W194XProp1 and S156insTProp1, both of which have intact DNA-binding domain, show a different DNA-binding activity to the Prop1-binding element in human Pit-1 gene

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W194XProp1 and S156insTProp1 have a different DNA-binding activity to the Prop1-binding element in human Pit-1 gene

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Abstract

Genetic alternation of transcription factors that are involved in the differentiation of pituitary gland and pituitary hormone expressions causes congenital combined pituitary hormone deficiency (CPHD) in humans. The mutation of Prop1 is major abnormality causing CPHD. In humans the prevalence of Prop1 mutation among the patients with CPHD has been reported at 48 %(16). Prop1 activates Pit-1 gene expression, which in turn stimulates GH, PRL, TSHb gene expressions. Therefore the patients with Prop1 mutation show GH, PRL, TSHb deficiency. In addition, LH and ACTH deficiencies are sometimes observed in the patients although the reason is not known.

Many of Prop1 mutations are reported to be located within the putative DNA bindings sites(16, 18). Therefore, it is thought the CPHD in the patients with Prop1 mutation is due to the inability of Prop1 to bind and activate the Pit-1 gene. However, this has not been directly tested since the Prop1 binding sites have not been identified in the human Pit-1 gene. Studies have been conducted showing defects in DNA binding activity of mutant Prop1 by EMSA using consensus elements (PRDQ9) of paired-like transcription factors (19-22) including Prop1.

It is recently reported that two patients with CPHD have mutations in the putative transactivation domain but not DNA binding domain of Prop1(27, 28). Out of the mutations, a mutation W194X Prop1 showed less DNA binding to PRDQ9 than the wild Prop1(27), suggesting the possibility that a domain other than DNA binding domain affects the binding affinity and low DNA binding affinity might be responsible for

CPHD even if the mutation is located in the domain other than DNA-binding domain. Recently we identified Prop1-binding element in human Pit-1 gene. In the present study, we assessed the function of W194X and 156M Prop1, both of them were found in the patients with CPHD, to bind and activate Pit-1 gene.

Materials and Methods

Cell culture

GH3 cells were obtained from American Type Culture Collection (Manassas, VA) and were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% (vol/vol) calf serum. Culture media contained penicillin G (100 μg/ml) and kanamycin (15.5 μg/ml).

Plasmids

PRDQ9 oligonucleotides (upper 5'-GAAGATCTTCTAGACTAATTGAATTAGCAGATCTTC-3', lower 5'-GAAGATCTGCTAATTCAATTAGTCTAGAAGATCTTC-3') were annealed and inserted into *Bgl II* site of multi-cloning site of pGL3 promoter vector (Promega Japan, Tokyo, Japan), which was named PRDQ9-Luc. -1340-Pit-1-Luc that has 1370bp of human Pit-1 gene (from -1340 to +30, when the translation start site was numbered as +1) was described previously. -1340-Pit-1-Luc was cut by *Kpn*I and *BssH*II and re-ligated after the removal of the flagment from -1340 to -143. The resulting reporter plasmid contained 172bp human Pit-1 gene (from -142 to +30) and was named -142-Pit-1-Luc. Flag-CMV-Prop1 that express Flag-tagged Prop1 was described elsewhere. Mutant Prop1 expression vectors, Flag-CMV-W194XProp1 and Flag-CMV-S156insTProp1, were made by using site-directed mutagenesis kit (Stratagene, La Jolla,

CA). In the site-directed mutagenesis to make Flag-CMV-W194XProp1, mutant sense primer (5'-CCAGTCTGAGGACTG**A**TACCCTACCTTGCACCC-3') and mutant antisense primer (5'-GGGTGCAAGGTAGGGTA**T**CAGTCCTCAGACTGG-3') were used. For making Flag-CMV-S156insTProp1, mutant sense primer (5'-GCTTGCCCCTATT**T**CTTACGCAGCACCACCA -3') and mutant antisense primer (5'-TGGTGGTGCTGCGTAAG**A**AATAGGGGCAAGC -3') were used. Bold characters indicate the mutated sites. W194XProp1 and S156insTProp1 cDNA that had been cut out from Flag-CMV-194Prop1M and Flag-CMV-S156insTProp1 were inserted into *PpuM*I and *Xcm*I sites of pcDNA3.1/Prop1 and the resulting plasmids were named pcDNA3.1/W194XProp1 and pcDNA3.1/S156insTProp1, respectively. All the DNAs amplified by PCR were sequenced with a DNA sequencer [model ABI PRISM 377; PerkinElmer Japan (Tokyo, Japan); Applied Biosystems Japan (Tokyo, Japan)] to confirm DNA sequence.

Transient expression assays

pcDNA3.1/Prop1 (1.2 μg) and Pit-1 reporter plasmids (0.4 μg) or PRDQ9 reporter plasmid (0.4μg) were transfected to GH3 cells using Lipofectamine 2000 (Invitrogen Japan, Tokyo, Japan). Five ng pRL-CMV containing the cDNA encoding Renilla luciferase (Promega) were also cotransfected to evaluate transfection efficiency. Cells were harvested 48 h after transfection, and luciferase activity was measured with Luminescencer-PSN (ATTO, Tokyo, Japan) using dual-luciferase assay system (Promega). The luciferase activity was normalized with the activity of cotransfected pRL-CMV. Values were expressed as multiples of induction relative to the activity of the pGL3 basic vector and represent mean ± SD of at least three determinations.

Prop1 protein

The Prop1 cDNA (National Center for Biotechnology Information accession no. NM_006261) was amplified by PCR using primers (forward, 5'-ATGGAAGCAGAAAGGAGGCG-3'; reverse, 5'-AGAGGATCCTCAGTTCCAGGACTTGGATG-3') and pcDNA3.1/Prop1 as a template. The resulting cDNA was inserted into *Eco*RV and *Bam*HI sites of the pTD1 (Shimadzu, Tokyo, Japan) to produce Prop1 mRNA. The cDNA was transcribed using Thermo T7 transcription kit (TOYOBO, Osaka, Japan) and was purified with DyeEx 2.0 spin kit (QIAGEN). The resulting mRNA was translated using the Transdirect insect cell (Shimadzu) according to the manufacturer's instruction. W194XProp1 and S156insTProp1 proteins were also made by the same method. The W194XProp1 cDNA was amplified by PCR using primers (forward, 5'-ATGGAAGCAGAAAGGAGGCG-3', reverse, 5'-CGCGGATCCTCAGTCCTCAGACTGGTGTG -3') and pcDNA3.1/W194XProp1 as a template. The 156M Prop1 cDNA was amplified by PCR using primers (forward, 5'-ATGGAAGCAGAAAGGAGGCG-3'; reverse, 5'-AGAGGATCCTCAGACTGGTGTGACAAAGC-3') and pcDNA3.1/156M Prop1 as a template.

EMSA

Mobility shift assays were performed to assess DNA-binding activity of wild and mutant Prop1s to PRDQ9 and the proximal Prop1 Binding Element (PBE) in human Pit-1 gene ( ). PRDQ9 is a sequence of paired-like transcription factors-binding motif,

and PBE is the DNA element from -88 to -43 of the human Pit-1. These oligonucleotides were labeled with digoxigenin (DIG) using terminal transferase. Binding reactions contained 62 fmol of the labeled probe, varying amounts of Prop1, 1 μg poly(deoxyinosine-cytosine), 0.1 μg poly-L-lysine, 20 mM HEPES (pH 7.6), 1 mM EDTA, 10 mm $(NH_4)_2SO_4$, 1 mM dithiothreitol, 2% Tween 20, and 30 mM KCl in a total volume of 15 μl. Reaction mixtures were incubated for 15 min at room temperature. For binding competition assays, 8 pmol unlabeled oligonucleotides were added to the reaction mixture. After incubation, the reaction mixtures were applied to nondenaturing polyacrylamide gel. After electrophoresis, DIG-labeled DNA fragments were transferred onto a Hybond-N-membrane (GE Healthcare, Tokyo, Japan) and detected using the DIG gel shift assay kit (Roche Diagnostics, Tokyo, Japan).

Statistical analysis

All data are presented as means ± SD. After ANOVA analysis where appropriate, Tukey-Kramer test was used to analyze differences between groups. $P < 0.05$ was considered as significant.

Results

Transient expression assay

pcDNA3.1/Prop1, pcDNA3.1/W194XProp1 and pcDNA3.1/S156insTProp1 increased luciferase activity of PRDQ9-Luc in GH3 cells by 2.6, 1.8 and 1.5 folds, respectively, compared with pcDNA3.1 (Fig.2A). On the other hand, pcDNA3.1/S156insTProp1 and pcDNA3.1/S156insTProp1 did not stimulate the expressions of -1340-Pit-1-Luc whereas pcDNA3.1/Prop1 markedly stimulated the expressions. -1340-Pit-1-Luc has

1340bp of 5'-flanking region of hPit-1 gene, in which PBE and PRD9-like elements are present ( ). -142-Pit-1-Luc has PBE but not a PRD9-like element. Next, we used -142-Pit-1-Luc as a reporter plasmid. pcDNA3.1/Prop1 and pcDNA3.1/W194XProp1 activated -142-Pit-1-Luc expression, although the activation by pcDNA3.1/W194XProp1 was modest compared with pcDNA3.1/Prop1 (Fig.2B). However, pcDNA3.1/S156insTProp1 did increase luciferase activity of -142-Pit-1-Luc (Fig.2C).

EMSA

To confirm the DNA binding activity of wild and mutant Prop1s to PRDQ9 and PBE, we performed EMSA. When PRDQ9 was used as a probe, wild Prop1 and W194XProp1 specifically bound to the probe. The density of shifted bands by W194Xprop1 was comparable to that by wild Prop1 (Fig.3A). Also, there was not a marked difference in the density of shifted bands by wild Prop1 and S156insTProp1 (Fig.3B). When PBE was used as a probe, either wild Prop1 or W194Xprop1 showed specific bounding to the probe (Fig.3C). However, S156insTProp1 did not show significant binding to PBE (Fig.3D).

Discussion

In the present study, we found that S156insTProp1 bound to PRDQ9 but did not bind to PBE. This was consistent with the result that S156insTProp1 had no stimulating activity for -1340-Pit-1-Luc and -142-Pit-1-Luc expressions while it stimulated PRDQ9-Luc expression. S156insT is located in C-terminal domain, putative trans-activating domain, not in DNA-binding domain. This finding suggests that C-terminal domain might affect

DNA-binding to PBE and that EMSA using PRDQ9 probe may not be suitable for the test of DNA binding of Prop1.

Analysis of protein structure of paired like homeodomain proteins that including Prop1 has indicated that Prop1 is separated to three domains; N-terminal, middle, and C-terminal domains. The middle domain is reported as DNA-binding domain. The mutation of this domain has been reported not to have DNA-binding activity. Almost all the Prop1 mutations found in the patients with CPHD have mutations in the middle, DNA-binding domain. Indeed mutant Prop1s in the domain have been shown to lose DNA-binding function. On the other hand, only two cases were reported with the Prop1 mutant in a domain other than DNA-binding domain. The mutants are W194XProp1 and S156insTProp1 and both mutant Prop1s have been supposed to have intact DNA binding domain and the defect of C-terminal transactivating domain is responsible for the loss of function.

Therefore, we, at first, thought that the loss of transactivating function of S156insTProp1 was due to the lack of C-terminal part of Prop1. However, EMSA using PBE probe showed no binding activity of S156insTProp1 and that the loss of the function of S156insTProp1 can be attributable to loss of DNA binding activity. S156insTProp1 showed the binding activity for PRDQ9. A minute conformational change in DNA-binding domain of S156insTProp1 may influence the binding to PRDQ9 and PBE.

W194XProp1 is also a mutant Prop1 that has mutation in transactivating domain. However W194XProp1 showed a marked binding activity to PRDQ9 and PBE as well as wild Prop1 did. W194XProp1 has nonsense mutant in codon 194 and the amino acids sequence until 193 is preserved whereas S156insTProp1 has different C-terminal amino

acid sequence from codon 156 to xxx due to frameshift by intertion of another T in codon 156. The difference in amino acid sequence following DNA-binding domain appears to influence the binding to PBE. The transactivating function of W194XProp1 is not so strong as that of wild Prop1, although W194XProp1 has a marked DNA-binding activity comparable to wild Prop1. This may be attributed to defect in transactivating domain not but DNA-binding domain.

Until now, many Prop1 mutations were reported in the patients with CPHD. The DNA-binding activity was examined with EMSA using PRDQ9 as a probe, because Prop1-binding element was not identified in human Pit-1 gene. Since we recently identified PBE in human Pit-1 gene, we assessed the DNA-binding function of wild and mutant Prop1s using PBE, a natural target element of Prop1. If PBE probe were not used, the difference of the DNA binding activity of mutant Prop1 could not been found.

In summary, we analyzed two Prop1 mutants whose mutation is located in transactivating domain. W194XProp1 showed a marked DNA-binding activity comparable to that of wild Prop1. S156insTProp1 did not bind PBE in contrast to wild Porp1 and W194XProp1. However, the activating function for Pit-1-Luc expression was lost or decreased in both mutant Prop1s. These findings suggest that two mutant Prop1 may result in decreased expression of Pit-1 gene; defect of transactivating domain in W194XProp1 and the loss of DNA-binding activity in S156insTProp1.

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Culture, and grants from the Japanese Ministry of Health, Welfare and Labor and Growth Science Foundation.

References

Figure Legends

Fig.2. Different activation among PRDQ9-Luc, -1340-Pit-1-Luc and -142-Pit-1-Luc by wild and mutant Prop1.

A. pcDNA3.1/Prop1, pcDNA3.1/W194XProp1 or pcDNA3.1/S156insTProp1 (1.2μg) was transfected with PRDQ9-Luc(0.4μg) to GH3 cells. pcDNA3.1/Prop1 significantly stimulated lucuferase activity of PRDQ9-Luc (2.64 ± 0.22). pcDNA3.1/W194XProp1 (1.83 ± 0.09) and pcDNA3.1/S156insTProp1 (1.46 ± 0.20) also significantly increased the activity, but the fold inductions were modest compared with pcDNA3.1/Prop1. *, $P<0.05$ vs. pcDNA3.1 (control). B. pcDNA3.1/Prop1, pcDNA3.1/W194XProp1 or pcDNA3.1/S156insTProp1 (1.2μg) was transfected with -1340-Pit-1-Luc (0.4μg) to GH3 cells. pcDNA3.1/Prop1 (3.67±0.27) and pcDNA3.1/W194XProp1 (1.20±0.13) stimulated lucuferase activity of -1340-Pit-1-Luc, but pcDNA3.1/S156insTProp1 (0.64±0.06) did not. *, $P<0.05$ vs. pcDNA3.1. C. pcDNA3.1/Prop1, pcDNA3.1/W194XProp1 or pcDNA3.1/S156insTProp1 (1.2μg) was transfected with -142-Pit-1-Luc (0.4μg) to GH3 cells. Whereas wild pcDNA3.1/Prop1 (17.2±0.95) and pcDNA3.1/W194XProp1 (5.68±0.39) significantly incrreased lucuferase activity of -142-Pit-1-Luc, pcDNA3.1/S156insTProp1 (1.22±0.18) did not enhance the activity. *, $P<0.05$ vs. pcDNA3.1

Fig.3. Different DNA-binding affinity to PRDQ9 and PBE probes of wild, W194X and S156insT Prop1.

A. Increasing amount of Prop1 was incubated with 62 fmol of DIG-labeled PRDQ9 in the presence or absence of 130 fold excess of unlabeled PRDQ9 (lane2-5). Increasing amount of W194X Prop1 was incubated with 62 fmol of DIG-labeled PRDQ9 in the presence or absence of 130fold excess of unlabeled PRDQ9 (lane6-9). Wild and W194X Prop1 specifically bound PRDQ9 and the affinity appeared similar. Lane1, DIG-labeled PRDQ9 probe was incubated without Prop1 and subjected. B. Increasing amount of wild (lane2-5) or S156insT Prop1 (lane6-9) was incubated with 62 fmol of DIG-labeled PRDQ9 in the presence or absence of 130 fold excess of unlabeled PRDQ9. Wild and S156insT Prop1 specifically bound PRDQ9 and the affinity appeared similar. Lane1, DIG-labeled PRDQ9 probe was incubated without Prop1 and applied. C. Increasing amount of wild Prop1 was incubated with 62 fmol of DIG-labeled PBE in the presence or absence of 130 fold excess of unlabeled PBE (lane2-5). Increasing amount of W194X Prop1 was incubated with 62 fmol of DIG-labeled PBE in the presence or absence of 130 fold excess of unlabeled PBE (lane6-9). Lane1, DIG-labeled PBE probe(62fmol) probe was incubated without Prop1 and subjected. D. Increasing amount of wild (lane2-5) or S156insT Prop1 (lane6-9) was incubated with 62 fmol of DIG-labeled PBE in the presence or absence of 130 fold excess of unlabeled PBE. Wild Prop1 showed a specific binding to PBE dose-dependently. In contrast, S156insT Prop1 did not bind to PBE. Lane1, DIG-labeled PBE probe was incubated without Prop1 and applied.

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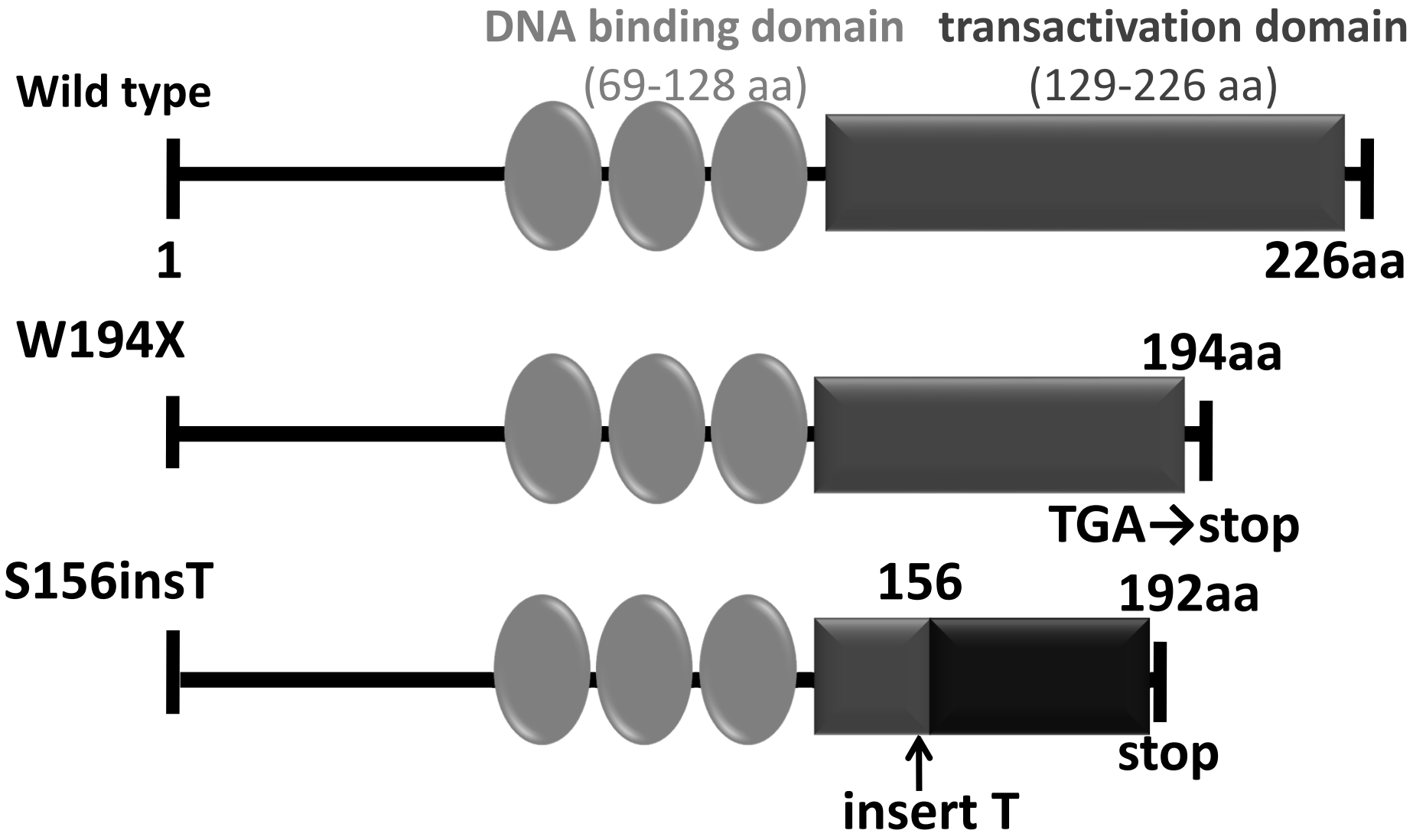
Figure1 候補


DNA binding domain
(69-128 aa)
transactivation domain
(129-226 aa)
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226aa
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194aa
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S156insT
156
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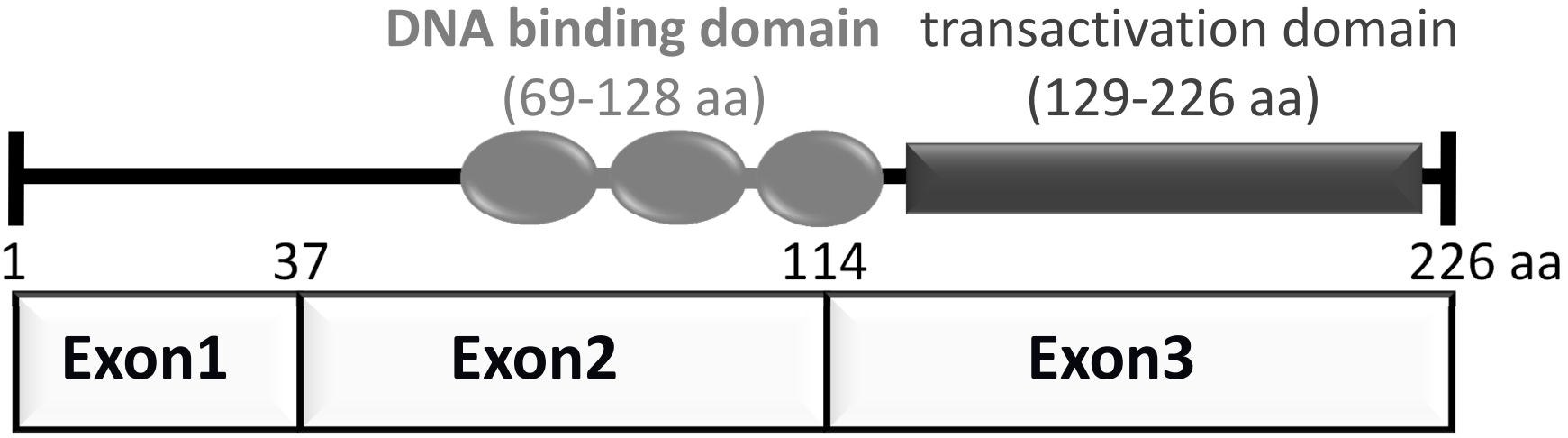
stop
insert T

Figure1 候補



Nucleotide change	Protein
2T→C	No translation
109+1G →T	Abnormal splicing
112-124del 13bp	Truncation at codon 480
149-150delGA	Truncation at codon 109
150delA	Truncation at codon 164
157delA	Truncation at codon 164
211 C→T	R71C
212G →A	R71H
217C →T	R73C
218G →A	R73H

Nucleotide change	Protein
247C →T	Q83X
263T →C	F88S
295C →T	R99X
296G →A	R99Q
301-302delAG	Truncation at codon 109
349T →A	F117I
358C →T	R120C
466-467insT	**Truncation at codon 192**
582G →A	**W194X**

Figure1 候補

Wild-type

5’-…(568)CAG TCT GAG GAC TGG TAC CCT ACC TTG CAC… -3’

Gln Ser Glu Asp Trp Tyr Pro Thr Leu His

W194X

5’-…CAG TCT GAG GAC TG**A** TAC CCT ACC TTG CAC …-3’

Gln Ser Glu Asp **Stop(194aa)**

Wild-type

5’-…(454)GCT TGC CCC TAT TCT TAC GCA GCA CCA CCA… -3’

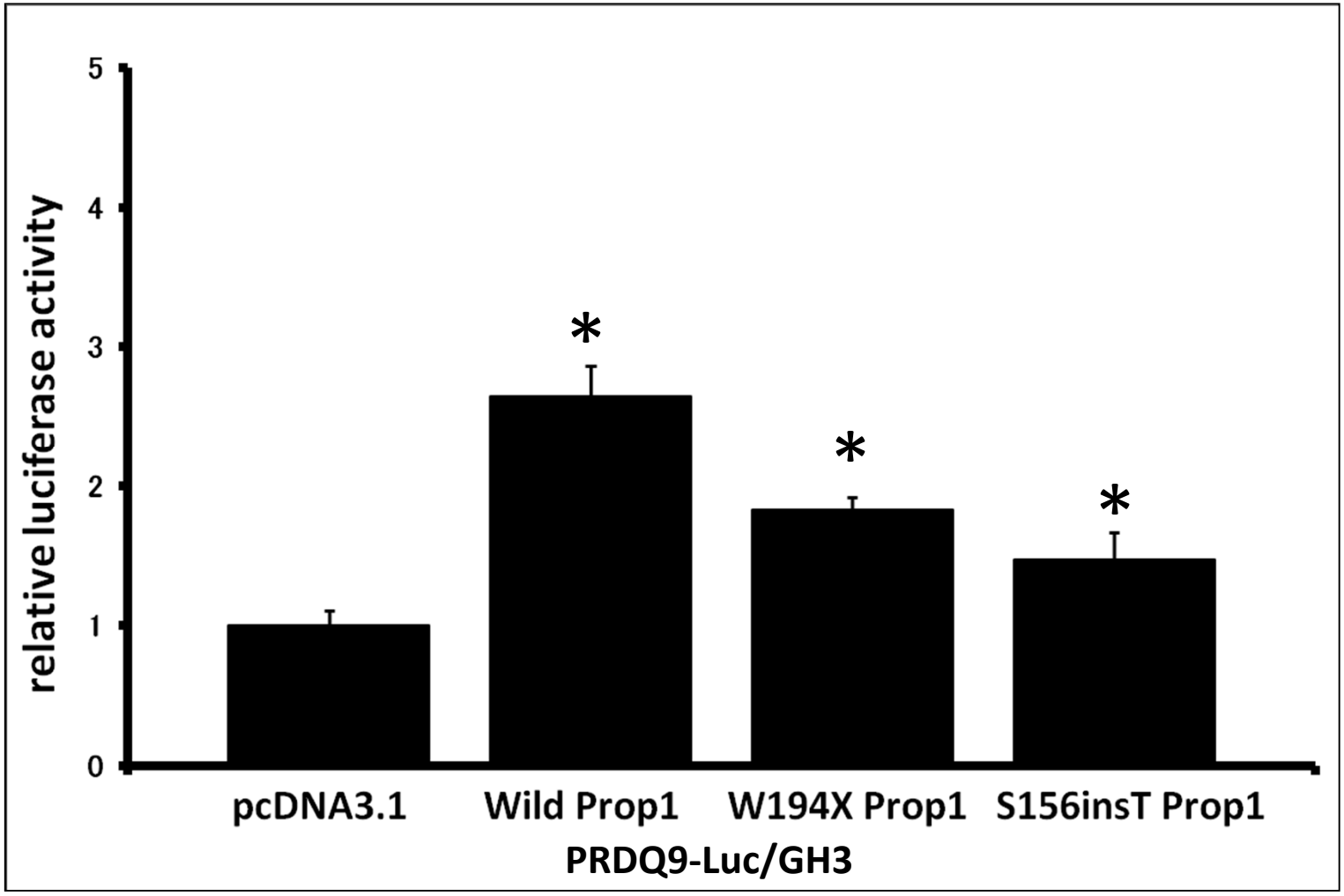
(152) Ala Cys Pro Tyr Ser Try Ala Ala Pro Pro…

S156insT

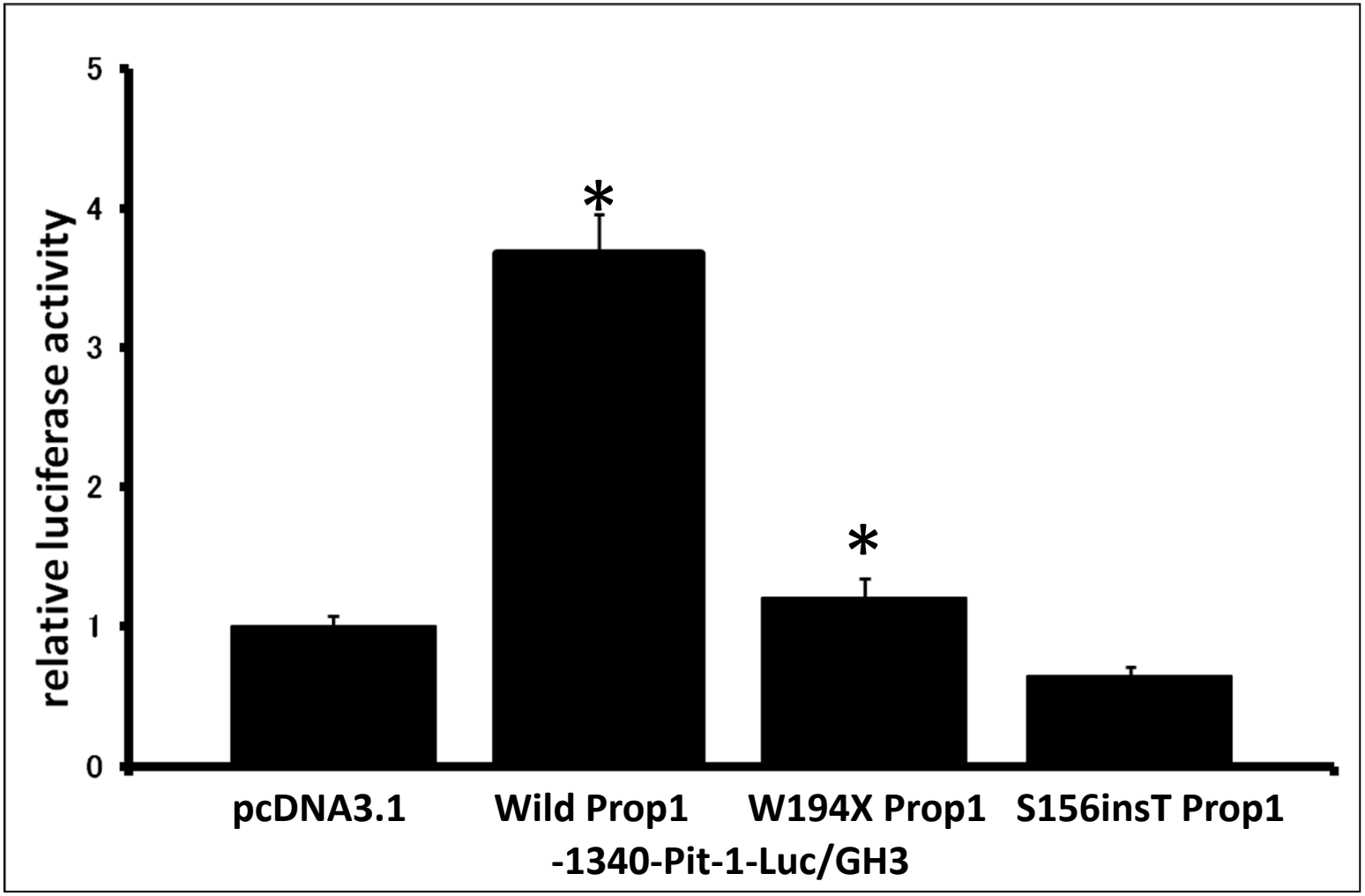
5’-…(454)GCT TGC CCC TAT T**T**C TTA CGC AGC ACC ACC A…….**TGA** GGA CTG…-3’

(152) Ala Cys Pro Tyr **Phe** Leu Arg Ser Thr Thr …….**stop(192aa)**

Figure 2 transient expression assays
A. Wild Prop1/pcDNA3.1, W194X Prop1/pcDNA3.1 or S156insT Prop1/pcDNA3.1(1.2μg) were transfected with PRDQ9-Luc(0.4μg) in GH3 cells. Wild Prop1(2.64±0.22) significantly stimulated lucuferase activity of PRDQ9-Luc, in contrast, W194X(1.83±0.09) and S156insT(1.46±0.20) slightly did.
*P<0.05 vs. pcDNA3.1



B. Wild Prop1/pcDNA3.1, W194X Prop1/pcDNA3.1 or S156insT Prop1/pcDNA3.1(1.2μg) were transfected with -1340-Pit-1-Luc(0.4μg) in GH3 cells. Wild Prop1(3.67±0.27) and W194X(1.20±0.13) stimulated lucuferase activity of -1340-Pit-1-Luc, but S156insT(0.64±0.06) didn't. *P<0.05 vs. pcDNA3.1



C. Wild Prop1/pcDNA3.1, W194X Prop1/pcDNA3.1 or S156insT Prop1/pcDNA3.1(1.2μg) were transfected with -142-Pit-1-Luc(0.4μg) in GH3 cells. Whereas wild Prop1(17.2±0.95) and W194X(5.68±0.39) significantly stimulated lucuferase activity of -142-Pit-1-Luc, S156insT(1.22±0.18) slightly did. *P<0.05 vs. pcDNA3.1

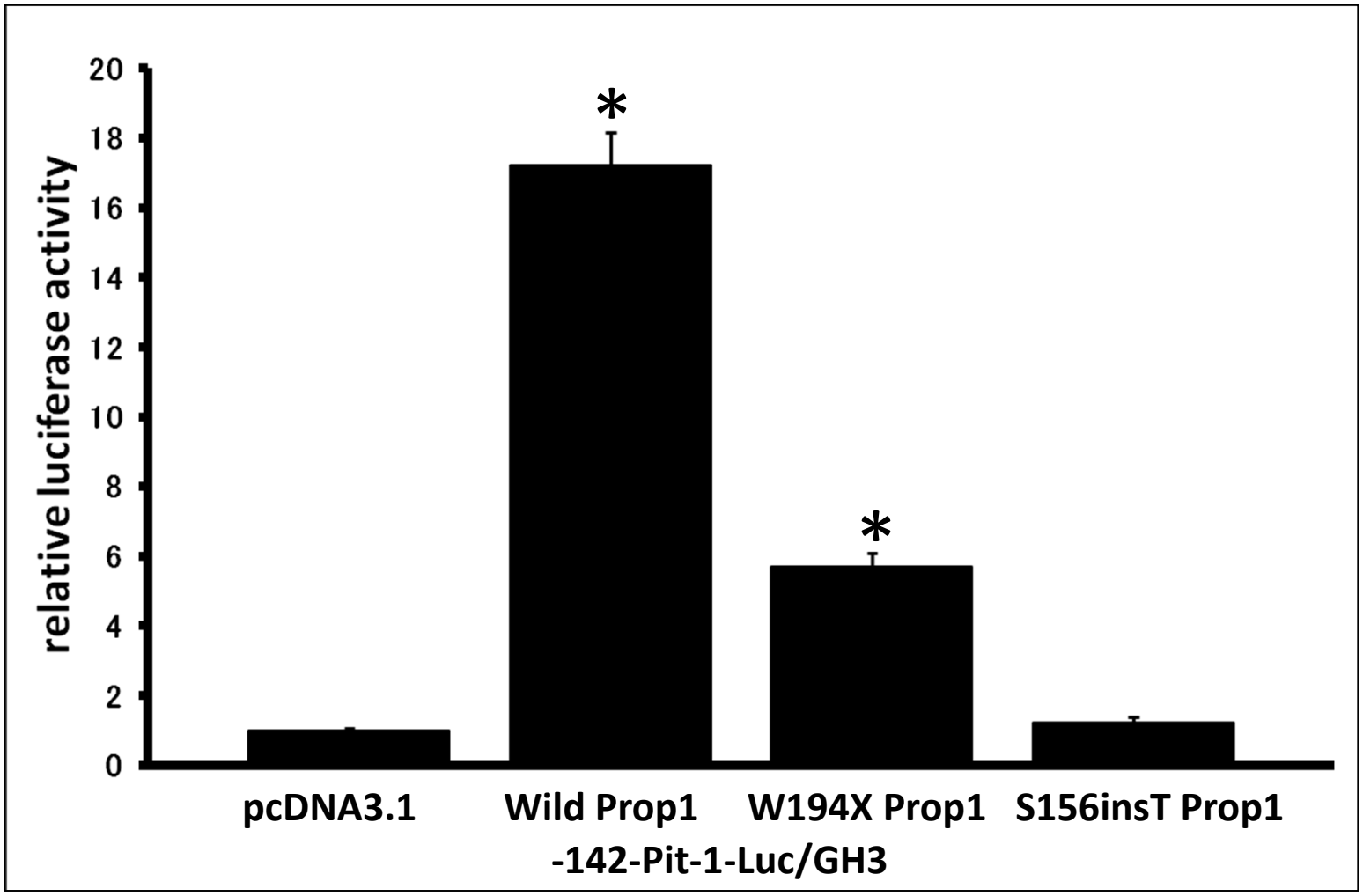
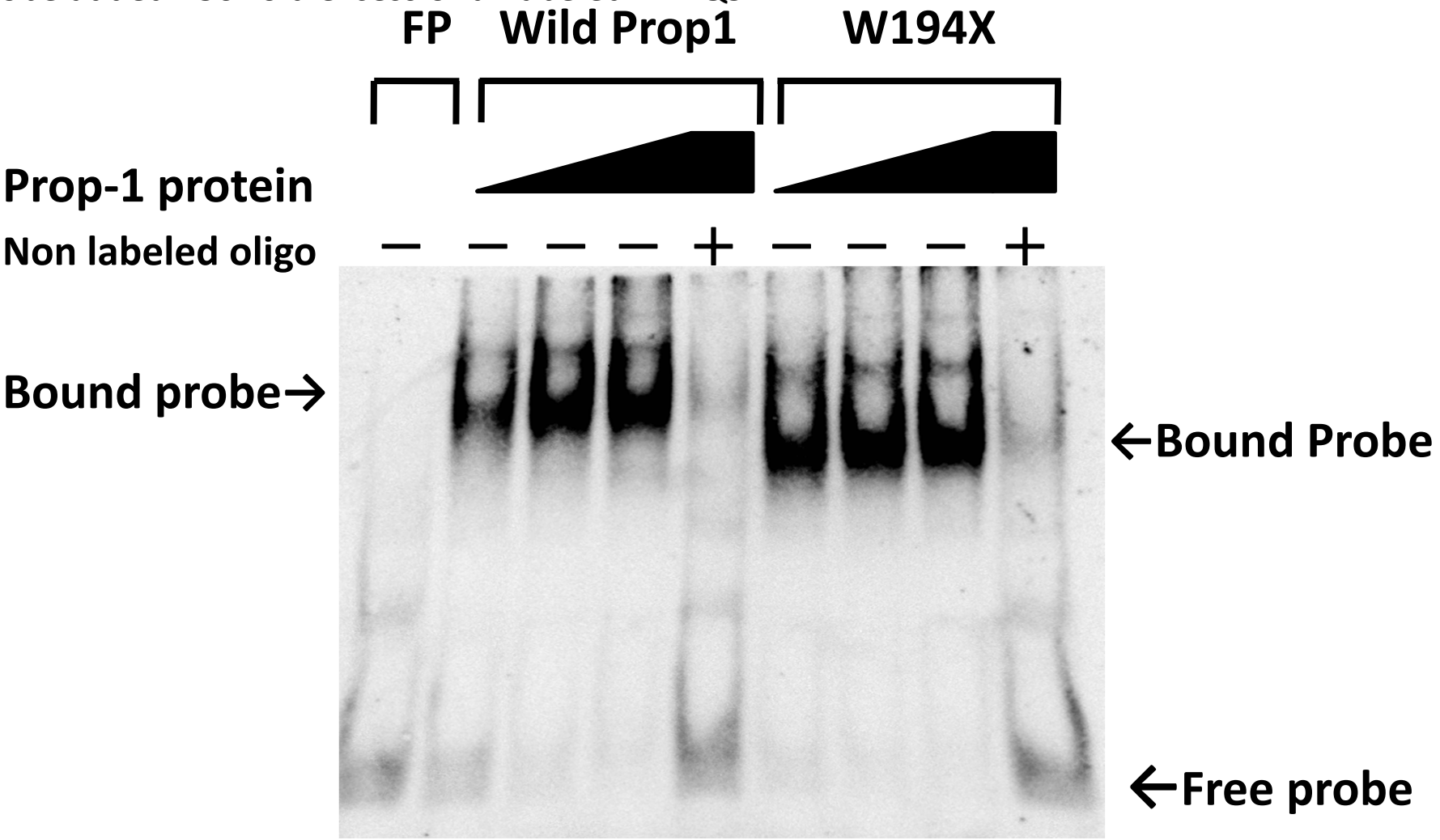
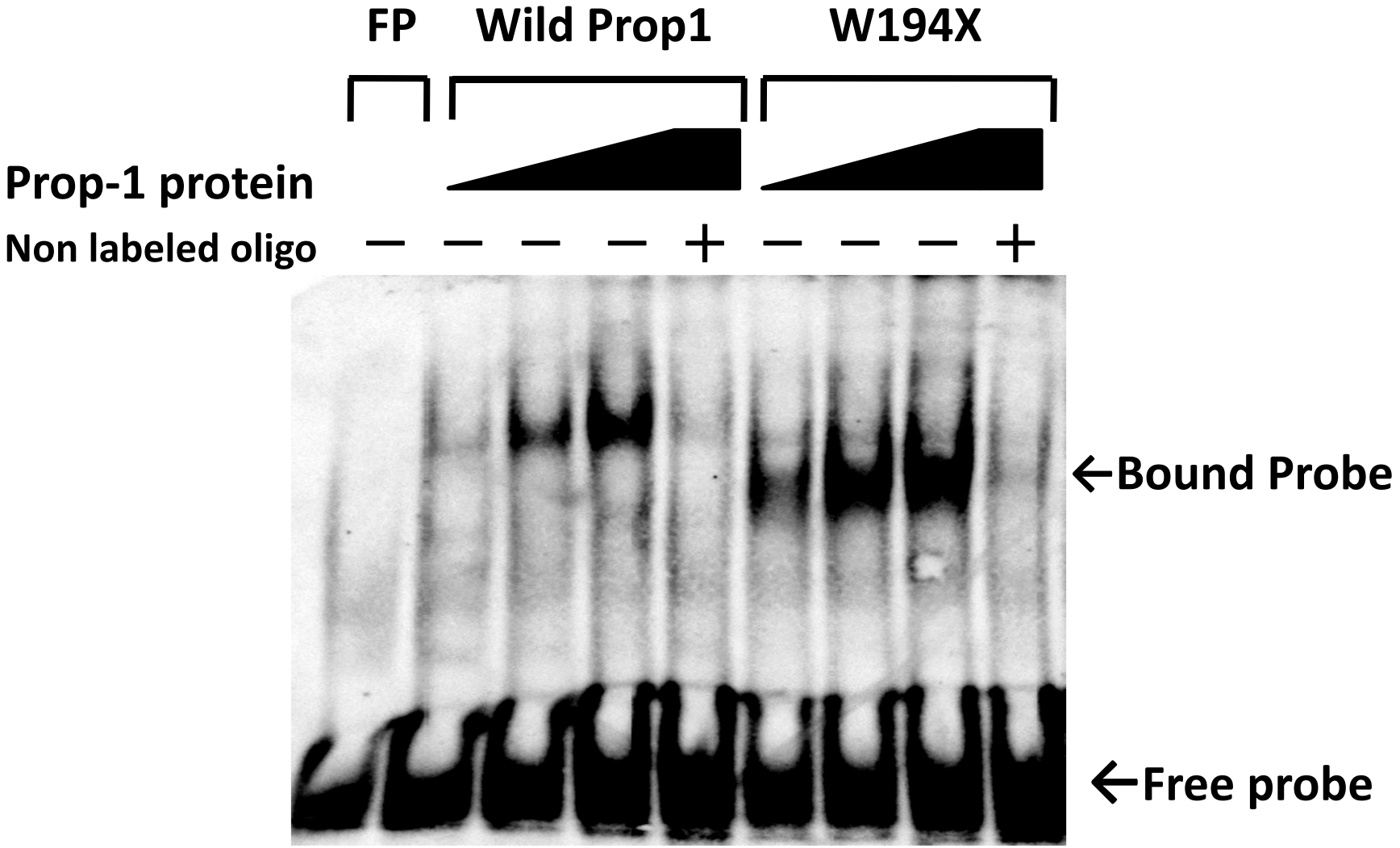


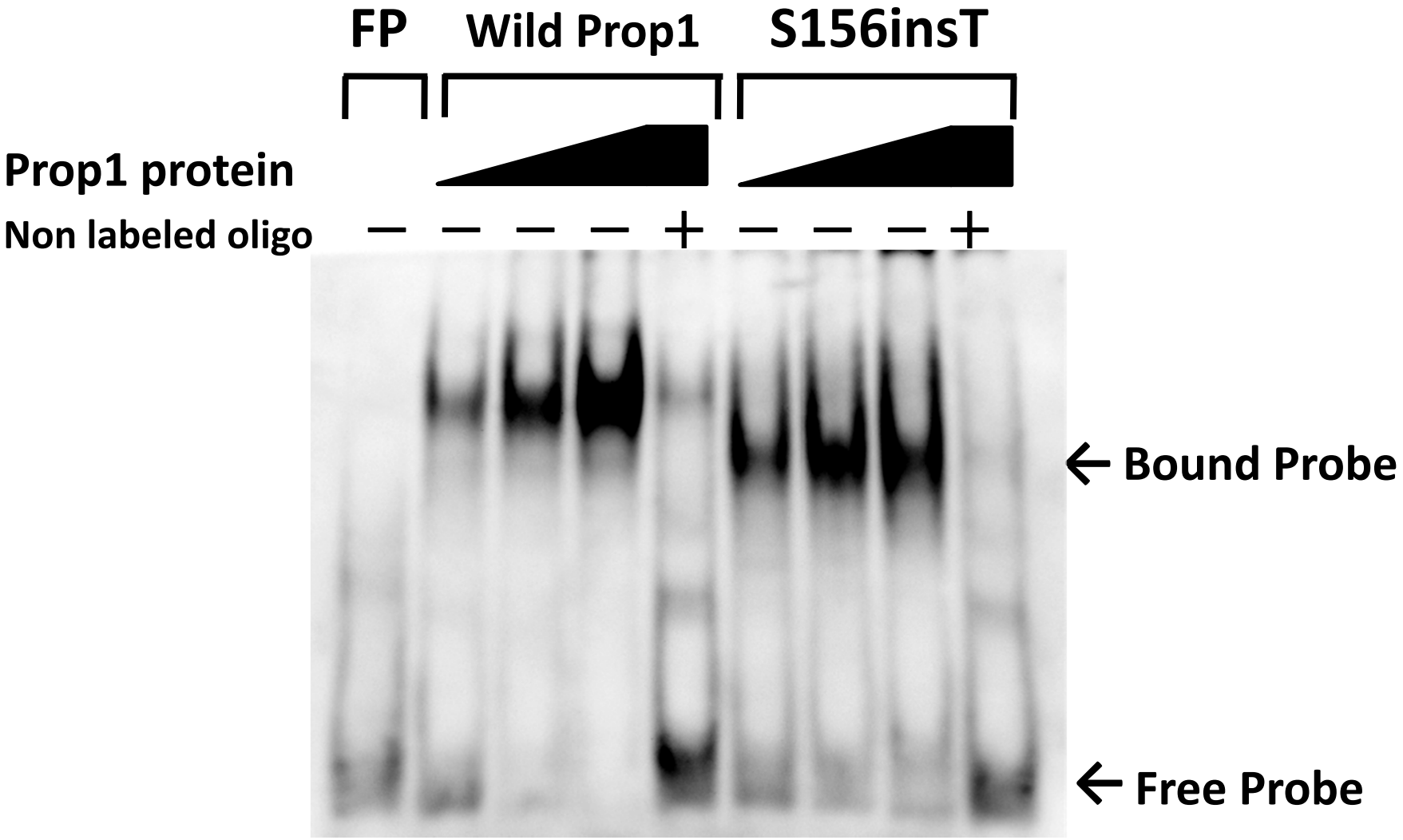
Figure3 EMSA A. Lane1, DIG-labeled PRDQ9 probe(62fmol). Lane2-4, Wild Prop1 protein(3.65, 7.3, 14.6μg) and DIG-labeled PRDQ9 probe. Lane5, Wild Prop1 protein(14.6μg) and DIG-labeled probe added 130-fold excess of unlabeled PRDQ9. Lane6-8, W194X Prop1 protein(3.65, 7.3, 14.6μg) and DIG-labeled PRDQ9 probe. Lane9, W194X Prop1 protein(14.6μg) and DIG-labeled probe added 130-fold excess of unlabeled PRDQ9.



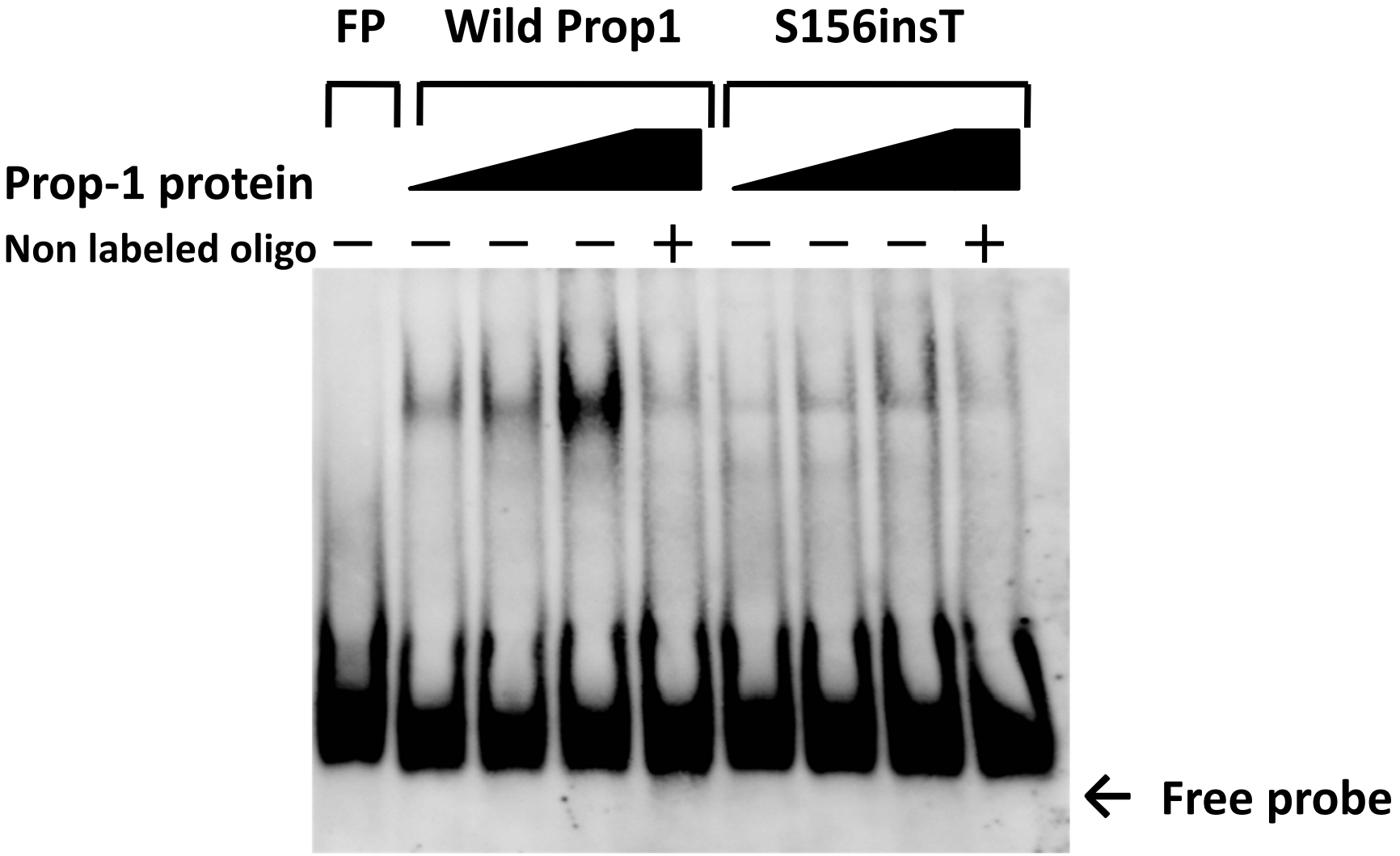
B. Lane1, DIG-labeled PBE probe(62fmol). Lane2-4, Wild Prop1 protein(3.65, 7.3, 14.6μg) and DIG-labeled PBE probe. Lane5, Wild Prop1 protein(14.6μg) and DIG-labeled PBE added 130-fold excess of unlabeled PBE. Lane6-8, W194X Prop1 protein(3.65, 7.3, 14.6μg) and DIG-labeled PBE probe. Lane9, W194X Prop1 protein(14.6μg) and DIG-labeled probe added 130-fold excess of unlabeled PBE.

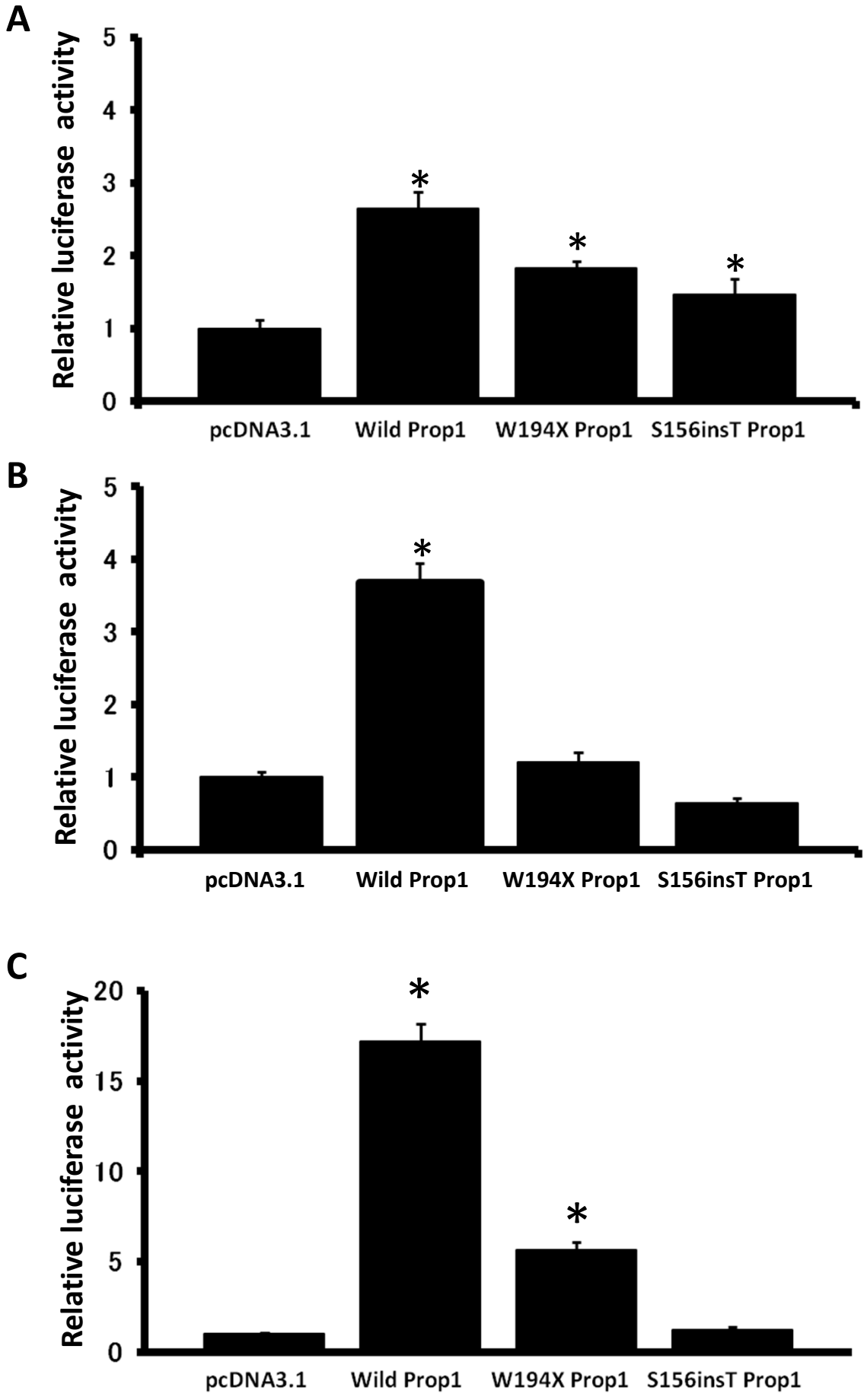


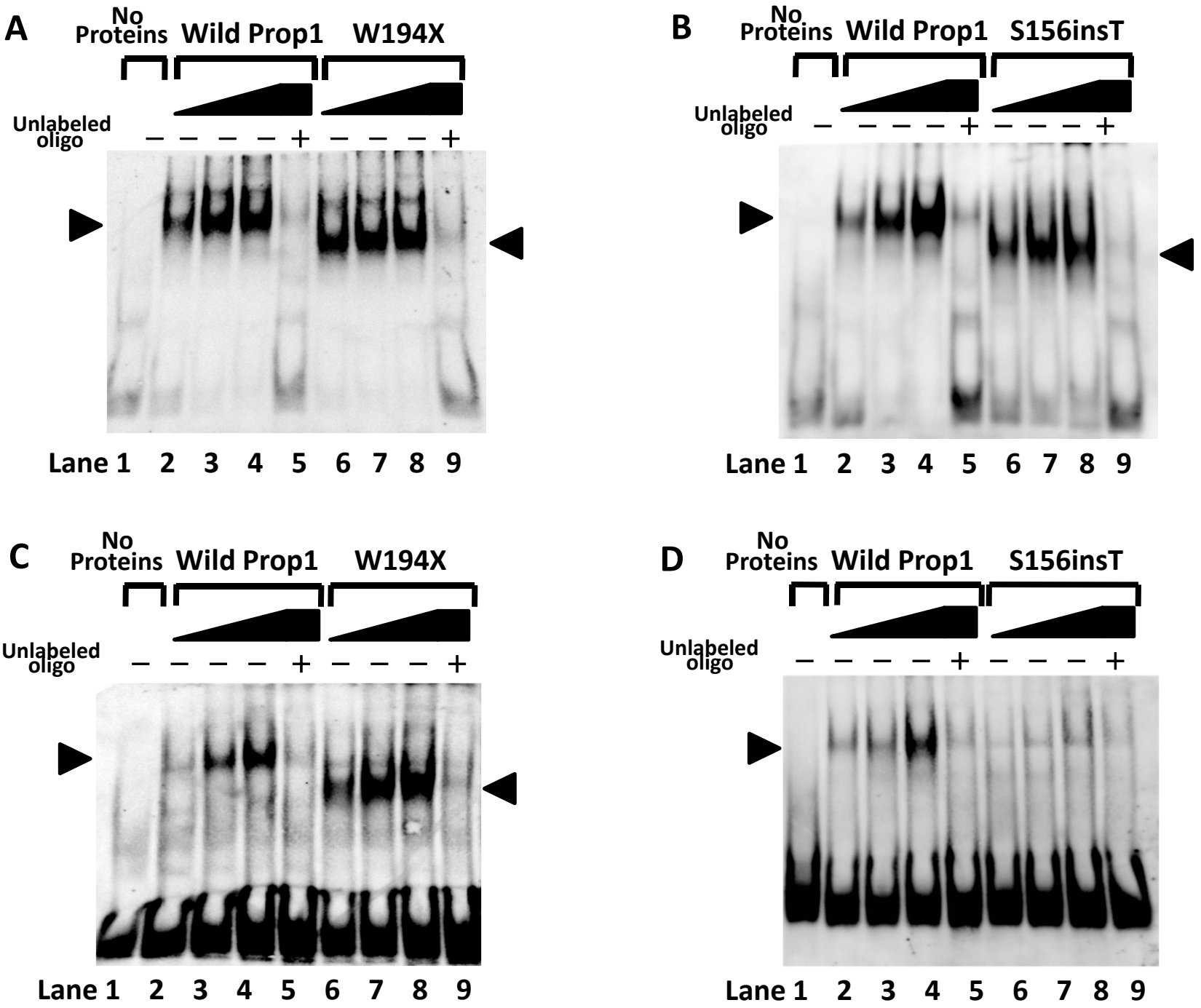
C. Lane1, DIG-labeled PRDQ9 probe(62fmol). Lane2-4, Wild Prop1 protein(3.65, 7.3, 14.6μg) and DIG-labeled PRDQ9 probe. Lane5, Wild Prop1 protein(14.6μg) and DIG-labeled PRDQ9 added 130-fold excess of unlabeled PRDQ9. Lane6-8, S156insT Prop1 protein(3.65, 7.3, 14.6μg) and DIG-labeled PRDQ9 probe. Lane9, S156insT Prop1 protein(14.6μg) and DIG-labeled probe added 130-fold excess of unlabeled PRDQ9.



D. Lane1, DIG-labeled PBE probe(62fmol). Lane2-4, Wild Prop1 protein(3.65, 7.3, 14.6μg) and DIG-labeled PBE probe. Lane5, Wild Prop1 protein(14.6μg) and DIG-labeled PBE added 130-fold excess of unlabeled PBE. Lane6-8, S156insT Prop1 protein(3.65, 7.3, 14.6μg) and DIG-labeled PBE probe. Lane9, S156insT Prop1 protein(14.6μg) and DIG-labeled probe added 130-fold excess of unlabeled PBE.




A
Relative luciferase activity
5
4
3
2
1
0
*
*
*
pcDNA3.1
Wild Prop1
W194X Prop1
S156insT Prop1
B
Relative luciferase activity
5
4
3
2
1
0
*
pcDNA3.1
Wild Prop1
W194X Prop1
S156insT Prop1
C
Relative luciferase activity
20
15
10
5
0
*
*
pcDNA3.1
Wild Prop1
W194X Prop1
S156insT Prop1


A
No Proteins
Wild Prop1
W194X
Unlabeled oligo
– – – – + – – – +
Lane 1 2 3 4 5 6 7 8 9
B
No Proteins
Wild Prop1
S156insT
Unlabeled oligo
– – – – + – – – +
Lane 1 2 3 4 5 6 7 8 9
C
No Proteins
Wild Prop1
W194X
Unlabeled oligo
– – – – + – – – +
Lane 1 2 3 4 5 6 7 8 9
D
No Proteins
Wild Prop1
S156insT
Unlabeled oligo
– – – – + – – – +
Lane 1 2 3 4 5 6 7 8 9