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Specific Interaction of pRB with a Rat Genomic DNA Fragment, REC11

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The retinoblastoma gene product (pRB) has been known to contain a sequence-nonspecific DNA binding activity. It is unknown whether pRB can recognize and bind to specific DNA sequences. We have recently identified a rat genomic DNA fragment, termed REC11, which can interact with rat Cdc37-related protein (RCdc37). In this study, we have found that pRB could interact with the REC11 DNA in a sequence-specific manner. A series of GST-RB deletion mutants was used in the gel shift assays to define the domain of pRB responsible for this interaction. GST-RB (385-611) and GST-RB (612-928) completely lost the binding activity, while GST-RB (555-682) retained an activity to associate with the REC11 DNA, indicating that the continuous spacer region of pRB might be important for this sequence-specific DNA binding activity.

The retinoblastoma antioncogene encodes a nuclear phosphoprotein (pRB) which is thought to play a key role in regulating mammalian cell growth (1). Biochemical approach has revieled that pRB displays a DNA-binding activity in a sequence-nonspecific manner (2), however it is unknown whether pRB can recognize a specific DNA sequence. Recently, it has been suggested that pRB can be involved in the regulation of gene expression. The expression of several cellular genes, including *c-fos, c-myc* and transforming growth factor β1 (TGF-β1) are shown to be regulated in either positive or negative manner, depending upon the cell types studied (3-5). Promoter deletion analysis defines a discrete element (retinoblastoma control element; RCE) within the promoter sequence required for the transcriptional regulation mediated by pRB (3, 5). Recently, it has been demonstrated that one of RCE-binding proteins is encoded by Sp-1 and functional interaction between pRB and Sp-1 might be required for RCE-dependent transcriptional regulation (6, 7).

As shown previously, we have identified a rat genomic DNA fragment, REC11, which exhibits a specific interaction with rat Cdc37-related protein (RCdc37) (8). Recent work in our laboratory has shown that RCdc37 could contain a possible transcriptional activation function and associate with pRB (9). Interestingly, there exist two copies of the putative RCE adjacent to the REC11 sequence, indicating some regulatory role(s) of this genomic region in pRB-mediated transcriptional control.

In the present study, we have found the specific interaction between pRB and REC11 sequence *in vitro.*

MATERIALS AND METHODS

GST-RB fusion proteins. GST-RB (385-928), GST-RB (385-611), GST-RB (612-928) and GST-RB (385-928; 706Y) were kindly provided by Dr. Y. Taya (National Cancer Center Research Institute, Tokyo, Japan). The GST-RB (555-682) was constructed by PCR reaction using a pair of the following oligonucleotides: 5′-GTCGAATTCATCATCGAATCATGGAATCCCTTGC-3′, 5′-GTCGAATTCGGGTCCAGATGATATGTTCTAATTC-3′. The PCR product was digested completely with EcoRI, purified by 5% polyacrylamide gel electrophoresis and subcloned into the EcoRI site of pGEX-2T (Pharmacia).

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  1 GATCATCCGTCTTTTGCTCATCTCTACCCTACACACTTTTCAAAGGCTCT
 51 TAGCAAGGCGGCAACTAACTTTAAGGCTATTTCTTATGAATAATAAAAAA
101 AAAACAGCCTTAAGAGAATTCAATTTTTGCACGGGCCTCTTTGTGGGTGT
151 TGCTATCTTCCATTTAAGCTGTCTGCCATAGAATTCACCTCCCGCTTCCC
201 TGGTAGGAGGTAGAGAAAACAGAGTTTGATTGTCTCTGGTCACAGTCAGC
251 AGGGGGTGCGGTTTGCTGTGGCACAGCAGGCCATAGGCACCCCCACCCCA
301 CCCCCCAATCCCTGGCATCAAGGCGGAATATGGATAGGGTCAGAATCTTT
351 CTGGATC
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FIG. 1. Nucleotide sequence of the REC11 DNA (underline) and its 5′- and 3′- flanking region. Two possible RCEs (retinoblastoma control element) are indicated by wavy lines.

Production of recombinant proteins in E. coli. The recombinant plasmids were introduced into *E. coli* JM109. The GST-RB fusion proteins were induced in the presence of 0.1mM IPTG (isopropyl-β-D-thiogalactopyranoside) and purified by glutathione-Sepharose 4B (Pharmacia), under conditions described previously (8).

Nucleotide sequence determination. The nucleotide sequence of the PCR product was determined in both strands by means of dideoxy chain termination method using T7 polymerase provided in the Sequenase version 2.0 kit (United States Biochemicals).

Gel mobility shift assay. GST or GST-RB fusion protein (0.1μg) was mixed with 0.5μg of poly[d(I-C)] (Boehringer Mannheim) in 20μl of a binding buffer containing 110mM KCl, 4mM $MgCl_2$, 4mM Tris-Cl pH 7.6, 4% glycerol, 0.05mM $ZnCl_2$ and 0.25% bromophenol blue (8). The reaction mixtures were kept at room temperature for 10 min. The ^{32}P-labeled probe DNA was then added and incubation was continued for another 20 min. The protein-DNA complexes were resolved by electrophoresis through neutral 5% polyacrylamide gels. The unlabeled probe DNA and the 123/124 bp restriction fragment of pBR322 were used as the specific and the nonspecific competitor, respectively.

RESULTS AND DISCUSSION

As described previously, a rat Cdc37-related protein (RCdc37) can interact specifically with the REC11 DNA (8) and associate with the retinoblastoma gene product (pRB) (9). Interestingly, there exist two copies of the putative retinoblastoma control element (RCE; 6, 7) in the 3′-flanking region of the REC11 sequence (293-298 and 298-303) (Fig. 1), suggesting a possible interaction of pRB with the REC11 DNA.

In order to examine a direct interaction of pRB with the REC11 DNA, we have carried out gel electrophoretic mobility shift assays using radio-labeled REC11 DNA and GST-RB fusion protein (385-928), which contains a pocket region with two noncontiguous domains in the carboxy-terminal 60kDa fragment of human pRB (10). As shown in Fig. 2, two retarded bands, designated complex I (upper) and II (lower), were detected in the presence of GST-RB (385-928), but not in the presence of GST. The specificity of this binding reaction was examined by band shift assays using a specific or a nonspecific competitor DNA. As shown in Fig. 3, the interaction between GST-RB (385-928) and the REC11 DNA was not affected by the unlabeled nonspecific competitor DNA (123/124 bp restriction fragment of pBR322), whereas the intensity of complex I and II was reduced in the presence of the unlabeled specific competitor DNA (REC11 sequence). These results suggest that pRB could directly bind to the REC11 DNA in a sequence-specific manner and the faster migrating band (complex II) might derive from the interaction of the REC11 DNA with the partial degradation product of pRB.

In order to define the region of pRB required for the specific interaction with the REC11 DNA, we have used two kinds of GST-RB fusion proteins in gel shift assays, GST-RB (385-611) and GST-RB (612-928) (10). GST-RB (385-611) is lacking the pocket B and the carboxy-terminal portion of pRB and GST-RB (612-928) is deprived of the pocket A (Fig. 4C). As

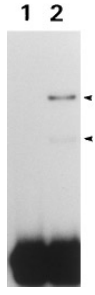

FIG. 2. Gel retardation assay showing the interaction between the REC11 DNA and pRB. Equal amount (0.1μg) of GST (lane 1) or GST-RB (385-928) (lane 2) was incubated in the presence of ^{32}P-labeled REC11 DNA (5,000cpm). The reaction mixture was analyzed by 5% polyacrylamide gel electrophoresis. Retarded bands (complex I and II) are indicated by arrowheads.

shown in Fig. 4A, both GST-RB (385-611) and GST-RB (612-928) completely lost the capacity to associate with the REC11 DNA. This result indicates that the critical region of pRB for the specific interaction with the REC11 DNA might exist within the continuous spacer region (573-645) which is disrupted both in GST-RB (385-611) and GST-RB (612-928). To investigate this possibility, we have generated a GST-RB fusion protein containing the region spanning amino acid residues 555-682 (Fig. 4C) by the PCR-based strategy as described in Materials and Methods and performed the gel shift assay in the presence of this construct. As shown in Fig. 4B, GST-RB (555-682) can form a complex with the REC11 DNA. Therefore, it could be speculated that the continuous spacer region of pRB is responsible for the specific interaction with the REC11 DNA. As reported previously, deletion of exon 21 (697-731) or 16 (475-499) of pRB can not affect the DNA-binding activity of pRB, when the native calf thymus DNA-cellulose column was used in the binding assays (11, 12). These observations are consistent with our present results, since regions encoded by exon 16 and 21 of pRB are located outside of the domain (555-682) required for the interaction with the REC11 DNA.

It has been known that there exist several mutant forms of pRB which contain naturally occuring single amino acid substitution at codon 706 and these mutants have been shown to lack the binding activity to oncoproteins such as SV40 large T antigen and E1A protein (13-15), indicating that this single amino acid substitution causes a loss of normal physiological function of pRB. In order to examine the effect of the point mutation on the REC11 DNA-binding activity of pRB, we have carried out the gel shift assay using the mutant form of pRB [GST-RB (385-928; 706Y)]. As shown in Fig. 5, GST-RB (385-928; 706Y)

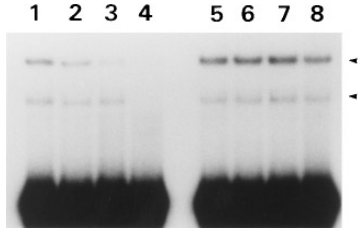

FIG. 3. Sequence-specific interaction of pRB with the REC11 DNA. Identical amount (0.1μg) of GST-RB (385-928) and the radiolabeled REC11 DNA (5,000cpm) were mixed and incubated with the increasing amounts of unlabeled specific (REC11 DNA) (lanes 1-4) or nonspecific (123/124 bp restriction fragment of pBR322) competitor DNA (lanes 5-8). The amount of competitor DNA was 0- (lanes 1 and 5), 1- (lanes 2 and 6), 10- (lanes 3 and 7) and 100-fold excess over the probe (lanes 4 and 8). Retarded bands are indicated by arrowheads.

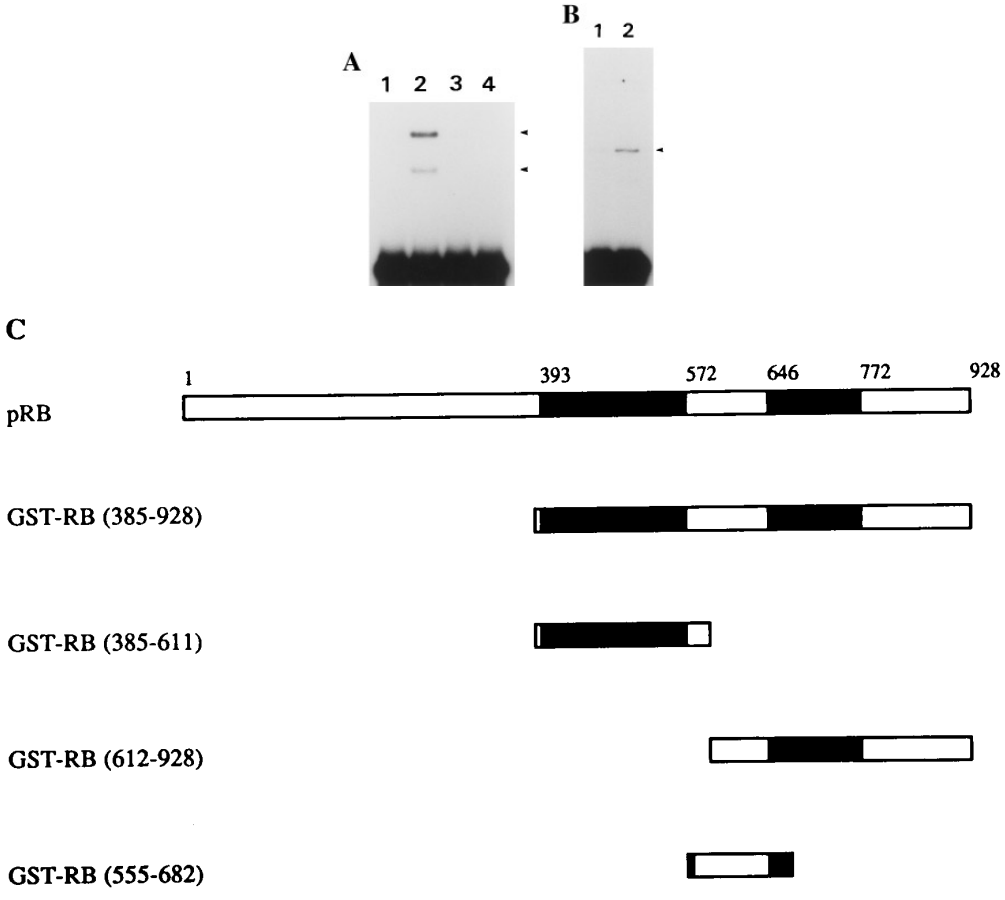

FIG. 4. Determination of the domain of pRB required for the interaction with the REC11 DNA. (A) Each of those GST-RB fusion proteins (0.1μg) was incubated in the presence of the radiolabeled REC11 DNA (5,000cpm). Free and protein-complexed DNA fragments were separated by 5% polyacrylamide gel. Lane 1, GST; lane 2, GST-RB (385-928); lane 3, GST-RB (385-611); lane 4, GST-RB (612-928). Retarded bands are indicated by arrowheads. (B) A similar experiment was carried out using GST (lane 1) or GST-RB (555-682) (lane 2). (C) The structure of GST-RB fusion proteins used in gel shift assays. The pocket region of pRB is shown by filled boxes.

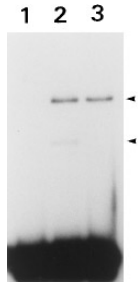

FIG. 5. Mutant form of pRB can associate with the REC11 DNA. Equal amount (0.1μg) of GST (lane 1), GST-RB (385-928) (lane 2) or mutant form of pRB [GST-RB (385-928; 706Y)] (lane 3) was incubated in the presence of ^{32}P-labeled REC11 DNA. The reaction mixture was analyzed by 5% polyacrylamide gel. Arrowheads indicate the position of retarded bands.

retains the binding activity to the REC11 DNA as efficiently as the wild type pRB. Although this result seems to suggest that the REC11 DNA-binding activity alone might not be sufficient for the tumor suppressive role of pRB, it is possible that the specific interaction of pRB with transcription factors such as E2F (1) might affect the efficiency of the REC11 DNA-binding activity of pRB.

It is interesting to note that there exist two copies of putative RCE in the 3′-side of the REC11 sequence (Fig. 1). It has been shown that Sp-1 could associate with RCE and the functional interaction of Sp-1 with pRB could be important for RCE-dependent transcriptional regulation (6, 7). Although the functional importance of the REC11 sequence is still unknown, it is intriguing to examine the possible role of the REC11 sequence in the pRB-mediated transcriptional regulation. Recently, we have cloned a 16-kb rat genomic DNA fragment containing the REC11 sequence and try to identify a gene whose expression, if any, might be regulated by pRB.

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