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Identification and characterization of mouse homologue to yeast Cdc7 protein and chromosomal localization of the cognate mouse gene Cdc7l

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Abstract. The Cdc7 kinase is required for the G1/S-phase transition during the cell cycle and plays a direct role in the activation of individual origins of replication in *Saccharomyces cerevisiae*. Here, we report the identification of a mouse cDNA, MmCdc7, whose product is closely related in sequence to *Saccharomyces cerevisiae* Cdc7 as well as their human, *Xenopus* and *Schizosaccharomyces pombe* homologues. The MmCdc7p contains the conserved subdomains common to all protein-serine/threonine kinases and three kinase inserts that are characteristic of members of the Cdc7 protein family. We have mapped the locus of the MmCdc7 gene to chromosome 5, band 5E. Conservation of structures among members of the Cdc7-related proteins suggests that these proteins play a key role in the regulation of DNA replication during the cell cycle in all eukaryotes.

Introduction

The initiation of DNA replication is a crucial event in the cell cycle and, therefore, is tightly regulated in eukaryotic cells. Initiation takes place at multiple origins of replication. At the end of mitosis, initiator proteins such as the origin recognition complex (ORC) (Bell and Stillman 1992), Cdc6 (Cocker et al. 1996), and the MCM proteins (Tye 1994) are assembled to form the pre-replicative chromatin (Dutta and Bell 1997). In budding yeast, in vivo footprinting experiments have shown that the ORC may be bound to origins in all stages of the cell cycle (Bell and Stillman 1992). However, different patterns of nucleotide contacts have been observed before and after DNA replication (Diffley et al. 1994; Diffley 1996). Therefore, it has been proposed that the ORC functions to recruit other initiator proteins to replication origins in a cell cycle-dependent manner and thereby establish replication-competent chromatin.

The assembly and activation of competent replication chromatin are under the control of the cell cycle-dependent kinase activities (Nasmyth 1996; Stillman 1996). The ORC proteins and other factors that interact with ORC and/or origins may be the targets of kinase activities. These factors include the MCM2–7 proteins as well as the Cdc6, Cdc7 and Dbf4 proteins (reviewed by Dutta and Bell 1997). The MCM2–7 proteins are essential for the initiation of DNA synthesis at the origins of replication (Yan et al. 1993). In *Saccharomyces cerevisiae*, binding of MCM proteins to chromatin appears to be essential for the transition of chromatin from the replication-incompetent to the replication-competent complex (Kubota et al. 1995). The loading of MCM2–7 to the chromatin is dependent on the binding of ORC and Cdc6 protein to the chromatin (Liang et al. 1995; Coleman et al. 1996). Recently, it was shown that MCM2–7 are localized at replication origins during G1-phase (Aparicio et al. 1997; Tanaka et al. 1997). Thus, the MCM proteins may determine the competence of replication origins. Changes in the phosphorylation states of the MCM proteins are crucial for the cell cycle-specific functions of these proteins (Todorov et al. 1995; Coue et al. 1996; Hendrickson et al. 1996; Young and Tye 1997), suggesting that protein kinases are involved in the regulation of MCM activities during the transition from the G1- to the S-phase.

The serine/threonine protein kinase Cdc7p (Hartwell 1973) is essential for the G1/S transition (Hollingworth and Scalfani 1992; Jackson et al. 1993). Although the expression level of Cdc7p remains constant through the cell cycle, Cdc7 kinase activity is activated at the G1/S boundary by the regulatory subunit Dbf4 (Jackson et al. 1993; Kitada et al. 1993). Dbf4, like cyclins, is expressed periodically during the cell cycle with transcript levels reaching the maximum at the G1/S-phase boundary (Jackson et al. 1993). Physical and functional inter-

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action between Cdc7-Dbf4 and replication proteins or replication origins has been observed (Dowell et al. 1994; Hardy and Pautz 1996; Hardy et al. 1997). These findings suggest that the Cdc7-Dbf4 protein complex is directly involved in initiation of DNA replication. The targets of the Cdc7-Dbf4 protein kinase are not known, but MCM proteins are favourite candidates. This was recently confirmed by the demonstration that Cdc7-Dbf4 phosphorylates Mcm2 at the G1 to S-phase transition (Lei et al. 1997). Moreover, it was shown that Cdc7-Dbf4 is also required for the activation of late-firing origins during S-phase (Bousset and Diffley 1997; Donaldson et al. 1997). Rather than acting as a global regulator of the G1-S transition, Cdc7 appears to play a more direct role in the firing of replication origins during S-phase.

Homologues of *S. cerevisiae* Cdc7 have been described for *Schizosaccharomyces pombe* (Hsk 1) (Masai et al. 1995), human (Jiang and Hunter 1997; Sato et al. 1997), and *Xenopus laevis* (Sato et al. 1997).

In this communication we report the isolation of a murine cDNA encoding a Cdc7-related kinase (MmCdc7). The mouse protein is closely related to *S. cerevisiae* Cdc7 as well as to their human and *Xenopus* homologues. We have mapped the locus of the murine *Cdc7* gene to chromosome 5, band 5E5.

Materials and methods

cDNA library screening and sequencing. A cDNA library from 9 day old mouse embryos (library no. 559, RessourcenZentrum, Berlin-Dahlem) was screened under low-stringency conditions. The 418 bp human Cdc7 cDNA probe used (EST clone No. IMAGp998F08588, RessourcenZentrum, Berlin-Dahlem) corresponded to amino acids 398–538 of the published sequence (Jiang and Hunter 1997; Sato et al. 1997). The probe was labeled by random priming (*redi*prime, Amersham). Filters were hybridized for 16 h with 5×SSC, 5×Denhardt's solution 0.5% SDS and 100 µg/ml calf thymus DNA. Filters were washed twice with 2×SSC, 0.1% SDS for 30 min, followed by 0.2×SSC, 0.1% SDS for 20 min and 0.1×SSC, 0.1% SDS for 20 min. Eight positive clones were identified with inserts of ~1.9 kb and ~3.0 kb. The larger cDNA clone (MPMGp559G19143Q3) was sequenced. The sequence is deposited with the EMBL Nucleotide Sequence Database under Accession No. AJ007661.

Fluorescence in situ hybridization (FISH). Chromosomal localization of mouse Cdc7 was performed by hybridizing a biotinylated full-length cDNA clone to metaphase chromosomes of the mouse WMP cell line in which all chromosomes are in the form of metacentric Robertsonian (Rb) chromosomes. The FISH experiments were performed following published procedures (Lichter et al. 1990). Briefly, denatured metaphase spreads were allowed to hybridize at 37°C overnight with the biotinylated murine Cdc7 cDNA probe (150 ng per slide) in a hybridization mixture consisting of 50% formamide, 10% dextran sulphate, 2×SSC and a 100-fold excess of sonicated denatured salmon sperm DNA. Slides were washed in 2×SSC containing 50% formamide at 37°C for 15 min and subsequently in 2×SSC for 15 min. Preparations were counter-stained simultaneously with propidium iodide and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) and hybridization signals were visualized under a Zeiss Axiophot microscope.

Results and discussion

In an attempt to obtain mouse sequences related to the human Cdc7 protein, a cDNA library from 9 day old mouse embryos (library no. 559, RessourcenZentrum, Berlin-Dahlem) was screened under low-stringency conditions. The 434 bp human Cdc7 cDNA probe corresponded to amino acids 398–538 of the published sequence (Jiang and Hunter 1997; Sato et al. 1997). Eight positive clones were identified with inserts of ~1.9 kb and ~3.0 kb. Sequencing of the largest cDNA clone (MPMGp559G19143Q3) revealed an open reading frame encoding a predicted protein of 564 amino acids (Fig. 1a), with an approximate molecular mass of 62,000. The initiator methionine is preceded by an untranslated leader sequence of 141 nucleotides that contains stop codons in all three reading frames. Multiple in-frame stop codons are present in the 1,196 nucleotide 3' UTR (untranslated region) downstream of the stop sequence that terminates the open reading frame. Also, a poly(A) tail is present.

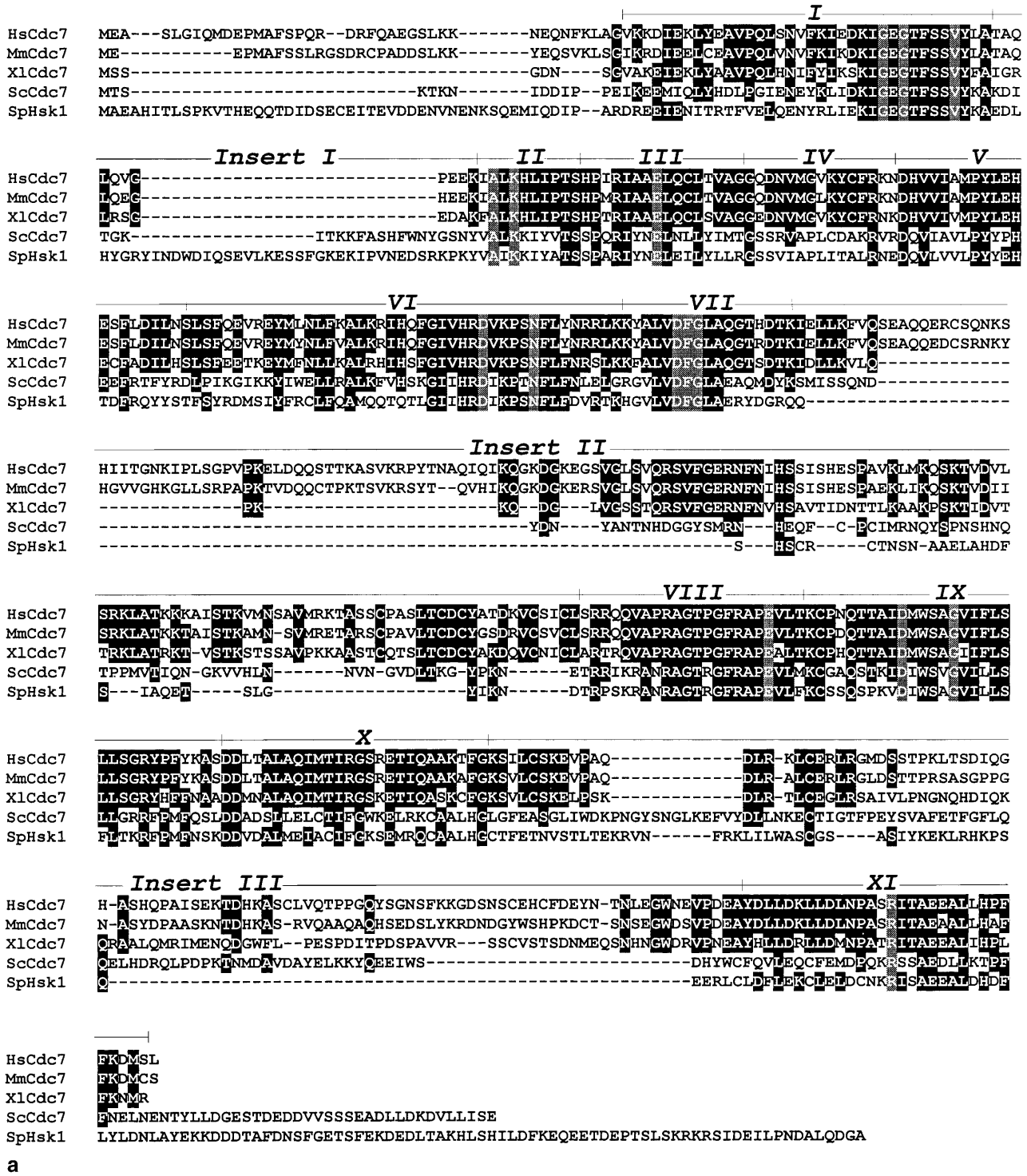
Analysis of the full-length cDNA demonstrated that MmCdc7 contains the 11 conserved subdomains found in all protein-serine/threonine kinases (Fig. 1a, and Hanks et al. 1988). Sequence identity analysis indicated that the kinase domain of MmCdc7 was closely related to Cdc7p of *S. cerevisiae* and its *S. pombe, Xenopus* and human homologues (Fig. 1a–c). As shown in Fig. 1b, the kinase domain of MmCdc7 has 94%, 77%, 44% and 43% identity in amino acid sequence with the Cdc7-related proteins of human and *Xenopus* and Cdc7 and Hsk1, respectively.

MmCdc7 also contains three "kinase insert" domains between kinase domains I and II, VII and VIII, and X and XI, designated as kinase inserts I, II and III, respectively. The other members of the Cdc7-related kinase group also have kinase inserts, of thus far undetermined biological function, at the same locations (Masai et al. 1995; Jiang and Hunter 1997; Sato et al. 1997). However, the sequences and lengths of these kinase inserts are not conserved among all the Cdc7-related kinases. In particular, kinase insert I is significantly longer in fission and budding yeasts compared with vertebrates (Fig. 1a).

A peculiar feature of both Cdc7p and Hsk1 is the presence of distinct C-terminal regions after the kinase domains that are rich in acidic residues. In *S. cerevisiae*, this C-terminal tail interacts with the Dbf4 protein (Jackson et al. 1993). MmCdc7, like the other Cdc7-related proteins of vertebrates (Masai et al. 1995, Jiang and Hunter 1997; Sato et al. 1997), does not contain similar C-terminal regions.

A region that conforms to the P-loop ATP/GTP-binding consensus sequence (Koonin 1993) was found (motif A, residues 430–437). Surprisingly, MmCdc7p does not include full-length consensus sites for phosphorylation by cyclin-dependent kinases (Jans et al. 1995). However, four partial (S/T)P sites are contained in MmCdc7p. It is highly probable that this protein could be phosphorylated at these positions by Cdks.

The chromosomal localization of mouse *Cdc7* was determined by hybridizing a biotinylated full-length cDNA clone to metaphase chromosomes of the mouse cell line WMP in which all chromosomes except chromosome 19 and the sex chromosomes are in the form of metacen-



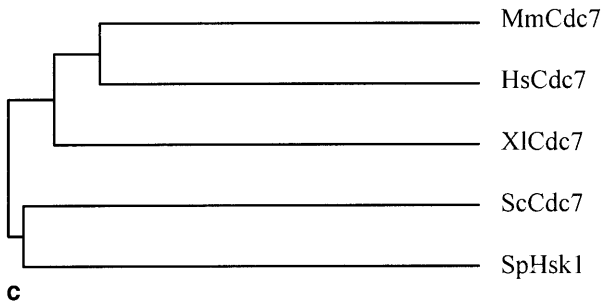
a

Fig. 1. a The amino acid sequence of murine Cdc7 aligned to the sequences of related proteins using the Clustal W program (NCBI). The *black* and *grey boxes* indicate identities between the mouse Cdc7 and the Cdc7 proteins from *Homo sapiens, Xenopus laevis, Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. The 11 conserved kinase domains common to all protein-serine/threonine kinases and the three kinase inserts are indicated by *bars*. *Grey boxes* indicate the locations of amino acid residues conserved in all protein-serine/threonine kinases (Hanks et al. 1988). **b** Per cent homology between Cdc7-related proteins from various species. **c** Hypothetical phylogenetic tree indicating the relative similarities between Cdc7-related proteins from various species

tric Rb chromosomes. Under low-stringency wash conditions, positive fluorescein isothiocyanate (FITC) signals were consistently noted on a pair of chromosomes that differed in arm length (Fig. 2). One of the chromosomes carrying the hybridization signal is the largest chromosome in the complement and its DAPI staining, revealing a faint Q-band pattern, indicated that this particular chromosome is the result of a complex translocation between chromosomes 5, 14 and 4 (Zörning et al. 1995). The long arm is composed of parts of chromosome 5 and translo-

Comparison	Identical or conserved residues	
	Total protein	Conserved kinase subdomains
Mouse vs human	80%	94%
Mouse vs *Xenopus*	55%	77%
Mouse vs. *S. cerevisiae*	38%	44%
Mouse vs *S. pombe*	39%	43%

b



c

Fig. 1b, c. (continued)

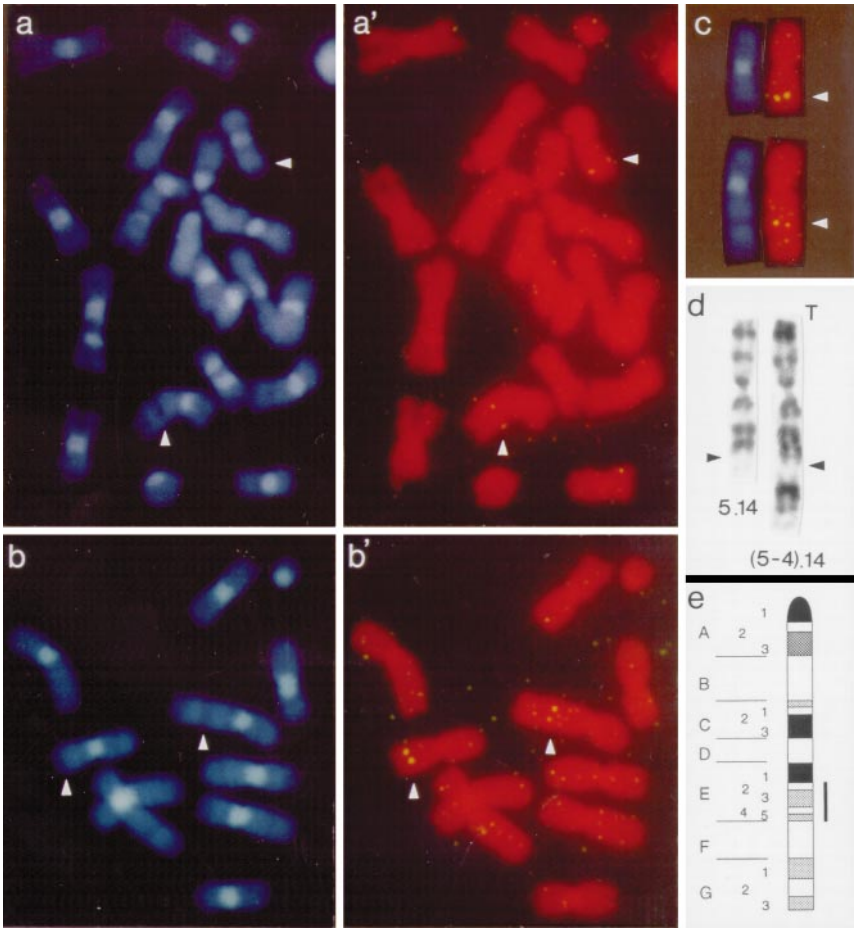


Fig. 2a–e. Chromosomal localization of the mouse *Cdc7* gene using fluorescence in situ hybridization (FISH). **a** 4', 6-diamidino-2-phenylindole (DAPI) counter-stained metaphase spread from a mouse WMP cell after hybridization to the Cdc7 probe. **a'** Same metaphase showing Cdc7-specific fluorescein isothiocyanate (FITC) signals on propidium iodide (PI) counter-stained chromosomes. The *arrowheads* point to the location of the hybridization signals confined to chromosome 5. **b** A partial metaphase spread of a WMP cell counter-stained with DAPI after hybridization to the Cdc7 probe. **b'** Same metaphase showing Cdc7-specific FITC signals on PI counter-stained chromosomes. The *arrowheads* point to the location of the hybridization signals confined to chromosome 5. **c** Selected cut-outs (*left* DAPI; *right* PI stained) showing the hybridization of the Cdc7 probe on chromosome 5 in the normal fusion chromosome as well as on the large submetacentric chromosome derived from the fusion of three different chromosomes. The DAPI banding pattern and the length of this marker chromosome allow the unambiguous identification of the complex rearranged chromosome in the metaphase spread. **d** G-banding of the chromosomes of WMP cells showing in detail the rearrangements of chromosome 5. Note that chromosome 5 is translocated to two different chromosomes. Both homologues of chromosome 5 are fused with chromosome 14, forming a standard fusion chromosome (*Rb 5.14*). However, one of these fusion chromosomes (*T*) is again fused to chromosome 4 at the distal long arm region, resulting in a complex rearranged chromosome [Rb (*5–14).4*]. The corresponding FISH signals of chromosome 5 on both rearranged chromosomes are indicated (*arrowheads*). **e** Schematic representation of a normal mouse chromosome 5 illustrating the location of the *Cdc7* locus at the E region

cated material from chromosome 4 (Fig. 2e). This particular rearranged chromosome can unequivocally be distinguished from other rearranged chromosomes in the metaphase spread by its characteristic morphology and DAPI banding pattern. The fluorescent signal on this chromosome was localized at the middle of the long arm. Because of the compound nature of the long arm of this particular chromosome it was initially difficult precisely to assign the locus either to chromosome 4 or to chromosome 5. However, the other copy of chromosome 5, which is fused to chromosome 14 (Rb 5.14), recurrently showed the fluorescent signal close to the distal region of its long arm (Fig. 2), allowing us to assign the mouse *Cdc7* locus to chromosome 5. Of the 20 metaphases screened, 19 showed signals on homologous regions of both chromosomes 5 and the hybridization signals appeared as twin spots, implying binding of the probe to both chromatids (Fig. 2). The FITC signal was more distal to the prominent DAPI fluorescing band of the long arm of the chromosome 5 but was never marked at the tip of the chromosome. From a detailed evaluation of the hybridization signal in ten metaphase plates and relating the hybridization signal location to the mouse G-band idiogram we could further refine the location of the *Cdc7* locus on chromosome 5 to region E.

Comparative gene mapping shows that considerable synteny exists between the distal region of mouse chromosome 5 and human chromosome 1p (Bell et al. 1995). The present assignment of the *Cdc7* locus to mouse chromosome 5 provides further evidence to support this synteny.

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