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BRIEF REPORT

Diversity of transactivation regions of DMRT1 in vertebrates

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Abstract

Background Doublesex and mab-3 related transcription factor (DMRT) 1, commonly found in all vertebrates, regulates the transcription of genes involved in the masculinization and maintenance of gonadal somatic cells and/or germline cell development. DMRT1 has a DNA-binding domain called the DM domain and a transcription regulatory region. Unlike the former, there is little knowledge about the latter transcription regulatory region. This study aimed to identify the transcription activation regions of DMRT1 from four species: humans and mice (mammals), leopard geckos (reptiles), and medaka (teleost fish), adding perspectives on evolutionary conservation and diversity.

Methods and results For each species, several expression plasmids of deletion mutants were constructed, and the resultant plasmid and a DMRT1-driven luciferase reporter were co-transfected into cultured cells to measure transactivation ability. The key point of this analysis is that the transactivation ability was normalized by quantifying the expression levels of DMRT1 variants using the HiBiT tag. As a result, two to three transactivation regions were suggested to exist in the C-terminal region of the DM domain in all four species. Among seven regions in DMRT1, the fourth region from the N-terminus contributed to transactivation common to the four species, and the sixth and seventh regions on the C-terminal side differed depending on the species.

Conclusions These findings indicated that the regions involved in the transactivation ability of DMRT1 could subtly change during evolution, indicating diversity in transactivation domains.

Keywords Transcription factor · Transactivation · DMRT1 · Vertebrate · Amino acid substitution

Abbreviations

DMRT Doublesex and Mab-3 Related Transcription Factor
TAD Transactivation Domain

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Introduction

dmrt1, belonging to *double sex and mab-3 related transcription factor* (*dmrt*) family, which encodes the DNA-binding domain (DM domain), is found only in vertebrates and is believed to have originated in the ancestors of vertebrates [1, 2]. Based on our previous analyses and those of other researchers, *dmrt1* is thought to have functioned in germ-line cells during early evolution of vertebrates, and during the evolution of gnathostomes, it may have acquired functions related to the masculinization and maintenance of somatic cells in undifferentiated gonads and testes, in addition to germ-line cells [1, 3–7]. Indeed, as a transcription factor, DMRT1 is involved in promoting or inhibiting the transcription of target genes in somatic cells of the reproductive system and gonads.

Many transcription factors that control the transcription of specific genes generally possess not only DNA-binding domains with sequence-specific binding to base pairs but

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also transcription-regulating domains (activation or repression). Regarding the former, multiple DNA-binding domains have been identified based on amino acid sequence similarities, and further analyses such as elucidation of their three-dimensional structures and specific DNA sequence binding have been actively conducted. However, concerning the latter, molecular insights, including analyses of binding affinities with essential coactivators or corepressors necessary for understanding transcription regulation, have been scarcely reported. Similarly, in the case of DMRT1, although the transcription activation domain is roughly reported to exist on the C-terminal side from the DM domain [8], precise identification of specific regions has not been achieved.

In this study, the aim was to identify the transcription activation domain of DMRT1. To achieve this, several deletion mutants of the C-terminal region were expressed in cultured cells for teleost fish, reptile, and mammalian DMRT1, and then transcriptional activity was examined using a luciferase reporter system. As a result, it was found that DMRT1 shares a common transcription activation domain consisting of about 30 amino acids and each species also possesses transcription activation domains in different regions. Furthermore, chimeric protein and amino acid substitution analyses suggested that the common activation domain had increased transcriptional activity during the evolution of mammals.

Materials and methods

Plasmid construction

5'-GTGAGCGGCTGGCGGCTGTTCAAGAAGATTAGC-3' encoding HiBiT-tag peptide, VSGWRLFKKIS, was inserted into EcoRV site of pcDNA3-FLAG [9]. The resultant plasmid vector, named pcDNA3-FLAG-HiBiT, was used for expression of various types of DMRT1 proteins in mammalian cells. cDNAs encoding human, mouse, leopard gecko, and medaka DMRT1, and their C-terminal truncated proteins were inserted in-frame between the FLAG and HiBiT-tag coding sequences of the vector. pcDNA3-FLAG-hDMRT1-full, -D7, -D(6–7), -D(5–7), and -D(4–7) contained the coding sequences for 1-373 aa, 1-317 aa, 1-299 aa, 1-236 aa, and 1-204 aa of human DMRT1, respectively. pcDNA3-FLAG-mDMRT1-full, -D7, -D(6–7), -D(5–7), and -D(4–7) contained the coding sequences for 1-374 aa, 1-315 aa, 1-297 aa, 1-234 aa, and 1-203 aa of mouse DMRT1, respectively.pcDNA3-FLAG-gDMRT1-full, -D7, -D(6–7), -D(5–7), and -D(4–7) contained the coding sequences for 1-349 aa, 1-293 aa, 1-269 aa, 1-206 aa, and 1-174 aa of leopard gecko DMRT1, respectively. pcDNA3-FLAG-gDMRT1-full, -D7, -D(6–7), -D(5–7), and -D(4–7) contained the coding sequences for 1-280 aa, 1-224 aa, 1-206 aa, 1-177 aa, and 1–51 aa of medaka DMRT1, respectively. Each protein ID was listed in Table S1.

On the base of Human DMRT1, chimeric proteins were constructed by exchanging its region 4 (205–236 aa) with the corresponding region 4 of gecko DMRT1 (175–206 aa), and by exchanging its region 7 (318–373 aa) with the corresponding region 7 of gecko DMRT1 (294–349 aa). Alternatively, on the base of gecko DMRT1, chimeric proteins were constructed by exchanging its region 4 (175–206 aa) with the corresponding region 4 of human DMRT1 (205–236 aa), and by exchanging its region 7 (294–349 aa) with the corresponding region 7 of human DMRT1 (318–373 aa). Construction of these four types of expression plasmids involved conducting PCR using pcDNA3-FLAG-hDMRT1-full or pcDNA3-FLAG-gDMRT1-full as templates, followed by assembling the resulting PCR fragments using In-Fusion technology.

To create four single amino acid substitution variants of human DMRT1 protein (S206V, F220Y, S232Q, L235V), the following primer pairs were used: 5'-ACCTGACCTGGTTGTGGACT-3'/5'-AGTCCACAACCAGGTCAGGT-3', 5'-AGCCGTCTCTGTATCCTTAT-3'/5'-ATAAGGATACAGAGACGGCT-3', 5'-AGTACCAGATGGCCTTGGCT-3'/5'-AGCCAAGGCCATCTGGTACT-3', and 5'-AGTACTCCATGGCCGTGGCT-3/5'-AGCcacGGCCATGGAGTACT-3'. PCR was performed using these primers with the pcDNA3-FLAG-hDMRT1-full template. Subsequently, the obtained PCR fragments were used to create expression plasmids using the In-Fusion method. To produce four single amino acid substitution variants of gecko DMRT1 protein (V175S, Y189F, Q202S, V205L), the following primer pairs were used: 5'-ACTTAGT*TTC*AGACTCCACC-3'/5'-GGTGGAGTCTGAAACTAAGT-3', 5'-CTGTTTCTTACTACAACAA-3'/5'-TTGTTGTAGTAAGAAACAG-3', 5'-AGTACTCCATGGCTGTGGCC-3'/5'-GGCCACAGCCATGGAGTACT-3', and 5'-AGTACCAGATGGCTTTGGCC-3'/5'-GGCCAAAGCCATCTGGTACT-3'. PCR was performed using these primers with the pcDNA3-FLAG-gDMRT1-full template. Subsequently, the obtained PCR fragments were used to create the expression plasmids using the In-Fusion method.

Luciferase reporter and HiBiT assay

24 h before transfection, HEK293T cells were plated at 1×10^5 per well in a 24 well plate. The cells were transfected with luciferase reporter plasmid p4×DMRT1-luc [4], DMRT1 expression plasmid, and *Renilla* luciferase vector pRL-SV40 (Promega, Wisconsin, USA) by polyethylenimine [10]. After 24 h, luciferase activities were measured in a Luminocounter 700 (Niti-ON). Firefly luciferase

activity was normalized by HiBiT activity for HiBiT-tagged DMRT1 proteins using the Nano-Glo HiBiT Lytic detection system (Promega, Wisconsin, USA).

Statistical analysis

Two-group or multiple group comparisons were performed by Student's t-test, or one-way ANOVA followed by Tukey HSD test, respectively. Significance for all test was set at $p < 0.05$.

Results

Conservation of amino acid sequences in seven regions of DMRT1 in vertebrates

A phylogenetic tree of amino acid sequences of DMRT1 from two mammalian species (human and mouse), two reptilian species (leopard gecko and painted turtle), and two bony fish species (medaka and zebrafish) was constructed using IQ-TREE (v1.6.12; http://www.iqtree.org/). Each protein ID is shown in Table S1. As a result, the phylogenetic relationships and topology of organisms were consistent (Fig. 1A). Next, the DMRT1 proteins from human, mouse, leopard gecko, and medaka were divided into seven domains based on information such as amino acid sequence prediction tools for secondary structure [11]. Figure 1B shows the amino acid numbers corresponding to each domain in each protein and the percentage identity of amino acid sequences between each domain of human DMRT1 and those of the other species. When comparing the three species, the mouse, which is evolutionarily closer to humans, showed the highest similarity in all domains, followed by the leopard gecko, while the evolutionarily distant medaka showed the lowest values. Comparing domains, the DM domain showed a high similarity of 83–98%, whereas other domains exhibited lower similarity compared to the DM domain, indicating lower conservation in amino acid sequences. Among domains other than the DM domain, 'domain 4' showed relatively higher conservation of amino acid sequences.

Identification of transcription activation regions in DMRT1 of four vertebrate species: in addition to the common region present in at least two locations

To identify the transcription activation region of DMRT1, we planned to compare and analyze the expression of DMRT1-driven luciferase reporter and effector proteins from human, mouse, leopard gecko, or medaka, each containing full-length DMRT1 or its deletion mutant protein, in cultured cells. In assays using a conventional luciferase reporter, it is often difficult to quantitate the expression of effectors, so the results are often presented in terms of relative activity corrected for transfection efficiency. However, in comparative analyses like this one between wild-type effector proteins and deletion mutant proteins, it is necessary to correct the reporter activity based on quantification of the effector protein. Therefore, for this purpose, we constructed plasmids with a HiBiT tag for quantification of expressed proteins in various DMRT1 expression plasmids (see Materials and Methods; Figs. 1B and 2). We confirmed that each construct produces proteins of the expected size by Western blot analysis (Fig. S1). We then co-transfected the DMRT1-driven firefly luciferase reporter plasmid and each effector expression plasmid or their empty vector, and *Renilla* luciferase reporter plasmid for transfection efficiency measurement in human embryonic fetal kidney HEK293T cells. After 24 h, cells were harvested, and firefly and *Renilla* luciferase activity as well as HiBiT activity were measured.

We first conducted a comparative analysis of the relative activity of firefly luciferase compared to *Renilla* luciferase. In all the four species of DMRT1, mutants with deletions from regions 1 to 3 (Δ [4–7]) showed values close to the basal level, similar to the empty vector, with no significant difference between them. However, mutants with deletions from regions 1 to 4 (Δ [5–7]) exhibited significantly higher relative values (Fig. S2). This suggests that regions 1–3 do not possess transcription activation ability, and a region consisting of approximately 40 amino acids ('Region 4') is indicated as a common transcription activation region among the four species. Additionally, in all four species, full-length DMRT1 showed higher relative values compared to regions 1–4 alone concerning *Renilla* luciferase, implying the possibility of transcription activation ability in regions other than 'Region 4'.

Next, we compared the relative values of firefly luciferase activity to HiBiT activity, which represents the expression level of full-length and various deletion mutants of DMRT1 within cells (Fig. 2). Consistent with the results of Fig. S2, 'Region 4' was indicated as the transcription activation region in all four species. Interestingly, in mammalian (human and mouse) DMRT1, their transcription activation abilities were both observed in 'Region 6', while in leopard gecko, it was observed in 'Regions 6–7', and in medaka, it was observed in 'Region 7'.

Transcriptional activity of chimeric proteins exchanging 'Region 4' or 'Region 7' between human and gecko DMRT1

In the above analysis of the transcription activation regions, while 'Region 4' is a common region in four species, the transcription activation ability of leopard gecko's DMRT1

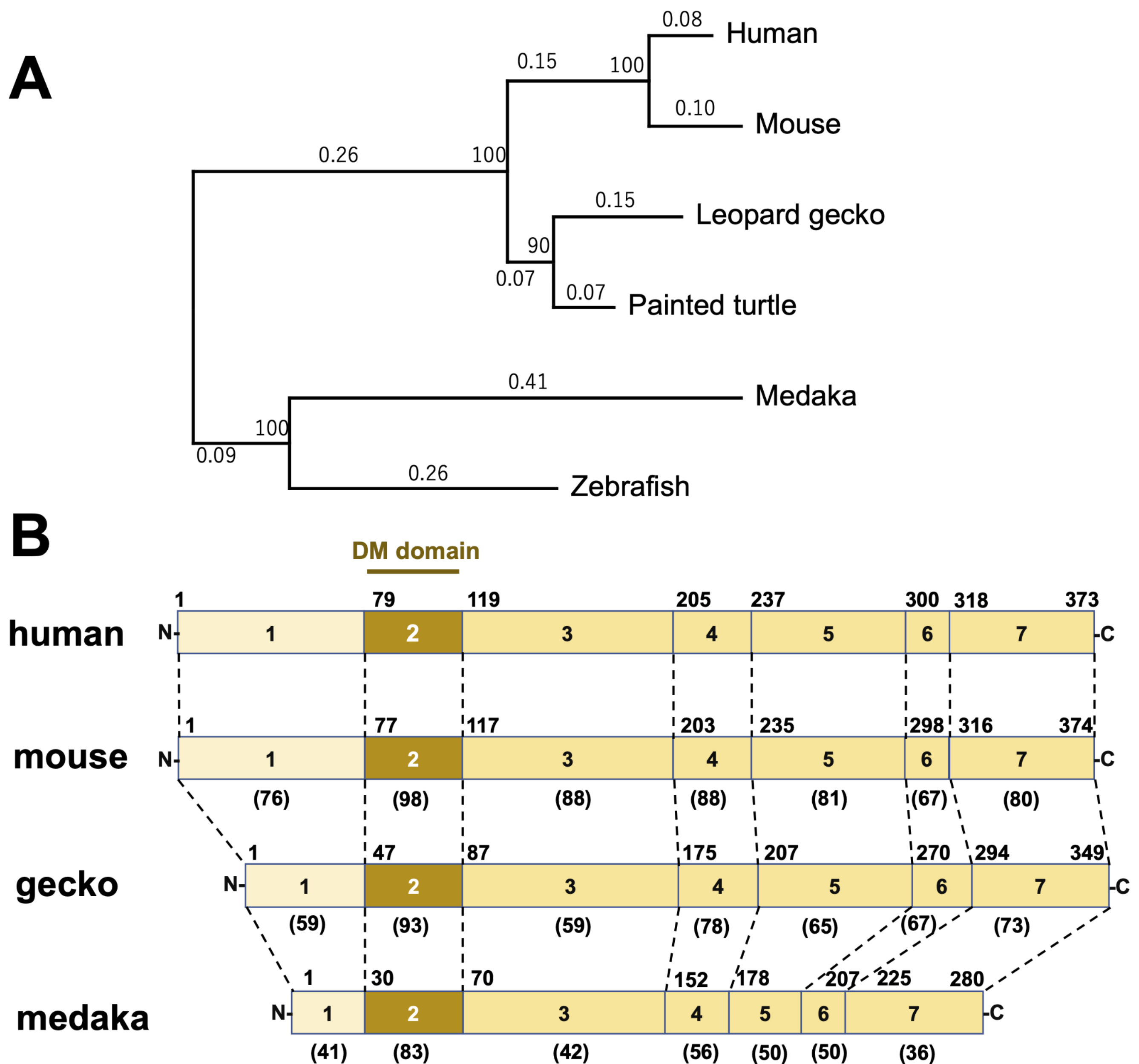

Fig. 1 Phylogenetic tree and structural schematic diagram of various vertebrate DMRT1s. The phylogenetic tree (**A**) was constructed using IQ-TREE. The numbers on the branches represent the estimated amino acid substitution rate per site, and the bootstrap values are shown next to the nodes. Using amino acid sequence prediction tools for secondary structure (SLiMPred; http://bioware.ucd.ie/~compass/biowareweb/Server_pages/slimpred.php), four DMRT1s were divided into seven regions (**B**), and the sequence identity (%) corresponding to the human sequence is shown below the schematic diagram

was suggested to have a greater contribution from 'Region 7' than 'Region 4' (Fig. 2). Then, plasmids expressing chimeric proteins exchanging Region 4 and Region 7 of human and leopard gecko DMRT1 were constructed, and comparative analyses of transcriptional activity were performed in the same procedure as in Fig. 2.

Concerning human DMRT1, when 'Region 4' was replaced with that of the leopard gecko, the activity decreased by about 24%, whereas when 'Region 7' was exchanged with that of the leopard gecko, approximately twice the activity was detected (Fig. 3A). On the other hand, regarding leopard gecko DMRT1, when 'Region 4' was replaced with that of human ≈, the activity increased by about 1.5 times, whereas when 'Region 7' was exchanged with that of human DMRT1, a decrease in activity of about 40% was observed (Fig. 3B). These results indicated that human 'Region 4' and 'Region 7' had higher and lower

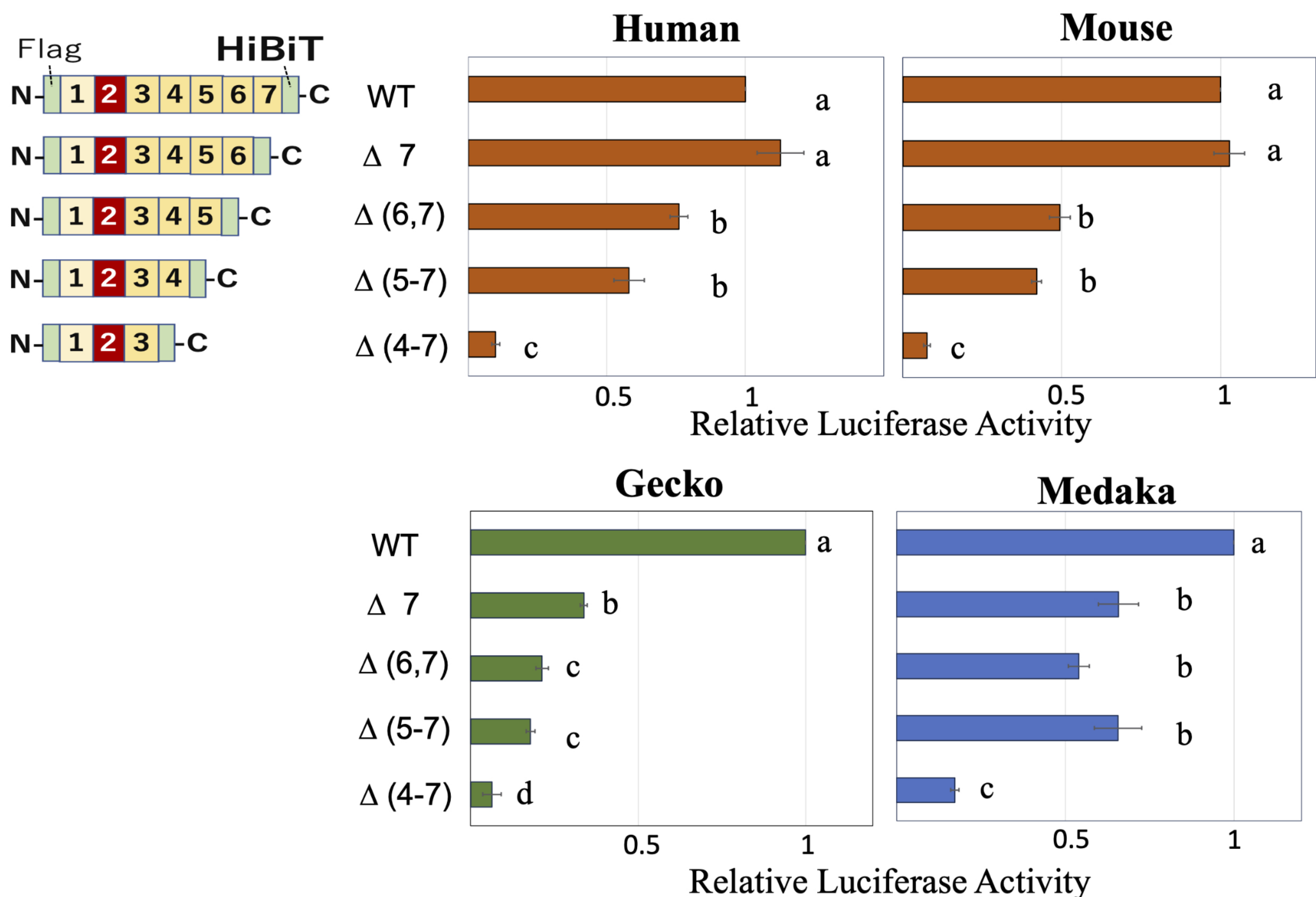

Fig. 2 Transcriptional activation ability of human, mouse, gecko, and medaka DMRT1 deletion mutants standardized by quantification of the HiBiT-tagged proteins. HEK293T cells were transfected with each HiBiT-tagged DMRT1 deletion expression plasmid and a DMRT1-driven firefly luciferase reporter plasmid. After 24 h, firefly luciferase activities were measured and standardized by quantification of HiBiT. a–d: means with different letters are significantly different from each other, as tested using one-way ANOVA, followed by the Tukey–Kramer HSD test ($P < 0.05$)

transcription activation abilities than those of leopard gecko, respectively.

The substitution of valine to serine on 'Region 4' of DMRT1 might contribute to the increase in its transcriptional activation ability during mammalian evolution

From the domain swapping experiments of 'Region 4' of human and leopard gecko DMRT1 (Fig. 3A), it is possible that some amino acid substitutions could contribute to transcriptional activation within 'Region 4' in the ancestor of humans. Additionally, since the mouse region 4 also exhibited similarly high transcriptional activation ability as humans (Fig. 2), evolutionary analysis was conducted on 'Region 4'. Using the amino acid sequences of 'Region 4' from six reptile species, eight mammalian species, and three amphibian species (Table S1), along with their corresponding nucleotide sequences, a branch-site model was employed to examine the d_N/d_S ratio and assess the possibility of amino acid substitutions under positive selection pressure. As a result, two amino acid substitutions with a d_N/d_S ratio greater than or equal to 1 within 'Region 4' were detected with a probability of more than 95% (Table S2). These substitutions occurred at serine residues corresponding to positions 205 and 232 of human DMRT1. At these two positions, serine was found in all therian mammals examined, while valine or glutamine was found in all reptilian species examined (Fig. 4A and Fig. S3).

Then, human DMRT1(S205V) or DMRT1(S232Q), in which serine at position 205 or 232 of human DMRT1 was replaced with valine or glutamine corresponding to gecko DMRT1, and conversely, gecko DMRT1(V175S) or DMRT1(Q201S), in which valine or glutamine at positions 175 or 201 of gecko DMRT1 were replaced with serine of human DMRT1, were expressed in HEK293T cells (Fig. 4). Transcriptional activity was examined using a luciferase reporter as in Figs. 2 and 3. As a result, human

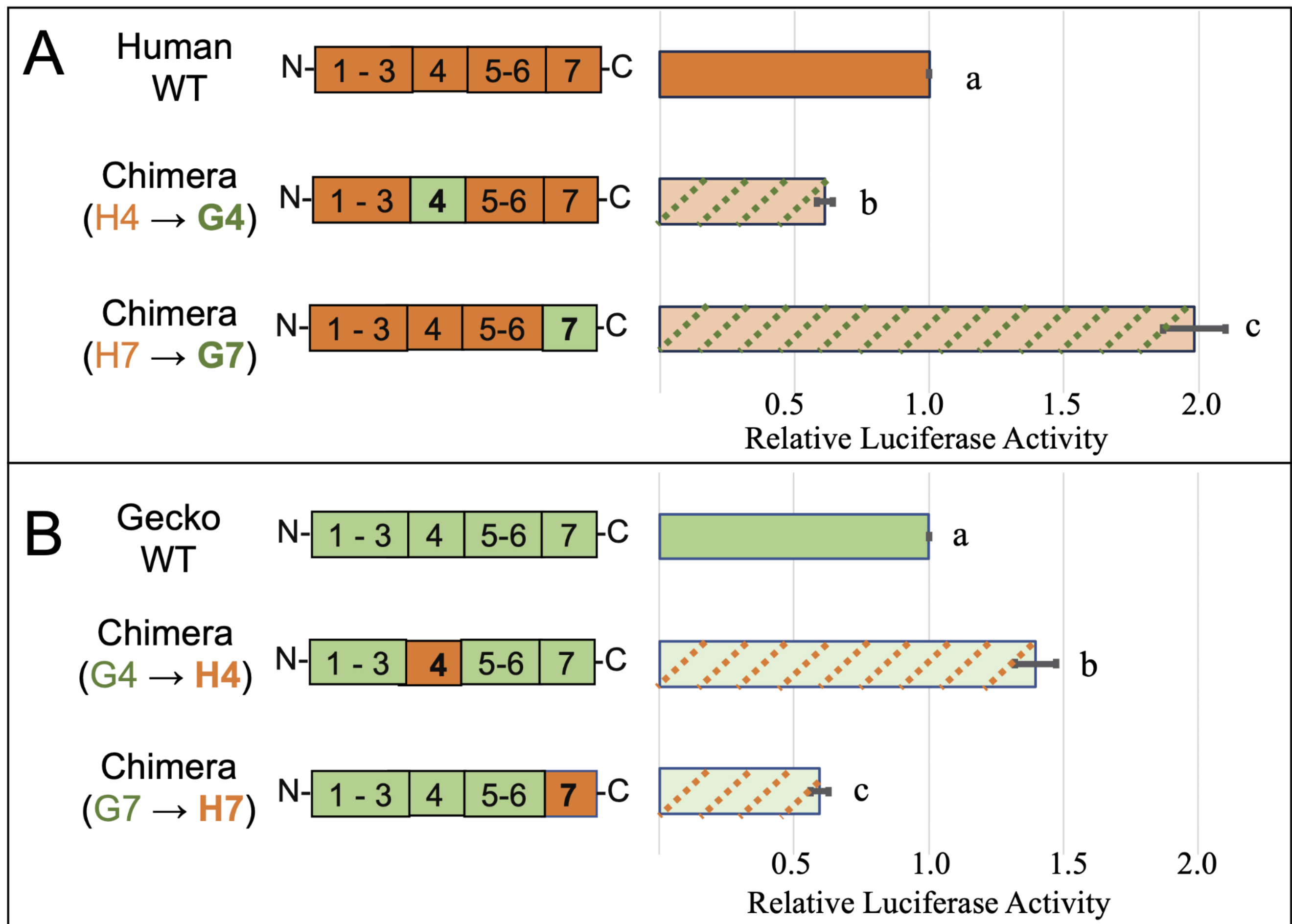

Fig. 3 Effects of swapping 'Region 4' or 'Region 7' between human and gecko DMRT1 on the transcriptional activation ability. HEK293T cells were transfected with each HiBiT-tagged DMRT1 chimeric expression plasmid and a DMRT1-driven firefly luciferase reporter plasmid. After 24 h, firefly luciferase activities were measured and standardized by quantification of HiBiT. a–c: means with different letters are significantly different from each other, as tested using one-way ANOVA, followed by the Tukey–Kramer HSD test ($P < 0.05$)

DMRT1(S205V) and gecko DMRT1(V175S) showed significant decreases and increases in activity, respectively, compared to the wild type (WT) (Fig. 4B-D). However, no significant change in activity due to the mutation was observed for human DMRT1(S232Q) or gecko DMRT1(Q201S) compared to the wild type.

From these findings, it was considered that the single amino acid mutation from valine to serine at the position, which occurred in the common ancestor of mammals, contributed to the increase in transcriptional activation ability of DMRT1 in 'Region 4' under positive selection.

Discussion

Many transcription factors have only one DNA binding domain with sequence specificity. On the other hand, with regard to transactivation domains (TADs), it has been reported that some transcription factors, including p53, have multiple TADs [12]. In this study, we analyzed the activation domains of the transcription factor DMRT1 and found that multiple TADs exist (Fig. 2). Interestingly, a comparison of DMRT1 from four species—human, mouse, the reptile leopard gecko, and the teleost fish medaka—suggested that the activation-related domains consist of a region common to all four species ('Region 4') and regions that differ depending on the species ('Region 6' and 'Region 7'). It is possible to speculate that the common TAD, 'Region 4', represents the evolutionary foundation of DMRT1's TADs, while 'Region 6' and 'Region 7' acquired additional transcriptional activation potential during species diversification.

The discovery of TADs dates back more than 30 years, but their functional mechanisms and molecular evolution remain largely unknown [13–15]. TADs are thought to recruit basic transcriptional machinery components, including coactivators, but they exhibit randomness in amino acid sequences and structural disorder, lacking the evolutionary conservation seen in sequence-specific DNA binding

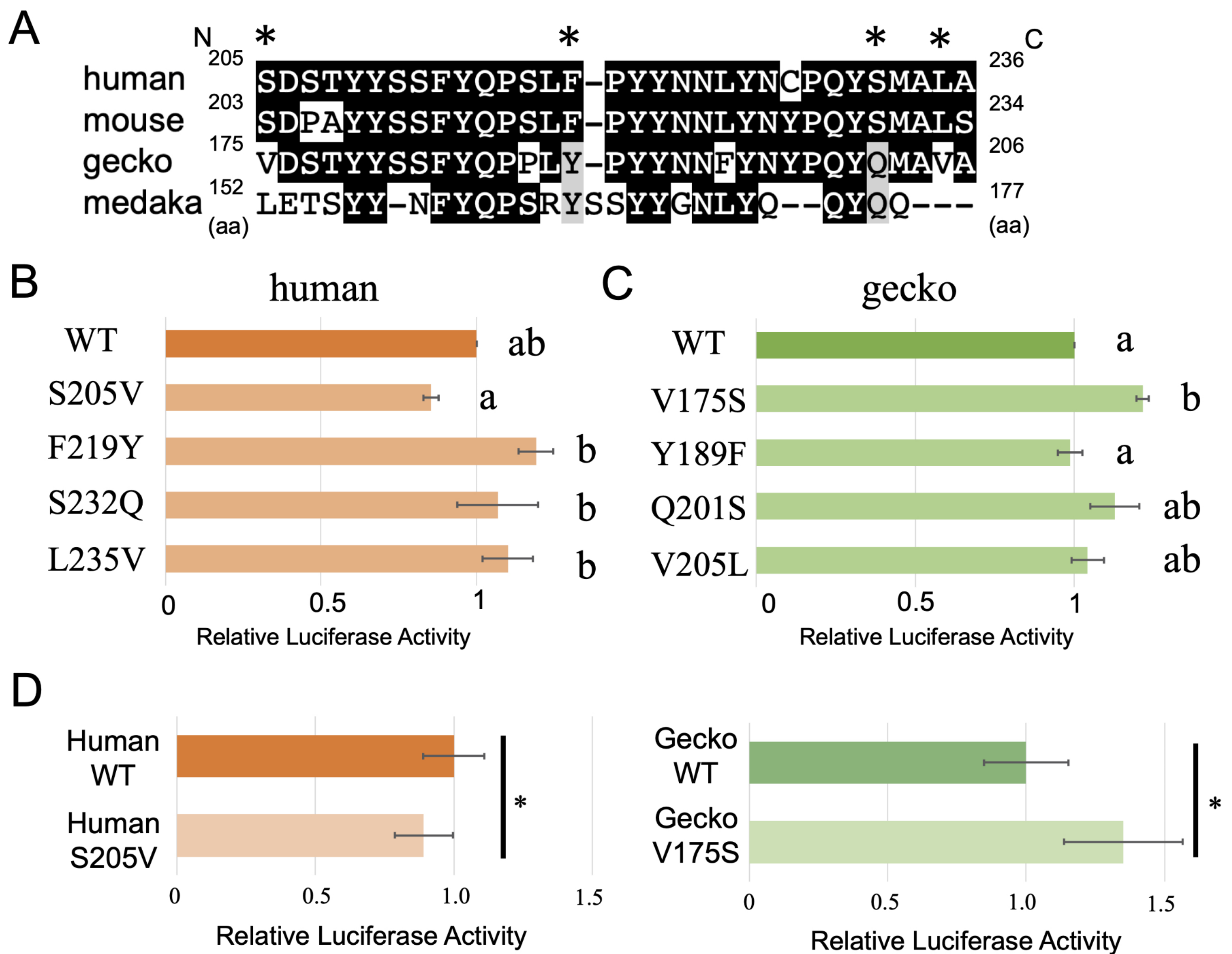

Fig. 4 Effects of four single amino acid substitutions within 'Region 4' on the transcriptional activation of human and gecko DMRT1. Amino acid sequence comparison in 'Region 4' is shown in (**A**). HEK293T cells were transfected with each HiBiT-tagged DMRT1 single amino acid substitution expression plasmid and a DMRT1-driven luciferase reporter plasmid. After 24 h, firefly luciferase activity was measured and standardized by quantification of HiBiT (**B**-**D**). Statistical significance was tested using multiple comparisons (**B**, **C**) and pairwise comparisons (**D**; * $P < 0.05$)

domains. Even within the same transcription factor, such as DMRT1 in this study or p53, there is no amino acid sequence homology among multiple TADs, suggesting that domain duplication from a single TAD is unlikely. Furthermore, the amino acid sequences of 'Regions 4, 6, and 7' identified in this study do not show any conservation with members of the DMRT superfamily, to which DMRT1 belongs. In the p53 family, consisting of three transcription factors—p53, p63, and p73—it is known that each has different TADs.

These observations suggest that many TADs in transcription factors might have been evolved not through strong evolutionary constraints, and some TADs emerged de novo during species diversification. Particularly in multicellular organisms, the presence of specialized epigenomes in various tissues, responding to developmental processes and environmental stimuli, implies that functional evolution or diversification might have occurred in response to the chromatin context of each cell.

Conclusion

The study demonstrated that the transcription activation regions of DMRT1 have evolved differently across species, with some regions being conserved and others showing diversity. The fourth region from the N-terminus contributes to transactivation in all species, while the sixth and seventh regions on the C-terminal side vary depending on the species. This indicates that the transactivation domains of DMRT1 have changed over the course of evolution, leading to diversity. While certain regions are conserved, other parts

differ between species, suggesting evolutionary diversity in the DMRT1 transcription activation regions.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11033-024-10006-9.

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Author contributions N.I., K.F., N.O. and M.I. designed the study; N.I and M.I. wrote the main manuscript text; N.I., K.F., N.O., S.H., N.S., S.I., H.O., K. Seki, and K. Suda performed the experiments; D.T., T.M., and K.T. analyzed and discussed the data. All authors reviewed the manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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