p180 and translocation into the nucleus, implying that p68 plays a pivotal role in the newly synthesized DNA polymerase α complex.

Chromosomal DNA replication in eukaryotes is a highly regulated process that requires a large number of replication factors, including distinct three types of DNA polymerases, α, δ, and ε. Studies on the in vitro replication of simian virus 40 (SV40) DNA have made possible the functional identification of several DNA replication factors, including DNA polymerases (4, 17, 35). To date, DNA polymerase α-primase has been considered to provide RNA-DNA primers for the initiation of leading-strand synthesis and Okazaki fragment synthesis on the lagging strand during SV40 DNA replication (36, 43, 44).

Mouse DNA polymerase α-primase complex was isolated from FM3A cells as a protein complex consisting of four subunits. The apparent molecular masses of these subunits based on their migration on sodium dodecyl sulfate (SDS) polyacrylamide gels are 180, 68, 54, and 46 kDa (39, 40). The two smaller subunits, p46 and p54, can be dissociated from the other subunits by treatment with 50% ethylene glycol (38) and possess DNA primase activity, as demonstrated by the synthesis of unit-length oligoribonucleotides (7, 27). The largest subunit, p180, is the catalytic subunit of DNA polymerase and exhibits intrinsic DNA polymerase activity, as revealed in the baculovirus expression system (6). When the human p180 subunit is expressed alone in insect cells, it displays similarities to the holoenzyme such as an identical K_m for the primer-template and deoxynucleoside triphosphate and similarities with respect to sensitivity to inhibitors, thermostability, DNA synthetic processivity, and fidelity (6). In contrast, the function of the second-largest subunit, p68, which is tightly bound to p180, remains unclear. To date, no enzymatic activity has been found for p68, and biochemical approaches have failed to dissociate p68 in a native form from p180 in mammalian cells. However, in all eukaryotes analyzed so far, the DNA polymerase α-primase complex consists of four subunits, including p68, and these subunits display significant homology in organisms ranging from yeasts to humans (5, 8, 23). Therefore, p68 has been considered to have a regulatory function conserved throughout evolution.

In *Saccharomyces cerevisiae*, p68 is encoded by a single and essential gene called *POL12* (12, 32). Recently, it was reported that p68 specifically plays an essential role at the initial stage of DNA synthesis, before the hydroxyurea-sensitive step, and that the subunit is phosphorylated and dephosphorylated in a cell cycle-dependent manner (11, 12). Furthermore, formation of the p180-p68 subcomplex appears to be a prerequisite for p68 phosphorylation (10). However, the physiological role of p68 phosphorylation has been refractory to investigation.

To explore the structure-function relationship of DNA polymerase α-primase, we previously constructed a cDNA overexpression system in cultured mammalian cell lines and analyzed a temperature-sensitive mutant which has a defective DNA polymerase α-primase complex (19). Using this system, we identified the nuclear localization signal (NLS) of DNA primase in the amino terminus of p54 and a piggyback binding transport mechanism of DNA primase (24). The finding that DNA primase possesses an independent NLS within its own sequence and that it can translocate into the nucleus in the absence of DNA polymerase α prompted us to investigate the subcellular distribution of the other subunits of DNA polymerase α, p180 and p68.

In this report, we describe a novel function for p68, identified by using a cDNA expression system. Coexpression of p68 with p180 markedly increased the protein level of p180, and as a result, exogenously expressed DNA polymerase activity increased considerably. In addition, coexpression of p68 with

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p180 markedly altered the subcellular distributions of these subunits; coexpressed p68 and p180 were exclusively colocalized in the nucleus, whereas p68 or p180 expressed alone was localized in the cytoplasm. Using several mutants containing deletion or substitution constructs, we found that mutual interaction is essential for the p180-p68 heterodimer to translocate into the nucleus. These results indicate that p68 plays a crucial and dual role in the function of p180 by allowing both its protein synthesis and translocation into the nucleus.

MATERIALS AND METHODS

Materials. All restriction enzymes and Klenow fragment were purchased from Takara (Ohtsu, Japan); phenylmethylsulfonyl fluoride (PMSF) was from Sigma; *Pfu* DNA polymerase was from Stratagene; horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse immunoglobulin G (IgG) antibodies were from MBL (Nagoya, Japan); fluorescein isothiocyanate (FITC)- or Texas red-conjugated goat anti-rabbit or anti-mouse IgG antibodies were from Vector Inc.; fetal bovine serum was from Nipro (Osaka, Japan); calf serum was from HyClone. The expression vector pcDEBΔ was a gift from Y. Nakabeppu (25). DNA polymerase α-specific hybridoma SJK132-20 was purchased from the American Type Culture Collection. Unless otherwise noted, all other chemicals and reagents were obtained from Wako Chemicals (Osaka, Japan).

Construction of expression vector. cDNAs for the four subunits of the mouse DNA polymerase α-primase complex were introduced into pcDEBΔ, which contains the SRα promoter (41), to generate plasmids pSRα46, pSRα54, pSRα68, and pSRα180 as described previously (19, 24).

The amino-terminal deletion series of the p68 subunit was constructed by PCR with a common 3′ primer (5′-CCTGCGCTGCCACGCTCA-3′) and different 5′ primers: p68Δ1-97 (5′-ATTGTTTCTAGAATGGAGCTAATTGAA-3′), p68Δ1-157 (5′-CCGAGTTCTAGAATGTCCCAGAAATAC-3′), andp68Δ1-208 (5′-CTGGCAGCTATCTAGAGATGTTTCAGCA-3′). PCR-amplified products were digested with *Xba*I and *Stu*I and were used to replace the original fragment in the p68 sequence.

For construction of carboxy-terminal truncation mutant p68Δ557-600, a stop codon and restriction enzyme sites were introduced by PCR using the amino-terminal primer 5′-CAGGTGGTCTAGATGCAGTTC-3′ and carboxy-terminal primer 5′-CATGATATCCTATTCCCGAAGG-3′. The PCR products were then digested with *Xba*I and *Eco*RV and subcloned into *Xba*I-*Eco*RV-digested pSRα68.

For construction of amino-terminal truncation mutant p180Δ1-191, the initiation methionine and restriction enzyme sites were introduced by PCR with primers 5′-CAGTAGATCTCATTGAGCTCGGTATCAATACA-3′ and 5′-TCCAAGCTCCTCGAGGAACT-3′. The PCR product was digested with *Xho*I and *Bgl*II and subcloned into *Xho*I-*Bgl*II-digested pSRα180. Then the *Sac*I/*Sac*I fragment of the subcloned plasmid was removed to adjust the initiation methionine.

Deletion mutants p180Δ1417-1465 and p180Δ1442-1465 were constructed by PCR with a common 5′ primer (5′-AAGCCGGGACACCATTG-3′) and different 3′ primers: p180Δ1417-1465 (5′-CTTCTTCAATAGATCTTACTCATGTTCAGT-3′) and p180 Δ1442-1465 (5′-CAGGACAAGATCTACTCTGCTATGTT-3′). PCR-amplified fragments were digested with *Bgl*II and *Bam*HI and were used to replace the original fragment in pSRα180.

Mutants with substitutions were constructed by PCR using the overlap extension technique (18). The following primer sets were used to introduce mutations: p180KK(45,46)QQ, 5′-GGAAGGATCCAGAACAATCTGCGATTG-3′ and 5′-CTTTGGTAAAATACTGAA-3′; p180KK(1421,1422)QQ, 5′-TATGAGCCACAGCAACAGGACCACATC-3′ and 5′-AGACTTTCCTTCCTCTGA-3′; and p180RKVK(1434-1437)NNVN, 5′-TATGAGCCACAGCAACAGGACCACATC-3′ and 5′-AGACTTTCCTTCCTCTGA-3′. The double mutant p180KK(1421,1422)QQ RKVK(1434-1437)NNVN was constructed by PCR using p180RKVK(1434-1437)NNVN as a template DNA and primers for p180KK(1421,1422)QQ.

The p180-green fluorescent protein (GFP) fusion constructs were produced by introducing the cDNA for GFP into the carboxyl terminus of pSRα180. The 1.0-kb *Nhe*I-*Mlu*I fragment of pEGFP-N1 (Clontech) was blunted by Klenow enzyme and subcloned into *Mlu*I site of pSRα180, which was generated by replacement of a 1.6-kb *Bam*HI fragment of pSRα180 with PCR-amplified products, using a 5′ primer (5′-AAGCCGGGACACCATTG-3′) and a 3′ primer (5′-GCAGATCTACGCGTAGTCTGGTACGTCGTACGGGTAGGACTTCCCAGCGT-3′).

The identity of each construct was confirmed by double-strand DNA sequencing using an ALF DNA sequencer (Pharmacia).

Cell culture and transfection. COS-1 and COS-7 cells, which were derived from the African green monkey kidney cell line CV-1 by transformation with an origin-defective SV40 isolate, were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in a 5% CO_2 incubator. Transfection was performed by electroporation as described previously (33). The level of protein expression was analyzed 48 h after transfection unless otherwise indicated.

To synchronize G_2/M phase, cells at 4 h after transfection with p180-GFP and pSRα68 were incubated with 10 mM thymidine for 22 h and then released with culture medium for 6 h. Cells enriched in S phase were then incubated in the presence of nocodazole (45 ng/ml) for 8 h and allowed to traverse the cell cycle.

Antibodies. The anti-p68 and anti-p180 polyclonal antibodies were generated in rabbits against glutathione *S*-transferase (GST) fusion proteins expressed in *Escherichia coli*. The 2.2-kb *Sma*I fragment of p68 cDNA or the 1.5-kb *Hin*dIII fragment of p180 was inserted into the expression vector pGEX2T (Pharmacia). The GST-p180 fusion construct was further excised and inserted into the vector pET22b (Novagen). These expression vectors encoding the fusion proteins were introduced into *E. coli* BL21 cells, and after induction by 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h, GST fusion proteins were precipitated as inclusion bodies, purified, and used as antigens.

For immunization, proteins were initially injected into rabbits with complete Freund's adjuvant and were subsequently inoculated with incomplete Freund's adjuvant. The anti-p68 antibody was purified by using antigen-immobilized columns as follows. An immunoglobulin fraction was precipitated from 30 ml of the antiserum with ammonium sulfate at 50% saturation, followed by centrifugation and suspension in 10 ml of phosphate-buffered saline (PBS)–0.1% NaN_3. After dialysis against the same solution, the protein fraction was applied to a recombinant p68-conjugated Sepharose column. The column volume was 3 ml. After extensive washing of the column with PBS, the antibody was eluted with 0.17 M glycine-HCl (pH 2.3), and the eluate was neutralized. The antibody fraction was passed over a GST affinity column to deplete the anti-GST antibody, followed by an *E. coli* whole-protein affinity column to remove anti-*E. coli* protein antibodies. This antibody fraction was dialyzed against PBS–0.1% NaN_3 and stored at −80°C. The anti-p180 antiserum was directly used for Western blot analysis, immunofluorescence analysis, and immunoprecipitation studies. The anti-p54 and anti-p46 polyclonal antibodies were as described previously (24).

Indirect immunofluorescence staining. Cells were grown in chamber slides (Nunc) coated with poly-L-lysine, washed with PBS, and fixed with 3.7% formaldehyde in PBS for 10 min on ice. Cells were then washed with PBS and permeabilized sequentially with 50, 75, and 95% ethanol on ice for 5 min each. The slides were then blocked with PBS containing 5% normal goat serum (blocking buffer) for 30 min at room temperature, incubated with anti-p68 (1.3 μg/ml in blocking buffer) or anti-p180 (diluted 1:3,000 in blocking buffer) antibody for 1 h at room temperature, and washed three times with PBS for 5 min each time. Cells were then incubated with FITC-conjugated secondary antibody for 1 h at room temperature, washed three times with PBS, and preserved in Vectorshield (Vector Inc.). DNA staining was performed by adding 1 μg of bisbenzimide (Hoechst 33258) per ml into the final PBS wash. The samples were examined under an Olympus PROVIS AX70 fluorescence microscope. For double-staining studies, monoclonal antibody SJK132-20 (ascitic fluid), antihemagglutinin (anti-HA) antibody, and Texas red-conjugated secondary antibody were used at a 1:400 dilution, 5 μg/ml, and 1.5 μg/ml, respectively.

For visualization of cells transfected with a GFP fusion construct, cells were fixed, permeabilized, and simultaneously stained with antibodies and Hoechst 33258 as described above.

Preparation of cell extracts and Western blot analysis. After transfection, COS-1 cells were washed with PBS, scraped from plates in PBS, centrifuged for 5 min, and resuspended in either Laemmli sample buffer (21), for whole-cell extracts, or extraction buffer as described previously (40). The samples were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred electrophoretically onto 0.45-μm-pore-size polyvinylidene difluoride membranes (Millipore). After incubation of the membranes with either anti-p46 (0.13 μg/ml), anti-p54 (0.3 μg/ml), anti-p68 (0.3 μg/ml), or anti-p180 (1:3,000) antibodies in TBS (Tris-buffered saline; 50 mM Tris-HCl [pH 7.5], 150 mM NaCl) containing 5% (wt/vol) dried milk for 1 h at room temperature, the membranes were washed three times with TBS containing 0.05% Tween 20. The membranes were then incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG in TBS containing 5% dried milk and washed again. Detection of the protein bands was performed with an enhanced chemiluminescence reagent (SuperSignal; Pierce) as instructed by the manufacturer. Kaleidoscope prestained standards (Bio-Rad) were used as molecular weight standards.

Northern blot analysis. Total RNA was isolated by using Isogen (Nippongene) from COS-1 cells which were transfected with expression plasmids. Ten micrograms of total RNA per lane was subjected to gel electrophoresis using a 1% agarose–6% formaldehyde gel, stained with SYBR green II (Molecular Probes), and transferred to a nylon membrane as described previously (23). The probe was generated by random priming with a 1.5-kb *Hin*dIII fragment of pSRα180.

Pulse-chase experiment. Transfected COS-1 cells were divided into two sets; half of the cells were used for Northern blot analysis, and the other half were used for pulse-chase experiments. Cells at 24 h after transfection were incubated for 1 h in methionine-cysteine-deficient Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% dialyzed calf serum and then labeled with 200 μCi of Tran^{35}S-label (ICN Pharmaceuticals, Inc.) per ml for either 10 or 30 min. The cells were subsequently washed in complete medium, chased with complete medium for indicated times, and lysed at 4°C in buffer A (50 mM Tris-HCl [pH 8.0]; 300 mM KCl; 10% glycerol; 1% Nonidet P-40; 1 mM PMSF; 0.2 μg of aprotinin, 0.2 μg of leupeptin, 0.1 μg of antipain, and 0.1 μg of pepstatin A per ml; 1 mM EDTA). In the case of pulse-labeling for 10 min, a chased medium contained a large excess of unlabeled methionine (300 μg/ml). After centrifugation, the protein concentrations of the supernatants were deter-

mined by the Bradford assay (Bio-Rad). Thirty micrograms of the supernatant was immunoprecipitated with 1 μl of anti-p180 polyclonal antiserum which had been preadsorbed to protein A-Sepharose CL-4B (Pharmacia) at 4°C for 6 h. After being washed with buffer A, precipitates were dissolved with 2× Laemmli sample buffer and then subjected to SDS-PAGE and fluorography using En^{3}Hance (Dupont NEN).

Coimmunoprecipitation analysis. Fifty-microgram aliquots of COS-1 cell extracts were immunoprecipitated with 1 μl of anti-p180 monoclonal antibody SJK132-20 (ascitic fluid) which had been preadsorbed to protein G-Sepharose (Pharmacia) for 4 h at 4°C in 200 μl of NET buffer (50 mM Tris-HCl [pH 7.5]; 150 mM NaCl; 0.1% Nonidet P-40; 1 mM EDTA; 0.25% gelatin; 1 mM PMSF; 0.2 μg of aprotinin, 0.2 μg of leupeptin, 0.1 μg of antipain, and 0.1 μg of pepstatin A per ml). After being washed with NET buffer, precipitates were dissolved with 30 μl of 2× Laemmli sample buffer and then subjected to SDS-PAGE and Western blot analysis using anti-p180 and anti-p68 polyclonal antibodies.

DNA polymerase assay. The DNA polymerase assay was carried out as described previously (38). Briefly, 5-μg aliquots of proteins were incubated with DNase I-activated calf thymus DNA (0.5 mg/ml) in a buffer containing 20 mM Tris-HCl (pH 8.0), 10% glycerol, 60 mM KCl, 5 mM $MgCl_2$, 3.3 mM 2-mercaptoethanol, 0.2 mg of bovine serum albumin per ml, 100 μM each dATP, dCTP, and dGTP, and 50 μM [^{3}H]dTTP (0.1 Ci/mmol). Incorporated [^{3}H]dTMP was measured by using a Whatman DE81 paper disc as described previously (37).

Glycerol density gradient sedimentation. COS-7 cells were transfected with either pcDEBΔ (16 μg), pSRα68 (16 μg), pSRα180 (16 μg), or pSRα180 (10 μg) and pSRα68 (6 μg), incubated for 72 h, and then harvested as described above. Extracts containing 250 μg of protein in 100 μl were layered onto a 2-ml linear 15 to 35% glycerol gradient in a buffer containing 25 mM potassium phosphate (pH 7.5), 300 mM KCl, 1 mM $MgCl_2$, and 0.5% Triton X-100. Centrifugation was for 16 h at 55,000 rpm at 4°C (Beckman TLS-55), and the gradient was collected from the top and divided into 29 fractions. DNA polymerase activity was assayed, and Western blot analysis of each fraction was performed.

RESULTS

Expression of mouse p68 and p180 subunits in COS-1 cells. To explore the functions of the four subunits of the DNA polymerase α-primase complex, we developed a cDNA expression system using cultured mammalian cells as hosts. Various combinations of cDNAs for the four subunits of the mouse complex were transiently transfected into monkey COS-1 cells, and the cells were cultured for 48 h. Proteins were extracted from the transfected cells, and DNA polymerase activity in the whole-cell extract was determined by using activated calf thymus DNA as a template. When pSRα180 alone was transfected, DNA polymerase activity increased moderately compared with the control extract in which only vector plasmid had been transfected (Fig. 1A). When pSRα180 was cotransfected with pSRα46 or pSRα54, a similar moderate increase in DNA polymerase activity was observed. However, cotransfection of pSRα180 with pSRα68 resulted in a marked increase in DNA polymerase activity. The effect of pSRα68 on the increase of DNA polymerase activity was dose dependent, as shown in Fig. 1A. Since there have been no reports about any contribution of p68 to DNA polymerase activity, we decided to further investigate this novel effect of p68. To determine the protein levels of exogenously expressed subunits, we prepared polyclonal antibodies against mouse p180 and p68 expressed in bacteria. The specificity of each of these antibodies was demonstrated by Western blot analysis of purified DNA polymerase subunits from mouse FM3A cells (data not shown) and with exogenously overexpressed mouse DNA polymerase subunits (Fig. 1B). The antiserum against mouse p180 scarcely cross-reacted with monkey p180. This was also the case for the antibody against p68, which cross-reacted even more weakly with the monkey subunit, as shown by Western blot analysis and immunofluorescence analysis (described below). The anti-p180 antibody reacted with a 180-kDa protein, corresponding to full-length p180, as well as with a 160-kDa protein which corresponds to the truncated p180 molecule. This truncated molecule was characterized previously (16, 40). Therefore, these antibodies were considered to be suitable for the detection of

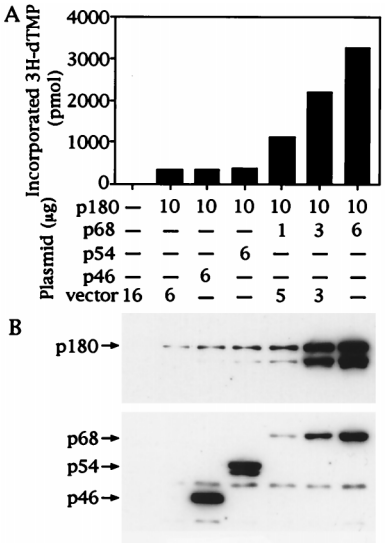

FIG. 1. Expression of mouse DNA polymerase α-primase subunits in COS-1 cells. cDNAs for the four subunits of mouse DNA polymerase α-primase were transfected individually or in combination into COS-1 cells. Forty-eight hours after transfection, the cells were lysed with a solution containing 20 mM potassium phosphate (pH 7.5), 300 mM KCl, 10% glycerol, 0.05% Triton X-100, and 0.1 mM EDTA. After centrifugation, the supernatants were assayed to estimate DNA polymerase activity and were subjected to Western blotting. (A) DNA polymerase activity of the COS-1 extract. COS-1 extracts (5 μg of protein) were incubated with [^{3}H]dTTP and DNase I-activated calf thymus DNA, and the incorporated radioactivity was measured as described in Materials and Methods. To estimate exogenously expressed DNA polymerase activity, endogenous DNA polymerase activity derived from COS-1 cells (approximately 20 pmol/μg/h) was determined in extracts from cells transfected with the vector control and subtracted from the total DNA polymerase activity of each sample. (B) Western blotting. Extracts (10 μg of protein) were subjected to SDS-PAGE followed by Western blot analysis with a mixture of antibodies against the four subunits of DNA polymerase α-primase.

ectopically expressed mouse DNA polymerase α-primase subunits in monkey COS-1 cells. The anti-p54 and anti-p46 antibodies have been characterized previously (24). These antibodies enabled us to estimate the protein levels of DNA polymerase α-primase subunits in transfected COS-1 cells. When p180 was cotransfected with p68, an intense signal for p180 was detected (Fig. 1B). The intensity of the p180 signal correlated with that of p68, showing that the effect of p68 was again dose dependent. Truncated 160-kDa protein was generated in proportion to the level of full-length p180. These results suggested that coexpression of p68 with p180 increased the protein level of p180, resulting in a marked increase in exogenous DNA polymerase activity. These findings were reproducible in other cell lines, including COS-7 and CV-1 cells (data not shown).

To determine whether the DNA polymerase activity of overexpressed p180 and p68 was derived from a heterodimer complex or the p180 subunit alone, glycerol gradient sedimentation analysis was carried out. The whole-cell extracts were loaded onto a 15 to 35% glycerol gradient; after centrifugation, the fractions were assayed for DNA polymerase activity and subjected to Western blot analysis. A single peak of DNA polymerase activity was observed irrespective of the presence of coexpressed p68 subunit (Fig. 2A). While DNA polymerase activity was detected in fractions 19 to 21 in the absence of p68, the activity sedimented at fractions 22 and 23 in the presence of p68, reflecting the increased molecular weight of the heterodimeric complex. Western blot analysis showed that p180 sedimented at a position that coincided precisely with the

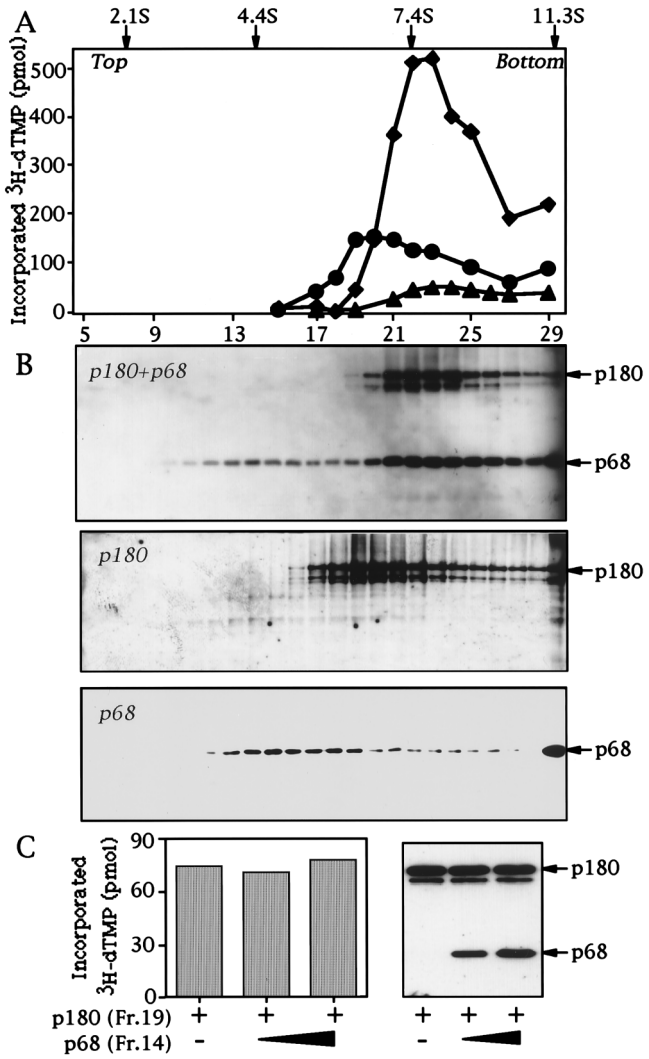

FIG. 2. Fractionation of overexpressed p180 in the presence or absence of p68 in COS-7 cells by glycerol density gradient sedimentation. Lysates of COS-7 cells transfected with pcDEBΔ (triangles), pSRα68 alone or pSRα180 alone (circles), or pSRα180 and pSRα68 (squares) were fractionated by 15 to 35% glycerol gradient sedimentation. The fractions were assayed for DNA polymerase activity and subjected to Western blotting as described in Materials and Methods. Protein markers run in a parallel gradient were chicken lysozyme (2.1S), bovine serum albumin (4.4S), yeast alcohol dehydrogenase (7.4S), and bovine catalase (11.3S). (A) DNA polymerase activity. Vector alone (triangles) represents endogenous DNA polymerase α-primase tetramer complex in COS-7 cells. (B) Western blotting using anti-p68 and anti-p180 antibodies. Because the expression level of singly expressed p180 was lower than that of heterodimeric p180-p68, the middle panel was obtained by a much longer exposure than that of the upper and lower panels. (C) Effect of singly expressed p68 on DNA polymerase activity. Fraction (Fr.) 19 from the gradient containing singly expressed p180 (middle gel of panel B) was mixed with fraction 14 of singly expressed p68 (lower gel of panel B), and then DNA polymerase activity was determined. In parallel, Western blot analysis was performed with anti-p68 and anti-p180 antibodies (right).

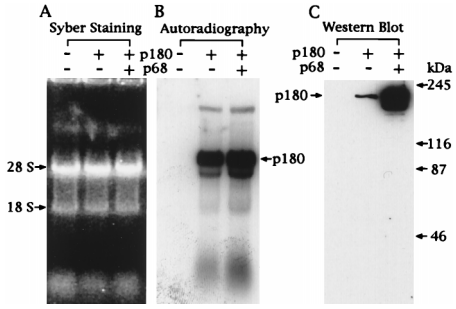

FIG. 3. Comparison of the mRNA and protein levels of p180 in the presence or absence of p68 by Northern blot and Western blot analyses. COS-1 cells were transfected with control vector, pSRα180, or pSRα180 with pSRα68, incubated for 48 h, and then harvested. Half of the cells were used for preparation of RNA, and the other half were used for Western blotting. Formaldehyde-denatured agarose gel electrophoresis was carried out on 10 μg of total RNA per lane, the gels were stained with SYBR green II, and then Northern blot analysis was performed. Western blot analysis was carried out with anti-p180 antiserum. (A) Staining pattern of total RNA with SYBR green II. (B) Autoradiogram to detect mRNA for transiently transcribed p180 in COS-1 cells. (C) Protein level of p180 by Western blot analysis using anti-p180 antibody. Ten micrograms of extract was subjected to SDS-PAGE as described for Fig. 1.

peaks of DNA polymerase activity seen in the presence or absence of p68 (Fig. 2B). The majority of p68 cosedimented with p180, indicating that coexpressed p180 and p68 formed a heterodimer. The position of the endogenous DNA polymerase α-primase tetrameric complex in COS-7 cells was shown in the gradient containing the control extract in which only vector plasmid had been transfected. Heterotetrameric DNA polymerase α sedimented at fractions 23 to 25, slightly faster than the heterodimer. The sedimentation coefficient of coexpressed p180 and p68 was 7.4S, which is close to the value of 7.1S observed for heterodimeric DNA polymerase purified from the tetrameric complex of FM3A cells (38). These results showed that coexpressed p180 and p68 were assembled into a heterodimeric complex, while singly transfected p180 existed as a monomer. An additional signal for p68 in the presence of p180 was found around fractions 13 to 17. Singly expressed p68 sedimented at a similar position, indicating that coexpressed p68 occurred in two forms, one as a heterodimeric complex with p180 and the other as free p68 whose sedimentation coefficient was 4.4S.

Since p68 not associated with p180 can be separated by glycerol density gradient sedimentation, we attempted to measure the effect of p68 on DNA polymerase activity. Fraction 19 from the gradient containing singly expressed p180 (Fig. 2B, middle) was mixed with fraction 14 of singly expressed p68 (Fig. 2B, bottom), and then DNA polymerase activity was determined. In parallel, Western blot analysis was performed with anti-p68 and anti-p180 antibodies (Fig. 2C, right). In the presence or absence of p68, DNA polymerase activity was almost equivalent (Fig. 2C, left), supporting the previous finding that p180 alone has full activity and properties similar to those of the tetrameric enzyme (6). Therefore, we conclude that p68-promoted DNA polymerase activity was caused by the increase in the p180 protein level.

Posttranscriptional control of the p180 protein level by coexpression of p68. To determine whether the accumulation of p180 caused by cotransfection of p68 occurs at the transcriptional level, we analyzed the mRNA level of the p180 gene in transfected cells. Transiently transfected COS-1 cells were divided into two parts; one half was harvested for the isolation of total RNA, and the other half was used for Western blot analysis and the pulse-chase experiment. Northern blot analysis revealed that the levels of p180 mRNA transcribed in p180-transfected cells were equivalent in the presence or absence of p68 coexpression (Fig. 3B). When the levels were normalized by comparison with 28S rRNA stained with SYBR green II, the mRNA levels of p180 were found to be 1 and 0.8 in the presence and absence of p68, respectively (Fig. 3A and B). In sharp contrast, the level of p180 protein as measured by Western blot analysis was 10 times higher in cells coexpressing both

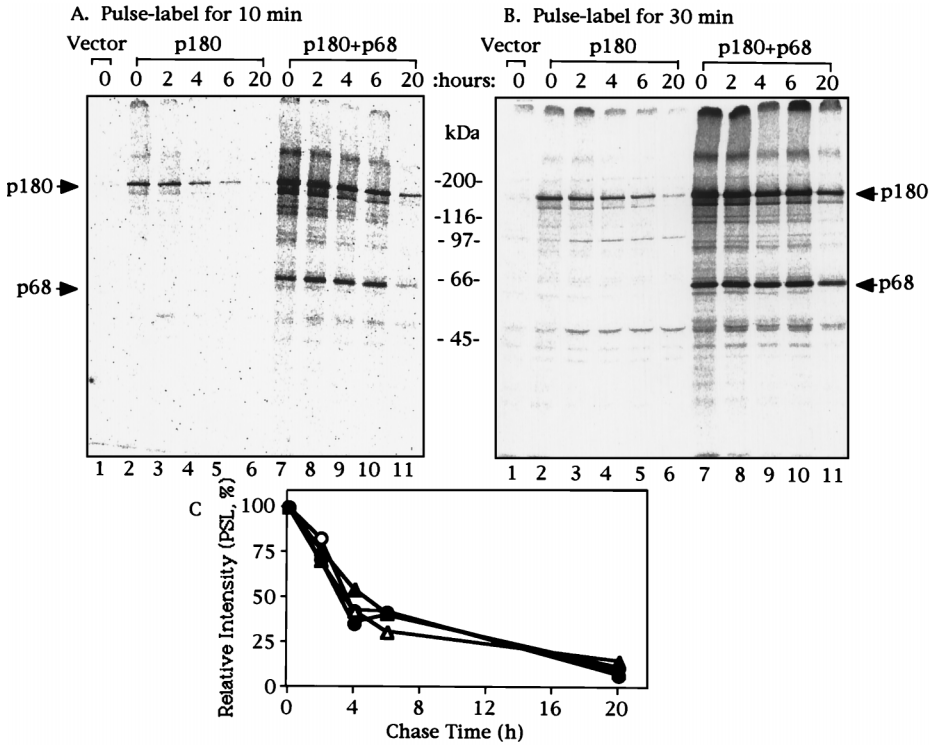

FIG. 4. Turnover of p180 in the presence or absence of p68 in COS-1 cells. (A and B) COS-1 cells, 48 h after transfection, were metabolically labeled with [^{35}S]methionine for 10 min (A) or 30 min (B) and either lysed immediately (0 h) or chased for 2 to 20 h in complete medium. p180 was immunoprecipitated from whole-cell extracts and analyzed by SDS-PAGE and fluorography. Equal amounts of protein were used in all immunoprecipitation reactions. (C) Densitometric data of p180 and p68 were quantified with a BAS2000 phosphorimager (Fuji). Quantified results were expressed relative to the 0-h value for each case. The values for photon-stimulated luminescence (PSL) of p180 at 0 h in the presence and absence of p68 were 904 (open circles) and 137 (open triangles) (A) and 6,980 (closed circles) and 671 (closed triangles) (B).

p180 and p68 than in cells expressing p180 alone (Fig. 3C). Since the marked increase of p180 levels could not be explained by the slight difference in the mRNA level, we conclude that the effect of p68 on the p180 protein level does not occur at the transcriptional level but involves some later step of either cotranslational or posttranslational control.

Next, we tried to measure the difference in protein turnover rates between singly expressed p180 and coexpressed p180 and p68 by using pulse-chase labeling and immunoprecipitation analysis. After incubation for 48 h, singly or doubly transfected COS-1 cells were pulse-labeled with L-[^{35}S]methionine for either 10 min (Fig. 4A) or 30 min (Fig. 4B) and then chased for 2, 4, 6, and 20 h. In both cases, the level of newly synthesized p180 coexpressed with p68 was approximately 10 times higher than that of p180 expressed alone. In addition, p68 could be coimmunoprecipitated with newly synthesized p180 immediately after pulse-labeling. In contrast, the half-lives of pulse-labeled p180 in either the presence or absence of p68 were almost the same, approximately 4.5 h (Fig. 4C). We found no difference between two experiments conducted with different pulse-labeling times. Taken together, these results indicate that p68 is rapidly assembled into a complex with p180 but that this does not influence the turnover rate of p180.

To address the mechanism of the increase in the p180 protein level promoted by p68, we measured the p180 protein synthesis rate. Transfected COS-1 cells were pulse-labeled with L-[^{35}S]methionine for 5, 10, 20, and 30 min, and then immunoprecipitation with anti-p180 antibody was performed. We found the difference in the p180 protein synthesis rates in the presence and absence of p68, and the difference corresponded exactly to that of the p180 protein levels (data not shown and Fig. 4A and B, lanes 2 and 7). These results suggest that the increase in the p180 protein level promoted by p68 is caused by enhancement of the synthesis rate of p180 during translation.

Subcellular distribution of p180 and p68 in transiently transfected COS-1 cells. The finding that coexpression of p68 and p180 markedly increased the protein level of p180 prompted us to examine the subcellular distribution of transiently transfected p68 and p180 by indirect immunofluorescence analysis. The affinity-purified anti-p68 antibody reacted with endogenous p68 in mouse NIH 3T3 cells and showed bright nuclear staining (data not shown). However, monkey p68 in COS-1 cells could be scarcely detected and was observed only after longer exposures (not shown). The anti-p180 monoclonal antibody SJK132-20 (1) reacted with monkey p180 as well as with mouse p180. The transiently transfected COS-1 cells were fixed 48 h after transfection, permeabilized, and incubated with anti-p68 polyclonal antibody or SJK132-20. In our transfection experiments, ectopic proteins were overexpressed in 10 to 20% of the cells. Unexpectedly, we found that p68 and p180 showed diffused cytoplasmic staining when each subunit was transfected individually (Fig. 5A, a and c). We scored 408 cells which displayed overproduction of ectopic p180 proteins. Among these cells, 403 cells showed cytoplasmic localization. In the case of p68, 329 of 441 transfected cells showed cytoplasmic localization. In sharp contrast, coexpression of both subunits markedly changed their subcellular distributions. As shown in Fig. 5A, e and f, coexpressed p68 and p180 were localized predominantly in the nucleus: 389 of 408 cotransfected cells showed nuclear localization. These findings

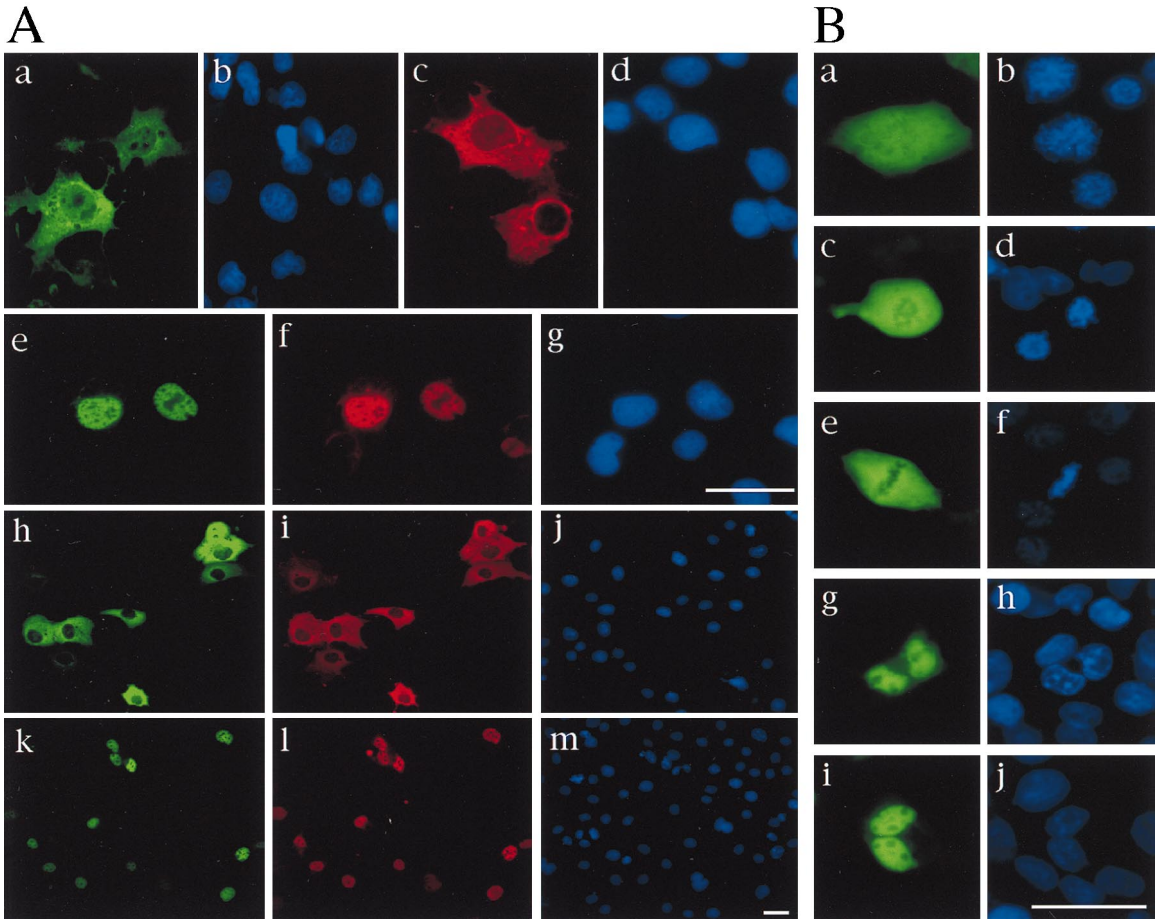

FIG. 5. Immunohistochemical analysis of transiently transfected mouse DNA polymerase α-primase in COS-1 cells. (A) COS-1 cells were transfected with pSRα68 (a and b), pSRα180 (c and d), pSRα68 and pSRα180 (e to g), p180-GFP (h to j), and p180-GFP and pSRα68 (k to m) and incubated for 48 h. Then the subcellular distribution of expressed proteins was examined by immunofluorescence microscopy using anti-p68 (a and e) or anti-p180 monoclonal SJK132-20 (c, f, i, and l) antibodies. Rabbit antibody was detected with FITC-conjugated anti-rabbit IgG antibody (a and e), while the monoclonal antibody was detected with Texas red-conjugated anti-mouse IgG antibody (c, f, i, and l). h and k, fluorescence derived from GFP; b, d, g, j, and m, Hoechst 33258 staining of the same cells to indicate the location of the nucleus. Bars, 40 μm. (B) Subcellular distribution of p180-GFP in the presence of p68 in mitotic cells. COS-1 cells transfected with p180-GFP and pSRα68 were synchronized as described in Materials and Methods. Cells representing progressive mitotic stages (a to d, prometaphase; e and f, metaphase; g to j, telophase or early G_1 phase) were photographed. a, c, e, g, and i, fluorescence derived from GFP; b, d, f, h, and j, Hoechst 33258 staining of the same cells to indicate the location of the nucleus. Bar, 40 μm.

were reproduced with HA-tagged p180 and anti-HA monoclonal antibody used in a double-staining experiment and in other cell lines, including COS-7, CV-1, and NIH 3T3 (data not shown). These results indicate that nuclear translocation of p180 and p68 depends on their mutual interaction.

To verify the effect of p68 on the subcellular distribution of p180, we designed another experiment using a chimeric construct with GFP. cDNA of GFP was fused with the carboxyl terminus of p180 and transfected into COS-1 cells in the presence or absence of p68. The subcellular distributions of p180 were determined on the basis of either fluorescence of GFP or immunofluorescence by anti-p180 antibody. The strong signal derived from GFP enabled us to determine the subcellular distribution of fusion protein efficiently and conveniently. In the absence of p68, p180-GFP was expressed exclusively in the cytoplasm (Fig. 5A, h to j), whereas in the presence of p68, p180-GFP localized in the nucleus (Fig. 5A, k to m). Thus, the effect of p68 was reproduced by using the GFP construct, indicating that (i) p68-dependent nuclear localization of p180 was not caused by artificial manipulations such as cell fixation or specificity of antibodies and (ii) the staining cells were not inadvertently selected from their particular appearance in the total population of randomly expressed cells.

To explore the relationship between nuclear translocation of p180-p68 and the cell cycle, cells transfected with p180-GFP and p68 were arrested in S phase by incubation with 10 mM thymidine for 20 h, released in culture medium for 8 h, and then treated with nocodazole (45 ng/ml) for 6 h. The cells enriched in G_2/M phase were allowed to traverse the cell cycle by release into culture medium. The fluorescence of GFP allowed us to detect the subcellular distribution of the p180-p68 heterodimer efficiently. In metaphase, proteins were distributed in the cytoplasm, while in telophase or early G_1 phase, p180-p68 localized in the nucleus (Fig. 5B). Such localization was in good agreement with the previous report by Nakamura et al. (26) concerning the endogenous human DNA polymerase α. Thus, transiently overexpressed subunits were translocated into the nucleus in the same manner as the endogenous polymerase α.

Identification of the domains of p68 implicated in the increase of p180 and nuclear translocation of the p180-p68 heterodimer. To date, the amino acid sequence of the second-

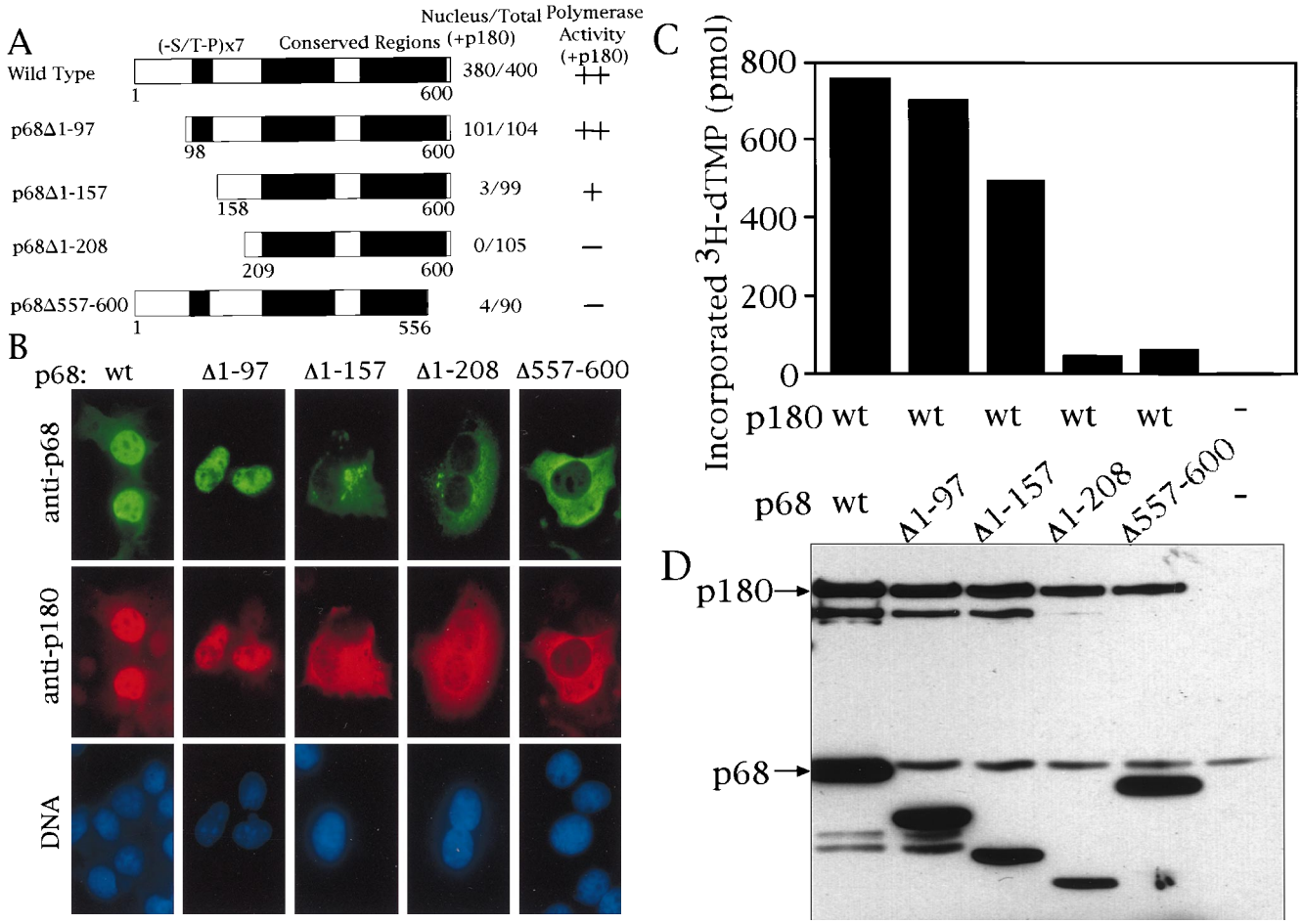

FIG. 6. Characterization of p68 deletion mutants. (A) Schematic representation of p68 mutant constructs. Closed and shaded boxes represent the cluster of -Ser/Thr-Pro- motifs and the highly conserved region, respectively. Numbers indicate amino acid positions of p68. Numbers of cells which showed nuclear localization are depicted at the right. (B) Subcellular distribution of mutant p68 and p180 by immunofluorescence analysis. p68 mutant constructs and pSRα180 were cotransfected into COS-1 cells, and expressed proteins were detected simultaneously by indirect immunofluorescence analysis using anti-p68 polyclonal antibody and FITC-conjugated anti-rabbit IgG antibody (upper row) or monoclonal antibody SJK132-20 and Texas red-conjugated anti-mouse IgG antibody (middle row). The lower row shows nuclear staining by Hoechst 33258. wt, wild type. (C) DNA polymerase activity. Five micrograms of COS-1 extract was incubated with [^{3}H]dTTP and DNase I-activated calf thymus DNA, and incorporated radioactivity was measured as described for Fig. 1. (D) Western blotting. Ten micrograms of extract was subjected to SDS-PAGE followed by Western blot analysis with anti-p68 and anti-p180 antibodies.

largest subunit of DNA polymerase α-primase complex has been identified in *S. cerevisiae*, *Drosophila*, mouse, and human cells. Alignment of the p68 sequences from these species reveals several features of the structure of p68. First, whereas the amino-terminal region is diverse and exhibits little homology, conserved amino acid sequences are distributed from the center to the carboxy-terminal region. Second, clusters of -Ser/Thr-Pro- motifs, which are considered to be putative phosphorylation sites for the cdc2 kinase family, are located in the amino-terminal region, as shown in Fig. 6A. However, the precise roles of these regions remain unclear. To confirm the role of p68 in regulating p180 protein levels and the nuclear translocation of the heterodimer, in addition to elucidating the structure-function relationship of p68, we constructed several deletion mutants of p68 (Fig. 6A). These mutant constructs were cotransfected into COS-1 cells with pSRα180, and the resultant mutant proteins were characterized by immunofluorescence analysis (Fig. 6B), DNA polymerase activity (Fig. 6C), and Western blot analysis (Fig. 6D). The amino-terminal deletion mutant p68Δ1-97 was almost identical to the wild type in terms of the increase in p180 protein level, DNA polymerase activity, and translocation into the nucleus. In contrast, the amino-terminal deletion mutant p68Δ1-208 and carboxy-terminal deletion mutant p68Δ557-600 lost the ability to increase the protein level of p180 and were localized in the cytoplasm. The amino-terminal deletion mutant p68Δ1-157, which lacks the clusters of -Ser/Thr-Pro- motifs, was found to be expressed in the cytoplasm even though it increased the p180 protein level more than the mutant p68Δ1-208. Taken together, these results indicate that p68-promoted increases in the p180 protein level and translocation of the heterodimer into the nucleus are supported by a broad region of p68, stretching from the center to the carboxyl terminus, which is highly conserved among eukaryotes.

Identification of the domain of p180 essential for nuclear translocation by using deletion and point mutants. Our finding that p68-promoted overproduction of p180 led to colocalization of both subunits in the nucleus prompted us to identify the domain of p180 essential for entry into the nucleus. Several motifs comprised of clusters of basic residues for the targeting of proteins to the nucleus, such as -KKKRK- for SV40 large T antigen (20, 22) and KR---------KKKK for nucleoplasmin (30),

have been identified. A search for sequences in p180 suggested that an authentic NLS might be located at residues 31 to 46 (23). To determine whether this putative NLS really functions as the NLS, we constructed a set of deletion mutants (Fig. 7A). Expression of each mutant in COS-1 cells was detected by Western blot analysis as shown in Fig. 7B. We found that all mutants of p180 singly transfected were localized in the cytoplasm in the absence of p68 (data not shown). However, the cells expressing p180Δ1-191 or p180Δ1442-1465, in which the amino terminus or carboxyl terminus, respectively, was truncated, showed intense nuclear localization similar to that seen with the wild-type p180 in the presence of coexpressed p68 (Fig. 7D, h and q). Substitution mutant p180KK(45,46)QQ, which lacked the basic residues of the putative NLS, was also exclusively localized in the nucleus in the presence of p68 (Fig. 7D, e). In contrast, cells expressing p180Δ1417-1465 exhibited diffuse cytoplasmic staining for both p68 and p180 (Fig. 7D, s and t). These findings suggest that (i) nuclear translocation of p180 is absolutely dependent on an interaction with coexpressed p68 and (ii) the amino-terminal region and a putative NLS found previously are not necessary for translocation of p180 into the nucleus.

To further define the region necessary for nuclear translocation of p180 and to eliminate problems arising from the marked changes that may occur in overall structure as a result of deletion mutations, we constructed several point mutants. In the carboxy-terminal region of p180, we noted that there were two clusters of basic residues. These sequences, KLKK (residues 1419 to 1422) and RKVK (residues 1434 to 1437), were then replaced by neutral amino acid residues, resulting in KLQQ and NNVN, respectively. Interestingly, both mutant proteins were found predominantly in the nucleus in the presence of p68 (Fig. 7D, k and n). In contrast, a double mutant which lacked both basic residues was shown to be localized predominantly in the cytoplasm even in the presence of p68 (Fig. 7D, v to x). All of these mutants were still associated with p68 as well as the wild-type p180 as demonstrated by coimmunoprecipitation analysis with anti-p180 antibody (Fig. 7C). These results indicate that basic residues besides the zinc finger motifs are important for the p180-p68 heterodimer to translocate into the nucleus.

DISCUSSION

Since baculovirus-derived recombinant p180 protein has been shown to possess intrinsic DNA polymerase activity not different from that of the four-subunit holoenzyme (6), the role of p68 has been an enigma. We used a cDNA expression system in cultured mammalian cells to study the influence of the noncatalytic subunit p68 on the subunit assembly and activity of DNA polymerase α. Our finding that p68 plays a role in the maintenance of the p180 concentration instead of in enzymatic regulation provides insight not only into the function of the p68 subunit but also into the mechanism of biosynthesis of this multisubunit complex.

The mechanism through which p68 increases the protein level of p180 is still speculative. We first found that coexpression of p180 with p68 markedly increased DNA polymerase activity in COS-1 cells. However, using Northern blot analyses and pulse-labeling experiments with Western blot analyses, we observed that the mRNA level and the turnover rate of recombinant p180 were not significantly affected by the presence of p68. In contrast, newly synthesized p180 was rapidly assembled into a heterodimeric complex with p68, and this resulted in the generation of much more p180 during the pulse-labeling period. Therefore, the p68-promoted increase in the p180 protein level probably occurs not at the transcriptional or posttranslational level but at the cotranslational level. In eukaryotic cells, although initiation control of transcription is the major step in the regulation of most genes, a variety of controls can intervene at the posttranscriptional level. Among these controls, translational control enables a cell to adjust the concentration of a protein rapidly and reversibly without rapid turnover of its mRNA. Such a mechanism has been reported for proto-oncogenes (28), ferritin (15), and many proteins during fertilization (46). In addition, pausing of translation elongation has been reported recently for eukaryotic cells. It was shown by van Wijk and Eichacker that translation elongation of chlorophyll-binding photosystem II center protein D1 was controlled strongly by light, and pausing resulted in the accumulation of translational intermediates in the dark (42). Young and Andrews reported that the α subunit of the signal recognition particle receptor has a strong pause site on its mRNA and that translation pausing facilitates the cotranslational membrane binding of this subunit (47). These are novel cases of cotranslational pausing, but such a regulatory mechanism may occur widely in eukaryotic cells (47). In the light of these data, it is tempting to speculate that p180 mRNA possesses a putative pausing site and that interaction with p68 facilitates progression of the translational apparatus through the pause site, resulting in the accumulation of a mature heterodimer complex containing p180 and p68.

The characterization of mammalian p68 was previously reported by Collins et al., who used bacterially expressed human p68, which was shown to serve as a molecular tether between DNA polymerase α and SV40 large T antigen in the SV40 replication system (5). On the other hand, mouse p68 expressed in the baculovirus system did not show any significant effect on the polyomavirus replication system (3). In both studies, the effect of p68 on production of p180 was not considered. In addition, the subcellular distribution of p68 has never been studied in mammalian cells. Collins et al. (5) demonstrated that the domain of p68 for interaction with the SV40 large T antigen was located in the amino-terminal region from amino acids 1 to 240. From these findings taken together with our results, the following domain structure of p68 emerges: the amino-terminal region of p68 (amino acids 1 to 240) serves as a binding site for other replicative proteins, whereas the center and the carboxy-terminal regions (157 to 600) are essential for interaction with p180. Interestingly, putative phosphorylation sites for cdc2 kinase are located in the amino-terminal region (115 to 157), implying that phosphorylation(s) of p68 may affect its interaction with other proteins, rather than being involved in intrinsic DNA polymerase activity or the biosynthetic pathway.

Our results strongly suggest that translocation of p68 and p180 into the nucleus is dependent on their mutual interaction. We tried to identify the essential domain required for nuclear translocation by deletion and substitution mutants and found that basic residues along with zinc finger motifs of p180 were critical for nuclear translocation. Although these basic charged residues were predicted by Dingwall and Laskey (9) to form a typical NLS, the function of the NLS is strictly dependent on interaction with p68. In general, an NLS is considered to consist of comparatively short sequences rich in basic amino acid residues, and such short sequences have been shown to be necessary and sufficient for translocation of proteins into the nucleus (9, 13, 14, 31). However, our result showing that the NLS of p180 is dependent on interaction with p68 is an exception to this rule. Since none of the constructs of p180 were able to enter the nucleus in the absence of p68, and all of the mutants of p68 were retained in the cytoplasm without p180

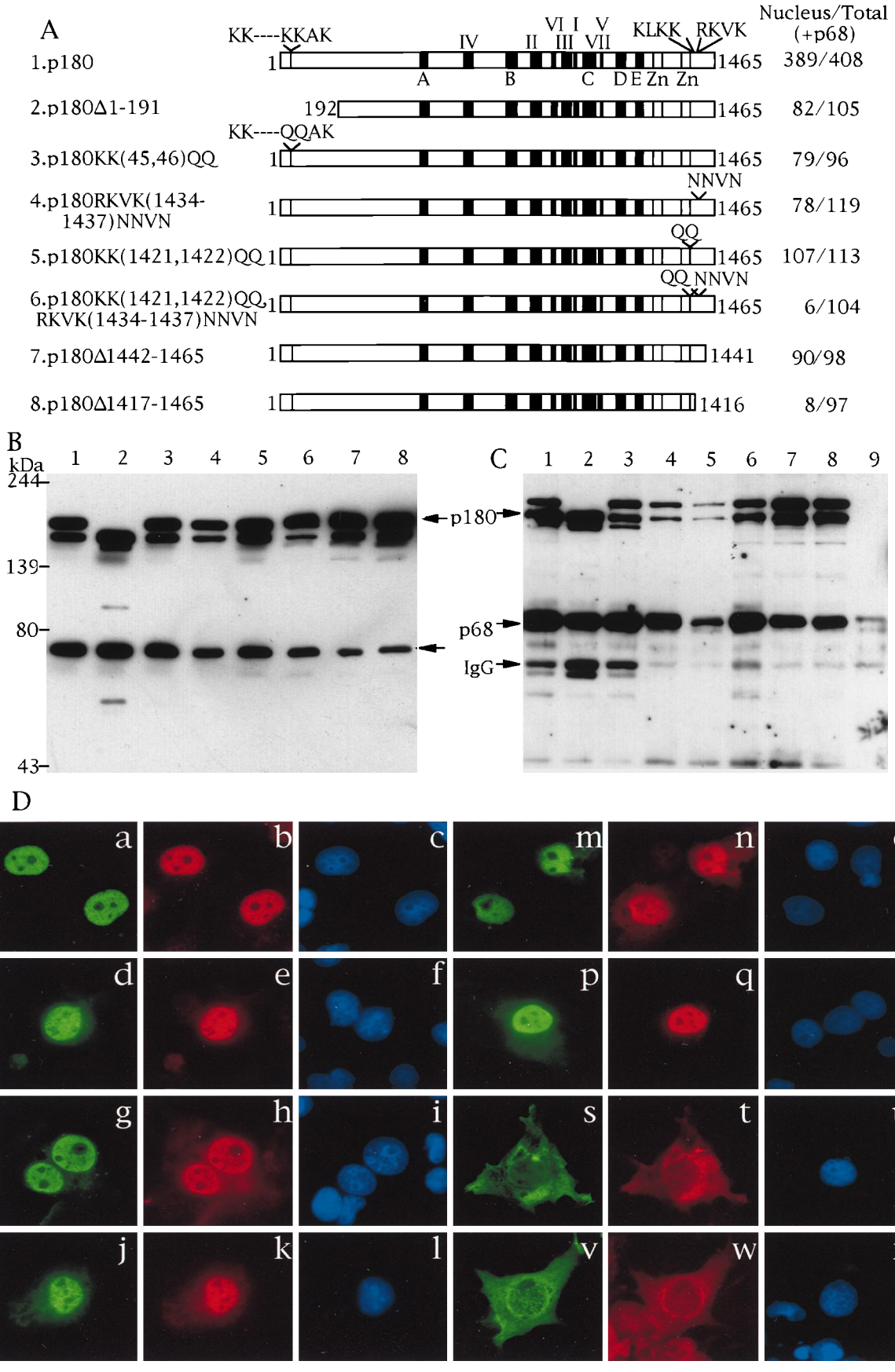
A
Nucleus/Total (+p68)
KK----KKAK
VI I V
IV II III VII
KLKK RKVK
1.p180
1
1465
389/408
A B C D E Zn Zn
2.p180Δ1-191
192
1465
82/105
KK----QQAK
3.p180KK(45,46)QQ
1
1465
79/96
NNVN
4.p180RKVK(1434-1437)NNVN
1
1465
78/119
QQ
5.p180KK(1421,1422)QQ
1
1465
107/113
QQ NNVN
6.p180KK(1421,1422)QQ, RKVK(1434-1437)NNVN
1
1465
6/104
7.p180Δ1442-1465
1
1441
90/98
8.p180Δ1417-1465
1
1416
8/97
B
kDa
1 2 3 4 5 6 7 8
244
139
80
43
C
1 2 3 4 5 6 7 8 9
p180
p68
IgG
D
a b c m n o
d e f p q r
g h i s t u
j k l v w x

FIG. 7. Identification of the p180 NLS in deletion mutants. (A) Schematic representation of p180 mutant constructs. The seven highly conserved regions in class B DNA polymerases (34, 45) are indicated by closed boxes with roman numerals (I to VII). The five conserved regions in eukaryotic DNA polymerase α (23) are indicated by hatched boxes with letters (A to E). Zinc finger motives are depicted by open boxes. Numbers indicate amino acid positions of p180. Numbers of cells which showed nuclear localization are depicted at the right. (B) Western blotting. Extracts (10 μg of protein) were subjected to SDS-PAGE followed by Western blot analysis with anti-p68 and anti-p180 antibodies. Lane numbers correspond to p180 mutant constructs shown in panel A. (C) Coimmunoprecipitation assay. Extracts (50 μg of protein) were immunoprecipitated with anti-p180 monoclonal antibody. One-tenth of each precipitate was subjected to Western blot analysis with anti-p68 and anti-p180 polyclonal antibodies. Lane numbers correspond to p180 mutant constructs in panel A except for lane 9, which represents a control extract with vector alone. (D) Subcellular distribution of mutant p180 and p68 by immunofluorescence analysis. pSRα68 and either pSRα180 (a to c), p180KK(45,46)QQ (d to f), p180Δ1-191 (g to i), p180KK(1421,1422)QQ (j to l), p180RKVK(1434-1437)NNVN (m to o), p180Δ1442-1465 (p to r), p180Δ1417-1465 (s to u), or p180KK(1421,1422)QQ RKVK(1434-1437)NNVN (v to x) were cotransfected into COS-1 cells, and the proteins expressed were detected simultaneously by indirect immunofluorescence analysis using anti-p68 polyclonal antibody and FITC-conjugated anti-rabbit IgG antibody (a, d, g, j, m, p, s, and v) or monoclonal antibody SJK132-20 and Texas red-conjugated anti-mouse IgG antibody (b, e, h, k, n, q, t, and w). c, f, i, l, o, r, u, and x, nuclear staining with Hoechst 33258.

(data not shown), the NLS of p180 may not be able by itself to present the requisite molecular surface for nuclear entry. For this to occur, binding to p68, and resultant conformational changes in the carboxyl terminus of p180, may be necessary to allow the cryptic NLS to appear on the molecular surface and bind to the NLS receptors. However, these results do not eliminate the possibility that p68 has a cryptic NLS of its own and that a conformational change in p68 on interaction with p180 activates this cryptic NLS. There is another possibility: that the p180-p68 heterodimer exhibits a new class of NLS which may be different from the authentic NLS composed of basic charged residues, such as the M9 domain of hnRNPA1 (14). To confirm that the NLS of p180 is localized in the carboxy-terminal domain and dependent on an interaction with p68 for activation, it will be necessary to examine the molecular conformation of the carboxyl terminus of p180 in the presence of p68 and binding of p180 to an NLS receptor such as mPendulin, mSRPI, or Rch1 (14, 29) in the presence of p68.

It was reported that in *S. cerevisiae*, a mutant strain overexpressing p68 contained this subunit in the nucleus (12). In addition, the NLS in p180 of *Schizosaccharomyces pombe* has recently been shown to be located in the amino-terminal region (2). However, compared with human and mouse p180, the corresponding region of the putative NLS of *S. pombe* p180 displays few conserved amino acid residues. Therefore, the nuclear translocation process of DNA polymerase α may be different between mammals and other eukaryotes, including *S. cerevisiae*, *S. pombe*, and *Drosophila melanogaster.*

In conclusion, we have demonstrated that the noncatalytic subunit p68 plays a crucial role in the biosynthesis of catalytic subunit p180. Since characterization of the in vivo function of p68 was hampered by the limited amount of DNA polymerase α in cells, the overexpression system provided a useful tool to understand the in vivo function of the four subunits of DNA polymerase α-primase. Continuation of this approach should allow a detailed analysis of the roles of the different subunits of DNA polymerase α in DNA replication in vivo and the nature of the protein-protein interactions between the subunits or with other replicative factors involved in the control of cell proliferation.

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