Deoxycytidyl Transferase Activity of the Human REV1 Protein Is Closely Associated with the Conserved Polymerase Domain*

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The REV1 protein is a member of the growing family of translesion DNA polymerases. A cDNA of the human REV1 gene that we had originally isolated encoded 1250 amino acids residues, which was one amino acid shorter than previously reported ones. The shorter form of REV1 was named REV1S. All individuals examined expressed equivalent amounts of REV1S and REV1 mRNA, suggesting that the REV1S mRNA is a splicing variant. We show that the REV1S protein also possesses deoxycytidyl transferase activity that inserts a dCMP opposite a DNA template apurinic/apyrimidinic site. Deletion and point mutation analysis of the REV1S protein revealed that the domain required for deoxycytidyl transferase and DNA binding activities of the REV1S protein are located in a conserved domain of the translesion DNA polymerases. This result indicates that the structure of the catalytic site of the deoxycytidyl transferase closely resembles that of the translesion DNA polymerases. Therefore, the molecular mechanism of the dCMP transfer reaction of the REV1S protein and maybe also the REV1 protein might be the same as that of the dNTP transfer reaction of the translesion DNA polymerases.

In yeast *Saccharomyces cerevisiae*, almost all induced mutations arise during translesion replication, a process that promotes elongation past sites of unrepairable lesions (1). The mutagenesis pathway (rev) mutants were initially isolated by their reduced mutations after UV treatment (2). The REV1, REV3, and REV7 genes are required for the major pathway for translesion replication in yeast (3–6). The REV1 gene encodes a deoxycytidyl transferase that incorporates dCMP opposite apurinic/apyrimidinic (AP)3 sites in the template (7). The REV3 and REV7 genes encode a translesion DNA polymerase, pol ζ (8, 9), which works together with the REV1 protein for translesion replication (7). Recently, the human orthologues of REV1 and REV3 genes are required for mutagenesis induced by UV light in humans (11, 12).

The REV1 gene is a member of a growing family including *umuC* (15) and *dinB/dinP* (16) of *Escherichia coli*; *RAD30* (17) of *S. cerevisiae*; and XPV/RAD30A (18, 19), *RAD30B* (20), and *DINB1* (21, 22) of humans. Recent studies have shown that those genes encode translesion DNA polymerases (18, 23–29). However, the REV1 protein does not possess such polymerase activity although it contains the conserved domain in translesion polymerases.

In this work, we made various mutants of the human REV1S protein. Biochemical analysis of those mutant proteins showed that the domain required for deoxycytidyl transferase activity and DNA binding is located in a conserved domain of human translesion DNA polymerases.

**EXPERIMENTAL PROCEDURES**

Isolation of Human REV1 cDNA

We found a partial sequence of a candidate of the human REV1 cDNA in a data base (accession number AJ131720). Based on the sequence, a portion of the cDNA fragment of the human REV1 was amplified by RT-PCR from human breast cancer using primers 5′-AA-TCTACTGGAATGCCTGGA-3′ and 5′-GTAAAACGACCTGAGGCGATGT-3′, and it was used as a probe for screening a human testis cDNA library (CLONTECH). In this library, the cDNA was inserted into a λ phage vector, ADR2 (CLONTECH). Four positive clones were obtained from 5 × 105 plaques. In all of the clones in these phages, the promoter-proximal side of the REV1 cDNA was truncated at an XbaI site, because XbaI had used to construct the cDNA library. One clone, named ARH2, was chosen for further analysis.

To obtain the promoter-proximal side of the REV1 cDNA, another portion of the cDNA fragment was amplified by RT-PCR from human breast cancer using primers 5′-TTTC tgtTTTTTGTCAAGGCTG-3′ and 5′-TTGGTTCTUTGGCGAGATTTC-3′, and it was used as a probe for screening the same library. One positive clone, named ARHT1, was obtained. This phage contained cDNA from the 5′-untranslated region of the XbaI site. Since the assembled cDNA encoded 1250 amino acid residues, 1 amino acid shorter than previously reported ones, we gave the name REV1S cDNA to the shorter form of the REV1 cDNA. The DDBJ/EMBL/GenBankTM accession number of the nucleotide sequence of the human REV1S cDNA is AB047646.

For construction of full-length REV1S cDNA, the BamHI/XbaI fragment of REV1S cDNA from ARH2 was inserted into the corresponding site of pBlueScript II SK (−). The resulting plasmid, pRH2IN, was digested with SacII and XbaI, and the SacII/XbaI fragment of REV1S cDNA from ARHT1 was inserted. The resulting plasmid, pREV1S, contained the fragment of the position 20–4227 of human REV1S cDNA (AB047646).

**Analysis of the REV1 and REV1S mRNA**

Total mRNA from human mononuclear cells was used as a source of RT-PCR (30, 31). First-strand cDNA synthesis was carried out with reverse transcriptase (RAV-2) (TaKaRa) from an oligo(dT) (12–18) primer (Life Technologies, Inc.). To amplify a portion of the human REV1/REV1S cDNA fragment, including the different region, the PCR primers 5′-GGGTCCGCAAGCCGGAGACAT-3′ and 5′-GGCTTGGCCTTGTCCC-3′ were used.

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† The abbreviations used are: AP, apurinic/apyrimidinic; EMSA, electrophoretic mobility shift assay; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR.
CATAACTACA-3' were used. After heating at 95 °C for 2 min, 35 cycles of amplification were performed according to the following conditions: 1 min of denaturation at 95 °C, 1 min of annealing at 58 °C, and 2 min of extension at 72 °C with TaKaRa Extaq™ polymerase (TaKaRa). The amplified 1618-base pair DNA fragment was digested with the primer 5’-GCCGAGAATCTGATTTCCCA-3’ using an ABI 373 DNA sequencing system (Applied Biosystems). To quantitate the ratio of the mRNA species, the amplified 1618-base pair cDNA was diluted and reamplified with Pyrobest™ DNA polymerase (TaKaRa) by PCR using the primers 5’-GCCGAGAATCTGATTTCCCA-3’ and 5’-3P-CCACGCTGAGGTTAGCCATATTACC-3’. The PCR products were separated on a 5% polyacrylamide gel containing 8 M urea. The radioactivity of each band of 96 and 93 bases, respectively, derived from REV1 and REV1S cDNA was measured using a Bio Imaging Analyzer BAS2000 (Fuji Photo Film Co., Ltd.).

Northern Blot Analysis of REV1 mRNA

To introduce site-directed mutations, we used a PCR-based method. DNA fragments of the limited portions were amplified by RCR and cloned. After confirming the nucleotide sequence, the small fragments were assembled with other fragments of the wild-type cDNA. All of the expression constructs are listed in Table I. Proteins were tagged with six histidines at their N termini.

Construction of Mutant REV1S Genes

To introduce a truncated protein containing extra amino acid residues, YR, at the C terminus (Table I).

The resulting plasmid, pET-C729, was inserted into the corresponding site of pBAD22A. The resulting plasmid was named pET-C729. A truncated protein was inserted. The resulting plasmid, pBADRN, was digested with BglII. The resulting plasmid was cloned into the pCR 2.1-TOPO vector (Invitrogen), and the resulting plasmid was named pET-C153 (Table I). The resulting plasmid was digested with BamHI and HindIII, and then the BamHI/HindIII fragment of pREV1S was inserted. The resulting plasmid, pET-C729, produces a truncated protein containing extra amino acid residues, GCRNSISSLSMISCQT, at the N terminus.

Construction of pET-C729—The 5’-portion of the REV1S gene was amplified by PCR with primers (5’-GAAGTCCCTAGATGAGG-3’ and 5’-TGCTTGGACCTGGCAGAGGA-3’) to introduce an Ndel site at the first ATG (position 173 of the cDNA). The PCR fragment was cloned into the pCR 2.1-TOPO vector (Invitrogen), and the resulting plasmid was named pRHFNde2. The PCR fragment was cloned into the pCR 2.1-TOPO vector (Invitrogen), and the resulting plasmid was named pET-C153 (Table I). The resulting plasmid was digested with BamHI and HindIII, and then the BamHI/HindIII fragment of pREV1S was inserted. The resulting plasmid, pET-C729, produces a truncated protein containing extra amino acid residues, YR, at the C terminus (Table I).

Construction of pET-C729—An XbaI/HindIII fragment that includes a ribosome-binding site and the 5’-portion of the REV1S gene of pET-C729 was inserted into the corresponding site of pBAD22A. The resulting plasmid, pBADRN, was digested with HindIII, and then the HindIII fragment of the 3’-portion of the REV1S gene of pREV1S was inserted. The resulting plasmid, pBAD-REV1S, produces a full-length REV1S protein (Table I).

Construction of pET-N245—To introduce an Ndel site at position 905 of the REV1S cDNA, PCR was carried out using primers (5’-TGGTGCAACAGCAGCAGAGGA-3’ and 5’-TACCAGCGCTGCAGCACAAC-3’) to introduce an Ndel site at position 2615 of the REV1S cDNA. The PCR fragment was cloned into the pCR 2.1-TOPO vector. The resulting plasmid was named p2620H-7. The resulting plasmid was digested with BamHI and HindIII, and then the BamHI/HindIII fragment of each of pET-N245 and pET-N407 was inserted into the corresponding site of pET15b (Table I). The expression of the h6-REV1S gene is induced by arabinose (32).

Construction of pET-N407—To introduce an Ndel site at position 1391 of the REV1S cDNA, PCR was carried out using primers (5’-ACACAGGACATATGTCACTAG-3’ and 5’-GATGGATCCGGTCTAGATGCTTTG-3’). The PCR fragment was cloned into the pCR 2.1-TOPO vector. The resulting plasmid, pRHNXN, was digested with ApaI and XbaI, and then the ApaI/XbaI fragment of pRH2IN was inserted. The resulting plasmid was named pRHXXN. The Ndel/BamHI fragment of pRHXXN was inserted into the corresponding site of pET15b. The resulting plasmid, pET-N407, produces a truncated protein starting from the 426th methionine (Table I).

Construction of pET-N407—To introduce an Ndel site at position 1391 of the REV1S cDNA, PCR was carried out using primers (5’-ACACAGGACATATGTCACTAG-3’ and 5’-GATGGATCCGGTCTAGATGCTTTG-3’). The PCR fragment was cloned into the pCR 2.1-TOPO vector. The resulting plasmid was named p2620H-7. The HindIII fragment of p2620H-7 was inserted into the HindIII site of pET-C885. The resulting plasmid produces a truncated protein at codon 810 (Table I).

Purification of the REV1S Protein and Its Derivatives

Five hundred ml of BL21 (DE3) (33) harboring pET-C885, pET-C810, pET-C729, pET-N425/C479, and pET-N407/C885 was grown in SB medium (32) at 15 °C. After heating at 95 °C for 2 min, 35 cycles of amplification were performed according to the following conditions: 1 min of denaturation at 95 °C, 1 min of annealing at 58 °C, and 2 min of extension at 72 °C with TaKaRa Extaq™ polymerase (TaKaRa). The amplified 1618-base pair DNA fragment was digested with the primer 5’-GCCGAGAATCTGATTTCCCA-3’ using an ABI 373 DNA sequencing system (Applied Biosystems). To quantitate the ratio of the mRNA species, the amplified 1618-base pair cDNA was diluted and reamplified with Pyrobest™ DNA polymerase (TaKaRa) by PCR using the primers 5’-GCCGAGAATCTGATTTCCCA-3’ and 5’-3P-CCACGCTGAGGTTAGCCATATTACC-3’. The PCR products were separated on a 5% polyacrylamide gel containing 8 M urea. The radioactivity of each band of 96 and 93 bases, respectively, derived from REV1 and REV1S cDNA was measured using a Bio Imaging Analyzer BAS2000 (Fuji Photo Film Co., Ltd.).
bation was continued for 10 h after the addition of L(+)-arabinose to 1% at an A_{600} value of 0.6. The cells were harvested by centrifugation, resuspended in 3 ml of buffer I (50 mM HEPES-NaOH, pH 7.5, 1 mM NaCl, 0.1 mM EDTA, 10 mM β-mercaptoethanol) per 1.5 g of cells, and frozen in liquid nitrogen. The cells were then treated on ice, added to phenylmethylsulfonyl fluoride to 1 mM, and lysed by adding 100 mM spermidine/lysozyme (4 mg/ml) in buffer I to 10 mM and 0.4 mg/ml, respectively. The cells were incubated on ice for 30 min, heated in a 37 °C water bath for 2 min, and then incubated on ice for 20 min. The cell lysate was clarified by centrifugation at 35,000 × g for 30 min at 4 °C. The following column chromatography was carried out at 4 °C using a SMART system (Amersham Pharmacia Biotech). Two ml of the lysate was applied at 0.1 ml/min to a 1-ml HiTrap chelating column (Amersham Pharmacia Biotech), which had been flushed with 2 ml of 0.1 mM NiSO_4 and equilibrated with buffer A (50 mM HEPES-NaOH, pH 7.5, 1 mM NaCl, 10% glycerol, 10 mM imidazole) containing 10 mM imidazole. The column was washed with 10 ml of equilibration buffer and then washed with 12 ml of buffer A containing 100 mM imidazole. The hREV1S and its derivatives were eluted with buffer A containing 300 mM imidazole. For further purification, 50 μl of the peak fraction was applied at 0.01 ml/min to a Superdex 200 PC 3.2/30 column (Amersham Pharmacia Biotech) equilibrated with buffer A, and 40-μl fractions were collected. Protein concentrations were determined by the Bio-Rad protein assay using bovine serum albumin (Bio-Rad) as the standard.

**(Deoxycytidyl Transferase Assays)**

Deoxycytidyl transferase assays were performed essentially as described by Nelson et al. (7) and Lin et al. (10). For the DNA substrate, a 5′-end ^32_P-labeled oligonucleotide primer 5′-CAGTCTAGCTTTAG-3′ was used for the deoxycytidyl transferase assay. Two ml of the lysate was applied at 0.1 ml/min to a 1-ml HiTrap chelating column (Amersham Pharmacia Biotech), which had been flushed with 2 ml of 0.1 mM NiSO_4 and equilibrated with buffer A (50 mM HEPES-NaOH, pH 7.5, 1 mM NaCl, 10% glycerol, 10 mM β-mercaptoethanol) containing 10 mM imidazole. The column was washed with 10 ml of equilibration buffer and then washed with 12 ml of buffer A containing 100 mM imidazole. The hREV1S and its derivatives were eluted with buffer A containing 300 mM imidazole. For further purification, 50 μl of the peak fraction was applied at 0.01 ml/min to a Superdex 200 PC 3.2/30 column (Amersham Pharmacia Biotech) equilibrated with buffer A, and 40-μl fractions were collected. Protein concentrations were determined by the Bio-Rad protein assay using bovine serum albumin (Bio-Rad) as the standard.

**(DNA Binding Assays by EMSA)**

The following binding method was adapted from the protocols of Masuda et al. (34, 35). As a substrate for binding measurements, the primers for deoxycytidyl transferase assay were used, but it was not treated with uracil-DNA glycosylase. Reaction mixtures (10 μl) contained 25 mM potassium phosphate buffer, pH 7.4, 5 mM MgCl_2, 0.1 mg/ml bovine serum albumin, 10% glycerol, 5 mM dithiothreitol, 0.5 nM substrate, and 1 μl of protein sample. The reaction products were resolved on 20% polyacrylamide gel containing 8M urea and autoradiographed at 80 °C. The amount of DNA present in each band was quantified using a Bio-Imaging Analyzer BAS2000 (Fuji Photo Film Co., Ltd.).

**(RESULTS)**

**(Cloning of the Human REV1S cDNA—By searching a data base, we found a human cDNA that encoded a homologue of the yeast REV1 protein. The cDNA has been isolated by yeast two-hybrid screening as a protein interacting with α-integrin (36). The gene was a good candidate for the human REV1 gene. Because the full-length cDNA was not available, we cloned partial cDNA from a human liver cDNA library to obtain a full-length cDNA. A clone isolated (accession number AB047646) looks like a truncated form, because it was not a full-length cDNA. The cDNA was amplified using PCR primers and separated on a 5% polyacrylamide gel containing 8M urea and autoradiographed at 80 °C. The amount of DNA present in each band was quantified using a Bio-Imaging Analyzer BAS2000 (Fuji Photo Film Co., Ltd.).)**

**(Fig. 1. Amino acid sequence of the REV1S protein. The open triangle indicates the position of the missing alanine residue in the REV1S protein. The underlined sequences show conserved regions in REV1 proteins of different species. The boxes show highly conserved sequences in translesion DNA polymerases. Vertical bars represent positions of truncation in the REV1S protein that were studied in this work. The closed triangle indicates the position of mutation of yeast rev1–1 mutant (15).)**

The human REV1 cDNA encodes an expected protein of 1250 residues, which is different from the recently reported REV1 protein of 1251 residues (Fig. 1). The former protein lacks an alanine residue at position 479 (Fig. 1). We denote here the shorter form of REV1 as REV1S. It was possible that the REV1S mRNA was a minority in the cell. To clarify this point, a portion of the REV1/REV1S cDNA fragments including position 479 was amplified by RT-PCR from mononuclear cells of 10 individuals and directly sequenced. The sequencing signals from all individuals are duplicated, since CTG and the intensity of both signals is a ratio of about 1:1, indicating that both forms exist in all individuals at similar amounts. DNA fragments including the region were amplified using a 5′-end ^32_P-labeled primer as one of the PCR primers and separated on a 5% polyacrylamide gel containing 8M urea and autoradiographed at 80 °C. The amount of DNA present in each band was quantified using a Bio-Imaging Analyzer BAS2000 (Fuji Photo Film Co., Ltd.).
cDNA. The expected nucleotide sequences of REV1 and REV1S are shown. The boxed sequence of the REV1 cDNA is duplicated in the boxed sequence of the REV1 S cDNA. A, direct sequencing of RT-PCR-amplified fragments of REV1 cDNA. The expected nucleotide sequences of REV1 and REV1S are shown. The boxed sequence of the REV1 cDNA is duplicated in the boxed sequence of the REV1 S cDNA. B, quantitation of REV1 and REV1S mRNA of 10 individuals. RT-PCR amplified fragments of REV1 cDNA were separated on 5% polyacrylamide gel containing 8 M urea. The positions of the 93- and 96-base fragments, which are derived from REV1S and REV1 cDNA, respectively, are indicated on the left. The band intensities were measured using a Bio-Imaging Analyzer BAS2000. Ratios of the shorter and longer bands, from 10 individuals was 1.25. We concluded that the REV1S mRNA is one of the major forms.

Expression of the REV1 Gene in Human Tissues—Expression of the human REV1 gene in various human tissues was examined by Northern blot analysis (Fig. 3). The human REV1/REV1S mRNA was detected as a 4.4-kilobase transcript in all tissues examined, indicating that the REV1 gene is ubiquitously expressed. Expression of the REV1 gene was relatively high in the testis and ovary and relatively low in the thymus and small intestine.

Deoxycytidyl Transferase Activity of the REV1S and Mutant Proteins—The human REV1 protein possesses a deoxycytidyl transferase activity (10). First, we examined the deoxycytidyl transferase activity of the REV1S protein. The REV1S protein was tagged with six histidine residues at its N terminus and expressed in E. coli cells. The tagged REV1S protein (h6-REV1S) was purified by affinity chromatography on a nickel chelating column. The purified protein was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 4, lane 1). Full-length h6-REV1S protein with M$_{r}$ 140,000 and lower bands were detected. The lower bands appeared specifically when the REV1S protein was induced (Fig. 4, compare lane 1 with lanes 3–9), indicating that the bands were degraded products of the h6-REV1S protein. This was confirmed by Western blot analysis (data not shown). Using a primed 30-mer DNA template containing an AP site, we assayed the transferase activity of the purified h6-REV1S. Transferase activity that extended the 32P-labeled primer by 1 nucleotide opposite the AP site was detected (Fig. 5B). This evidence proves that the REV1S protein, as well as the REV1 protein, possesses deoxycytidyl transferase activity (10).

Next, we made a series of deletion mutants with six histidine residues at their N termini (Table I, Figs. 1 and 5A) and purified the proteins (Fig. 4, lanes 3–9). As shown in Fig. 5B, the ΔN245 and ΔC885 proteins as well as the full-length h6-REV1S protein were active, indicating that 244- and 365-amino acid residues of the N and C termini, respectively, are dispensable for the transferase activity. Consistently, the ΔN245/ΔC885 protein, which has both truncations of N and C termini, sustained the activity. ΔC810 and ΔC729 proteins, which have further truncation of the C terminus, were inactive. The transferase activity of ΔA407 protein was dramatically decreased, but significant activity was still detected. However, the protein ΔN407/ΔC885, which has both truncations of 406 residues of the N terminus and 365 residues of the C terminus, was inactive.

Johnson et al. (37) introduced a mutation in the highly conserved SIDE sequence in yeast Rad30, in which the aspartate and glutamate residues had each been changed to alanine, and they found that the mutant Rad30 protein lacks DNA polymerase activity. We also changed the corresponding residues to alanines (D569A/E570A) (Table I, Figs. 1 and 5A). The protein was purified like the wild-type h6-REV1S protein (Fig. 4, lane 2). In striking contrast to the transferase activity of the wild-type h6-REV1S protein, the D569A/E570A protein showed no transferase activity (Fig. 5B).

The proteins were further analyzed by gel filtration chromatography (Fig. 6). Interestingly, the apparent molecular weight of each protein was much larger than the calculated molecular weight except for the ΔN407/ΔC885 protein (Table II). The peak of each protein (Fig. 6A) corresponded to the peak of the activity (Fig. 6B), indicating that the activity is intrinsic to
each protein. This result confirmed the former conclusion. In ΔN245 and ΔN407, two peaks of activity were detected (Fig. 6B). The second peaks of activity were co-eluted with C terminus-truncated polypeptides (data not shown). The elution profile of the D569A/E570A protein is identical to that of the wild-type protein (Fig. 5A and Table II), suggesting that the amino acid replacements do not affect the global structure of the REV1S protein. This chromatography successfully separated the degraded fractions. Using the fractions of the leading edges of the chromatography, the specific activities of mutant proteins were quantitatively compared (Table III). The relative edges of the chromatography, the specific activities of mutant proteins were quantitatively compared (Table III). The relative specific activity of ΔN245 was slightly higher than that of the wild type, and those of ΔC885 and ΔN245/ΔC885 proteins were slightly lower than the wild type level. The activity of the ΔN407 protein was about 0.1% of the wild type level (Table III).

**DNA Binding Activity of REV1S and Mutant Proteins**—The DNA binding activities of the peak fractions of gel filtration chromatography were analyzed by EMSA. To detect a protein-DNA complex on the gel, the primed template for the deoxycytidyl transferase assay was used as a substrate for EMSA. However, because an AP site is unstable in gels (38), the uracil nucleotide immediately downstream from the annealed primer was uracil, not an AP site. The uracil template is a good substrate as well as the AP site for deoxycytidyl transferase activity (7, 10). To elucidate the molecular mechanism for the deoxycytidyl transfer reaction, we made several deletion and point mutants of the REV1S cDNA and purified recombinant proteins as histidine-tagged forms (Fig. 4). We showed

**DISCUSSION**

The REV1 gene has been isolated and identified as one of the genes responsible for damage-induced mutagenesis in yeast. In humans, the pathway of damage-induced mutagenesis is largely unknown. Mutagenesis is a key event in carcinogenesis and genetic diseases. To elucidate the pathway of mutagenesis in humans, we cloned and characterized the human orthologue of the REV1 gene.

A human REV1 cDNA that we isolated encoded a one-amino acid shorter protein than recently reported clones (Fig. 1) (10, 11). This shorter form of the REV1 protein was named REV1S. Gibbs et al. (11) also reported a shorter form of the REV1 mRNA and suggested that this mRNA resulted from slippage at the 3′ splice site of an intron rather than a polymorphism. We examined the distribution of REV1S mRNA in a human population. Direct sequencing of RT-PCR-amplified fragments from normal human mononuclear cells of 10 individuals showed that all individuals expressed both of the REV1 and REV1S mRNA (Fig. 2). The ratio of REV1 and REV1S mRNA was about 1:1 (Fig. 2). These results support the proposal of Gibbs et al. (11). The alternative possibility that humans have two copies of the gene is unlikely for the following reasons: (i) the position of the alanine codon is an exon-intron junction (11); (ii) the human REV1 gene was mapped to only one locus (10); (iii) the nucleotide sequence of REV1 cDNA reported by Gibbs et al. (11) is identical to that of REV1S cDNA except for the insertion of the alanine codon; and (iv) only one species of DNA fragment containing introns was amplified by genomic PCR (data not shown).

Lin et al. (10) showed that expression of the REV1 gene was ubiquitous in human tissues using the RT-PCR method. The results of Northern blot analysis confirmed their observation (Fig. 3). Moreover, we found that expression of the gene was relatively high in the testis and ovary and relatively low in the thymus and small intestine (Fig. 3).

It has recently been shown that the human REV1 gene encodes a deoxycytidyl transferase (10). Although the REV1 protein is a member of a family of translesion DNA polymerases, the REV1 protein does not possess any DNA polymerase activity (7, 10). To elucidate the molecular mechanism for the deoxycytidyl transfer reaction, we made several deletion and point mutants of the REV1S cDNA and purified recombinant proteins as histidine-tagged forms (Fig. 4). We showed

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that the REV1S protein also possesses deoxycytidyl transferase activity. The value of specific activity, 320 pmol of dCMP transferase per mg of protein, is lower than that previously reported for the REV1 protein (10). However, we believe that our results from mutation analysis of the REV1S protein are applicable to the case of the REV1 protein. We are currently preparing REV1 protein to compare its activity with that of REV1S.

The domain conserved in translesion DNA polymerase is located in the central region of the REV1 protein (motifs II to VII) (Fig. 1). The BRCT domain and motif I are specific to REV1 but not DNA binding. These results indicated that the BRCT domain is dispensable for transferase activity in vitro. The activity of the ΔN407 protein was dramatically decreased, and that of the ΔC810 protein was under the detection limit, although the protein contains the total polymerase domain (motifs II–VII) (Figs. 1 and 5). On the other hand, the ΔC885 protein, which is 75 residues longer than the ΔC810 protein, retains deoxycytidyl transferase activity at the wild-type level (Fig. 5). This result suggests that the region consisting of 75 residues of the C terminus of the ΔC885 protein is required for the activity. In this region, we found a motif (motif VIII) that is well conserved among the REV1 proteins of different species (Fig. 1). Johnson et al. (37) reported that the replacement of DE residues in motif VIII of the Rad30 protein abolished the polymerase activity of the Rad30 protein, because these residues also play a critical role in the polymerase activity of other polymerases (23, 25, 27, 28). Motif III of the Rad30 protein corresponds to motif IV of the REV1 protein (Fig. 1). In the REV1S protein, we made a mutant, D569A/E570A, that has mutations in motif IV, and we found that D569A/E570A completely lacks deoxycytidyl transferase activity (Fig. 5). It is possible that the global structure of D569A/E570A is not disrupted by the amino acid replacements, because the elution profile from gel filtration chromatography (Fig. 6) and the DNA binding property of D569A/E570A (Fig. 7) are identical to those of the wild-type protein. Therefore, we believe that the ED residues are essential for catalysis of the deoxycytidyl transferase reaction of the REV1/REV1S protein but not DNA binding. These results indicated that the structure of the catalytic site of the deoxycytidyl transferase closely resembles that of the translesion DNA polymerases. Therefore, the molecular mechanism of the dCMP transfer reaction of the REV1/REV1S protein might be the same as that of the dNTP transfer reaction of the translesion DNA polymerases.
EMSA results indicated that the DNA binding domain was located in the conserved polymerase domain (motifs II–VII). We found that the affinities of the inactive proteins were slightly decreased. Since the lack of transferase activity might be a result of the decreased affinity to DNA, the transferase activity of ΔN407 was measured at increased concentrations of substrate DNA. However, even the highest substrate concentration (1 μM) did not improve the decreased transferase activity. This substrate concentration is 70 times higher than the apparent binding constant of the protein. Therefore, we believe that the loss of deoxycytidyl transferase activity for the mutant proteins did not directly result from a decrease in the affinity to substrate DNA. Rather, the stability of the protein-DNA complex of the inactive proteins seems to decrease. The result of EMSA showed that the size of the h6-REV1-DNA complex seemed to gradually increase depending on the protein concentration (Fig. 7). This observation suggests that the REV1 proteins might multimerize on the DNA. The multimerization might play an important role in the transferase activity. The molecular nature of the REV1-DNA complex is currently being investigated.

One of the characteristic features of the structure of the REV1 protein is the BRCT domain. The BRCT domain is a characteristic motif of DNA repair and cell cycle checkpoint proteins (39). A mutation was found in the BRCT domain of the yeast rev1-1 mutant, which lacks damage-induced mutability (Fig. 1) (15). However, the Rev1-1 protein retains a significant fraction of its deoxycytidyl transferase activity (40). These findings suggest the existence of a regulatory mechanism of deoxycytidyl transferase activity (40). These findings might play an important role in the transferase activity. The molecular nature of the REV1-DNA complex is currently being investigated.

It is very likely, considering the following observations, that the REV1 protein exists as a dimer in solution. (i) The apparent molecular weight of each protein is roughly twice the calculated molecular weight, except for the ΔN407/ΔC885 protein (Table II). The apparent molecular weight of the ΔN407/ΔC885 protein is close to the calculated molecular weight (Table II), suggesting that only the ΔN407/ΔC885 protein is a monomer. (ii) The mobility of the ΔN407/ΔC885-DNA complex is much faster than that of others on gels (Fig. 7A). This supports the idea that only ΔN407/ΔC885 exists as a monomer and that the REV1S protein forms a dimer.
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