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The Second Largest Subunit of Mouse DNA Polymerase ε, DPE2, Interacts with SAP18 and Recruits the Sin3 Co-Repressor Protein to DNA[1]

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DNA polymerase ε is essential for cell viability and chromosomal DNA replication in budding yeast. In addition, DNA polymerase ε may be involved in DNA repair and cell-cycle checkpoint control. The enzyme consists of at least four subunits in mammalian cells as well as in yeast. The largest subunit of DNA polymerase ε is responsible for polymerase activity. To date, the functions of the other subunits have remained unknown. With a view to elucidating the functions of the second largest subunit of mouse DNA polymerase ε (DPE2), yeast two-hybrid screening was performed to identify mouse proteins that interact with this subunit. SAP18, a polypeptide associated with co-repressor protein Sin3, was identified as an interacting protein. A part of the N-terminal region of DPE2 (comprising amino acids 85–250) was found to be responsible for the interaction with SAP18. The interaction induced repression of transcription in reporter plasmid assays, which was inhibited by trichostatin A. These results indicate that DPE2 may recruit histone deacetylase (HDAC) to the replication fork to modify the chromatin structure.

Key words: B subunit, DNA polymerase ε, histone deacetylase, SAP18, Sin3.

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More than ten DNA polymerases have been identified in eukaryotic cells (*1, 2*). Three of them are involved in nuclear DNA replication, specifically, DNA polymerases α, δ, and ε (Pol α, δ, and ε) (*3, 4*). Among them, the absence of primase activity and the higher processivity in the case of Pol ε suggest that it is involved in the elongation step of DNA synthesis on the leading and/or lagging strand. Pol ε is essential for cell viability and chromosomal DNA replication in yeast (*5, 6*). However, the catalytic domain of Pol ε is dispensable for cell viability in budding yeast (*7, 8*). Moreover, there is evidence suggesting that Pol ε is involved in DNA repair and cell-cycle checkpoint control in eukaryotic cells (*5*). Therefore, the precise roles of Pol ε in eukaryotic cells remain to be elucidated.

The three replicative DNA polymerases share common subunit structures in that their catalytic subunits are associated with at least one smaller polypeptide, namely, the B subunit (*9*). Genetic evidence indicates that the B subunits of Pol α, δ, and ε are essential for the viability of *Saccharomyces cerevisiae* (*10–13*). Although comparison of the B subunits of the three replicative DNA polymerases from various organisms and DNA polymerases of archaebacteria has revealed the presence of 12 conserved motifs (*9*), none of them correspond to any known functional motif, and no enzymatic activity has been specifically assigned to date. In this study, we searched for proteins that interact with the B subunit of mouse Pol ε (DPE2) in order to clarify the function of this enzyme.

Yeast two-hybrid screening using the Matchmaker LexA Two-hybrid system (Clontech) was performed to search for mouse proteins that interact with DPE2. Prior to the publication of the human and mouse DPE2 sequences (*14, 15*), we also identified a full length mouse DPE2 cDNA in a mouse FM3A cDNA library. The full-length mouse DPE2 cDNA was used as a bait to screen a mouse embryonic fibroblast cDNA library (Clontech) as a prey. Out of 105 positive clones (8.6×10^5 clones were screened), 8 were independently identified as SAP18 (Sin3-associated polypeptide 18) by DNA sequencing of the prey plasmids present in positive clones, and all contained the full coding sequence. SAP18 is a component of the Sin3 complex (*16*), a co-repressor of the Mad-Max heterodimer, and a homolog of yeast global-transcriptional repressor Sin3p (*17, 18*). The Sin3 complex consists of SAP18, SAP30, RbAp48, RbAp46, HDAC1, and HDAC2. The interaction between DPE2 and

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SAP18 was confirmed *in vitro* by performing histidine-tag pull-down assays with TALON resin. DNA constructs expressing hexa-histidine-tagged T7-tagged DPE2 (His-T7-DPE2) or HA-tagged SAP18 (SAP18-HA) from the SRα promoter were transfected into COS-7 cells individually with Lipofectamine Plus reagent (Gibco BRL). After incubation for 48 h, cells were harvested and whole cell extracts were prepared, as described previously (*19*). The cell extracts were mixed and incubated with TALON resin at 4°C for 1 h in the presence of 10 mM imidazole. After washing the resin with buffer containing 10 mM imidazole, proteins were recovered by suspension in Laemmli sample buffer, and then subjected to SDS-PAGE and Western blot analysis with anti-T7 or anti-HA monoclonal antibodies. Consistent with the results of the yeast two-hybrid screening, SAP18-HA was co-precipitated with His-T7-DPE2. The mixing of cell extracts of SAP18-HA-transfected cells with control vector-transfected cell extracts did not result in the precipitation of SAP18-HA with TALON (Fig. 1A). Co-expression of His-T7-DPE2 and SAP18-HA using the IRES-containing vector in COS-7 cells gave the same results (data not shown). The DPE2 domains involved in binding SAP18 were determined by means of TALON pull-down assays (Fig. 1, B and C). We constructed a series of DPE2 deletion mutants using the information on the positions of the conserved regions in DPE2 (*9*). These mutant constructs were transfected into COS-7 cells and then cell extracts were prepared, as described above. Significant variability in the expression and/or solubility of these mutant proteins was observed (see Fig. 1C, for example), so only soluble mutants were used for further experiments. Truncated DPE2 (1–255) and DPE2 (85–527) bound SAP18-HA as efficiently as the full-length protein (Fig. 1C). On the other hand, another DPE2 deletion mutant, DPE2 (177–440), bound SAP18-HA weakly in spite of the large excess of the protein in the assay, and the mutant DPE2 (250–527), that is present in a larger amount in the assay compared to truncated DPE2 (1–255) and DPE2 (85–527), scarcely bound SAP18-HA, suggesting that amino acids 85–250 play an important role in the interaction between DPE2 and SAP18. This region contained conserved regions I–III.

We next examined the biological significance of the interaction between DPE2 and SAP18. Since SAP18 is associated with mammalian transcriptional repressor Sin3, the interaction of DPE2 with SAP18 might be responsible for the recruitment of the histone deacetylase activity of the Sin3 complex at or near the replication fork. To examine this possibility, the effects of DPE2 on transcription repression were examined in a reporter assay. We constructed

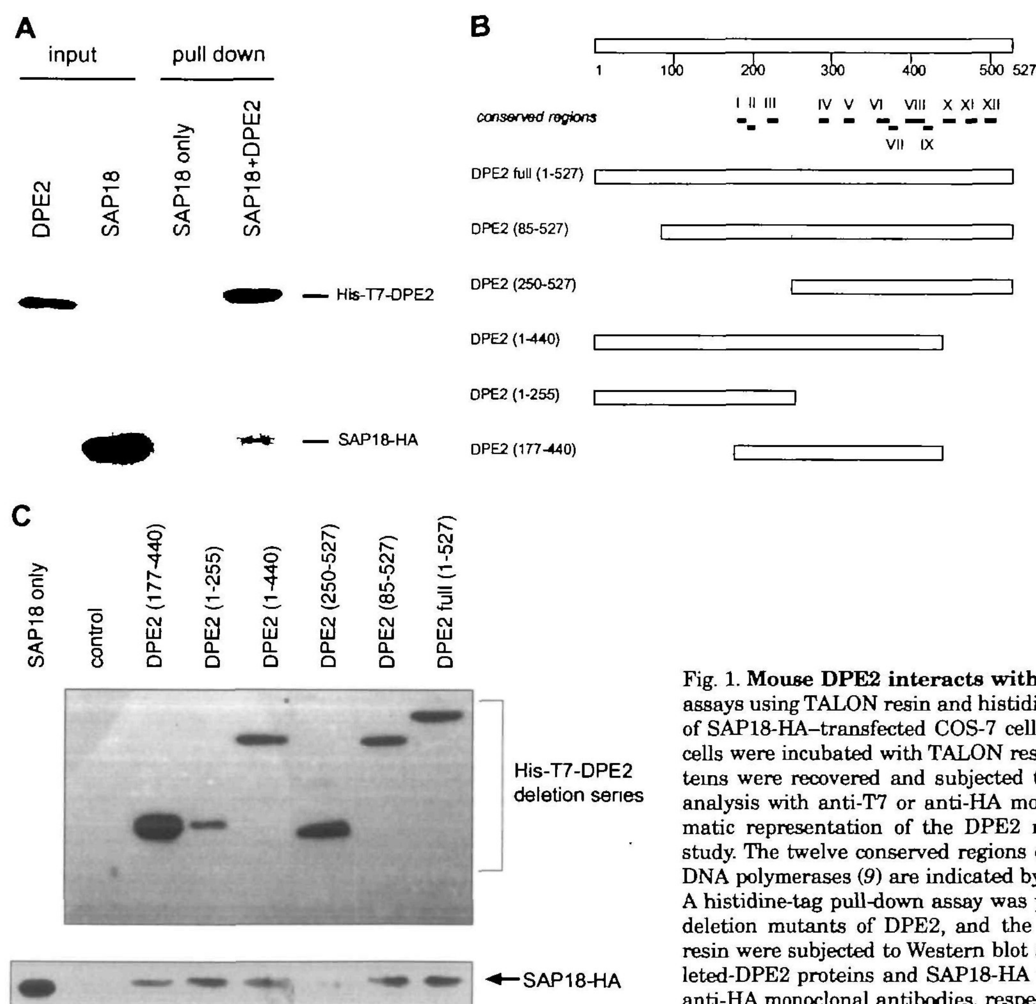

Fig. 1. **Mouse DPE2 interacts with SAP18 *in vitro*.** (A) Pull-down assays using TALON resin and histidine-tagged proteins. Cell extracts of SAP18-HA–transfected COS-7 cells and His-T7-DPE2–transfected cells were incubated with TALON resin. After washing the resin, proteins were recovered and subjected to SDS-PAGE and Western blot analysis with anti-T7 or anti-HA monoclonal antibodies. (B) A schematic representation of the DPE2 mutant constructs used in this study. The twelve conserved regions of the second subunit of class B DNA polymerases (*9*) are indicated by Roman numerals (I to XII). (C) A histidine-tag pull-down assay was performed, using His-T7–tagged deletion mutants of DPE2, and the proteins bound to the TALON resin were subjected to Western blot analysis as described in (A). Deleted-DPE2 proteins and SAP18-HA were detected with anti-T7 and anti-HA monoclonal antibodies, respectively.

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luciferase reporter plasmids with or without five Gal4 DNA-binding sites located upstream of the thymidine kinase (TK) promoter (Fig. 2A) (*20*). SAP18 and DPE2 were fused to the GAL4 DNA-binding domain in the pM vector (Clontech), the resulting vectors being designated as pM-SAP18 and pM-DPE2, respectively. Another expression vector, pIRES (dbdDPE2+SAP18), was constructed, in which DPE2 was fused to the GAL4 DNA-binding domain (dbdDPE2), and SAP18 was cloned into the same pSRα-IRES vector (*21, 22*). The vectors were co-transfected into NIH3T3 cells with the reporter plasmid to examine the effect of the interaction between DPE2 and SAP18 on transcription. As shown in Fig. 2B, targeting of DPE2 to the promoter led to repression of transcription (pM-DPE2). Targeting of SAP18 to the promoter also repressed transcription (pM-SAP18), but the effect was weaker than with pM-DPE2. The effect of SAP18 on transcription is consistent with the published data (*16*). When DPE2 containing the GAL4 DNA-binding domain and SAP18 were co-expressed from the pIRES (dbdDPE2+ SAP18) plasmid, stronger repression was observed than that with pM-DPE2 or pM-SAP18 alone. Transformation of vectors containing SAP18 without the GAL4 DNA-binding domain had a minimal effect on repression. These results indicate that DPE2 and SAP18 co-operatively repress transcription.

To determine whether or not the transcriptional repression was due to the histone deacetylase activity associated with Sin3, cells were treated with trichostatin A (TSA), an inhibitor of this activity (*23*). As shown in Fig. 2C, TSA inhibited the transcriptional repression caused by pM-DPE2 and pM-SAP18. However, TSA inhibition was the strongest, corresponding to twofold greater luciferase activity compared to in the control without TSA, when the pIRES (dbdDPB2+SAP18) vector was employed. Although the repression caused by pM-DPE2 was not fully inhibited by TSA, it was demonstrated that some part of this repression is dependent on HDAC activity. These results suggest that DPE2 alone can recruit HDAC activity to DNA, and that SAP18 enhances the recruitment of HDAC by interacting with Sin3, resulting in transcriptional repression.

In eukaryotic cells, newly-synthesized DNA must be rapidly assembled into the proper chromatin configuration to foster or inhibit transcription. Acetylation of histone neutralizes the positive charge of the histone tail, resulting in relaxation of the chromatin structure, and therefore allows for easier interactions between DNA and proteins (*24*). Conversely, deacetylation of histone represses transcription through the reverse reaction (*25, 26*). Recently, a novel histone acetylase that binds to the origin recognition complex (ORC) was identified and designated as HBO1 (histone acetylase binding to ORC) (*27*). Our present study shows for the first time that there is a physical and functional

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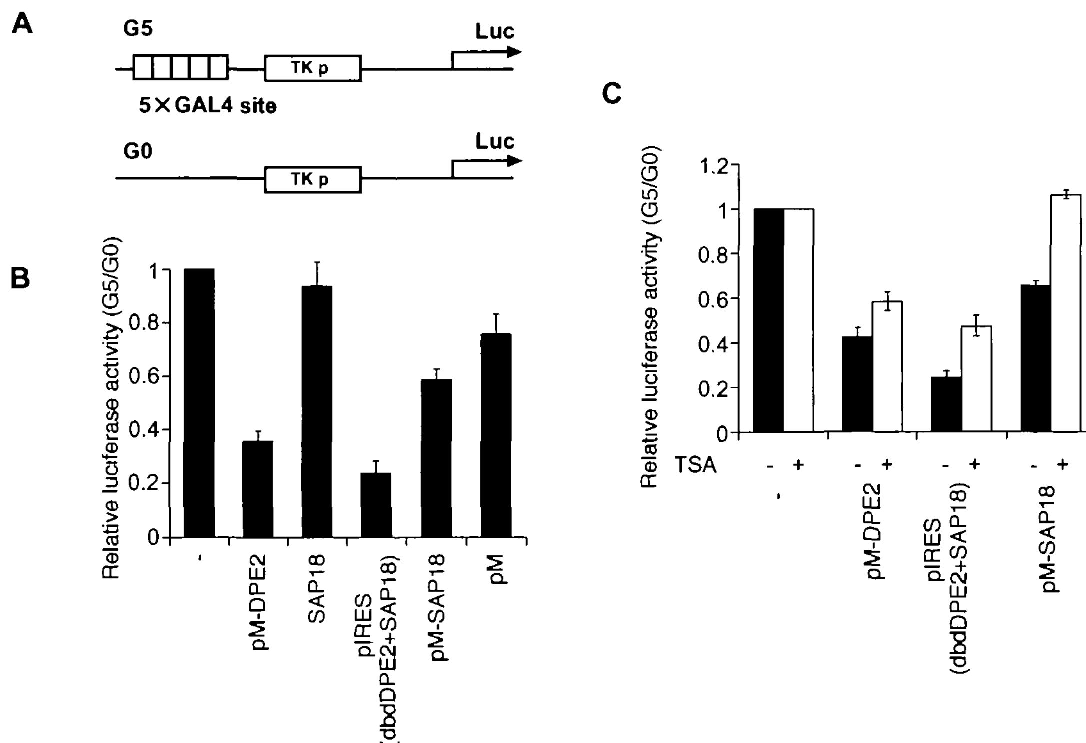

Fig. 2. **DPE2 functionally interacts with SAP18 *in vivo*.** (A) A schematic representation of the luciferase reporter plasmids used in this study. The reporter plasmid contains five (G5) or no (G0) Gal4-binding sites upstream of the thymidine kinase promoter. (B) NIH3T3 cells were transfected with a luciferase reporter plasmid and effective vectors. The effective vectors encoding DPE2 and SAP18 fused to the GAL4-DNA binding domain in the pM vector were designated as pM-DPE2 or pM-SAP18, respectively. Another vector, pIRES (dbdDPE2+SAP18), encoded DPE2 fused to the GAL4 DNA-binding domain (dbdDPE2) and non-tagged SAP18 containing the IRES sequence. Forty-eight hours after transfection, cell extracts were prepared and then luciferase activity was measured. Transfection efficiencies were normalized, using the β-galactosidase assay. Luciferase activity was expressed as the ratio of G5 and G0, and error bars represent standard deviation. Luciferase activity in cells without effective vectors was set at 1. (C) Inhibition of transcription repression by TSA. Forty hours after transfection, cells were treated with 100 nM TSA or mock-treated with an equal amount of ethanol for 8 h.

interaction between the subunit of a replicative DNA polymerase and an HDAC complex, Sin3. Thus, it seems likely that HBO1 may function before, while Sin3 may work after DNA replication.

Recently, the third and fourth subunits of human Pol ε were identified, and both subunits contain histone fold motifs (*28*) which are similar to CCAAT binding factors (CBF). The histone fold motifs of the CBF contribute to the interaction between CBF and histone acetyltransferase (*29*). It is possible that Pol ε containing DPE2 and these histone fold motif subunits are involved in regulation of the histone acetylation status.

Another mechanism involved in the overall regulation of gene expression or transcriptional silencing is DNA methylation (*30*). Heterochromatic regions (including pericentromeric and telomeric repeats), inactive X chromosome and transcriptionally silent alleles of selected imprinted genes are associated with a lack of transcriptional activity, heavy CpG methylation and compact chromatin with hypoacetylated histones. They also tend to replicate in the late S phase. Hypermethylated regions of DNA have to be remethylated as soon as possible after DNA replication to maintain the proper pattern of gene expression, coupled with histone deacetylation on chromatin. Recently, it was found that the key enzyme that maintains mammalian DNA methylation, DNMT1, binds HDAC2 and a novel corepressor, DMAP1 (DNMT1-associated protein), to mediate transcriptional repression (*20*). DMAP1 is targeted to replication foci throughout the S phase by DNMT1, whereas HDAC2 joins DNMT1 and DMAP1 only during the late S phase, providing a platform for the deacetylation of histones in heterochromatin after replication (*20*). Thus, eukaryotic cells apparently possess multiple mechanisms that ensure the re-establishment of the proper chromatin status after each round of DNA replication.

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