The Human POLD1 Gene

IDENTIFICATION OF AN UPSTREAM ACTIVATOR SEQUENCE, ACTIVATION BY Sp1 AND Sp3, AND CELL CYCLE REGULATION

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The promoter of the human POLD1 gene encoding the catalytic subunit of DNA polymerase δ is GC-rich and does not contain a TATA box. Transient transfection analysis in HeLa cells employing POLD1-luciferase chimeric plasmids revealed a core promoter region extending 328 base pairs (bp) from the major transcription initiation site. Multiple elements in this region including two 11-bp direct repeats located between nucleotide positions −92 and −22, play an important role in POLD1 promoter activity. Deletion or linker-replacement mutations of the repeats drastically reduced the promoter activity. A 70-bp DNA fragment containing the two repeats could stimulate the expression of the POLD1 or a heterologous promoter in an orientation-independent manner. DNase I footprinting and band-shift assays showed that HeLa nuclear extracts contained proteins specifically binding to the repeat sequences. Southwestern blot and UV cross-linking analyses identified Sp1 and two 85-kDa proteins that bound to the repeats. Additionally, screening of HeLa cDNA expression libraries for the sequence-specific DNA-binding protein using the 11-bp repeat sequences as the probe, identified a cDNA that corresponds to Sp3, a member of the Sp1 family. Cotransfection studies in Drosophila SL2 cells showed that both Sp1 and Sp3, but not Sp2, could activate the POLD1 promoter through the repeat sequences. The POLD1 promoter activity was induced about 4-fold at the late G1/S boundary in serum-stimulated cells. The 11-bp repeats together with an E2F-like sequence, located adjacent to the major transcription initiation site, were important for the stimulation. Taken together, this study provides a direct evidence for transcriptional regulation of the human POLD1 gene.

The DNA replication of eukaryotic chromosomes is a complex but highly regulated process. Through the cooperation of multiple protein factors and enzymes including DNA polymerases, each chromosome replicates once during the S phase of the cell cycle (reviewed in Ref. 1). Presently, the mechanisms underlying this cell cycle regulation of DNA replication are not completely understood.

DNA polymerase δ (pol δ) is one of the major enzymes involved in the synthesis of mammalian nuclear DNA (2, 3). It was reported as a new type of DNA polymerase with an intrinsic 3' to 5’ exonuclease activity (4), suggesting that it possesses exonuclease proofreading ability (5, 6). Purified pol δ is composed of a 125-kDa catalytic subunit and an associated 48-kDa small subunit whose function has not been defined (7). In addition, a 36-kDa factor was shown to convert the pol δ activity from low to high processivity (8). This factor was subsequently shown to be identical to the proliferating cell nuclear antigen (9, 10).

Previously, we, in collaboration with Lee’s group (11), and others (12) isolated the full-length cDNA for the catalytic subunit of human pol δ. In addition, the coding sequences of the pol δ catalytic subunit have been isolated from bakers’ yeast (13), fission yeast (14), malaria parasite (15, 16), calf thymus (17), and mouse cells (18). Sequence analysis showed that the catalytic subunit of human pol δ contains 1,107 amino acids and is related to other eukaryotic DNA polymerases (11). Recently, we also cloned the gene for the catalytic subunit of human pol δ (POLD1) and its 5’-flanking sequences (19). The human POLD1 gene contains 27 exons and 26 introns. Transcription of the gene appears to be initiated at multiple sites with the major initiation site 53 nucleotides upstream of the ATG start codon. As found in the promoters of many mammalian housekeeping genes (for reviews, see Refs. 20–22), the POLD1 promoter is GC-rich and does not contain a TATA box. Several potential binding sites for transcription factors AP2, CTF, Ets1, GCF, MBF-1, NF-E1, and Sp1 are present in the 5’-flanking region of the POLD1 gene (19). However, the significance of these elements in regulating POLD1 promoter activity is presently not known.

Studies on the expression of the genes involved in nucleotide metabolism and DNA synthesis reveal the presence of multiple regulatory elements including the GC boxes, which are required for maximal promoter activity (23–30). The transcription factor Sp1 (31, 32) has been shown to regulate the transcription through the GC boxes. Recently, several Sp1-related proteins, Sp2, Sp3, and Sp4, that bind to the GC box or GT motif were identified (33–35); however, their role in promoter regulation is less defined. Sp3 and Sp4 share a high degree of amino acid sequence homology with Sp1 (33). The high degree of structural conservation among Sp1, Sp3, and Sp4 suggests that these transcriptional regulators act through binding to

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The abbreviations used are: pol δ, DNA polymerase δ; PCR, polymerase chain reaction; HSVtk, herpes simplex virus thymidine kinase gene; UTR, untranslated region; ORF, open reading frame; DMEM, Dulbecco’s modified Eagle’s medium; PBS, fetal bovine serum; SL2, Drosophila Schneider line 2 cells; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; PBS, phosphate-buffered saline; bp, base pair(s); kb, kilobase pair(s); Ubx, Ultrabithorax gene.
similar DNA elements. Like Sp1, both Sp3 and Sp4 can bind to the GC box or GT motif, and Sp4 can activate several Sp1-responsive promoters (34, 36, 37). However, the Sp4 mRNA is detected only in brain (33). On the other hand, Sp3 is ubiquitously expressed and can either activate or repress transcription (34, 36–38). In addition, Sp3 can repress Sp1-mediated activation (34, 36, 39). Another Sp1-family member, Sp2, appears to share less homology with other members and has not been shown to act as an activator or a repressor (35). In addition, the binding affinity of Sp2 to the GT motif is much weaker than that of Sp1 and Sp3. Nonetheless, it has been hypothesized that these Sp1-family transcription factors may play a concerted role in regulating different GC or GT sequence in the promoter (36). Since the POLD1 promoter contains potential Sp1-binding sites, it would be important to examine if the Sp1-family proteins can regulate POLD1 transcription.

POLD1 expression is induced in serum-stimulated cells, consistent with its involvement in DNA replication (11). Northern blot analysis of RNA from populations of human Molt 4 cells resistant with its involvement in DNA replication showed that blot analysis of RNA from populations of human Molt 4 cells consistent with its involvement in DNA replication (11). Northern blot analysis of RNA from populations of human Molt 4 cells consistent with its involvement in DNA replication (11).

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POLD1 promoter linked to the luciferase expression cassette from pGL2-basic (Promega) has been described (19). A series of 5′-unidirectional deletion mutants within the POLD1 promoter DNA of pGL2-8′–1758) was created using the Erase-a-Basesystem (Promega) or the Bal31 nuclease (44). Some 5′ and 3′ deletion mutants of the POLD1 promoter were constructed by deleting appropriate restriction fragments or by polymerase chain reaction (PCR; Ref. 45). The linker replacement mutants within the 11-bp repeat region were constructed by ligation an XhoI linker to the proper deletion clones created by Bal31 deletion or by PCR. To construct the POLD1 promoter linked to the luciferase expression cassette, the chimeric promoter plasmids, pGL2-8R2R1-HSVtkbasal and pGL2-8R1R2-HSVtkbasal, were created by inserting the 70-bp PstI-SacI fragment containing the entire Sp2 coding region into the 5′-flanking region of the POLD1 promoter DNA 5′ to the HSVtk basal promoter. The exact locations of the deletion end points or appropriate fusions in all of these plasmids were determined by sequencing with POLD1-specific primers or primers derived from the pGL2 vector (46).

The Sp1 expression plasmid, pPacSp1 (47), was generously provided by Dr. Robert Tjian of University of California at Berkeley. It contains a 2.6-kb Drosophila actin 5C promoter, a 700-bp Ubx cDNA fragment consisting of the 5′ untranslated region (UTR) plus the first eight codons of the Ubx open reading frame (ORF), a 2.1-kb Sp1 cDNA fused with the Ubx ORF, and a 1.1-kb 3′ UTR of the actin 5C gene providing the polyadenylation (polyA) signal sequence. Expression plasmids for Sp2 and Sp3 were generated based on pPacSp1. To construct the Sp2 expression plasmid, a BamHI linker was inserted immediately 5′ to the Sp2 translation initiation codon in the Sp2 cDNA plasmid, Sp2-pKS(−) (American Type Culture Collection; Ref. 35), and an XhoI linker was inserted into the SacI site at nucleotide position 2062. The BamHI-XhoI fragment containing the entire Sp2 coding region was excised and used to replace the BamHI-XhoI fragment in pPacSp1 to generate the pPacSp2 plasmid. To construct the Sp3 expression plasmid, an Sp3 cDNA clone was isolated from the screening of a cDNA expression library (see below). A 2.1-kb EcoRI-XhoI Sp3 cDNA fragment was excised and used to replace the EcoRI-XhoI Sp3 cDNA in pPacSp1 to generate the pPacSp3 plasmid.

Cell Culture, Transfection, Promoter Activity, and Western Blot Analysis—Human HeLa cells and mouse C3H10T1/2 cells (American Type Culture Collection) were routinely grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). Drosophila Schneider line (SL2) cells (48) were grown in Schneider medium (Life Technologies, Inc.) supplemented with 10% FBS at 25 °C. Transfection assays were performed by the calcium phosphate technique (49) with slight modifications (50). A 106 SL2 cells (50) (POLD1-luciferase plasmid 10 μg) was transfected into a 100-mm dish of actively growing HeLa cells. To standardize the transfection efficiency, 2 μg of the pCH110 plasmid (Pharma Biotech Inc.), containing the SV40 early promoter driving β-galactosidase gene, were also included as an internal control in each transfection. Calcium phosphate-DNA coprecipitate mixtures were prepared and added to cells in the presence of medium. Following overnight incubation, the medium was replaced with fresh growth medium. At 48 h after transfection, cells were harvested in reporter lysis buffer and the luciferase and β-galactosidase activities were measured using the reporter assay system (Promega). For all transfection assays, at least three independent experiments were performed. The luciferase activity expressed from each promoter construct was normalized to the β-galactosidase activity for transfection of Drosophila SL2 cells, 5 μg of each POLD1-luciferase plasmid and various amounts of the Sp1, Sp2, or Sp3 expression plasmid were used. The total amounts of DNA were compensated to 20 μg with the control pPacU vector (47). An equal amount of cell extracts from each transfection was used for measuring the luciferase activity. To detect the expression of the Sp1-family protein, the same amount (80 μg) of protein extracts from each transfection of SL2 cells was used for electrophoresis in an 8% SDS polyacrylamide gel and blotted onto a PVDF-Plus filter (Micron Separations) using a Hoefer Transphor electrophoresis unit (44). The filter slice containing extracts from cells transfected with each Sp1-family expression plasmid was stained with the corresponding antibody against each expressed protein (anti-Sp1 (ICB), anti-Sp2 (K-20), or anti-Sp3 (D-20) antibody from Santa Cruz), followed by a secondary antibody conjugated with alkaline phosphatase using the Protoblot AP system (Promega).

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts from HeLa cells were prepared as described (51). Briefly, HeLa cell nuclei were extracted with 0.35 M KCl and nuclear extracts were dialyzed against the buffer containing 10 mM HEPES, pH 7.9, 100 mM KCl, 20% glycerol, 1.5 mM MgCl2, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol. The protein concentration of the extract was determined by using the Bio-Rad protein assay dye reagent and was approximately 5 mg/ml. For preparing nuclear extracts from transfected SL2 cells, a rapid micropreparation method was used (52). SL2 cells were transfected with 5 μg of the Sp1, Sp2, or Sp3 expression plasmid, and nuclear extracts were prepared 48 h after transfection. EMSA (53) was performed according to the Gel Shift Assay System (Promega) with minor modifications. Briefly, various amounts of nuclear extracts were incubated with 1 μg of poly(dI-dC)-poly(dI-dC) (Pharmacia) in 10 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, and 4% glycerol in a total volume of 18 μl. After incubation at room temperature for 5 min, 2 μl of 32P-labeled double-stranded DNA probe (0.8 ng) was added and the mixture incubated for an additional 20 min. The reaction products were electrophoresed in a native gel (acylamide:bisacylamide ratio = 60:1). The probes used were the 70-bp PolI-Sacl fragment (position −92 to −23 relative to the major transcription initiation site) containing the two 11-bp repeats of the POLD1 promoter or the linker replacement mutants with one or both copies of the repeats replaced by the XhoI linker. For antibody supershift experiments, 1 μg of the anti-Sp1 or anti-Sp3 antibody (Santa
Cruz) was added to the binding reaction described above, and the reaction mixture was incubated overnight at 4°C prior to electrophoresis. DNase I Footprinting Analysis—The POLD1 promoter DNA containing the sequence between −261 and +49 relative to the major transcription initiation site (designated as pGL2-92d) by E. coli DNA polymerase I (44). The end-labeled DNA was digested with XhoI to remove 21-bp DNA from the distal end of the POLD1 promoter. The resulting DNA was labeled with T4 polynucleotide kinase or 32P-labeled with ice-cold 70% ethanol. After washing with PBS containing 1% bovine serum albumin, fixed cells were incubated in propidium iodide (PI) staining solution (10 μg/ml of PI and 0.5 mg/ml of RNase A) at 37°C for 30 min. and then in 4°C in the dark for at least 1.5 h. Flow cytometry analysis was performed on a Coulter EPICS Elite flow cytometer. For detection of cell cycle, cells were stained with 2 μg/ml of propidium iodide for 30 min, and then 4°C in the dark for at least 1.5 h. Flow cytometry analysis was performed on a Coulter EPICS Elite flow cytometer.
268 positively regulates the POLD1 promoter expression, while there is a negative regulatory sequence located between positions 268 and 258. Further analysis showed that an additional deletion to position 248 resulted in a similar level of promoter activity as that of the 258 deletion (Fig. 2A). In contrast, deletion to position 240 led to a large reduction (7%) of the promoter activity and deletion to position 222 decreased the promoter activity to about 5% of that produced by pGL2-d(292). These results suggest the existence of another positive regulatory sequence located around position 248 to 240. Interestingly, examination of the sequences within the two positive regulatory regions revealed the presence of two identical 11-bp sequences (5'TGGCGTGGCC-3', located at position 281 to 271 and position 249 to 239), arranged in the same orientation in each region (Fig. 2A).

To test if the two 11-bp repeats are essential for POLD1 promoter activity, several linker replacement mutations, with an XhoI linker replacing part of the 11-bp repeat sequence in pGL2-d(292), were constructed and tested (Fig. 2B). As compared to the promoter activity expressed from pGL2-d(292), mutants with a linker replacing either the left (d(292)M2R1–1) or right half (d(292)M2R1–2) of the 5’ copy of the 11-bp repeat sequence, reduced the promoter activity by about 2-fold. Similarly, a mutant with a linker replacing the middle portion of the 3’ copy of the 11-bp repeat (d(292)R2M1) also reduced the promoter activity by about 2-fold. Interestingly, mutations in both copies of the 11-bp repeats resulted in drastic reduction of the promoter activity by 10–12-fold (d(292)M2M1–1 and d(292)M2M1–2 in Fig. 2B). Additionally, the promoter activities of these double replacement mutants were similar to that of the deletion mutant without both repeats (compared to pGL2-d(292) in Fig. 2A). All of these results indicate that the two 11-bp repeats are important transcriptional regulatory elements for the POLD1 promoter.

The POLD1 11-bp Direct Repeats Can Enhance Promoter Activity in an Orientation-independent Manner—The role of the two 11-bp direct repeats as a positive regulatory sequence was further investigated by placing them upstream of a heterologous promoter. A 70-bp POLD1 DNA containing the two repeats was inserted in both orientations into the plasmid containing the luciferase reporter gene controlled by the HSVtk basal promoter (Fig. 3). The resulting plasmids were transfected into HeLa cells and tested for their promoter activities. As expected, the HSVtk basal promoter expressed relatively low activity since it contained only the TATA sequence and 60-bp upstream DNA (compare pGL2-HSVtkbasal to pGL2-d(292) in Fig. 3). Insertion of the two 11-bp repeats in the sense orientation upstream of the HSVtk basal promoter resulted in stimulation of the expression by about 16-fold (compare pGL2-d(292)M2R1-HSVtkbasal to pGL2-d(292) in Fig. 3). Insertion of the two repeats in the antisense orientation upstream of the HSVtk basal promoter also stimulated the
expression by about 7-fold (compared pGL2-dR1R2-HSVtkbasal to pGL2-HSVtkbasal). These results indicate that the two 11-bp direct repeats can function as an activator on a heterologous promoter in an orientation-independent manner. Consistently, the 70-bp POLD1 DNA containing the two direct repeats also stimulated the POLD1 promoter activity when inserted in the antisense orientation (compare pGL2-δ(92)R1R2 to pGL2-δ(22)). However, it should be noted that the two 11-bp direct repeats, when placed in the original orientation, appeared to activate the POLD1 or HSVtk basal promoter to a higher extent than when inserted in the opposite orientation (Fig. 3).

Specific Nuclear Proteins Bind to the POLD1 Promoter DNA—To localize the protein binding sites on the POLD1 promoter DNA, DNase I footprint analysis was performed using...
ing crude HeLa nuclear extracts and a 310-bp POLD1 DNA (nucleotide position −261 to +49) as the substrate (Fig. 4). When the sense strand of the POLD1 DNA was examined, the nuclear extracts protected three regions (Fig. 4A). Protected region I extended from position −117 to −97. Analysis of the sequence in this region revealed the presence of a putative Sp1-binding site. Protected region II extended from position −85 to −66 and protected region III extended from position −52 to −34. Remarkably, the 11-bp activator sequence is located at the center of the protected region II and III (designated R2 and R1; see Fig. 4A). Analysis of the antisense strand of the POLD1 DNA demonstrated protection of the same three regions (Fig. 4B). These results indicate that the specific nuclear proteins bind to the POLD1 promoter DNA, including the two 11-bp repeats.

To corroborate the results of the DNase I footprint analysis and to examine the specificity of the binding proteins, electrophoretic mobility shift assays were carried out using HeLa nuclear extracts and the 70-bp POLD1 DNA (position −92 to −23, designated R2R1), containing the two 11-bp repeat sequences (Fig. 4A). When the upstream Sp1 site detected by the DNase I footprint analysis from pGL2-β (−82 to −92), as the probe (Fig. 5). With low amounts of nuclear extracts, one major (designated C1) and one minor (designated C1v) protein-DNA complexes were observed (Fig. 5A, lanes 2 and 3). With increasing amounts of nuclear extracts, four complexes, designated C1–C4, were identified. With excess amount (12 µg) of nuclear extracts, the C4 complex became the predominant species, while the C1 complex was the minor species (lane 4). The specificity of the protein-DNA interaction was indicated by the disappearance of the shifted bands in the presence of a 50-fold molar excess of unlabelled R2R1 DNA (lane 6). In contrast, incubation with a 50-fold excess of a DNA fragment containing the POLD1 initiator region (position −22 to +49, designated DI) did not result in any decrease of the intensity of the shifted species (lane 7).

To further examine the characteristics of the four complexes, the 70-bp DNA fragments containing one or two mutated copies of the 11-bp repeat from the linker replacement mutants (d4d−92)R2R1−2, d4d−92)R2M1, and d4d−92)M2M1−2 in Fig. 2B) were used in the EMSA. When the M2R1 DNA with the linker-replacement mutation in the 5’ copy of the 11-bp repeat sequence was used as the probe, the C1 complex was detected as the major species even when excess amounts of HeLa nuclear extracts were used (Fig. 5A, lanes 8–11). Similarly, when the R2M1 DNA with a mutation in the 3’ copy of the 11-bp repeat sequence was used as the probe, the C1 complex remained as the major species, while some slow-migrating complexes could also be detected when excess amounts of nuclear extracts were used (lanes 12–15). These results suggest that the C1 complex was formed only when one copy of either repeat was occupied by the protein. In contrast, no protein-DNA complex was detected when the M2M1 DNA containing mutations in both copies of the repeat sequences was used (lanes 16–19). All of these results indicate that the 11-bp repeats in the POLD1 promoter are bound by specific nuclear proteins.

Since the left half of the 11-bp repeat sequence (5’-GCGCCGCTGCCCC-3’) resembles the consensus Sp1-binding site (5’-GCGCCG-3’; Refs. 92 and 96), we also conducted an antibody supershift experiment to determine if the specific protein-DNA complexes detected in EMSA contained the Sp1 protein. By adjusting the amount of HeLa nuclear extracts and the R2R1 DNA as the probe, four protein-DNA complexes similar to those observed above (Fig. 5A) were detected (Fig. 5B). Addition of an anti-Sp1 antibody resulted in supershifting of most, but not all, of these complexes. When either M2R1 or R2M1 DNA was used as the probe, addition of the anti-Sp1 antibody also led to supershifting of most of the C1 complex (Fig. 5B). The addition of an anti-E2F antibody as a control (50) had no effect on the complexes (data not shown).
Transcription Analysis of the Human POLD1 Gene

A Doublet of the 85-kDa Nuclear Proteins Can Also Bind to the Two 11-bp Repeats—To further characterize the 11-bp repeat-binding proteins, Southwestern blot analysis of HeLa nuclear extracts was carried out using the same 70-bp R2R1 DNA containing the two repeats as the probe (Fig. 6). In addition, a double-stranded 22-bp oligonucleotide with an Sp1 site (5'-ATTCGATCG-3'; Ref. 57) was also used as a probe in a comparative analysis. When the R2R1 DNA was used, a doublet of major DNA-binding proteins with molecular masses of around 85 kDa and a minor DNA-binding protein of 105 kDa were detected (Fig. 6A, lane 1). However, when the Sp1 site-containing oligonucleotide was used, a major DNA-binding protein of 105 kDa, which corresponds to the size of the reported Sp1 protein (57), was identified (Fig. 6A, lane 2). Note that this Sp1 protein co-migrated with the 105-kDa band detected by the R2R1 probe (compare lane 2 to lane 1 in Fig. 6A), suggesting that the 105-kDa band is the Sp1 protein. In addition, the Sp1 oligonucleotide also detected a minor band of around 53 kDa in the analysis.

We also conducted a Southwestern blot experiment using the mutant POLD1 DNA probes containing one or two mutated copies of the 11-bp repeat from the linker replacement mutants, like those used in the previous EMSA (Fig. 5). In contrast to R2R1, the M2R1 DNA with the linker-replacement mutation in the 5' copy of the 11-bp repeat sequence or the R2M1 DNA with the mutation in the 3' copy only detected the 105-kDa protein (Fig. 6B). In addition, the intensity of this 105-kDa band detected by these single-replacement mutant DNAs was much stronger than that detected by the wild-type R2R1 DNA. When the M2M1 DNA containing mutations in both copies of the repeats was used, only the 105-kDa band with reduced intensity, was observed (Fig. 6B). These results suggest that the binding of the 85-kDa proteins requires both copies of the 11-bp repeat sequences.

Because Southwestern blot employs a denaturation step, some DNA-binding proteins sensitive to this treatment might not be detected in this analysis. To exclude this possibility and to confirm the results obtained from the Southwestern blot analysis, UV cross-linking experiments were conducted. The same 32P-labeled R2R1 DNA containing the two 11-bp repeats was mixed with HeLa nuclear extracts and binding was allowed to reach equilibrium. The mixture was then subjected to UV irradiation for various time and the cross-linked material analyzed by electrophoresis. A doublet of major bands migrating at about 110 kDa and a minor band with slightly higher molecular mass were detected (Fig. 7). The pattern of these bands is similar to that observed in the Southwestern blot analysis (Fig. 6), albeit with slower mobilities due to the presence of the probe DNA in the cross-linked complexes. A band with molecular mass of around 64 kDa was also seen. The intensity of all of these bands increased linearly with increasing time of irradiation, consistent with a simple interaction between the activated DNA and closely associated proteins. In addition, production of all of these bands could be inhibited by competition with the unlabeled homologous DNA (data not shown). Taken together, these results indicate that in addition to Sp1, the 85-kDa proteins in HeLa nuclear extracts can bind specifically to the 11-bp repeat sequences of the POLD1 promoter DNA.

Sp1 and Sp3, but Not Sp2, Can Activate the POLD1 Promoter—To identify the gene encoding the protein that binds to the 11-bp repeat sequences, we screened HeLa cDNA expression libraries with a concatenated 70-bp R2R1 DNA, which contains the two 11-bp repeats as the probe. One positive clone containing a 2.1-kb cDNA insert was obtained, and sequence analysis showed that the cDNA sequence is identical to that of Sp3, a member of the Sp1 family (33, 35). The sequence of the 2.1-kb cDNA covers almost the entire Sp3 ORF (33, 35), starting at position +46, relative to the first nucleotide of the published Sp3 cDNA sequence designated as +1 (33), and ending at +2109, 15 bp downstream of the translation termination codon.

To test if the Sp1-family proteins can activate the POLD1 promoter, expression plasmids for Sp1, Sp2 (35), and Sp3 were...
When the useful in studying the function of the Sp1-family proteins (34, 47, 58). When the useful in studying the function of the Sp1-family proteins (34, 47, 58). When the useful in studying the function of the Sp1-family proteins (34, 47, 58).

SL2 cells, which lack endogenous Sp factors and have been peats (Fig. 8)

p85s oligonucleotide (labeled as lane 2)

The positions of the Sp1 protein, the 85-kDa binding proteins (p85s), and molecular size markers (Life Technologies) are marked. B, binding of the 85-kDa proteins requires both copies of the 11-bp repeats. R2R1 or its mutant derivatives, M2R1, R2M1 or M2M1, was used as the probe. The positions of the molecular size markers are indicated.

Deletion of a doublet of 85-kDa proteins and a 105-kDa protein that interact with the 11-bp repeats (Fig. 8 B) drastically reduced the ability of Sp1 or Sp3 to stimu-

FIG. 6. Identification of the proteins that interact with the POLD1 11-bp repeats by Southwestern blot analysis. (A) Detection of a doublet of 85-kDa proteins and a 105-kDa protein that interact with the 11-bp repeats. HeLa nuclear extract were resolved by SDS-PAGE and then transferred to a PVDF-Plus membrane. One half of the blot was probed with the 70-bp POLD1 DNA containing the two repeats (R2R1, labeled as lane 1), and the other half was probed with an Sp1 oligonucleotide (labeled as lane 2). The positions of the Sp1 protein, the 85-kDa binding proteins (p85s), and molecular size markers (Life Technologies) are marked. B, binding of the 85-kDa proteins requires both copies of the 11-bp repeats. R2R1 or its mutant derivatives, M2R1, R2M1 or M2M1, was used as the probe. The positions of the molecular size markers are indicated.

FIG. 7. UV cross-linking of HeLa nuclear extracts to the POLD1 DNA containing the 11-bp repeats. The 70-bp R2R1 DNA probe containing the two repeats was incubated with nuclear extracts and subjected to UV irradiation for various times as indicated. The positions of a doublet of major bands migrating at about 110 kDa and a minor band with slightly higher molecular mass are identified with arrows. Also, the positions of the molecular size markers are indicated.

constructed (Fig. 8A) and used in cotransfection of Drosophila SL2 cells, which lack endogenous Sp factors and have been useful in studying the function of the Sp1-family proteins (34, 47, 58). When the POLD1-luciferase construct pGL2-ð−92) containing the two 11-bp repeats was cotransfected with increasing amounts of each Sp1-family expression vector, both Sp1 and Sp3, but not Sp2, stimulated the POLD1 promoter activity (Fig. 8B). Maximal stimulation was detected when 5 μg of the Sp1- or Sp3-expression plasmid was used. To determine if the stimulation of the POLD1 promoter activity by Sp1 and Sp3 was mediated through the two 11-bp repeats, the POLD1-luciferase plasmid with (pGL2-ð−92) or without (pGL2-ð−22)) the two repeats was cotransfected with 5 μg of each Sp1-family expression plasmid into SL2 cells. In addition, the pGL2-Basic plasmid was used as the vector control. As expected, only the Sp1- and Sp3-expression plasmid stimulated the promoter activity of pGL2-ð−92) containing the two repeats (Fig. 8C). Deletion of the two 11-bp repeats (pGL2-ð−22)) drastically reduced the ability of Sp1 or Sp3 to stimu-

late the POLD1 promoter activity. Intriguingly, both Sp1 and Sp3 also stimulated the luciferase activity expressed by the control pGL2-Basic plasmid (Fig. 8C), suggesting that this reporter plasmid contains cryptic promoter elements responsive to Sp1 and Sp3 stimulation in Drosophila SL2 cells. Nonetheless, by comparing to the level of stimulation for each reporter construct, the luciferase activity of the pGL2-ð−22) plasmid without the two 11-bp repeats was stimulated to the extent similar to that of the pGL2-Basic plasmid by Sp1 or Sp3, while in the presence of the two repeats (pGL2-ð−92)) a much larger stimulation of the luciferase activity was observed (Fig. 8C). It should be pointed out that all three Sp1-family proteins were expressed at the similar level in transfected SL2 cells (Fig. 5D). Taken together, these results indicate that Sp1 and Sp3, but not Sp2, can activate the POLD1 promoter through the two 11-bp repeats.

To examine if the stimulation of the POLD1 promoter activity by Sp1 and Sp3 correlates with their ability to bind to the 11-bp repeat sequence, nuclear extracts from transfected SL2 cells expressing each Sp1-family protein were tested for the binding ability to the POLD1 R2R1 DNA. As compared with the protein-DNA complexes detected in HeLa extracts (Fig. 9A, lanes 2 and 3), extracts from SL2 cells transfected with the control pPacU vector gave rise to two fast-migrating complexes with relatively low intensity (Fig. 9A, lane 4), representing some endogenous binding activities from the Drosophila cells. On the other hand, extracts from Sp1- or Sp3-expressing SL2 cells produced multiple slow-migrating complexes, which migrated to similar positions as those seen in HeLa extracts (Fig. 9A, lanes 5 and 6). In contrast, extracts from Sp2-expressing SL2 cells did not give rise to any slow-migrating complexes and only showed endogenous binding activities (Fig. 9A, lane 7).

Two additional experiments were carried out to confirm that the slow-migrating complexes are resulted from the binding of the POLD1 R2R1 DNA by Sp1 or Sp3 expressed in transfected SL2 cells. First, an antibody supershift experiment using the
anti-Sp1 or anti-Sp3 antibody was conducted. Similar to those observed in HeLa extracts (Fig. 5A), multiple protein-DNA complexes were detected with increasing amounts of extracts prepared from Sp1- or Sp3-expressing SL2 cells (Fig. 9B). Addition of the anti-Sp1 antibody to the binding reaction containing Sp1-expressing SL2 extracts led to supershift all of the slow-migrating complexes to form higher-ordered complexes (compare lane 4 with lane 5 in Fig. 9B). Similarly, addition of the anti-Sp3 antibody to the binding reaction containing Sp3-expressing SL2 extracts also led to supershift all of the slow-migrating complexes to form higher-ordered complexes (compare lane 8 with lane 10 in Fig. 9B). Second, a Southwestern blot using the R2R1 DNA as the probe was also conducted. When extracts from Sp1-expressing SL2 cells were used, a 75-kDa DNA-binding protein was detected (Fig. 9C), consistent with the calculated molecular mass from the Sp1 ORF in the expression plasmid. Note that the molecular mass of this SL2 cell-expressed Sp1 is smaller than that of the reported Sp1

**Fig. 8. Trans-activation of the POLD1 promoter by the Sp1-family proteins.** A, schematic diagram of the expression plasmids for Sp1, Sp2, and Sp3. The cDNA of Sp1, Sp2, or Sp3 was inserted into the pPacU vector containing the Drosophila actin 5C promoter, the Ubx 5’ UTR (leader), and the actin 5C poly(A) signal sequence (47). B, activation of the POLD1 promoter by Sp1 and Sp3, but not Sp2. The POLD1-luciferase plasmid pGL2-8 (−92), which contains the two 11-bp repeats but not the upstream Sp1 site, were cotransfected with various amounts of the expression plasmid for Sp1 (○), Sp2 (○), or Sp3 (△) into Drosophila SL2 cells. The pPacU vector was also used as a background control. The level of activation of the POLD1 promoter by each Sp1-family protein is calculated as the luciferase activity expressed from cells cotransfected with pGL2-8 (−92) and the Sp1-family expression plasmid divided by that from cells transfected with pGL2-8 (−92) and pPacU. C, the POLD1 11-bp repeats are required for the activation by the Sp1-family proteins. The POLD1-luciferase plasmid pGL2-8 (−22) containing the two repeats (represented as the solid bar), the pGL2-8 (−22) plasmid without the two repeats (represented as the shaded bar), or the control pGL2-Basic plasmid (represented as the open bar) was cotransfected with each Sp1-family expression vector into SL2 cells, and the luciferase activity was measured as before. D, expression of the Sp1-family proteins in Drosophila SL2 cells. Western blot analysis for detecting the Sp1, Sp2, or Sp3 protein was conducted as described under “Materials and Methods.” Extracts from cells transfected with the pPacU vector were stained with a mixture of antibodies against Sp1, Sp2, and Sp3, serving as a background control. The positions of Sp1 (●), Sp2 (○), and Sp3 (△) relative to the molecular size markers are indicated.
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Fig. 9. Sp1 and Sp3, but not Sp2, can bind to the 11-bp repeat sequences. A, binding of the Sp1-family protein to the 11-bp repeats of the POLD1 DNA. EMSA was conducted as described previously using the POLD1 R2R1 probe and the extracts from untransfected HeLa cells or SL2 cells transfected with each Sp1-family expression plasmid or the pPacU vector. B, antibody supershift experiments confirmed the binding specificity of Sp1 or Sp3 to the R2R1 DNA. Note that the anti-Sp1 antibody could supershift the Sp1 complexes but not the Sp3 complexes. The anti-Sp3 antibody could supershift the Sp3 complexes, but gave rise to a smear pattern when added to the Sp1 complexes. C, Southwestern blot analysis using extracts from transfected SL2 cells detected the binding of Sp1 or Sp3 to the R2R1 DNA. The positions of Sp1 (○), Sp3 (▲), and an endogenous binding protein of SL2 cells (*) are marked.

Protein, presumably due to N-terminal 82-amino acid truncation of the Sp1 ORF in the expression plasmid (47). Similarly, when extracts from Sp3-expressing SL2 cells were used, a 90-kDa DNA-binding protein was detected (Fig. 9C), consistent with the calculated molecular mass from the Sp3 ORF (33, 35) in the expression plasmid. In addition, the molecular mass of the Sp1 or Sp3 protein detected here is identical to that observed in the Western blot analysis (Fig. 8D). Intriguingly, although both Sp1 and Sp3 bind well to the R2R1 DNA in EMSA (Fig. 9, A and B), the intensity of the Sp3 band detected in this Western blot is much weaker than that of Sp1 (Fig. 9C). In contrast, when extracts from SL2 cells transfected with the pPacSp2 or the control pPacU plasmid were used, only an endogenous 50-kDa protein with weak intensity was detected (Fig. 9C). From all of these analyses, we conclude that Sp1 and Sp3, but not Sp2, can bind to the 11-bp repeat-containing R2R1 DNA.

The POLD1 Promoter Is Regulated during the Cell Cycle—Yang et al. (11) previously showed that the human POLD1 mRNA level is increased 10-fold in serum-stimulated IMR90 cells. To test if the POLD1 promoter can respond to serum stimulation, the POLD1-luciferase plasmid pGL2-δ(−1758) containing the 1.8-kb POLD1 promoter was transfected into C3H10T1/2 cells. After transfection, cells were growth-arrested and then restimulated into the cell cycle with 10% serum. The stimulated cells were harvested for reporter enzyme assays at various time points after serum addition. As shown in Fig. 10A, the 1.8-kb POLD1 promoter was induced during the cell cycle and the increase in the POLD1 promoter activity began at 8–12 h after serum stimulation and reached to the highest level (about 4-fold) at 24–28 h, corresponding to the late G1/S phase as indicated by flow cytometry analysis (Fig. 10B).

To define the cis-acting elements responsible for serum stimulation of the POLD1 promoter during the cell cycle, several promoter deletion mutant derivatives of pGL2-δ(−1758) were studied. Deletion of the 5’ upstream sequence between nucleotide positions −1758 and −92 appeared to have little or no effect on serum stimulation of the POLD1 promoter activity. Further deletion to position −22 (pGL2-δ(−22)), which removes the two 11-bp repeats, reduced but still showed some response to serum stimulation (about 2-fold; Fig. 10B). These results indicate that the sequence between position −92 and −22 containing the two repeats is important for serum stimulation of the POLD1 promoter. To confirm the role of the two 11-bp repeats in serum response, linker-replacement mutations in both copies of the repeats were introduced into pGL2-δ(−1758) (Fig. 11A). The resulting pGL2-δ(−1758/M2M1 mutant was studied for the serum response as described before. Similar to the deletion mutant pGL2-δ(−22) without the two repeats, pGL2-δ(−1758/M2M1) also showed reduced response to serum stimulation (Fig. 11B).

Since the POLD1 promoter without the two repeats (pGL2-δ(−22)) still gave rise to some response to serum stimulation (Fig. 10B), we examined if there was any additional element involved in the regulation. Examination of the POLD1 sequence between position −22 and +49 in pGL2-δ(−22) revealed two sequences that resemble the binding site for the transcription factor E2F, known to be involved in cell-cycle regulation of many S-phase genes (21, 59, 60). One such sequence is located adjacent to the major transcription initiation site at position −13 to −6 with the sequence of 5’-TTG CGCGC-3’ (one nucleotide (underlined) differs from the consensus sequence of the E2F-binding site E2F, known to be involved in cell-cycle regulation of many S-phase genes (21, 59, 60). One such sequence is located adjacent to the major transcription initiation site at position −13 to −6 with the sequence of 5’-TTG CGCGC-3’, where S = G(C). The other is located in the 5’ UTR at position +37 to +30 with the sequence of 5’-TTTCCGC-3’, which is arranged in the antisense orientation and completely matches with the consensus sequence of the E2F-binding site (Fig. 11B). To examine if these two sequences are important for serum
stimulation of the POLD1 promoter, mutation in each sequence was introduced into pGL2-β (−1758) (Fig. 11A). Mutation of the putative E2F-binding sequence in the leader region (pGL2-β (−1758)Lm) did not affect the response of the POLD1 promoter to serum stimulation, while mutation of the E2F-like sequence at the initiator region (pGL2-β −92) (M1) and pGL2-β −22 (M2) reduced the response to about 2-fold, suggesting that the E2F-like sequence at the initiator region is also important for serum stimulation.

To examine if both the 11-bp repeats and the E2F-like sequence at the initiator region can confer full serum response of the POLD1 promoter, the double mutant pGL2-β (−92)M2M1/Im containing mutations in both of these sequences was constructed (Fig. 11A) and analyzed. As shown in Fig. 11B, this mutant plasmid did not show any response to serum stimulation. Taken together, these results indicate that the human POLD1 promoter is induced during serum stimulation, and both the 11-bp repeats and an E2F-like sequence adjacent the major transcription initiation site are important for the regulation.

**DISCUSSION**

In this paper, we have analyzed the promoter of the gene for the catalytic subunit of human DNA polymerase β (POLD1). We have demonstrated that the core promoter of the POLD1 gene is located within 328-bp DNA upstream from the major transcription initiation site. Multiple elements including two 11-bp direct repeats located between nucleotide position −92 and −22, play an important role in POLD1 promoter activity.

Several lines of evidence indicate that the two 11-bp repeats
function as an activator sequence for the POLD1 promoter. First, deletion of one copy of the two repeats reduced the POLD1 promoter activity by about 2–4-fold, while deletion of both copies nearly abolished the promoter activity. Second, linker-replacement mutations in either one or both copies of the two repeats gave rise to a similar effect on the promoter activity as the deletion mutants. Third, a 70-bp POLD1 DNA containing the two 11-bp repeats can enhance the expression of both the POLD1 and the heterologous HSVtk basal promoters in an orientation-independent manner.

The sequence of the 11-bp repeat (5'-GGGCGTGGCC-3') does not show complete identity with any known transcription factor-binding sites (61–63). However, the left half of the repeat sequence (5'-GGGGCGTGGCC') resembles the consensus sequence of the Sp1-binding site (5'-GGGCGG-3'; 31, 32). Indeed, several experiments indicated that Sp1 can bind to the 11-bp repeat sequence of the POLD1 promoter DNA. Southwestern blot analysis showed that a 105-kDa protein, comigrating with the Sp1 protein, could bind to the repeat sequence. An anti-Sp1 antibody could supershift most of the protein-DNA complexes containing the 11-bp repeats in EMSA. In addition, the Sp1 protein expressed in Drosophila S2 cells could bind to the repeat sequence. Similar to Sp1, another Sp1-family protein, Sp3, could also bind to the 11-bp repeats. A cDNA clone encoding Sp3 was obtained by screening the HeLa cDNA expression library for the sequence-specific DNA-binding protein using the 11-bp repeats as the probe. Also, the Sp3 protein expressed from this cDNA could bind to the repeat sequence. The role of these Sp1-family proteins in regulating the POLD1 promoter activity was demonstrated by the fact that both Sp3 and Sp1 can activate the POLD1 promoter through the two 11-bp repeats. In contrast, Sp2 can not bind to the repeat sequence and as a consequence, has no effect on the POLD1 promoter activity.

In addition to the Sp1-family proteins, a doublet of 85-kDa protein was found to bind to the 11-bp repeats in both the Southwestern blot and UV cross-linking analyses. Consistently, although a linker-replacement mutation in the left half of the 5' copy of the repeat sequence that resembles the Sp1-binding site reduced the POLD1 promoter activity, mutation in the right half produced a similar effect. In addition, deletion of the entire 5' copy of the 11-bp repeat resulted in a greater reduction of the promoter activity, suggesting the requirement of the entire 11-bp repeat sequence and the possible involvement of the 85-kDa proteins for POLD1 promoter regulation. It should be pointed out that these 85-kDa proteins are not degradation products of Sp1, since they were not detected by the Sp1 site-containing oligonucleotide DNA. Additionally, the molecular weights of these proteins are not the same as those of the Sp1-family members (33–35) or the small GT/GC box-binding proteins BTEB1, BTEB2, and EKLF (64–66).

Intriguingly, the 85-kDa binding proteins were the predominant species that bind to the 11-bp repeats in Southwestern blot analysis, while most, but not all, of the protein-DNA complexes detected by EMSA could be supershifted by the anti-Sp1 antibody. Similarly, Sp3 appears to bind well to the 11-bp repeat sequence in EMSA but only binds weakly in Southwestern blot analysis as compared to Sp1, which shows strong binding in both assays. It is possible that Sp1 may have stronger binding affinity to the 11-bp repeat sequence in EMSA, while the conditions in Southwestern blot analysis allow the detection of other subtle DNA-binding proteins such as the...
Transcriptional level. Posttranscriptional regulation has been catalytic subunit of human pol
sequence motif (5'-CACTTG-3') at position 63 to 58 shares similarity to the binding sites of the basic region helix-loop-helix and basic region helix-loop-helix-zipper proteins, defined as the E-box sequence located immediately upstream to the major transcriptional regulation of the human POLD1 gene through this element for its timely expression during the cell cycle. It is possible that Sp1 can bind and activate the POLD1 promoter through the 11-bp repeat sequence, and both the 11-bp repeats and the E2F-like sequence near the initiator region are involved in serum stimulation of the POLD1 promoter activity, we are presently conducting experiments to examine if Sp1 and E2F can cooperatively regulate POLD1 expression. Nevertheless, the present study provides direct evidence for transcriptional regulation of the human POLD1 gene.

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