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

Yuji Masuda, Mamoru Takahashi, Noriko Tsunekuni, Tomoyuki Minami, Masaharu Sumii, Kiyoshi Miyagawa, and Kenji Kamiya‡

* 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 acid 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 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 unrepaird 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) 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 (10, 11), REV3 (12, 13), and REV7 (14) have been identified. It has been shown that the human REV1 protein possesses a deoxycytidyl transferase activity (10) and that the human 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 DNB1 (21, 22) of humans. Recent studies have shown that these 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 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-CTCTAGCTGGACGCTTGA-3' and 5'-GTAAAACACCTGAGACATG-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 × 10^9 plaques. In all of the clones in these phages, the promoter-proximal side of the REV1 cDNA was truncated at an XhoI site, because XhoI had used to construct the cDNA library. One clone, named ΔREV2, 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'-TTCTGTTTTTTGTCAGGGCTG-3' and 5'-TTGTTCTCGACAGGATTTC-3', and it was used as a probe for screening the same library. One positive clone, named ΔREV1T1, was obtained. This phage contained cDNA from the 5'-untranslated region to the XhoI 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/GenBank accession number of the nucleotide sequence of the human REV1S cDNA is AB047646.

For construction of full-length REV1S cDNA, the BamHI/BstXI fragment of REV1S cDNA from ΔREV2 was inserted into the corresponding site of pBluescript II SK (-). The resulting plasmid, pRH2IN, was digested with SacI and XhoI, and the SacI/BstXI fragment of REV1S cDNA from ΔREV1T1 was inserted. The resulting plasmid, pREV1S, contained the fragment of the position 20–427 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'-GGGTCCGCAAGGCCGGAGAA-3' and 5'-GCTTGGCCCTGAGACATG-3'.
Catalytic Activity of Human REV1 in the Polymerase Domain

The BglII/XbaI fragment from the human REV1S cDNA was used as a probe. Human multi-tissue Northern filters (MTN1 and -2, CLONTECH) were hybridized with the probe in ExpressHyb™ Hybridization Solution (CLONTECH) at 65 °C and washed with 0.1 × SSC and 0.1% SDS at 65 °C. The hybridized human REV1/REV1S mRNA was visualized by autoradiography at ~80 °C.

**Construction of Mutant REV1S Genes**

To introduce site-directed mutations, we used a PCR-based method. DNA fragments of the limited portions were amplified by PCR 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 histidine residues at their N termini.

**Construction of pET-C729—** The 5′-portion of the REV1S gene was amplified by PCR with primers (5'-GGCAGAGATCTGGATTCTCCACCA-3' and 5'-TCATGCTATGCCAGAGGA-3') to introduce an Ndel site at the first ATG (position 173 of the REV1S cDNA). The PCR fragment was cloned into the pCR 2.1-TOPO vector (Invitrogen), and the resulting plasmid was named pRHNXB6. (Table I). The PCR fragment was digested with BamHI and HindIII, and then the BamHI/HindIII fragment of pREV1S was inserted into the corresponding site of pBAD22A. The resulting plasmid, pBAD-REV1S, produces a full-length REV1S protein starting from the 407th methionine (Table I).

**Construction of pET-N407—** To introduce an Ndel site at position 905 of the REV1S cDNA, PCR was carried out using primers (5'-TTGGTGCGCACTACAGAGCAG-3' and 5'-TGATTCTGGATCCGAGGAGA-3') to introduce the Ndel site at the first ATG (position 173 of the REV1S cDNA). The PCR fragment was cloned into the pCR 2.1-TOPO vector (Invitrogen), and the resulting plasmid was named pRHNX. The PCR fragment was digested with BamHI and HindIII, and then the BamHI/HindIII fragment of pREV1S was inserted into the corresponding site of pET-C153. The resulting plasmid, pET-C810 (Table I), was digested with BamHI and HindIII, and then the BamHI/HindIII fragment of pREV1S was inserted into the pET-C729 vector. The resulting plasmid, pET-C885, produces a truncated protein containing extra amino acid residues, GCRNISISSLSMISCQT, at the C terminus (Table I).

**Construction of pET-N245—** To introduce an Ndel site at position 579 of the REV1S cDNA, PCR was carried out using primers (5'-TTGGTGCGCACTACAGAGCAG-3' and 5'-TGATTCTGGATCCGAGGAGA-3') to introduce the Ndel site at the first ATG (position 173 of the REV1S cDNA). The PCR fragment was cloned into the pCR 2.1-TOPO vector, and the resulting plasmid was named pET-N245/C885. The PCR fragment was cloned into the pET-C729 vector, and the resulting plasmid was named pET-N245/C479. The PCR fragment was cloned into the pET-C153 vector, and the resulting plasmid was named pET-N245/C479. The resulting plasmid was named pET-N245/C479 (Table I).

**Construction of pET-N245/C885 and pET-N407/C885—** To introduce an Ndel site at position 905 of the REV1S cDNA, PCR was carried out using primers (5'-TTGGTGCGCACTACAGAGCAG-3' and 5'-TGATTCTGGATCCGAGGAGA-3') to introduce the Ndel site at the first ATG (position 173 of the REV1S cDNA). The PCR fragment was digested with BamHI and HindIII, and then the BamHI/HindIII fragment of pREV1S was inserted into the corresponding site of pET-C153. The resulting plasmid was named pET-C810 (Table I). The PCR fragment was digested with BamHI and HindIII, and then the BamHI/HindIII fragment of pREV1S was inserted into the corresponding site of pET-C729. The resulting plasmid was named pET-N407/C885 (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-N245/C885, and pET-N407/C885 was grown in LB broth supplemented with ampicillin (100 µg/ml) at 37 °C with aeration until the culture reached an A600 value of 0.6. isopropyl β-D-thiogalactopyranoside was added to 0.2 mM, and the incubation was continued for 1 h. BL21 (DE3) harboring pET-N245 and pET-N407 was grown at 15 °C, and incubation was continued for 10 h after the addition of isopropyl β-D-thiogalactopyranoside at an A600 value of 0.6. For the full-length hREV1S induction, BL21 (DE3) harboring pBAD-REV1S and pBAD-REV1SA was grown in SB medium (32) at 15 °C, and inducible proteins were tagged with six histidine residues at the N terminus. The numbers indicate the positions of amino acid residues of the expected proteins.

**Amino acid residues aspartate 569 and glutamate 570 were changed to alanines.**

**Extra amino acid residues, AANKARKEAELAAATAEQ, were attached at the C terminus.**

**Extra amino acid residues, YR, were attached at the C terminus.**

**Extra amino acid residues, AANKKEAEALAAATAEQ, were attached at the C terminus.**
bation was continued for 10 h after the addition of L-(+)-arabinosine to 1% at an A600 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 added to 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 cell lysate was applied at 0.1 ml/min to a 1-ml HiTrap chelating column (Amersham Pharmacia Biotech), which had been washed with 2 ml of 0.1 M NiSO4 and equilibrated with buffer A (50 mM HEPES-NaOH, pH 7.5, 1 M NaCl, 10% glycerol, 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 hi-REV1S and its derivatives were eluted with buffer A containing 300 mM imidazole. For further purification, 50 μl of the peak fraction was applied to 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 32P-labeled oligonucleotide primer 5′-CAGTCGACTGATG-T3′ annealed to an oligonucleotide template, 5′-CTCGTACGTCCTTCAU-CATACGTCAGCATCTTCAU-3′, was used (18). The substrate was treated with 0.1 units of E. coli uracil-DNA glycosylase (New England Biolabs) at 30 °C for 30 min in a reaction mixture just before adding enzyme. The reaction mixture (25 μM) contained 25 mM potassium phosphate buffer, pH 7.4, 5 mM MgCl2, 0.1 mM dCTP, 100 nM substrate, and 1 μl of protein sample. The protein samples had been diluted with buffer A containing 0.1 mg/ml bovine serum albumin as indicated. After incubation at 30 °C for 30 min, reactions were terminated with 10 μl of stop solution (30 mM EDTA, 94% formamide, 0.05% bromphenol blue, 0.05% xylene cyanole). The reaction products were resolved on 20% polyacrylamide gel containing 8M urea and autoradiographed at 80 °C.

**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 primer 5′-end deoxycytidyl transferase assay was used, but it was not treated with uracil-DNA glycosylase. Reaction mixtures (10 μl) contained 25 mM potassium phosphate buffer, pH 7.4, 0.2 mg/ml bovine serum albumin, 5 mM dithiothreitol, 0.1 mM dCTP, 100 mM substrate, and 1 μl of protein sample. The mixtures were incubated on ice for 15 min and loaded on a prerunning 4% polyacrylamide gel (79:1, acrylamide/bisacrylamide). The electrophoresis buffer contained 6 mM Tris-HCl (pH 7.5), 5 mM sodium acetate, and 0.1 mM EDTA, and the gels were subjected to a constant voltage of 8 V/cm applied for 2 h at 6 °C. Following gel electrophoresis, the gels were dried and autoradiographed at ~80 °C. The amounts of the binding fraction and free DNA were 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 yeast REV1 proteins of different species. The boxes show highly conserved sequences in translesion DNA polymerases. Vertical bars represent positions of truncation in the REV1 derivatives that were studied in this work. The closed triangle indicates the position of mutation of yeast rev1−1 mutant (15).

**DNA Binding Assays by EMSA**

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 in the REV1 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 REV1 derivatives that were studied in this work. The closed triangle indicates the position of mutation of yeast rev1−1 mutant (15).
m urea (Fig. 2B). A short band, 93-base product, was derived from the REV1S cDNA, and a band 3 bases longer was derived from REV1 cDNA. The average ratio of the shorter and longer bands, REV1/REV1S, from 10 individuals was 1.25. We concluded that 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 \(^{32}\)P-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 \( \Delta N245 \) and \( \Delta C885 \) 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 \( \Delta N245/\Delta C885 \) protein, which has both truncations of N and C termini, sustained the activity. \( \Delta C810 \) and \( \Delta C729 \) proteins, which have further truncation of the C terminus, were inactive. The transferase activity of \( \Delta 407 \) protein was dramatically decreased, but significant activity was still detected. However, the protein \( \Delta N407/\Delta C885 \), 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 \( \Delta N407/\Delta C885 \) 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
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 REVIS 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 REVIS mRNA (Fig. 2). The ratio of REV1 and REVIS 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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Footnote: 
2 Y. Masuda, M. Sumii, and K. Kamiya, unpublished data.
that the REV1S protein also possesses deoxycytidyl transferase activity.

The specific activities of the indicated fractions of gel filtration chromatography were determined in standard reaction conditions.

The relative activities were calculated using the specific activity and the calculated molecular weight of each protein. The relative activities of ΔN245, ΔN407, ΔC885, ΔC810, ΔC729, and ΔN407/ΔC885 proteins were less than 0.03% of that of the h6-REV1S protein.

Table II

Summary of the deoxycytidyl transferase activities of the h6-REV1S and mutant proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Fraction no.</th>
<th>Specific activity [pmol/mg]</th>
<th>Relative activity [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>h6-REV1S</td>
<td>9</td>
<td>550</td>
<td>100</td>
</tr>
<tr>
<td>ΔN245</td>
<td>11</td>
<td>630</td>
<td>160</td>
</tr>
<tr>
<td>ΔN407</td>
<td>11</td>
<td>3.0</td>
<td>0.1</td>
</tr>
<tr>
<td>ΔC885</td>
<td>12</td>
<td>230</td>
<td>50</td>
</tr>
<tr>
<td>ΔN245/ΔC885</td>
<td>14</td>
<td>460</td>
<td>80</td>
</tr>
</tbody>
</table>

Table III

Apparent molecular weight of the h6-REV1S protein and its derivatives by gel filtration chromatography

The Superdex 200 PC 3.2/30 column was calibrated with ferritin (440 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), and ribonuclease A (13.7 kDa), and a standard curve was produced. Apparent $M_r$ was determined using the standard curve.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Calculated $M_r$</th>
<th>Apparent $M_r$</th>
</tr>
</thead>
<tbody>
<tr>
<td>h6-REV1S</td>
<td>140,000</td>
<td>320,000</td>
</tr>
<tr>
<td>ΔN245</td>
<td>113,000</td>
<td>260,000</td>
</tr>
<tr>
<td>ΔN407</td>
<td>95,000</td>
<td>200,000</td>
</tr>
<tr>
<td>ΔC885</td>
<td>102,000</td>
<td>180,000</td>
</tr>
<tr>
<td>ΔC810</td>
<td>92,000</td>
<td>165,000</td>
</tr>
<tr>
<td>ΔC729</td>
<td>84,000</td>
<td>165,000</td>
</tr>
<tr>
<td>ΔN245/ΔC885</td>
<td>73,000</td>
<td>110,000</td>
</tr>
<tr>
<td>ΔN407/ΔC885</td>
<td>57,000</td>
<td>65,000</td>
</tr>
</tbody>
</table>

The specific activities of the indicated fractions of gel filtration chromatography were determined in standard reaction conditions.

The relative activities were calculated using the specific activity and the calculated molecular weight of each protein. The relative activities of ΔN245, ΔN407, ΔC885, ΔC810, ΔC729, and ΔN407/ΔC885 proteins were less than 0.03% of that of the h6-REV1S protein.
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 of the REV1 protein by the BRCT domain in vivo. Our results showed the removal of the BRCT domain instead slightly increased the activity (Table III, compare h6-REV1S and ΔC885 with ΔN245 and ΔN245/ΔC885). This might suggest that the BRCT domain negatively regulates the transferase activity in vivo and that the in vivo role of the BRTC domain of the REV1 protein is conserved from yeast to humans.

**Acknowledgments**—We are grateful to Dr. Saburo Fukuda, Dr. Hiroaki Yasumoto, and Dr. Jun Teishima for generating figures. We thank Kumiko Mizuno, Hatsue Wakayama, and Emi Yagi for laboratory assistance.

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**FIG. 7.** DNA binding activity of the h6-REV1S and mutant proteins. A, titration of proteins. Reaction mixtures, each containing 0.5 nM of labeled DNA and the indicated concentration of protein, were incubated and analyzed as described under "Experimental Procedures." The protein concentrations were calculated as monomers. The arrowheads indicate the positions of origins of the electrophoresis. F, free DNA; C, protein-DNA complexes. B, quantitation of EMSA results. The intensities of free DNA and total shifted fractions were measured using a Bio-Imaging Analyzer BAS2000, and the fraction of DNA shifted was calculated as the amount of total shifted fractions divided by total DNA and normalized to 100%.

**TABLE IV**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Affinity&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>h6-REV1S</td>
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</tr>
<tr>
<td>D569A/E570A</td>
<td>2.5</td>
</tr>
<tr>
<td>ΔN245</td>
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<tr>
<td>ΔN407/ΔC885</td>
<td>8.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> The protein concentration at which 50% of the DNA was bound by protein. The protein concentrations were calculated as monomers. The experimental errors in these determinations are less than 10%.
1646–1649
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