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Site-directed mutagenesis study on DNA binding regions of the mouse homologue of Suppressor of Hairless, RBP-Jκ

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ABSTRACT

To map regions important for DNA binding of the mouse homologue of Suppressor of Hairless or RBP-Jκ protein, mutated mouse RBP-Jκ cDNAs were made by insertion of oligonucleotide linkers or base replacement. DNA binding assays using the mutated proteins expressed in COS cells showed that various mutations between 218 Arg and 227 Arg decreased the DNA binding activity drastically. The DNA binding activity was not affected by amino acid replacements within the integrase motif of the RBP-Jκ protein (230His-269His). Replacements between 291Arg and 323Tyr affected the DNA binding activity slightly but reproducibly. These results indicate that the region encompassing 218Arg-227Arg is critical for the DNA binding activity of RBP-Jκ. This region did not show any significant homology to motifs or domains of the previously described DNA binding proteins. Using a truncation mutant protein RBP-Jκ was shown to associate with DNA as a monomer.

INTRODUCTION

The mouse RBP-Jκ protein was isolated from the nuclear extract of a mouse pre-B cell line as a DNA binding protein specific to the VDJ-recombination signal sequence (RS) with a 23-base spacer (Jκ type RS)(1). The amino acid sequence predicted from cDNA encoding the mouse RBP-Jκ protein has revealed the presence of a 40-residue motif which shares homology to the catalytic domains of a group of site specific recombinases named integrase family (2). The integrase family includes lambda phage integrase, fimbriae switch recombinase of *Escherichia coli*, yeast Flp recombinase and other recombinases found in phage and bacteria (3,4). No other regions of the RBP-Jκ protein than the integrase motif were significantly related to previously described motifs found in many DNA binding proteins such as leucine zipper, helix-turn-helix, basic helix-loop-helix, and zinc fingers (2,5).

Later, the mouse RBP-Jκ protein was shown to be expressed in nuclei of almost all mouse tissues and culture cells at the almost same level (6). We have also shown that the RBP-Jκ gene is highly conserved in *Drosophila* (7), mouse, and human (8). The human and mouse RBP-Jκ proteins are 98% identical in their amino acid sequence. Between mouse and *Drosophila* RBP-Jκ, the N- and C-terminal regions are quite diverged but the central 250 residues are highly conserved with 93% of identical amino acids (7). The region flanking the N-terminal side of the integrase motif is especially highly conserved in *Drosophila* and mouse; only one amino acid is changed among 46 residues, suggesting the functional significance of this region. The *Drosophila* RBP-Jκ protein binds to various Jκ-RS mutant probes with the same specificity to that of the mouse counterpart. *Drosophila* RBP-Jκ turned out to be identical to the gene for Suppressor of Hairless (Su(H)) mutation, a gene known to be involved in the development of the peripheral nervous tissues (9,10). The Su(H) gene (or RBP-Jκ), and the gene for Hairless mutation, antagonistically regulate the formation of sensory bristles (11–14). The ubiquitous expression in adult mouse, strong conservation even in invertebrate and the involvement in the development of *Drosophila* peripheral nervous system suggest that the RBP-Jκ gene may have functions other than VDJ-recombination.

In fact, our recent study on the DNA binding specificity of the mouse RBP-Jκ protein have indicated that the purified mouse RBP-Jκ protein recognizes a unique consensus sequence that partially overlaps but is not precisely same as the heptamer of VDJ-RS (15). The core consensus sequence consists of an octanucleotide of CGTGGGAA and some nucleotides on both sides of the core seem to contribute to the maximal binding.

In order to understand the structural characteristics of this unique DNA binding protein, we introduced various mutations to the mouse RBP-Jκ protein and measured DNA binding activities. The results described in this paper indicate that two regions flanking the integrase motif are important for DNA binding.

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MATERIALS AND METHODS

Materials

Restriction enzymes, the Klenow fragment of *Escherichia coli* DNA polymerase I, and other enzymes used were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan); radiolabeled nucleotides and ^{125}I-labeled anti-rat IgG antibody were from Amersham Corp.. COS 7 cells were cultured in Dulbecco's modified Eagle's minimum essential medium supplemented with 10% fetal calf serum. Preparation of the rat monoclonal antibodies against the mouse RBP-Jκ protein has been described (6).

Expression vectors

To express the full-length wild type RBP-Jκ protein, CDM8-RBP-2 plasmid was used. This plasmid contained, between the *Hind*III and *Xba*I sites of the CDM8 vector, the 1.6 kb *Spl*I−*Hinc*II fragment of the mouse RBP-Jκ mRNA (clone RBP-2, ref. 2) and an oligonucleotide linker which contained 5′ end of the RBP-Jκ mRNA from 3 base pairs (bp) upstream of the initiation codon to the *Spl*I site.

To make linker insertion mutants, CDM8-RBP-2 was digested with restriction enzymes within the cDNA insert (see Fig. 1a for restriction sites), treated with the Klenow fragment to make blunt ends, and then ligated with *Sal*I linkers of appropriate sizes to generate in-frame fusions. C-terminal deletion mutants were constructed by inserting oligonucleotides containing stop codons in all three reading frames between the newly generated *Sal*I sites and the *Xba*I site at the 3′ end of the inserted cDNA. To make N-terminal deletion mutants, fragments between the *Spl*I site and other restriction sites were removed from CDM8-RBP-2, and the remaining fragments were treated with the Klenow fragment to make blunt ends and then circularized by inserting *Sal*I linkers of appropriate sizes so as to restore the reading frames.

Oligonucleotide-directed *in vitro* mutagenesis to construct replacement mutants was carried out using polymerase chain reaction (PCR) by the modified method of Kammann et al. (16). First, in order to facilitate subsequent subcloning, the nucleotide sequence at amino acid 143 (Leu) in CDM8-RBP-2 was changed so that a unique *Xho*I site was generated maintaining the correct amino acid sequence. A 401 bp *Xho*I−*Hind*III fragments and a 287 bp *Hind*III−*Apa*I fragment of the RBP-Jκ cDNA were cloned into the BlueScript KS$^+$ vector and used as the templates of PCR. The first step PCR for mutagenesis was carried out with a primer complementary to the T3 or T7 promoter region of the vector and a second oligonucleotide primer (32−38mer) containing mutated nucleotides (see below for structures of the oligonucleotides). The mutated oligonucleotides were designed so that the two successive amino acids were changed to ArgSer or GlySer generating a new *Bgl*II or *Bam*HI site, respectively, and thereby facilitating identification of mutants. After the first PCR, the amplified products were purified by agarose gel electrophoresis. The purified DNAs were subjected to the second step PCR to regenerate the complete fragments as described (16) using primers complementary to the T3 and T7 promoter sites of the vector. The amplified products were digested with *Xho*I and *Hind*III or with *Hind*III and *Apa*I. The corresponding fragments in CDM8-RBP-2 were replaced by the mutated fragments. Four other mutants with single amino acid changes (R218H, R220G, H230G, and Y267A) were constructed by the same method. The structures of all the mutant RBP-Jκ cDNAs were confirmed by sequencing using Sequenase kits (United State Biochemical Corp.). The Y267A mutant was found to contain an additional, accidental mutation at 194Ser to Ala.

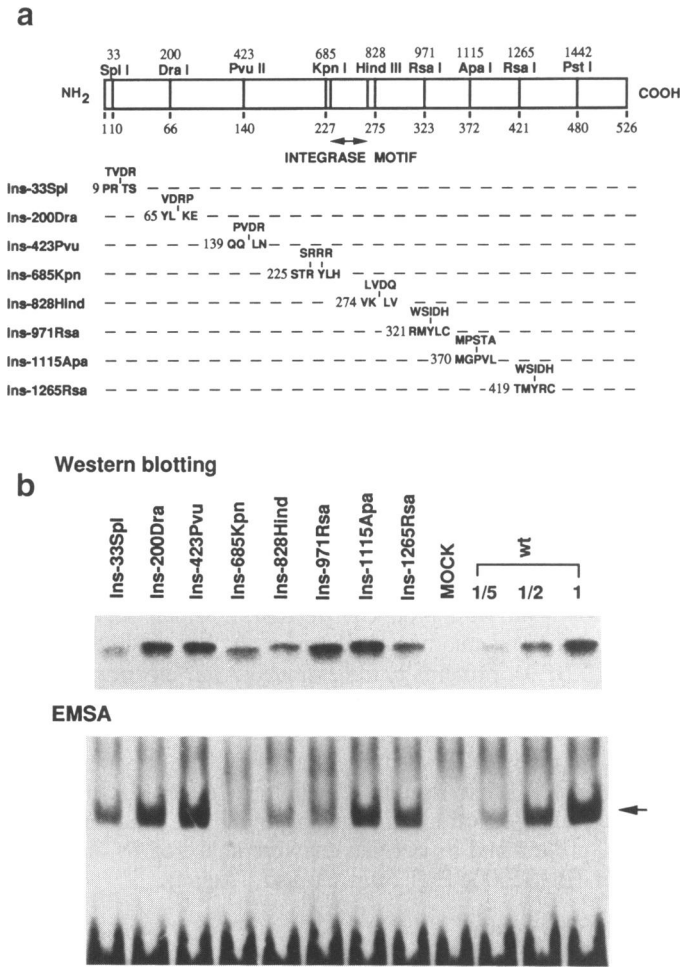

Figure 1. DNA binding activities of linker insertion mutants. Panel **a**, structure of linker mutants. At the top, structure of the mouse RBP-Jκ protein is diagrammed. The restriction enzyme sites are shown along with nucleotide positions above and corresponding amino acid positions below the diagram. Inserted amino acids of each mutant are shown above the wild type sequence. Panel **b**, Western blotting and EMSA of the nuclear extracts from COS cells transfected with the mutants and wild type RBP-Jκ cDNAs.

Sequences of the synthetic oligonucleotide primers used are:

RK162GS/RS; TCTGATTCAGACAAG^{G}/$_{A}$GATC^{T}/$_{C}$CATTTCATGTTGTCT
KR185GS/RS; GGTGTGTTCCTCAGC^{G}/$_{A}$GATC^{T}/$_{C}$ATAAAGGTCATCTCC
KK196GS/RS; TCCAAACCCTCCAAA^{G}/$_{A}$GATC^{T}/$_{C}$CAGTCACTGAAGAAT
KV212GS/RS; ATTGCTTCAGGAACG^{G}/$_{A}$GATC^{T}/$_{C}$GCACTGTTCAATCGC
R218H; GCACTGTTCAATC<u>A</u>CCTT<u>AGATCT</u>CAGACAGTTAGTACC
R220G; CTGTTCAATCGCCTT^{C}/$_{G}$^{A}/$_{G}$CTCCCAGACAGTTAGT
RY227GS/RS; CAGACAGTTAGTACC^{G}/$_{A}$GATC^{T}/$_{C}$CTGCATGTAGAAGGA
H230G; AGTACCAGGTACCTG^{C}/$_{G}$^{G}/$_{T}$TGTAGAAGGAGGGAAT
GA244GS/RS; AGTTCACAACAGTGG^{G}/$_{A}$GATC^{T}/$_{C}$TTTTACATCCATCTC
EE259GS/RS; GACGAGTCGGAAGGA^{G}/$_{A}$GATC^{T}/$_{C}$TTCACAGTTAGAGAT
Y267A; ACAGTTAGAGATGGC^{G}/$_{T}$^{C}/$_{T}$CATCCATTACGGGCAG
RL287GS; GGCATGGCACTCCCA^{G}/$_{A}$GATC^{T}/$_{C}$ATAATT^{A}/$_{G}$GGAAAGTT
RK291GS; CCAAGATTGATAATT^{G}/$_{A}$GATC^{T}/$_{C}$GTTGATAAGCAGACG
KQ295GS; ATT^{A}/$_{G}$GGAAAGTTGAT^{G}/$_{A}$GATC^{T}/$_{C}$ACGGCATTACTGGAT
DD303GS; GCATTACTGGATGCA^{G}/$_{A}$GATC^{T}/$_{C}$CCTGTATCACAACTC
FY314GS; CCACAAATGTGCA<u>GGATCC</u>CTTAAGGATACAG

ER329GS; GCCTTTCTCAAGGATCCATAATCCAATTTCAGGCC
KE341GS/RS; GCCACTCCATGTCCA$^{G}/_{A}$GATC$^{T}/_{C}$CAAAATAAGGAAATG

Underlines indicate the sequence changed or restriction sites newly introduced.

Transfection and preparation of nuclear extracts

COS 7 cells were plated at a density of 10^6 cells per 10 cm dish. After 20–24 hr of culture, cells were transfected with 2 μg of DNA by the DEAE-dextran method as described previously (17), and harvested 66–72 hr later. Mock transfection was carried out using the CDM8 vector without insert cDNA. The nuclear extract was prepared by the method of Dignam *et al.* (18). The typical nuclear extract contained 5mg/ml of protein, and 0.1–2 μl of aliquot was used for Western blotting and DNA binding assays.

Western blotting analysis

One-μl samples of the nuclear extracts of COS cells transfected with mutated CDM8-RBP-2 were applied to a SDS-gel. Also applied were 0.1–2 μl samples of the nuclear extract of the cells transfected with wild type CDM8-RBP-2 which served as a reference for quantitative comparison of the contents of the mutated RBP-Jκ proteins in the extracts. After electrophoresis, separated proteins were transferred to a nitrocellulose filter. The filter was probed with a mixture of rat monoclonal antibody against the mouse RBP-Jκ protein as described previously (6). The filter was then incubated with ^{125}I-labeled anti-rat IgG antibody, exposed on an imaging plate and the amount of the RBP-Jκ protein and its derivatives were analyzed by an image analyzer (BAS2000, Fuji Film Co.Ltd., Japan).

DNA binding assay

The DNA probe used for the DNA binding assay was the oligonucleotide derived from the m8 gene promoter region of the *Drosophila* Enhancer of split gene cluster (19). The m8 promoter contains the best naturally occurring binding sequence (shown with underline in the following sentence) for the mouse RBP-Jκ protein (20). The oligonucleotide with the sequence of 5′-GATCCGTCGACGGGGCACTGTGGGAACGGAAAGAGT-3′ was cloned between *BamHI* and *XbaI* sites of the pUC19 vector. The DNA fragment containing the m8 probe sequence was isolated by digestion of the resulting plasmid with *EcoRI* and *HindIII*, labeled, and used for the electrophoretic mobility shift assay (EMSA) as described previously (1). The DNA binding reaction was carried out using 2 ng of the ^{32}P-labeled m8 probe and 1 μl (approx. 5 μg protein) of the nuclear extract. After electrophoresis, the gel was dried, exposed on an imaging plate and analyzed by the BAS2000 image analyzer. The DNA binding activities were calculated from the radioactivities in the shifted bands after correction with the amounts of the RBP-Jκ protein derivatives in the nuclear extracts. Western blotting and DNA binding assay of each mutant protein was repeated 2–4 times and the representative data were shown in the figures in this paper.

RESULTS

DNA binding activity of deletion mutants

To locate regions essential to DNA binding, we first constructed a series of truncated RBP-Jκ cDNA utilizing 9 restriction enzyme sites (see Fig.1a for restriction sites). The truncated cDNAs were cloned into the CDM8 vector and expressed in COS cells. The N-terminal and C-terminal truncated proteins except for CΔ1442Pst and CΔ1115Apa were not expressed in COS cells (data not shown), suggesting that the precise three dimensional structure might be important for the stability of the RBP-Jκ protein. CΔ1442Pst which had a deletion of 46 amino acids in the C-terminus was expressed at the same level as the wild type protein, and retained the complete DNA binding activity (Fig. 4). Although CΔ1115Apa with a deletion of 156 amino acid in the C-terminus was expressed at 1/5 of the wild type protein, this protein did not bind to the DNA probe (data not shown).

We have shown that the mouse RBP-Jκ gene produces two types of mRNA (corresponding to cDNA clones RBP-2 and RBP-2N) by alternative splicing (20). The two proteins derived from the two mRNAs differ in the amino acid sequence encoded by exon 1 but have the same DNA binding activity. Therefore, the N-terminal 46 amino acids of the RBP-Jκ protein (clone RBP-2) is not involved in DNA binding. Together with the above result with the truncated mutants, we concluded that the N-terminal 46 and C-terminal 46 amino acids were not necessary for the DNA binding activity. Deprivation of the DNA binding activity in CΔ1115Apa may be due to modification of three dimensional structure by this deletion because of an extremely low yield of the mutant protein (also see below).

Table 1. DNA binding activities of insertion mutants

Mutant	Protein amount in extract	DNA binding activity in extract	Specific activity
Ins-33Spl	26	43	165
Ins-200Dra	74	74	100
Ins-423Pvu	81	105	130
Ins-685Kpn	45	10	22
Ins-828Hind	40	24	60
Ins-971Rsa	88	38	43
Ins-1115Apa	97	62	64
Ins-1265Rsa	48	46	96

The amounts and the DNA binding activities of the mutant proteins were determined by densitometric analysis of the autoradiograms in Fig. 1b and shown as values relative to those for the wild type RBP-Jκ protein (=100%). Both western blotting and DNA binding activities were quantitated within a linearly increasing range. The specific binding activity of each mutant protein was calculated from the DNA binding activity divided by the amount of the mutant protein in the nuclear extract. Experiments using different lots of DNA probes were repeated several times and essentially similar results were obtained.

DNA binding activity of linker insertion mutants

We then introduced linker mutations by inserting appropriate *SalI* recognition oligonucleotide linkers at various restriction sites of the RBP-Jκ cDNA (Fig. 1a). Ins-685Kpn which disrupted the normal amino acid sequence at 227Arg decreased the DNA binding activity to about 20% of the wild type protein (Fig. 1b and Table 1). Ins-971Rsa with disruption at 323Tyr decreased the DNA binding activity marginally but reproducibly to approximately 40% of the wild type protein. Ins-828Hind (disruption at 275Lys) showed an almost normal DNA binding activity (60% of the wild type protein). Mutations at *ApaI* [1115] and *RsaI* [1265] sites (disruptions at 372Pro and 421Tyr, respectively) did not significantly change the DNA binding activity in support of the above conclusion that loss of the DNA binding activity in CD1115Apa might be due to conformation change by a large deletion. Linker mutations in the N-terminal

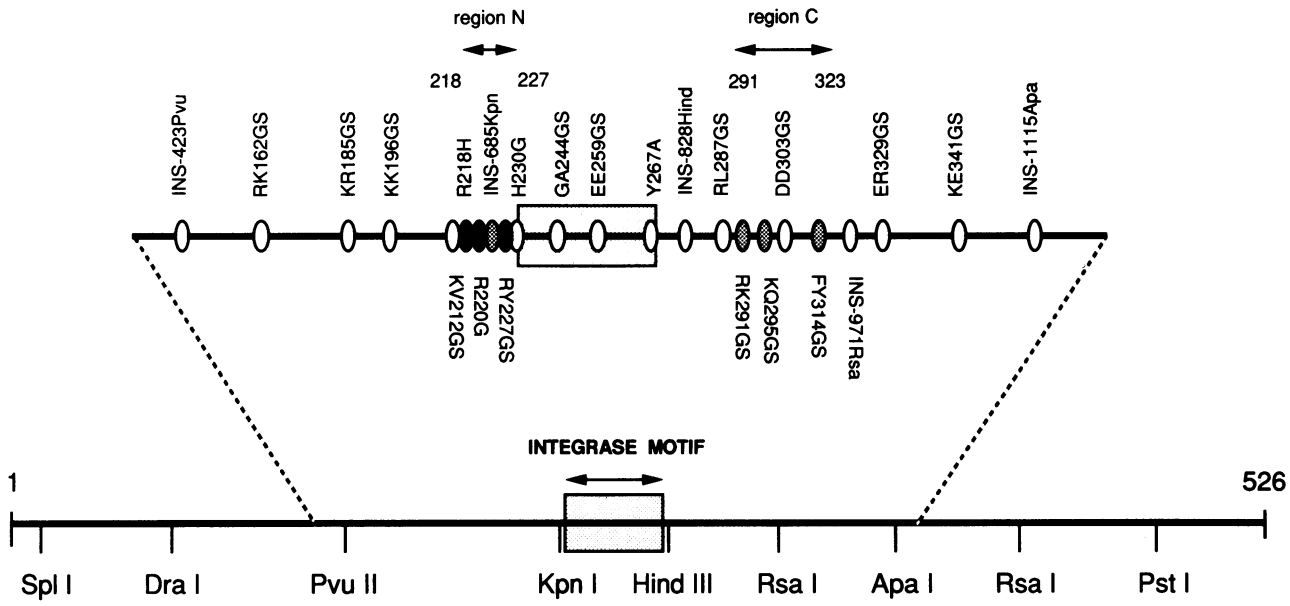

Figure 2. Positions of mutations with amino acid changes. Also shown are the positions of linker insertion mutants between PvuII[423] and ApaI[1115]. Open, shaded, and solid marks indicate mutations with normal, marginally decreased (20% −40% of the wild type RBP-Jκ protein), and significantly decreased (less than 20%) DNA binding activity, respectively. Regions N and C (see text) are indicated by horizontal arrows with residue numbers.

region (Ins-33Spl, Ins-200Dra, and Ins-423Pvu) did not decrease the DNA binding activity. These results suggest that regions important for the DNA binding may be localized in the middle of the protein around the integrase motif (between 230 His and 269 His). We, therefore, focused our analysis to the region between the *Pvu*II [423] and *Apa*I [1115].

DNA binding activity of replacement mutants

We chose mainly charged amino acid residues in the *Pvu*II[423]−*Apa*I[1115] region, and replaced each one of the chosen residues and its C-terminal adjacent residue to ArgSer or to GlySer (Fig. 2). Replacement to ArgSer at all positions examined did not alter the DNA binding activity of the RBP-Jκ protein (data not shown). But the DNA binding activity was decreased when the replacement to GlySer was introduced at 227ArgTyr, 291ArgLys, 295LysGln, or 314PheTyr (Fig. 2 and 3).

Since the replacement at 227ArgTyr (RY227GS) decreased the DNA binding activity drastically to 6%, three point mutations with a single amino acid change were made between 212Lys and 230His to analyze this region further (Fig. 3a). Two of such mutations (R218H and R220G) decreased the DNA binding activity to 2 and 8% of the wild type, respectively (Fig. 3b and Table 2). The other mutation (H230G) within the integrase motif affected the DNA binding activity only slightly (46% of the wild type). The linker mutant Ins-685Kpn in this region affected the binding activity as described above (Table 1). The R218H, R220G, and RY227GS mutant proteins were expressed in COS cells at high levels (160% or higher as compared to the wild type protein) (Table 2), suggesting that the mutation in this region may not affect the stability, and probably the conformation of the RBP-Jκ protein. Taken together, we conclude that the structure of the region containing 218Arg and 227ArgTyr is essential to the DNA binding of the mouse RBP-Jκ protein.

Amino acid changes at 244GlyAla (GA244GS) and 259GluGlu (EE259GS) to GlySer within the integrase motif (230His-269His) did not affect the DNA binding activity (Fig. 3b and Table 2). Amino acid changes at the same sites to ArgSer also did not affect the DNA binding activity either (data not shown). Another point mutation (Y267A) was introduced within the integrase motif. Both H230G and Y267A, mutations at two of the three residues which are strictly conserved among integrase family recombinases (3,4), did not decrease the DNA binding activity significantly (Table 2). These results indicate that the integrase motif itself does not contribute to the binding to DNA.

The amino acid change at 291ArgLys, 295LysGln or 314PheTyr to GlySer (RK291GS, KQ295GS or FY314GS) decreased the DNA binding activity to approximately 30% of the wild type protein (Fig. 3b and Table 2). The expression level of the RK291GS protein was always very low (less than 20% of the wild type protein in repeated experiments) and, therefore, the effect of this mutation was ambiguous. The replacement at 287ArgLeu or 329GluArg to GlySer (RL287GS or ER329GS), however, did not affect the DNA binding. As described above, the linker mutant Ins-971Rsa (with disruption at 323Tyr) had the decreased binding activity (Table 1). Taken together, the RBP-Jκ protein seems to have a second region that influences the DNA binding activity. This region is located between 291ArgLys and 323Tyr. The effects on the DNA binding activities of mutations within this region were much smaller than those of mutations in the 218Arg−227ArgTyr region.

RBP-Jκ protein binds to DNA as a monomer

To test whether the RBP-Jκ protein binds to DNA as a dimer or not, we co-transfected COS cells with the CΔ1442Pst mutant and wild type RBP-Jκ cDNAs and analyzed the electrophoretic mobilities of the probe-bound forms of the protein products (Fig. 4). The probe-bound CΔ1442Pst protein migrated faster than the

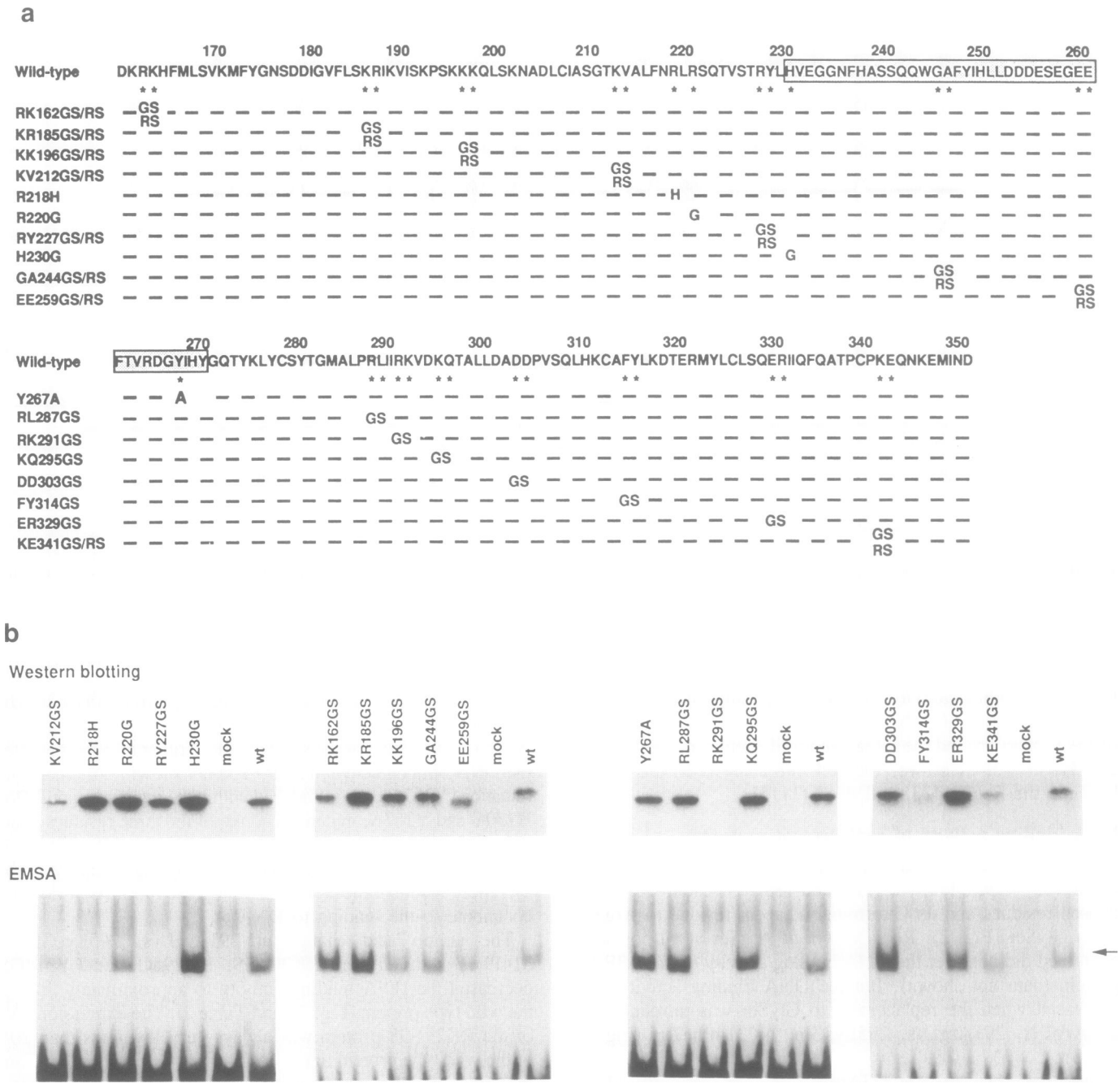

Figure 3. DNA binding activities of mutants with amino acid changes in the region between amino acid residues 161 and 350. Panel **a**, positions and structures of mutants. Box indicates the integrase motif. Panel **b**, Western blotting and EMSA of the nuclear extracts of COS cells transfected with the mutants and the wild type RBP-Jκ cDNA.

probe-bound wild type protein. Co-expression of these two proteins did not give rise to a new shifted band with an intermediate mobility, indicating that the CΔ1442Pst and wild type proteins could not form a complex. We tentatively concluded, therefore, that the RBP-Jκ protein binds DNA as a monomer, although we can not completely exclude the formal possibility that the C-terminal truncation prevents the complex formation with the wild type protein but not with the mutant itself.

DISCUSSION

The data presented in this study provide evidence that the full DNA binding ability of the mouse RBP-Jκ protein requires two separate regions on both sides of the integrase motif (Fig. 2); the region N (218Arg−227Arg) and the region C (291ArgLys−323Tyr). These two regions are within the most highly conserved regions of RBP-Jκ proteins between *Drosophila*

Table 2. DNA binding activities of mutants with amino acid changes.

Mutant	Protein amount in extract	DNA binding activity in extract	Specific activity
RK162GS	97	246	254
KR185GS	334	283	85
KK196GS	189	134	71
KV212GS	31	15	48
R218H	603	10	2
R220G	556	47	8
RY227GS	160	9	6
H230G	516	235	46
GA244GS	174	140	80
EE259GS	105	89	85
Y267A	74	111	150
RL287GS	114	115	101
RK291GS	18	5	28
KQ295GS	165	53	32
DD303GS	177	289	163
FY314GS	70	24	34
ER329GS	282	200	71
KE341GS	92	94	102

The amounts and the DNA binding activities of the mutant proteins were determined by densitometric analysis of the autoradiograms in Fig. 3b and shown as values relative to those for the wildtype RBP-Jκ protein (=100%). The specific binding activity of each mutant protein was calculated from the DNA binding activity divided by the amount of the mutant protein in the nuclear extract. As described in Table 1 all data were calculated within a linear range and repeated at least several times using different lots of DNA probes.

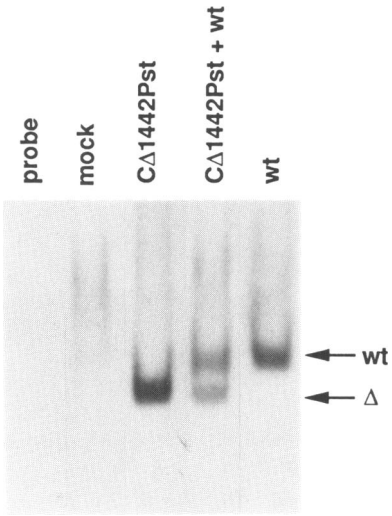

Figure 4. The RBP-Jκ protein binds to the DNA probe as a monomer. COS cells were transfected with CDM8-RBP-2 and CΔ1442Pst either separately or together, and the nuclear extracts were examined by EMSA. Arrows indicate the positions of the wild type (wt) and truncated (Δ) RBP-Jκ proteins associated with the DNA probe.

and mouse (7). The region N is located within the especially highly conserved region; among 46 amino acids between 184Ser and 229Leu, only one amino acid (212Lys) in the mouse RBP-Jκ protein was replaced to Asn in the *Drosophila* RBP-Jκ protein. In fact, the replacement of 212LysVal to GlySer in the mouse RBP-Jκ protein decreased the DNA binding activity only slightly (Table 2). Among 55 amino acid residues between 275Lys and 329Glu of the mouse RBP-Jκ protein, only four amino acids are different from the *Drosophila* RBP-Jκ protein. Since the DNA binding specificity of the *Drosophila* RBP-Jκ protein was almost the same to that of the mouse RBP-Jκ protein as examined with various Jκ RS mutant probes (7), it is, quite reasonable to assume that these two regions are important for the DNA binding activity of the RBP-Jκ protein.

Needless to say, the loss of function by site-directed mutagenesis does not demonstrate that the mutated residue is directly involved in that particular function. We have tried to produce various partial polypeptides of RBP-Jκ which carries the DNA binding activity in Cos cells or *E.coli* without success. As described above N- or C-terminal truncation of RBP-Jκ appears to perturb the stable conformation of RBP-Jκ. Although we could not provide the direct evidence that region N interacts with DNA, the following reasons made us to propose that this is the case; 1) three independent replacement mutations within this region almost completely abolished the DNA binding activity; 2) the amounts of these mutant proteins were not reduced, suggesting little change of the stability and conformation of the proteins; 3) no other mutations had such drastic loss of function except for large trunctions which usually caused instability of the proteins. The region C only partially contributes to the DNA binding ability, suggesting that the region C may be important for the correct conformation of the region N. We searched the database for proteins containing sequences similar to these regions, but could not find any. The prediction of the secondary structure by two algorithms (21, 22) revealed that the region N would not have any characteristic structure but that the region C could form two α-helices connected by a turn structure. The DD303GS mutant which retained the full DNA binding activity had the amino acid changes within the putative turn. Four other region C mutations with decreased DNA binding activity all fell within the putative α-helix regions. This finding suggests that the helix structure in the region C may be important for the DNA binding activity.

The present data show that the integrase motif of the mouse RBP-Jκ protein is not involved in DNA binding. The integrase motif of some members of the integrase family have been shown to be dispensable for the target sequence recognition (23, 24). In the yeast Flp recombinase, the DNA binding domain was localized within the region around Arg at position 191 that is 115 residues N-terminal to the integrase motif (24). The function of the integrase motif in the RBP-Jκ protein is not clear. The integrase motif of the RBP-Jκ protein lacks the fourth Arg residue that is strictly conserved in all other members of the family, while the RBP-Jκ protein retains other two strictly conserved (230His and 267Tyr) and one highly conserved (269His) amino acids in the integrase motif (6). The fourth Arg residue in the integrase motif of the yeast Flp recombinase was shown to be important for the DNA cleavage activity that is essential for the recombination reaction (25). To explore the function of the integrase motif of the RBP-Jκ proteins, we have analyzed a *Drosophila* RBP-Jκ mutant (called HG36) (13), in which a Glu residue within the integrase motif is replaced with Lys (9). This amino acid change does not affect the DNA binding activity of the mutant (S. Maruyama and T. H., unpublished data) although the mutation causes loss-of-function. It is thus apparent that the integrase motif of the RBP-Jκ protein at least in *Drosophila* has physiological function other than DNA binding.

The regulatory role of *Drosophila* RBP-Jκ in the development of the peripheral nervous tissue implies that the *Drosophila* RBP-Jκ protein may function as a transcriptional regulator (9−14). Recently, we could show an activation of transcription by the

Drosophila RBP-Jκ protein in a co-transfection assay (T. F. and T. H., unpublished data). The integrase motif of the RBP-Jκ protein contains a cluster of negatively charged amino acids, that is characteristics of transactivation domains of a number of transcription factors (26). Furthermore, *Drosophila* HG36 mutant has the Glu to Arg change within this stretch. These findings suggest the possibility that the integrase motif of the RBP-Jκ protein may function as a transactivation domain. The integrase motifs of other members of the integrase family do not have such stretches of negatively charged amino acids (3,4).

The mouse RBP-Jκ protein has other characteristic structures. The region corresponding to the exon 1 of the RBP-2 cDNA clone (2) is rich in proline (8 prolines in 46 residues). This Pro rich region is not present in the protein encoded by the RBP-2N mRNA which is produced by alternative splicing from the same gene (20). There is a Ser/Thr rich region in its C-terminus (residues 460–526). The Ser and Thr content of this region is 38%. The Pro rich and Ser/Thr rich regions are not conserved in the *Drosophila* RBP-Jκ protein and are replaced by Asn/Gln rich sequences in *Drosophila*. The two regions are not required for DNA binding. These two regions might function as modification sites such as proteolytic cleavage, phosphorylation or glycosylation, but the exact functions of these regions remain to be elucidated. The RBP-Jκ protein is one of the most highly conserved protein from *Drosophila* to human (2,7,8) and indispensable for mouse development because RBP-Jκ knock-out mice are homozygously lethal (C. Oka, T. Nakano and T. H., unpublished data). The RBP-Jκ protein is a novel DNA binding protein probably involved in gene regulation during development of various tissues in mouse. In order to clearly show the physiological function and mode of action of the RBP-Jκ protein, it is necessary to identify target genes, to which the mouse RBP-Jκ protein binds specifically.

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