Biochemistry (Moscow), Vol. 66, No. 6, 2001, pp. 623-627. Translated from Biokhimiya, Vol. 66, No. 6, 2001, pp. 768-774.

Effect of Two Conserved Amino Acid Residues on DREB1A Function

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Received August 17, 2000
Revision received January 20, 2001

Abstract—Transcription factors of the DREBP subgroup and the EREBP subgroup contain conserved DNA-binding domains called AP2/EREBP domains, which specifically bind to DRE *cis*-element and GCC-box, respectively. The 14th and 19th amino acid residues of AP2/EREBP domains are absolutely conserved in the transcription factors of the DREBP subgroup as well as in the EREBP subgroup. However, these two residues of transcription factors of the DREBP subgroup are different from those of the EREBP subgroup. To assess the functional significance of these two residues in binding to the target sequence, the Val (14th residue) and Glu (19th residue) of the AP2/EREBP domain of DREB1A (a transcription factor of the DREBP subgroup) were mutated individually or doubly to Ala and Asp, respectively. This made the 14th and 19th amino acid residues of mutant DREB1A identical to the corresponding residues of transcription factors of the EREBP subgroup. Yeast *in vivo* analysis showed that: 1) on a selective medium plate of SD/His^- Ura^- Trp^- + 30 mM ~ 60 mM 3-AT, the growth of yeast cells containing *HIS* and *lacZ* double reporter genes was normal in the transformation of the 19th singly mutated DREB1A, obviously inhibited in the transformation of the 14th singly mutated DREB1A, and seriously inhibited in the transformation of the 14th/19th doubly mutated DREB1A; 2) quantitative assay of β-galactosidase activity showed that the intensities of *lacZ* expression decreased in the transformations of the 14th singly mutated and 14th/19th doubly mutated types. The experimental results revealed that the 19th site mutation did not affect the binding of the DREB1A transcription factor to the DRE *cis*-element; the 14th site mutation obviously inhibited their binding; and the double mutation of the 14th/19th sites seriously inhibited their binding. This suggests that the conserved Val (14th) and Glu (19th) residues are crucial in the regulation of the binding activity of DREB1A to the DRE *cis*-element.

Key words: DREB1A transcription factor, AP2/EREBP domain, DRE *cis*-element

Recently the AP2/EREBP transcription factor, a novel type of transcription factor, has been isolated from a variety of higher plants. AP2/EREBP-related genes form a large family which consists of many members found in several plant species, such as *Arabidopsis thaliana*, tobacco, tomato, rice, maize, castor bean, etc. [1, 2]. All proteins of the AP2/EREBP family contain one (or two) conserved DNA-binding domain(s) composed of 57-70 amino acid residues, which is named the AP2/EREBP domain. All AP2/EREBP proteins possess typical structural characteristics of transcription factors such as a DNA-binding domain (i.e., the AP2/EREBP domain), nuclear localization signals (i.e., basic amino acid sequences), and putative transcription activation domains (i.e., acidic region, serine-rich region, etc.).

The members of the AP2/EREBP family can be divided into at least two groups, the AP2 group and the EREBP group, according to the number of the AP2/EREBP domain. The AP2 group includes AP2 and ANT found in *Arabidopsis*, Glossy 15, and ids1 found in maize. The genes of this group encode proteins that contain two AP2 domains [3-7], which may function as transcription regulators and thus play important roles in developmental processes of the plant.

The genes assigned to the EREBP group encode proteins possessing only one AP2/EREBP domain. Many members, such as EREBP1-4, Pit4-6, DREB1 A-C (CBF1-3), DREB2A-B, AtERF1-5, and AtEBP belong to this group [8-16]. Some of the members have been shown to function as transcription factors (see figure). The members of this group can be further divided into three subgroups based on their binding to different *cis*-acting elements.

As shown in the figure, the members in the first subgroup (EREBP) include EREBP1-4 of tobacco, Pit4-6 of tomato, and AtERF1-5 and AtERP of *Arabidopsis*, etc. These transcription factors have been experimentally shown to recognize and bind to the GCC box. The core sequence of the GCC box is AGCCGCC, which is the

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a

EREBP subgroup

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consensus  ..RGVR.RPWG..AAEIRD......RVWLGT......AA.AYD.AA...RG..A..NF.
EREBP1     HYRGVRRRPWGKFAAEIRDPAKNGARVWLGTYETDEEAALAYDKAAYRMRGSKAHLNFP
EREBP2     HYRGVRQRPWGKFAAEIRDPAKNGARVWLGTYETAEEAALAYDKAAYRMRGSKALLNFP
EREBP3     HYRGVRKRPWGRYAAEIRDPGK-KSRVWLGTFDTAEEAAKAYDTAAREFRGPKAKTNFP
EREBP4     HYRGVRQRPWGKFAAEIRDPNRKGTRVWLGTFDTAIEAAKAYDRAAFKLRGSKAIVNFP
ATERF-1    HYRGVRQRPWGKFAAEIRDPAKNGARVWLGTFETAEDAALAYDRAAFRMRGSRALLNFP
ATERF-2    HYRGVRQRPWGKFAAEIRDPAKNGARVWLGTFETAEDAALAYDIAAFRMRGSRALLNFP
ATERF-3    RFRGVRKRPWGRFAAEIRDPWK-KARVWLGTFDSAEEAARAYDSAARNLRGPKAKTNFP
ATERF-4    RYRGVRKRPWGRYAAEIRDPGK-KTRVWLGTFDTAEEAARAYDTAARDFRGAKAKTNFP
ATERF-5    HYRGVRQRPWGKFAAEIRDPNKRGSRVWLGTFDTAIEAARAYDEAAFRLRGSKAILNFP
ATERF-6    HYRGVRMRPWGKFAAEIRDPTRRGTRVWLGTFETAIEAARAYDKEAFRLRGSKAILNFP
ERF1       SYRGVRRRPWGKFAAEIRDSTRNGIRVWLGTFESAEEAALAYDQAAFSMRGSSAILNFS
AtEBP      VYRGIRKRPWGKWAAEIRDPRK-GVRVWLGTFNTAEEAAMAYDVAAKQIRGDKAKLNFP
Pti4       HYRGVRQRPWGKFAAEIRDPAKNGARVWLGTYETAEEAAIAYDKAAYRMRGSKAHLNFP
Pti5       KYRGVRRRPWGKYAAEIRDWARHGARVWLGTFETAEEAALAYDRAAFRMRGAKALLNFP
Pti6       KFRGVRQRPWGRWAAEIRDPTR-GKRVWLGTYDTPEEAAVVYDKAAVKLKGPDAVTNFP
OPBP1      SFRGVRRRPWGKFAAEIRDSTRNGVRVWLGTFDSPEAAALAYDQAAFLMRGTSAILNFP
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DREBP subgroup

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consensus        ..RGVR.R..GKWV.E.REP....R.WLGTF.TA..AA.A.D.AA.A..G..A.LNF.
DREB1A(CBF3)     IYRGVRRRNSGKWVCEVREPNKKTRIWLGTFQTAEMAARAHDVAALALRGRSACLNFA
DREB1B(CBF1)     IYRGVRQRNSGKWVSEVREPNKKTRIWLGTFQTAEMAARAHDVAALALRGRSACLNFA
DREB1C(CBF2)     IYRGVRQRNSGKWVCELREPNKKTRIWLGTFQTAEMAARAHDVAAIALRGRSACLNFA
DREB2A           SFRGVRQRIWGKWVAEIREPNRGSRLWLGTFPTAQEAASAYDEAAKAMYGPLARLNFP
DREB2B           SFRGVRQRIWGKWVAEIREPKIGTRLWLGTFPTAEKAASAYDEAATAMYGSLARLNFP
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RAV subgroup

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consensus  KYKGVVPQPNGRWGAQIYEKHQRVWLGTFNE..EAAR.YD.A..RFR.RDAV.NFK
RAV1       KYKGVVPQPNGRWGAQIYEKHQRVWLGTFNEEDEAARAYDVAVHRFRRRDAVTNFK
RAV2       KYKGVVPQPNGRWGAQIYEKHQRVWLGTFNEQEEAARSYDIAACRFRGRDAVVNFK
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b

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EREBP      ..RGVR.RPWG..AAEIRD......RVWLGT......AA.AYD.AA...RG..A..NF.
DREBP      ..RGVR.R..GKWV.E.REP..-..R.WLGTF.TA..AA.A.D.AA.A..G..A.LNF.
consensus  ..RGVR.R.WG..*.E.R*......R.WLGT......AA...D.A.........A..NF.
           ---------- β-sheet ----------  ---------- α-helix ----------
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Alignment of the AP2/EREBP domain of EREBP group proteins. a) Amino acid sequences of the AP2/EREBP domain of the EREBP group. b) Comparison of the consensus sequence of AP2/EREBP domains between the DREBP and EREBP subgroups. Amino acid residues shown by NMR analysis [23] to contact DNA bases are underlined. Regions of β-sheet and α-helix are indicated. Amino acids labeled with asterisks in the consensus indicate the conserved amino acid residues (14th and 19th). Their physicochemical properties are absolutely conserved within each subgroup but differ between the DREBP and EREBP subgroups.

ethylene-responsive element identified from the genes encoding β-1,3-glucanase, PR-1, and PR-5d proteins of tobacco. It has been shown that GCC box is present in the 5′ untranscribed regions of almost all basic pathogenesis-related genes. EREBP transcription factors of tobacco have been shown to function in signal transduction under biotic and abiotic stresses [2, 15, 17].

The second subgroup (DREBP) mainly consists of DREB1A-C (CBF1-3) and DREB2A-B of *Arabidopsis*. DREB1A-C and DREB2A-B specifically recognize and bind to DRE (dehydration-responsive element) *cis*-element or DRE-like *cis*-elements involved in plant responses to drought, high-salt, and cold stresses [11]. DRE or DRE-like *cis*-elements exist widely in promoters of plant genes such as *rd29A*, *rd17*, *kin1*, etc., which are induced by dehydration, high-salt, and cold stresses. The core sequence of DRE or DRE-like *cis*-elements is A/GCCGAC.

The third subgroup, the RAV subgroup, includes two members: RAV1 and RAV2 [18]. Their N-terminal regions are homologous to the AP2/EREBP DNA-binding domain. It has been shown that the proteins encoded by *RAV1* and *RAV2* can bind to the CAACA motif *in vitro* [18, 19].

In our previous report, the amino acid sequences of the AP2/EREBP domains between the DREBP and EREBP subgroups were compared, and consensus amino acids were found [11]. All of the consensus amino acids are conserved in these two subgroups except that the 14th and 19th amino acids are valine (Val) and glutamate (Glu) in the DREBP subgroup and alanine (Ala) and aspartic acid (Asp) in the EREBP subgroup. Apparently, these two amino acids might be significant regarding the specificity of binding to target sequences of *cis*-elements. To test this inference, we mutated the 14th and 19th amino acids of the AP2/EREBP domain of DREB1A individually and doubly and analyzed their roles in binding to target *cis*-element. The doubly mutated DREB1A in which Val (14th) and Glu (19th) residues of the AP2/EREBP domain were replaced by Ala and Asp to be identical with the AP2/EREBP domain of the EREBP subgroup showed greatly decreased affinity for DRE *cis*-element, the target sequence of transcription factor of the DREBP subgroup.

MATERIALS AND METHODS

To examine transactivation activity of DREBP protein, 4-tandem-copies of a 75 bp fragment containing a DRE *cis*-element from the promoter of the *rd29A* gene were inserted into *Eco*RI-*Mlu*I sites of MCS in pHISi-1 expression vector carrying the *HIS3* reporter gene and *Eco*RI-*Sal*I sites of MCS in pLacZi expression vector carrying the *lacZ* reporter gene and *URA3* gene (MATCHMAKER One-Hybrid System, Clontech), respectively (see [11]). Two recombinant reporter vectors were integrated into the same yeast YM4271 strain used for reporter vector integration for transactivation experiments. The genotype of YM4271 strain is *MATa*, *ura3-52*, *his3-200*, *ade2-101*, *lys2-801*, *leu2-3*, *112*, *trp1-903*, *tyr1-501*.

The DREB1A fragments of the 14th singly mutated (V14A), the 19th singly mutated (E19D), and the 14th/19th doubly mutated (V14A/E19D), in which the 14th V (valine) and the 19th E (glutamate) of the AP2/EREBP domain were individually or doubly replaced with A (alanine) and D (aspartic acid), were obtained by PCR. The primer sets used for the preparation of mutated DREB1A fragments were designed as follows:

1) V14A, 5′-GTAATACGACTCACTATAGGGC-3′ (T7 primer) and 5′-TGAGCTCGAGCTGCCATCTCAGCGGTTTGAAATGT
TCCGAGCCAAATCCTTGTTTTCTTGTTTGTTCTCTAACCTCACA<u>CG</u>CCCACT-3′;

2) E19D, T7 primer and 5′-TGAGCTCGAGCTGCCATCTCAGCG
GTTTGAAATGTTCCGAGCCAAATCCTTGTTTTCTTGTTTGG<u>G</u>TCTCTA-3′;

3) V14A/E19D, T7 primer and 5′-TGAGCTCGAGCTGCCATCTCAGCGGTTT
GAAATGTTCCGAGCCAAATCCTTGTTTTCTTGTTTGG<u>G</u>TCTCTAACCTCACA<u>CG</u>CCCACT-3′ (the mutated bases were underlined).

Three mutated PCR fragments were cloned to the *Xho*I site of MCS in pBluescript SK^+ vector for sequencing, respectively. The wild type of DREB1A inserted into the *Eco*RI site of MCS in pBluescript SK^+ was digested with *Xho*I and then the 5′ *Xho*I-digested fragment was displaced by a mutated PCR fragment.

The three full-length DREB1A mutant sequences (V14A mutation, E19D mutation, and V14A/E19D mutations) were constructed into the *Not*I site in YepGAP expression vector containing a *TRP* gene, respectively [11]. The yeast YM4271-His^+Ura^+ reporter containing dual *HIS* and *lacZ* reporter genes was transformed by LiAc–polyethylene glycol according to the protocol of the One-Hybrid System (Clontech) by using 1 μg of YepGAP plasmid with V14A, E19D, or V14A/E19D mutations. Transactivation experiments were made on a selective medium plate of SD/His^- Ura^- Trp^- with 0, 15, 30, 45, and 60 mM 3-AT.

Quantitative β-galactosidase assays were performed as described by Guarente [20].

Transactivation experiments were based on the following principle. If the transcription factor expressed in yeast can bind to the target sequence (i.e., DRE *cis*-element) and then activate the transcription of downstream reporter genes of *HIS* and *lacZ*, the yeast cells can grow in minimal medium which is short of histidine, uracil, and tryptophan (SD/His^- Ura^- Trp^- + 15 mM ~ 60 mM 3-AT), and the activity of β-galactosidase can be examined out.

Table 1. Examination of the growth of yeast cells transformed with vectors containing V14A mutation, E19D mutation, V14A/E19D mutations, or wild type DREB1A, respectively

Reporter strains	3-AT concentration				
	0 mM	15 mM	30 mM	45 mM	60 mM
YepGAP vector	+++++	+			
YepGAP vector + V14A/E19D	+++++	++	+		
YepGAP vector + V14A	+++++	++++	+++	++	+
YepGAP vector + E19D	+++++	+++++	+++++	++++	+++
YepGAP vector + DREB1A	+++++	+++++	+++++	++++	+++

Note: Mutant and wild type DREB1A sequences constructed in YepGAP were transformed to yeast YM4271-His^+Ura^+ reporter containing dual *HIS* and *lacZ* reporter genes, respectively. Yeast cells were grown on selective medium plate of SD/$His^-Ura^-Trp^-$ with 3-AT at indicated concentrations. Growth of the colonies was evaluated after 6 days. "+", growth (more + indicates better growth).

3-AT (3-aminotriazole) is a competitive inhibitor of the yeast His3 protein; it can inhibit the background of the expression of the *HIS* reporter gene. If the transcription factor expressed in yeast cannot bind to the target sequence, the yeast cells cannot grow on the selective medium plate of SD/His^- Ura^- Trp^- in the presence of 13 mM 3-AT.

RESULTS

Table 1 shows the growth of the yeast cells in transactivation experiments of the 14th singly mutated DREB1A (V14A mutation), the 19th singly mutated DREB1A (E19D mutation), the 14th/19th doubly mutated DREB1A (V14A/E19D mutation), and the wild type of DREB1A. As a control, when the YepGAP vector was transferred to yeast recombinants containing dual report genes (*HIS* and *lacZ*), the yeast cells could normally grow in a selective medium plate of SD/His^- Ura^-Trp^- and were completely inhibited in the presence of 15 mM 3-AT. When vectors with V14A, E19D, or V14A/E19D mutations were transferred to the yeast cells containing *HIS* and *lacZ* reporter genes, the growth of the yeast cells showed significantly different results in the presence of 15-60 mM 3-AT. Yeast cells could normally grow in the transformants with E19D mutation, but the growth of the yeast cells was obviously inhibited in the transformants with the V14A mutation and was seriously inhibited in the transformants with the V14A/E19D mutation under the same experimental conditions.

Table 2. *LacZ* expression in yeast cells transformed with V14A mutation, E19D mutation, V14A/E19D mutations, or wild type DREB1A, respectively

Reporter strains	β-Galactosidase activity, u*	Relative activity, %
YepGAP vector + DREB1A	7.270 ± 0.956	100.0
YepGAP vector + E19D	7.175 ± 0.814	98.7
YepGAP vector + V14A	1.650 ± 0.118	22.7
YepGAP vector + V14A/E19D	0.125 ± 0.014	1.7
YepGAP vector	0.035 ± 0.008	0.5

Note: Mutant and wild type DREB1A sequences constructed in YepGAP were transformed to yeast YM4271-His^+Ura^+ reporter containing dual *HIS* and *lacZ* reporter genes, respectively. Yeast cells were grown on a selective medium plate of SD/$His^-Ura^-Trp^-$ for 18 h ($n = 6$; ± indicates standard error).

* Miller units.

Quantitative assay of β-galactosidase activity showed that the intensities of *lacZ* expressions decreased in the transformants with V14A or V14A/E19D mutations. Comparing with the control of wild type DREB1A, the relative β-galactosidase activities were 98.7, 22.7, and 1.7% in the transformants with E19A, V14A, and V14A/E19D mutations, respectively (Table 2).

The experimental results showed the 19th site mutation (from Glu to Asp) did not affect the binding affinity of DREB1A transcription factor to DRE *cis*-element; the 14th site mutation (from Val to Ala) obviously inhibited the binding affinity; and double mutations of the 14th and 19th (from Val and Glu to Ala and Asp) seriously inhibited the binding.

DISCUSSION

AP2/EREBP domains have recently been shown to be present in numerous gene products of unknown function in various plants. This strongly suggests that the

AP2/EREBP domain is an evolutionarily conserved element that is essential for the proteins to function [1, 2]. Ohme-Takagi and his colleagues showed that the regions flanking the DNA binding domain are not critical for mediating the protein–DNA interaction *in vitro* [15, 21, 22]. Therefore it could be predicted that the AP2/EREBP domain is essential and sufficient to confer the DNA binding activity of the EREBP-like group proteins.

Detection of a functional site in a regulatory protein is of great interest. In general, the specific properties of a given protein result from a limited number of key amino acid residues [24-31]. To understand the molecular mechanisms of binding specificity of the DREBP and EREBP subgroups, we compared the consensus amino acid residues of the AP2/EREBP domain within and between two subgroups. We found two residues whose physicochemical properties were absolutely conserved within each subgroup but differed between the two subgroups, i.e., the 14th and 19th amino acid residues are valine (Val) and glutamate (Glu) in DREBP and alanine (Ala) and aspartic acid (Asp) in EREBP, respectively.

In this study, we reported the functional significance of two highly conserved amino acids of the AP2/EREBP domain of DREB1A. The 14th Val and 19th Glu of the AP2/EREBP domain in DREB1A were singly or doubly mutated, and we analyzed the effects of the mutations on DREB1A binding to the target sequence (DRE *cis*-element). As shown in Tables 1 and 2, the E19D single mutation did not affect the binding ability of DREB1A with the DRE *cis*-element. The V14A single mutation made both 3-AT resistance and β-galactosidase activity decrease significantly. Interestingly, the decrement of the binding ability of DREB1A to DRE *cis*-element caused by the V14A/E19D double mutation surpassed that caused by the V14A mutation. This suggests that the conserved 14th Val is crucial to regulate the binding activity of DREB1A to the DRE *cis*-element. Although the single mutation of the 19th Glu did not obviously affect DREB1A function, the V14A/E19D double-mutation resulted in much lower binding ability of DREB1A to DRE *cis*-element compared to that shown by the V14A single mutation. This suggests that the 19th amino acid residue could also play an important role. In summary, the conserved Val (14th) and Glu (19th) of the AP2/EREBP domain are crucial to the regulation of the binding activity of DREB1A to the DRE *cis*-element.

This work was supported by NKBRSF, P. R. China (G1999011703 and G1998010100) and the Natural Science Foundation of Tsinghua University.

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