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Dbf4 Motifs: Conserved Motifs in Activation Subunits for Cdc7 Kinases Essential for S-Phase

Hisao Masai*,[1] and Ken-ichi Arai*,†

**Department of Molecular and Developmental Biology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan; and †CREST, Japan Science and Technology Corporation (JST), Tokyo, Japan*

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Dbf4 and its related molecules were originally identified as cyclin-like partners for Cdc7 kinases, essential for S-phase. Recent reports and database search indicate the presence of multiple Dbf4-related molecules with distinct functions. We have identified three stretches of amino acids which are conserved in various Dbf4-related molecules and possibly play distinct functions in binding to and activation of the catalytic subunits as well as in interactions with other proteins. Discovery of conserved motifs for this possible new protein family would serve as a useful framework for future identification of new members of this family as well as for probing their functions.

Cdc7-Dbf4 kinase complexes, originally identified in *Saccharomyces cerevisiae,* play pivotal roles in initiation of DNA replication (1, 2). Recent studies have demonstrated the presence of related kinase complexes, composed of Cdc7-related catalytic subunit and Dbf4-related regulatory subunit, in varieties of eukaryotes including distantly related fission yeast and human (3–11). Furthermore, recent reports and database search indicate the presence of multiple Dbf4-related molecules with distinct functions, suggesting the presence of a novel "Dbf4-family."

Studies of Cdc7-Dbf4 related kinase complexes in various eukaryotes pointed to striking conservation in expression and functions of this essential kinase complex (3). Similar to Cdk-cyclin, kinase activity of Cdc7 is generally strictly dependent upon the activation subunit (12, 13), except for fission yeast where the catalytic subunit alone exhibits intrinsic kinase activity which is stimulated by the activation subunit. Cdc7-Dbf4 kinase complexes appear to be associated with chromatin (14), suggesting that components in the replication complex may be the targets of Cdc7-Dbf4 kinase. ORC (origin recognition complex), Cdc6, and MCM (minichromosome maintenance) proteins constitute prereplicative complex (preRC) at each cell cycle which acts as a landing pad for subsequent assembly of an active replication complex and is prerequisite for initiation of DNA replication (15). Biochemical and genetic evidence has indicated that MCM2 is the primary target of Cdc7 kinase during S phase (5, 7, 16).

Cdc7-Dbf4 acts as a molecular switch for activation of replication origins (3). The activity of Cdc7 kinase is largely determined by the level of the activation subunits, which oscillates during cell cycle. The protein levels of the regulatory subunits are regulated at both transcriptional and post-translational levels. In yeast, the protein stability is regulated by APC (anaphase promoting complex), and the Dbf4 protein amount increases at the G1/S boundary and stays at a high level throughout S through G2 phases (17, 18).

Recently, a potentially novel Cdc7-Dbf4-related kinase complex which plays roles distinct from known S phase regulation was reported in fission yeast (19), and search of database indicates the presence of other Dbf4-related molecules on the genome of higher eukaryotes (data not shown). Through comparison of the primary structures of the regulatory subunits for Cdc7-related kinases and Dbf4-related molecules, we have identified three conserved motifs and two of them bear similarity to the BRCT (BRCA1 carboxyl terminal domain) motif and a CCHH-type zinc-finger motif.

Abbreviations used: ORC, origin recognition complex; MCM, minichromosome maintenance; preRC, prereplicative complex; APC, anaphase promoting complex; BRCT, BRCA1 carboxyl terminal domain.

[1] To whom correspondence should be addressed. Fax: 81-3-5449-5424. E-mail: hisao@ims.u-tokyo.ac.jp.

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MATERIALS AND METHODS

The multi-sequence alignment program "DIALIGN2.1" (20) was used to obtain the best alignment of multiple polypeptide sequences. Protein patterns were searched by "Proscan" program (Pole Bio-Informatique Lyonnais).

RESULTS AND DISCUSSION

Three Conserved Motifs in Dbf4-Related Molecules

In spite of striking similarity to cyclins in its periodic expression and kinase activation, Dbf4 and its functional homologues do not bear any sequence similarity to known cyclin molecules. Therefore, the mechanism of kinase activation of Cdc7 by Dbf4 may be quite different from that of Cdk by cyclins. The structures of the Cdc7 catalytic subunits are also unique in that they contain insert sequences which split kinase conserved motifs, although the kinase domains share about 45% identity between yeasts and human (6). In contrast, known activation subunits for Cdc7-related kinases share only a low degree of sequence similarity. Dbf4 and Him1/Dfp1, the fission yeast Dbf4 homologue, share only 26% identity in the central 254 amino acids (from 165 to 416 of Him1/Dfp1 protein) in addition to the C-terminal 69 amino acids (465–534) with 39% identity (7, 10), and no overall sequence similarity was identified between the human homologue ASK and yeast counterparts (9, 11).

We have identified three small stretches of amino acids which are present in all the known Dbf4-related molecules from various eukaryotes as well as in coding frames identified in database. We have named these conserved motifs, in the orientation from N-terminus to C-terminus, Dbf4-motif-N, Dbf4-motif-M and Dbf4-motif-C, respectively, since they are always found in this orientation on the polypeptides (Fig. 1). These motifs are found in Spo6 (20), a second fission yeast Dbf4-related protein whose functions are specific for meiosis. Recently, Spo4 (Database Accession No. AB036342), another Cdc7-like kinase in fission yeast, was identified and was found to form a complex with Spo6 (21). This, together with the presence of unidentified open reading frames containing similar Dbf4 motifs in the database (data not shown), suggests the presence of a novel family of Cdc7-Dbf4-related kinase complexes with distinct cell cycle functions for each member.

Dbf4-Motif-N Related to BRCT

Dbf4-motif-N shows some similarity to the domain I of BRCT, which was shown to be present on wide varieties of repair and DNA damage/replication checkpoint proteins (22, Fig. 2). Sequences similar to Dbf4-motif-N were found in fission yeast Cut5/Rad4 (23) and its budding yeast homologue Dpb11 (24). These sequences are parts of previously identified BRCT motifs in these proteins. Dbf4-motif-N is dispensable for mitotic functions but plays essential roles in DNA replication checkpoint and recovery from DNA damages (10). Thus, this motif may interact with checkpoint regulators to transduce checkpoint signaling from the replication forks to cell cycle and/or repair machinery. Studies on budding yeast Dbf4 indicated its sequence-specific interaction with replication origins (25), possibly through association with replication machinery at the origins. Dbf4-motif-N may also interact with components of replication complexes to facilitate recognition of its substrates and/or to promote responses to stalled replication forks.

Dbf4-Motif-M, a Proline-Rich Motif, Involved in Protein-Protein Interaction

Dbf4-motif-M is characterized by the presence of conserved prolines and aromatic residues. In spite of extensive search of database by using Profile Scan or PATTINPROT search as well as by other programs, no similar motifs have been identified on database. Thus, Dbf4-motif-M may represent a novel protein interaction domain unique to the Dbf4 protein family. Functional significance of Dbf4-motif-M is strongly indicated by the existence of amino acid substitutions of conserved prolines in this motif in some of the naturally occurring *dbf4(ts)* mutants of *S. cerevisiae* (Fig. 1). We have recently shown that an isolated Dbf4-motif-M polypeptide can bind to Hsk1 (Ogino *et al.*, unpublished result). Thus, this motif functions for association with and activation of the catalytic subunit.

Dbf4-Motif-C, a CCHH-Type Zinc Finger-like Motif

Dbf4-motif-C bears the highest degree of conservation among the three, and it contains the highly conserved $GXCEXC(X)_9H(X)_5H(X)_2FA$ motif, which resembles the CCHH-type zinc finger motif (consensus: $C\text{-}N_{2-4}\text{-}C\text{-}N_3\text{-}U\text{-}N_8\text{-}H\text{-}N_{3-5}\text{-}H$ where N and U represent any residue and hydrophobic or aromatic residues, respectively) (Fig. 1, 26). Dbf4-motif-C is also essential for functions of Him1/Dfp1, since C-terminally truncated mutants of Him1 protein lacking a portion or all of this motif cannot exhibit full mitotic functions. An isolated Dbf4-motif-C polypeptide of 65 amino acid can bind to Hsk1 (Ogino *et al.*, unpublished result). Consistent with this result, the 50 amino acid segment of Him1/Dfp1 protein (10) and the C-terminal 123 amino acids of Dbf4 (25, 27), both of which contain the Dbf4-motif-C, were shown to be sufficient for interac-

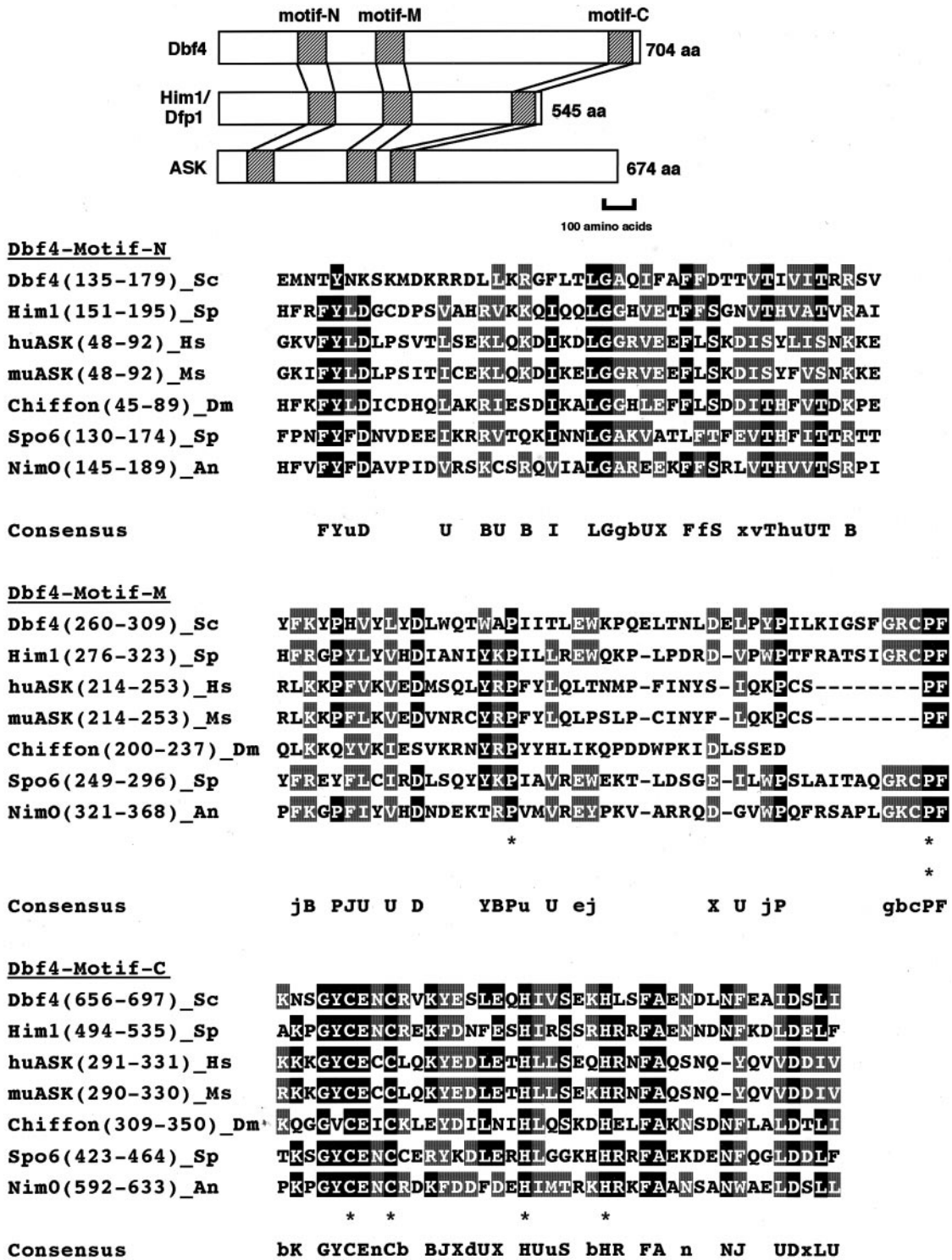

FIG. 1. Alignment of three conserved Dbf4-motifs. The upper drawing shows relative locations of each motif on the Cdc7 regulatory subunits from three species, shown by striped boxes (Dbf4 from budding yeast, Him1/Dfp1 from fission yeast and ASK from human). The white letters in black backgrounds represent those amino acids conserved in more than five members of the seven Dbf4-related proteins, Dbf4, Him1/Dfp1, huASK, muASK, Chiffon, Spo6 and NimO. The white letters in gray backgrounds represent the positions at which similar or identical amino acids are found in more than half of the members. In the consensus, the residues conserved in more than five members are indicated by large capitals, while those conserved in four members are shown with small letters. In Dbf4-motif-M, the two proline residues indicated by asterisk were shown to be mutated in *dbf4* temperature-sensitive mutants (the proline with one asterisk to leucine in *dbf4-1,* and the proline with two asterisk to leucine or serine in *dbf4-2* or *dbf4-3,* respectively; Sugino *et al.*, submitted). In Dbf4-motif-C, the conserved cysteine and histidine residues predicted to constitute a CCHH zinc-finger structure are indicated by asterisks. The data base

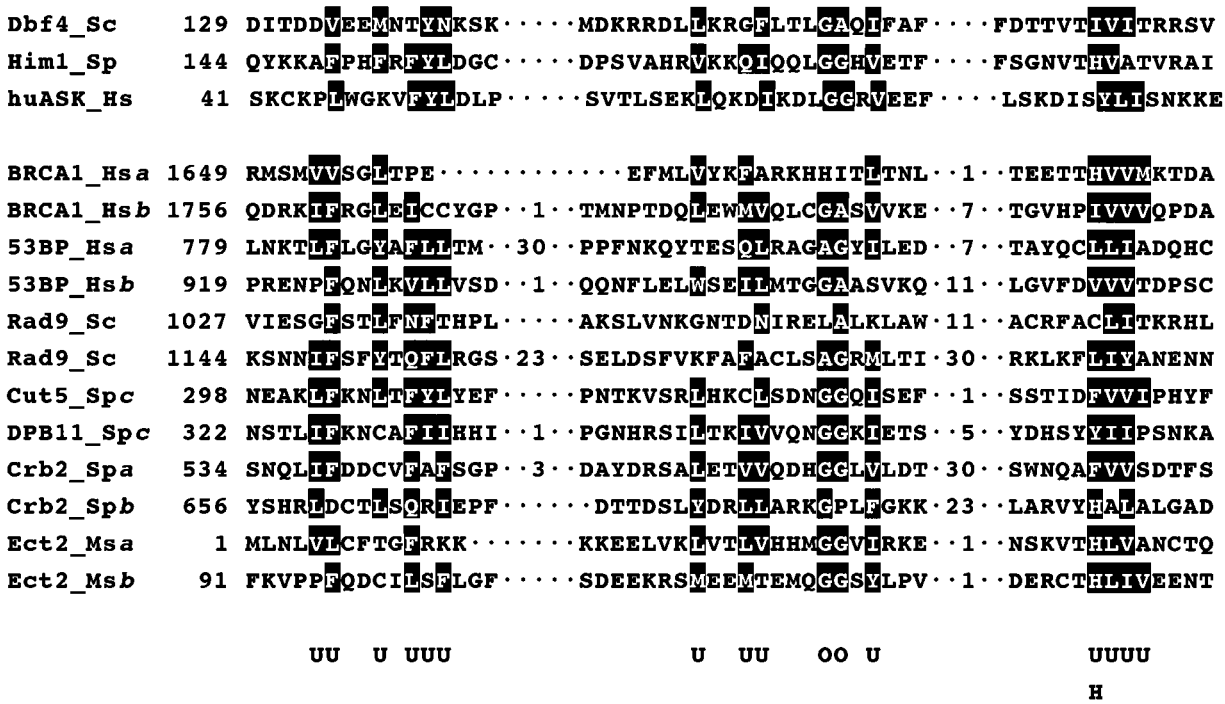

FIG. 2. Alignment of Dbf4-motif-N with sequences within the representative BRCT domains. The residues which conform with the consensus for the BRCT domain (22), shown at the bottom of the alignment, are shown in white letters with black background. Only the motif-I of the BRCT domain is shown. U, hydrophobic residues; O, small residues; H, histidine. The italic letters following the name of the protein sequence indicate consecutive copies of the BRCT domain present within the same protein. The numbers at the beginning of the sequences indicate the position of the first residue. The numbers within the gaps of the sequences indicate those of the additional residues present within the gaps. Sc, *Saccharomyces cerevisiae;* Sp, *Schizosaccharomyces pombe;* Hs, human; Ms, mouse.

tion with Hsk1 and Cdc7, respectively, in two-hybrid assays.

Regions on Dbf4-Related Proteins Other Than Dbf4-Motifs

The regions N-terminal to Dbf4-motif-N are diverged both in terms of lengths and sequences among species and are unlikely to play significant roles. The distances between Dbf4-motif-N and Dbf4-motif-M are relatively constant; 86 amino acids in Dbf4 and Him1/Dfp1, and 127 amino acids in ASK. The sequences of this region are well conserved between Him1/Dfp1 and Dbf4. Therefore, this segment, which may not be essential for mitotic functions, still play some role in functions of Him1/Dfp1 protein. On the other hand, the distances between Dbf4-motif-M and Dbf4-motif-C are variable with very little sequence conservation; 357, 182 and 40 amino acids in Dbf4, Him1/Dfp1 and ASK, respectively. This region may be involved in species-specific interactions between regulatory and catalytic subunits. Alternatively, it may not be essential for kinase activation nor mitotic functions and serve merely as a "bridging segment" which connects the two conserved motifs for kinase activation.

Identification of conserved motifs on Dbf4-related molecules will provide important framework to further elucidate details of protein-protein interactions as well as mechanisms of kinase activation involving this important cell cycle regulator, and will also help identify novel members of "Dbf4 family."

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accession numbers are as follows; Dbf4, Siss-prot DBF4_YEAST; Him1, TrEMBL 059836; huASK, TrEMBL Q9Y2M6; muASK, gp: MMDBF4_1; Chiffon, gp:AF158178_1; Spo6, gp:AB020809; NimO, TrEMBL 093843. The abbreviations for species from which each member is derived are as follows; Sc, *Saccharomyces cerevisiae;* Sp, *Schizosaccharomyces pombe;* Hs, human; Ms, mouse; Dm, *Drosophila melanogaster;* An, *Emericella nidulance.* Amino acids are indicated by regular one letter representation in addition to the following denotations; U, hydrophobic residues; J, aromatic residues; B, basic residues; X, acidic residues. Some of the calculated E values of each motif in comparison with Him1 are as follows. Motif-N: huASK, 0.39; muASK, 0.51; Chiffon, 5e-07; Spo6, 0.005; NimO, 0.005. Motif-M: Dbf4, 0.001; Spo6, 8e-8; Nim0, 6e-08. Motif-C: Dbf4, 7e-07; huASK, 3e-04; muASK, 3e-04; Chiffon, 0.002; Spo6, 7e-07; NimO, 3e-10.

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