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Human and *Xenopus* cDNAs encoding budding yeast Cdc7-related kinases: *in vitro* phosphorylation of MCM subunits by a putative human homologue of Cdc7

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Saccharomyces cerevisiae **Cdc7 kinase is essential for initiation of DNA replication, and Hsk1, a related kinase of *Schizosaccharomyces pombe*, is also required for DNA replication of fission yeast cells. We report here cDNAs encoding Cdc7-related kinases from human and *Xenopus* (huCdc7 and xeCdc7, respectively). The cloned cDNA for huCdc7 contains an open reading frame consisting of 574 amino acids with a predicted molecular weight of 63 847 that possesses overall amino acid identity of 32% (54% including similar residues) to Cdc7 and Hsk1. huCDC7 is transcribed in the various tissues examined, but most abundantly in testis. Three transcripts of 4.4, 3.5 and 2.4 kb in length are detected. The 3.5 kb transcript is the most predominant and is expressed in all the tissues examined. A cDNA containing a 91 nucleotide insertion at the N-terminal region of huCDC7 is also detected, suggesting the presence of multiple splicing variants. The huCdc7 protein is expressed at a constant level during the mitotic cell cycle and is localized primarily in nuclei in interphase and distributed diffusibly in cytoplasm in the mitotic phase. The wild-type huCdc7 protein expressed in COS7 cells phosphorylates MCM2 and MCM3 proteins *in vitro*, suggesting that huCdc7 may regulate processes of DNA replication by modulating MCM functions.**

Keywords: CDC7/cell cycle/DNA replication/ MCM proteins/serine-threonine kinase

Introduction

Initiation of chromosomal replication in eukaryotes is tightly regulated during the cell cycle. Genetic and biochemical studies in the yeast *Saccharomyces cerevisiae* have yielded considerable information on molecular interactions between replication machinery and cell cycle regulators (Coverley and Laskey, 1994; Huberman, 1995; Kearsey *et al.*, 1996). The replication origins of *S.cerevisiae*, which are composed of the conserved 11 bp 'A' or core sequence and auxiliary 'B' elements, exist as nucleoprotein structures which involve origin recognition complex (ORC), MCM, Cdc6 and probably other proteins (Campbell and Newlon, 1991; Walker *et al.*, 1991; Bell and Stillman, 1992; Diffley and Cocker, 1992; Marahrens and Stillman, 1992; Bell *et al.*, 1993; Diffley *et al.*, 1994; Rao *et al.*, 1994; Rowley *et al.*, 1994, 1995; Theis and Newlon, 1994; Fox *et al.*, 1995; Liang *et al.*, 1995; Loo *et al.*, 1995; Rao and Stillman, 1995). It was reported previously that these protein–DNA complexes may alternate between two distinct states during the cell cycle; one that exists prior to the S phase and the other that is detected during the G_2 and M phases. The former pre-replicative complex contains ORC and Cdc6 as well as MCM licensing factor, while the latter post-replicative complex may contain only ORC (Liang *et al.*, 1995; Cocker *et al.*, 1996; Donovan and Diffley, 1996). The pre-replicative complex may be activated by regulatory molecules which trigger the initiation of S phase. Genetic study of *S.cerevisiae* has implicated serine-threonine kinases in this step, among which the Cdc7–Dbf4 kinase complex may turn on the ultimate 'START' signal for the S phase (Kitada *et al.*, 1992; Jackson *et al.*, 1993; Dowell *et al.*, 1994; Sclafani and Jackson, 1994; Bell, 1995). In the presence of active Cdc7 kinase, the S phase can be completed in the absence of protein synthesis (Hartwell, 1974). Cdc7, whose kinase activity peaks at the G_1/S boundary, activates DNA replication machinery in conjunction with Dbf4, which not only stimulates its kinase activity but may also tether Cdc7 at the origins of replication (Jackson *et al.*, 1993; Yoon *et al.*, 1993; Dowell *et al.*, 1994).

The structures of DNA replication origins and modes of their activation have been elusive in higher eukaryotes (Hamlin and Dijkwel, 1995). However, identification of genes related to ORC and MCM components in *Xenopus*, *Drosophila* and mammals has strongly indicated that the basic components required for initiation of chromosomal replication may be conserved in higher eukaryotes (Thommes *et al.*, 1992; Hu *et al.*, 1993; Chong *et al.*, 1995; Ehrenhofer-Murray *et al.*, 1995; Gavin *et al.*, 1995; Gossen *et al.*, 1995; Kimura *et al.*, 1995; Kubota *et al.*, 1995; Treisman *et al.*, 1995; Carpenter *et al.*, 1996). We previously reported *hsk1*$^+$, whose product is a putative *Schizosaccharomyces pombe* homologue of Cdc7 kinase (Masai *et al.*, 1995). *hsk1*$^+$ is essential for viability of *S.pombe* cells, and analyses of DNA content and morphology of germinating spores containing *hsk1* null alleles indicated that *hsk1*$^+$ is required for DNA replication as well as for coupling of the M phase to S phase initiation. The presence of the structurally and functionally related kinases in two distantly related yeast species suggested the possibility that eukaryotic DNA replication may be regulated through a conserved mechanism which involves Cdc7-related kinases.

We report here isolation of human and *Xenopus* cDNAs encoding Cdc7-related kinases (huCdc7 and xeCdc7, respectively). We show that huCdc7 is a nuclear protein kinase expressed at a constant level throughout the cell cycle. We also report that huCdc7 expressed in COS7 cells phosphorylates MCM components *in vitro*, suggesting possible regulation of MCM functions by Cdc7-related kinase.

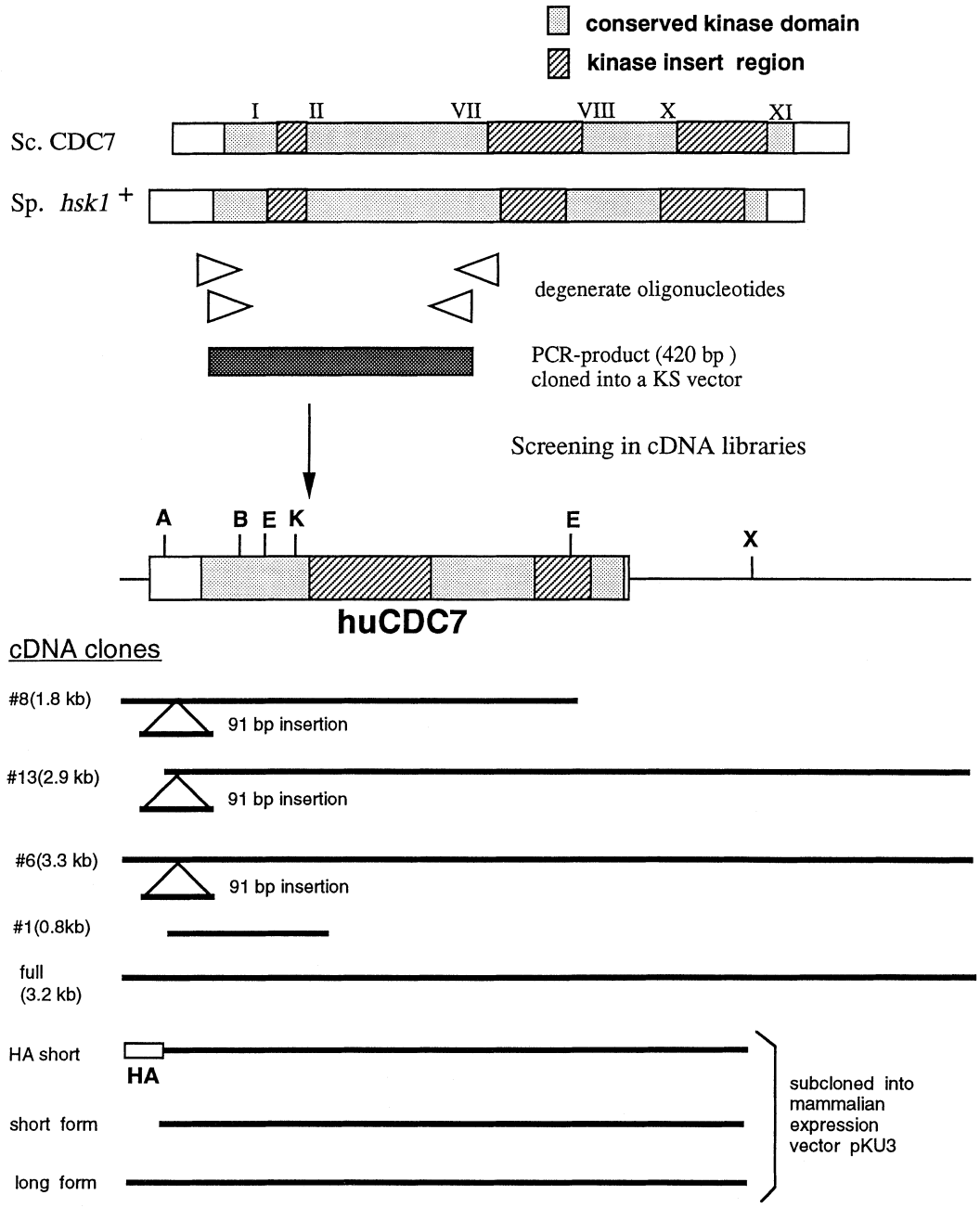

Fig. 1. Strategy for PCR cloning of CDC7-related kinases and cloned cDNAs. Two sets of degenerate primers indicated by open arrowheads were designed on the basis of the conserved amino acid sequences between the products of *S.cerevisiae* (Sc.) *CDC7* and *S.pombe* (Sp.) *hsk1*$^+$. Shaded and striped regions represent conserved kinase domains and kinase insert sequences, respectively, whereas open areas indicate N- and C-terminal tail sequences. A, B, E, K and X represent the sites for *Age*I, *Bcl*I, *Eco*RI, *Kas*I and *Xba*I, respectively. HA indicates a haemagglutinin epitope sequence. The thick bars indicate the segments present in the isolated cDNAs or those subcloned into vectors. Details are described in the text.

Results

Isolation of human and Xenopus cDNAs related to S.cerevisiae CDC7 and S.pombe hsk1$^+$

In order to isolate *CDC7*/*hsk1*$^+$-related genes from higher eukaryotes, we performed RT–PCR on mRNA isolated from murine embryonic stem (ES) cells, using degenerate oligonucleotide probes designed on the basis of amino acid sequence homology between the products of *CDC7* and *hsk1*$^+$. A PCR product ~420 bp long, generated with a set of the primers derived from the conserved amino acid sequences of kinase subdomains I and VII, was isolated and used as a template for the second nested PCR with primers derived from the conserved domains I and VI. The second PCR gave rise to one major band of expected size (~350 bp). Therefore, the 420 bp long PCR product from the first PCR was subcloned into the T vector (Marchuk *et al.*, 1991) and its sequence was determined.

Sequencing of this DNA fragment revealed the presence of a reading frame that showed substantial homology (52 identical amino acids from 138 residues) to both Cdc7 and Hsk1. Screening of human and *Xenopus* cDNA libraries using this DNA fragment as a probe led to isolation of three human and three *Xenopus* clones, and the inserts of these positive clones were subcloned into KS vectors for further characterization (Figure 1).

The restriction mapping of the 1.8 and 2.9 kb inserts from the human clones #8 and #13 (both obtained from a fetal liver library), respectively, indicated the presence of an overlapping region between the two inserts. The nucleotide sequence of 1756 bp long #8 cDNA revealed the presence of a 334 bp 5′-untranslated region and a 1422 bp stretch of a coding region. The clone #13 cDNA did not contain 5′-untranslated region, but possessed a 1416 bp stretch of a coding region and a 1334 bp 3′-untranslated region together with poly(A) sequences. Although a 535 amino acid long coding region could be deduced by combining #8 and #13 cDNAs (#6), a start codon ATG was not found upstream of the kinase subdomain I. The N-terminal region of clone #8 was identical to the portion of the sequence of #1 (obtained from a testis library), except that #1 cDNA had a 91 bp deletion. This 91 bp deletion would give rise to an open reading frame (ORF) that contains three possible ATG codons, among which the first (from the 5′ end) is used to initiate translation of huCdc7 protein (see below). The full-length huCDC7 cDNA contains a 1722 bp long ORF, encoding a 574 amino acid long protein with a predicted mol. wt of 63 847 (Figure 2).

Xenopus clones #25 and #28, obtained from a *Xenopus* oocyte library, were identical and the nucleotide sequence of the 1467 bp insert of #25 cDNA showed a 1425 bp long ORF frame and a 42 bp 3′-untranslated region. Clone #23, obtained from another *Xenopus* oocyte library, contained an additional eight amino acids at the N-terminus including the putative ATG initiation codon, and a 1449 bp long ORF, encoding a 483 amino acid protein with a predicted mol. wt of 53 509, was deduced by combining the three clones.

Primary structures of Cdc7-related kinases from higher eukaryotes

The predicted primary structures of human and *Xenopus* Cdc7-related kinase indicate that their kinase domains are highly homologous to those of Cdc7 and Hsk1 (Figure 3A). When confined to the kinase conserved domains, 44% identity at the amino acid level (62% including similar residues) is detected between Cdc7 and huCdc7 or xeCdc7, whereas 80% identity (90% including similar residues) is detected between human and frog. Amino acid sequence alignments of the kinase domains of Cdc7-related kinases were calculated together with other representative serine/threonine kinases, using the ClustalW program, and a hypothetical phylogenetic tree was drawn. In the phylogenetic tree, Cdc7-related kinases were classified into a subfamily distinct from other kinases, including CDK, CKII or MAPK (Figure 3B), indicating that they are members of a unique ‘Cdc7’ kinase family.

Cdc7 and Hsk1 are characterized by the presence of three ‘kinase-insert’ sequences between the kinase domains I and II, VII and VIII, and X and XI, designated as kinase insert I, II and III, respectively. huCdc7 and xeCdc7 also contain two amino acid insertions at the same locations (corresponding to kinase inserts II and III), although the presence of kinase insert I was not obvious in the human and frog clones. The lengths and sequences of the kinase inserts are not conserved between yeasts and higher eukaryotes as they are not between the two yeast species, although weak homology is identified between the human

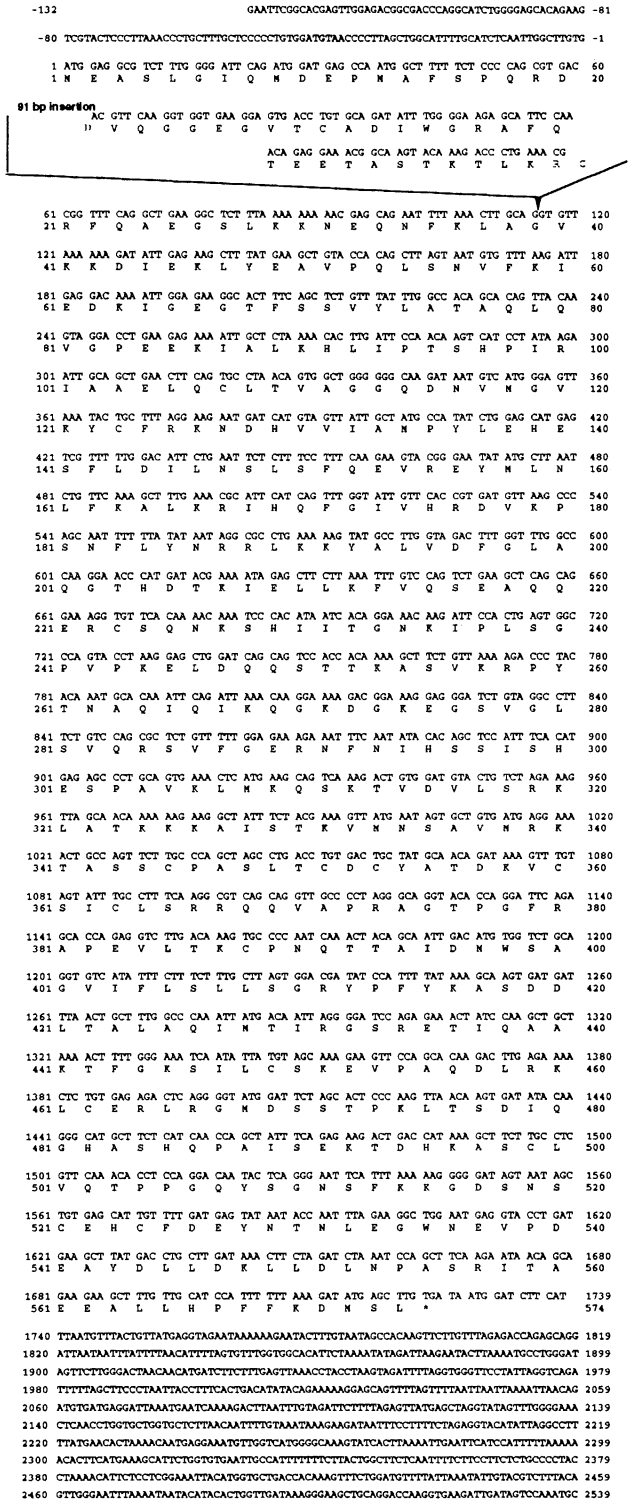

Fig. 2. Nucleotide sequences of a full-length huCDC7 cDNA and predicted amino acid sequences. The A residue of the putative first methionine codon is taken as +1. The position and nucleotide sequence of the 91 bp insertion is also shown. The poly(A) addition signal is double-underlined.

A

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                                                                                <I>
1 huCdc7 MEASLGIQMDEPMAF SPQRDRFQAEGSLKK NEQNFKL-------- ---AGVKKDIEKLYE AVPQLSNVFKIEDKI GEGTFSSVYLATAQL    79
2 xeCdc7 ---------------- ------------MSS GD-N----------- ---SGVAKEIEKLYA AVPQLHNIFYIKSKI GEGTFSSVYFAIGRL    48
3 ScCdc7 ---------------- --------MTSKTKN IDDIP---------- ---PEIKEEMIQLYH DLPGIENEYKLIDKI GEGTFSSVYKAKDIT    54
4 SpCdc7 -MAEAHITLSPKVTH EQQTDIDSECEITEV DDENVNENKSQEMIQ DIPARDREEIENITR TFVELQENYRLIEKI GEGTFSSVYKAEDLH    89
                                                          ..  .      .   . .  ** ********* *

          kinase insert 1                    <II>         <III>         <IV>                <V>
1 huCdc7 Q-------------- ----VG--------- --PEEKIALKHLIPT SHPIRIAAELQCLTV AGGQDNVMGVKYCFR KNDHVVIAMPYLEHE   140
2 xeCdc7 R-------------- ----SG--------- --EDAKFALKHLIPT SHPTRIAAELQCLSV AGGEDNVMGVKYCFR NKDHVVIVMPYLEHE   109
3 ScCdc7 G-------------- ----KITKKFASHFW NYGSNYVALKKIYVT SSPQRIYNELNLLYI MTGSSRVAPLCDAKR VRDQVIAVLPYYPHE   126
4 SpCdc7 YGRYINDWDIQSEVL KESSFGKEKIPVNED SRKPKYVAIKKIYAT SSPARIYNELEILYL LRGSSVIAPLITALR NEDQVLVVLPYYEHT   179
                                        *.*..  * * * **  **  * .   *   .  .  . *   *.*.  .**  *

                                  <VI>                        <VII>          kinase insert 2
1 huCdc7 SFLDILNSLSFQEVR EYMLNLFKALKRIHQ FGIVHRDVKPSNFLY NRRLKKYALVDFGLA QGTHDTKIELLKFVQ SEAQQERCSQNKSHI   230
2 xeCdc7 CFADILHSLSFEETK EYMFNLLKALRHIHS FGIVHRDVKPSNFLF NRSLKKFALVDFGLA QGTSDTKIDLLKVLQ ---------------   184
3 ScCdc7 EFRTFYRDLPIKGIK KYIWELLRALKFVHS KGIIHRDIKPTNFLF NLELGRGVLVDFGLA EAQMDYKSMISSQND ---------------   201
4 SpCdc7 DFRQYYSTFSYRDMS IYFRCLFQAMQQTQT LGIIHRDIKPSNFLF DVRTKHGVLVDFGLA ER------------- ---------------   241
          *               *   * .*..  .   **.***.**.***.       .  ******* .


1 huCdc7 ITGNKIPLSGPVPKE LDQQSTTKASVKRPY TNAQIQIKQGKDGKE GSVGLSVQRSVFGER NFNIHSSISHESPAV KLMKQSKTVDVLSRK   320
2 xeCdc7 ------------PKK QD------------ --------------- GLVGSSTQRSVFGER NFNVHSAVTIDNTTL KAAKPSKTIDVTTRK   234
3 ScCdc7 ---------------- YD------------ --------------- -NYANTNHDGGYSMR N---HEQFCPCIMRN QYSPNSHNQTPPMVT   244
4 SpCdc7 ---------------- YD------------ --------------- ------------GRQ Q--SHSCRCTNSNAA ELAHDFSIAQ-----   269
                         *                                          *   .

                                                      <VIII>                      <IX>
1 huCdc7 LATKKKAISTKVMNS AVMRKTASSCPASLT CDCYATDKVCSICLS RRQQVAPRAGTPGFR APEVLTKCPNQTTAI DMWSAGVIFLSLLSG   410
2 xeCdc7 LATR-KTVSTKSTSS AVPKKAASTCQTSLT CDCYAKDQVCNICLA RTRQVAPRAGTPGFR APEALTKCPHQTTAI DMWSAGIIFLSLLSG   323
3 ScCdc7 IQNG-KVVHLNNVNG VDLTKG--------- ---YPKNE------T RRIKRANRAGTRGFR APEVLMKCGAQSTKI DIWSVGVILLSLLGR   315
4 SpCdc7 --------ETSLG-- ---------------- ---YIKND------T RPSKRANRAGTRGFR APEVLFKCSSQSPKV DIWSAGVILLSFLTK   325
                                             *  . * **** *** *** * **  *.  . *.**.*.* ** *

               <X>                  kinase insert 3
1 huCdc7 RYPFYKASDDLTALA QIMTIRGSRETIQAA KTFGKSILCSKEVP- ---------AQDLRK LCERLRG---M---- DSSTPKLTSDIQGHA   483
2 xeCdc7 RYHFFNAADDMNALA QIMTIRGSKETIQAS KCFGKSVLCSKELP- ---------SKDLRT LCEGLRSAIVLPNGN QHDIQKQRAALQMRI   403
3 ScCdc7 RFPMFQSLDDADSLL ELCTIFGWKELRKCA ALHG----LGFEASG LIWDKPNGYSNGLKE FVYDLLNKECT---- IGTFPEYSVAFETFG   397
4 SpCdc7 RFPMFNSKDDVDALM EIACIFGKSEMRQCA ALHG----CTFETN- ---------------- ---------------- VSTLTEKRVNFR---   377
         *.  . . **  .*  .. .* *  *  ...    *       *

                                                                  <XI>
1 huCdc7 SHQPAISEKTDHKAS CLVQTPPGQYSGNSF KKGDSNSCEHCFDEY -NTNLEGWNEVPDEA YDLLDKLLDLNPASR ITAEEALLHPFFKDM   572
2 xeCdc7 MENQ------------ DGWFLPESPDITPDS PAVVRSSCVSTSDNM EQSNHNGWDRVPNEA YHLLDRLLDMNPATR ITAEEALIHPLFKNM   482
3 ScCdc7 FLQ------------ --QELHDRMSIEPQL PDPKTNMDAVDAYEL KKYQEEIWSD-HYWC FQVLEQCFEMDPQKR SSAEDLLKTPFFNEL   472
4 SpCdc7 ---------------- ---------------- KLILWASCGSASIYK EKLRHKPSQE-ERLC LDFLEKCLELDCNKR ISAEEALDHDFLYLD   436
                                                          .    *..  ..    *  .**. *


1 huCdc7 SL-------------- ---------------- ---------------- ---------------- -----------    574
2 xeCdc7 R--------------- ---------------- ---------------- ---------------- -----------    483
3 ScCdc7 NENTYLLD------- --GES---TDEDD-- -----VVSSSEADLL DKDVLLISE------ -----------    507
4 SpCdc7 NLAYEKKDDDTAFDN SFGETSFEKDEDLTA KHLSHILDFKEQEET DEPTSLSKRKRSIDE ILPNDALQDGA    507
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B

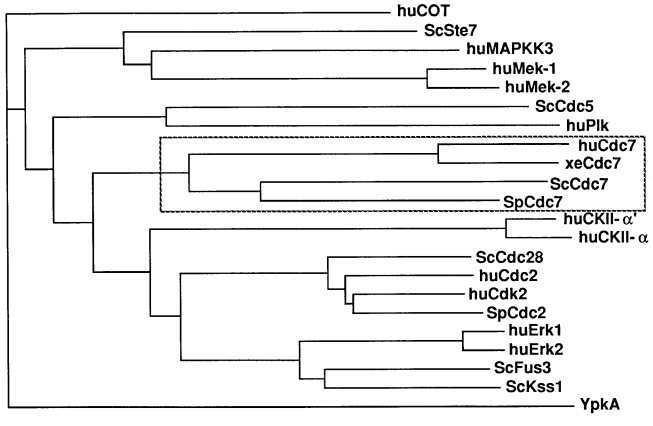

Fig. 3. Comparison of CDC7-related kinases from various organisms. (**A**) The predicted amino acid sequences of four CDC7-related kinases are aligned for maximum homology. Amino acid numbers are shown on the left. The roman figures above the sequences indicate the locations of the conserved kinase domains as previously proposed (Hanks *et al.*, 1988), which are boxed in grey. The asterisks indicate the amino acid residues conserved in all four proteins. (**B**) A hypothetical phylogenetic tree. Amino acid sequence alignments were calculated using the ClustalW program, and a phylogenetic tree was drawn by the SINCA program (Zuckerlandl and Pauling and neighbour-joining methods). huCdc7, xeCdc7, ScCdc7 and SpCdc7 indicate the Cdc7-related kinases from human, *Xenopus*, *S.cerevisiae* and *S.pombe*, respectively. COT, *cot* oncogene product; YpkA, enterobacterial protein kinase.

and *Xenopus* proteins. The kinase insert II of huCdc7 or xeCdc7 is 163 or 108 amino acid long, respectively, and 57 amino acids are identical, with 11 additional similar residues. The kinase insert III of huCdc7 or xeCdc7 is 98 or 95 amino acids long, respectively, of which 34 amino acids are identical and 14 additional amino acids are similar.

Another feature of Cdc7 and Hsk1 is the presence of

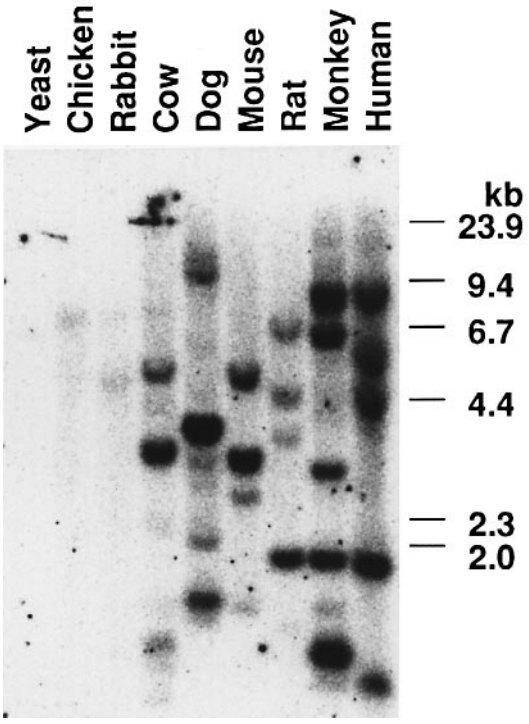

Fig. 4. Genomic Southern analysis of *CDC7*-related genes in various species. Genomic DNAs were digested with *Eco*RI. Membrane was hybridized using huCDC7 cDNA (probe A, nucleotide residues –132 to +1535; see the legend to Figure 5) as a probe, and washed in 0.1× SSC and 0.1% SDS at 50°C.

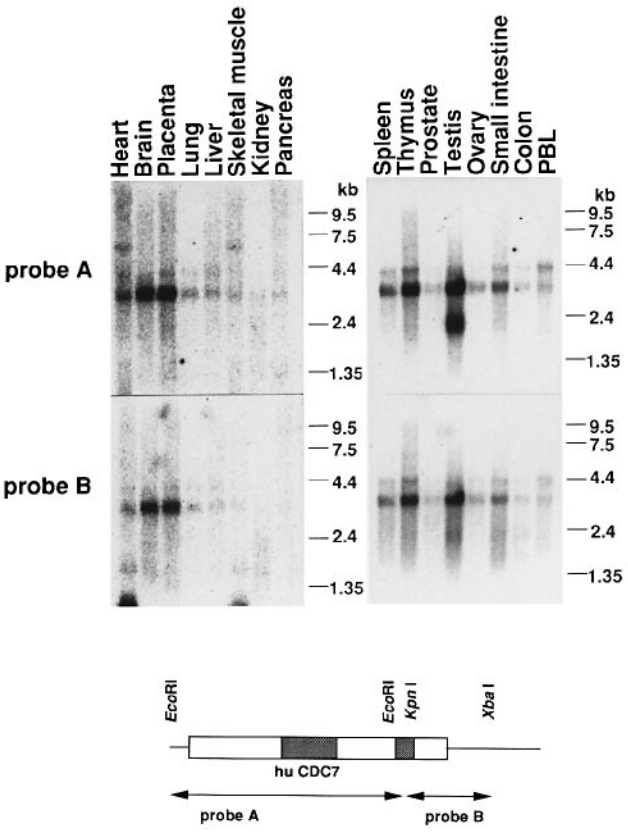

Fig. 5. Expression of huCDC7 mRNA in various human tissues. Membranes were hybridized with probe A (upper) or probe B (lower). Probes A and B include the huCDC7 cDNA fragment from nucleotide residue –132 to +1535 and that from +1611 to +2202, respectively. The shaded regions indicate the kinase insert sequences.

the C-terminal regions which are rich in acidic residues. Two-hybrid assays indicated that Dbf4 protein could interact with this C-terminal tail of Cdc7 protein in budding yeast (Jackson *et al.*, 1993). The C-terminal regions of both Cdc7 and Hsk1 are essential for the functions of these two kinases (our unpublished data). Unexpectedly, huCdc7 and xeCdc7 did not contain similar C-terminal tails (see discussion below).

Genomic Southern and Northern analysis

Southern analyses of genomic DNA from various eukaryotic species using two *Eco*RI fragments (0.7 and 1.1 kb) derived from huCDC7 cDNA containing the amino acids 1–511 as a probe (probe A) indicated that they hybridized with DNA fragments of human, monkey, rat, mouse, dog, cow, rabbit and chicken under a stringent washing condition (0.1× SSC and 0.1% SDS at 50°C), but that they did not hybridize with those of budding yeast even under a relaxed washing condition (2× SSC and 0.1% SDS at 42°C) (Figure 4).

Northern analyses of mRNA from various tissues (Figure 5) showed that huCDC7 transcripts, which were detected in most tissues examined, are expressed at a high level in testis and at a moderate level in thymus, spleen, placenta, brain and heart. Three transcripts of 4.4, 3.5 and 2.4 kb in length were detected, among which the 3.5 kb transcript was ubiquitously detected. The 2.4 kb transcript was seen only in testis, and did not hybridize with a probe specific to the C-terminal region of huCDC7 cDNA (*Kpn*I–*Xba*I fragment containing the amino acids 538–574; probe B), indicating that it is an alternatively spliced form lacking the C-terminal coding region. Transcription of a mouse Cdc7-related kinase also exhibited similar tissue distribution, with the highest expression in testis (our unpublished data).

The 3.5 kb transcript was predominant and ubiquitously detected in various human cell lines such as HL60, K562, MOLT4, Raji, SW480 and HeLa (S3) (data not shown). The level of huCDC7 transcription was similar between these cancer cell lines. The 4.4 kb transcript was also detected in most cell lines, albeit at a much lower level.

Identification of endogenous huCdc7 protein

Endogenous huCdc7 was detected by specific antibodies, which were raised against GST fusion proteins containing segments of huCdc7 protein. The mouse monoclonal antibody 4A8 specifically recognized a single protein which was expressed by the huCdc7-expressing plasmid in COS7 cells and migrated with an apparent mol. wt of 68 kDa (Figure 6A). huCdc7 protein was immunoprecipitated by the 4A8 antibody from the cell lysates prepared from a factor-dependent myeloid leukaemia cell line, TF-1, and was identified by Western blot analysis with the rabbit polyclonal antibody #1 (Figure 6B). In order to determine which of the three possible ATG codons is utilized to initiate translation of the huCdc7 coding frame, we expressed both long and short forms of huCdc7 (pKU-long-huCdc7 and pKU-short-huCdc7 initiated from the first and third ATG, respectively; Figure 1) and compared their migration on a gel with that of the endogenous

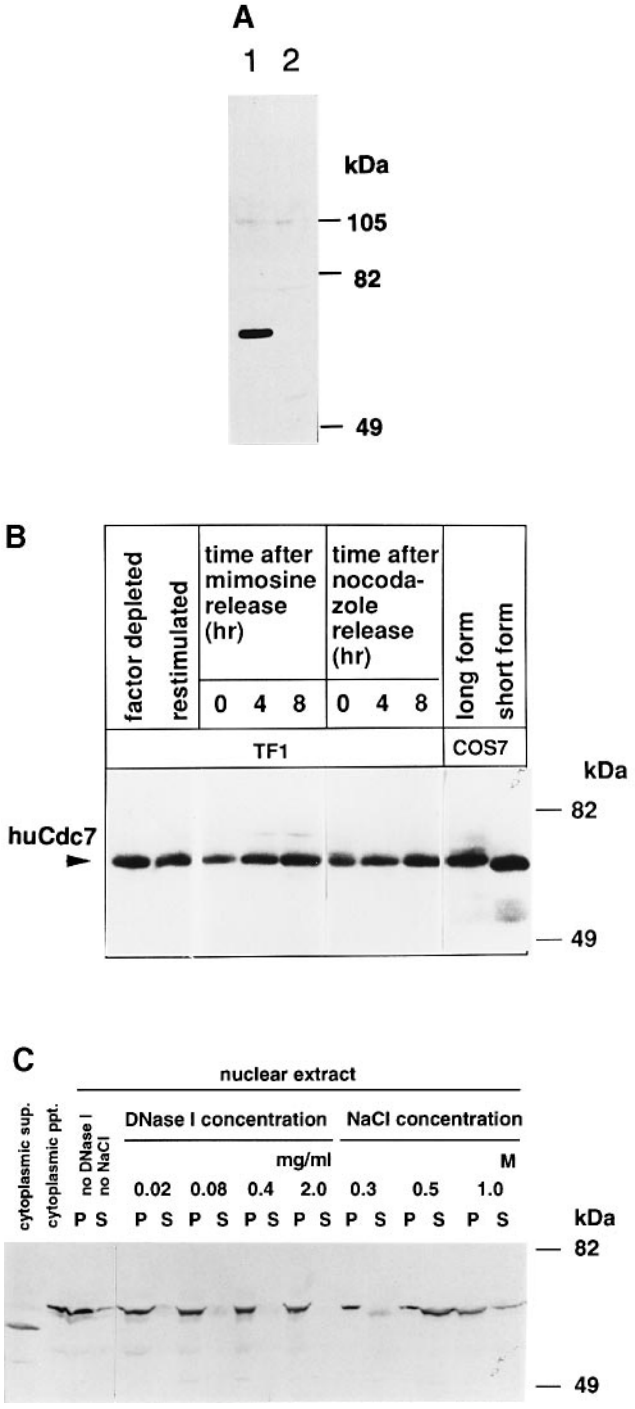

Fig. 6. Expression of huCdc7 protein in tissue culture cells. (**A**) Specificity of the monoclonal antibody, 4A8. Nuclear extracts prepared from COS7 cells transfected with pKU-long-huCdc7 (lane 1) or with the vector (lane 2) were separated on SDS–PAGE and blotted in 1 μg/ml monoclonal antibody 4A8. (**B**) Expression of huCdc7 protein at different cell cycle stages. The whole cell lysates were prepared from 10^7 TF-1 cells or from 1.5×10^6 COS7 cells which had been transfected with 1 μg of long or short form of huCDC7 expression vector and had been harvested 2 days after transfection. huCdc7 protein was immunoprecipiated with the monoclonal antibody 4A8. (**C**) Salt and DNase I extraction of nuclear huCdc7 protein. K562 cells were extracted with hypotonic buffer and nuclei were extracted with buffer containing DNase I or NaCl at the concentrations indicated. The supernatant (S) and pellet (P) were separated by centrifugation. Immunoprecipitates (B) and protein fractions (C) were separated on an 8% SDS–PAGE, transferred onto Immobilon paper and were blotted with anti-huCdc7 polyclonal antibody #1.

protein. The endogenous huCdc7 protein co-migrated with the long form, indicating that the first ATG initiates translation of huCdc7 (Figure 6B).

The amount of huCdc7 stayed relatively constant at various phases of the mitotic cell cycle in TF-1 cells synchronized by mimosine or nocodazole or after depletion of a growth factor, human granulocyte–macrophage colony-stimulating factor (GM-CSF) (Figure 6B).

Nuclear localization of huCdc7

huCdc7 is localized mainly in nuclei in the interphase and is present diffusibly in the cytoplasm in the mitotic phase of HeLa cells, as indicated by immunofluorescence analyses using the monoclonal antibody 4A8 (data not shown). In order to examine whether the nuclear localization of huCdc7 changes at the G_1/S transition, HeLa cells were synchronized at the G_1/S boundary by double thymidine block and the S phase cells were obtained at 4 h after the release into the cell cycle. In this experiment, soluble proteins and phospholipids were extracted with Triton X-100 before fixation (Fey *et al.*, 1984). Cells were stained by anti-bromodeoxyuridine (BrdU), anti-proliferating cell nuclear antigen (PCNA), anti-mouse CDC21 (MCM4) and anti-huCdc7 (4A8) antibodies. BrdU was incorporated into 100% of the S phase cells, indicating that DNA was being synthesized synchronously (Figure 7B). PCNA accumulated in nuclei as the S phase progressed (Figure 7D). On the other hand, MCM protein was localized in nuclei before DNA synthesis, and disappeared from nuclei during the S phase, as expected from its licensing function required for 'once and only once' replication in the S phase (Figure 7E and F) (Blow and Laskey, 1988; Blow, 1993; Kimura *et al.*, 1994; Chong *et al.*, 1995; Kubota *et al.*, 1995). huCdc7 was found to be localized in nuclei from the G_1 through the S phase, and no obvious relocation of huCdc7 upon progression into the S phase was detected (Figure 7G and H). More than 50% of the nuclear huCdc7 protein was extracted in buffer containing 0.5 M NaCl, but remained in the pellet after digestion with 2 mg/ml DNase I (Figure 6C). Similarly, MCM3 was extracted by salt but not by DNase I, as previously reported (data not shown; Kimura *et al.*, 1994). Therefore, it is likely that the majority of huCdc7 prepared from a random culture binds to some nuclear structures rather than to chromatin.

huCdc7 phosphorylates MCM proteins

Studies on yeast and *Xenopus* DNA replication indicated that eukaryotic replication origins may be associated with ORC and MCM protein complexes, and functions of these origin-associated proteins may be regulated in a cell cycle-specific manner (Blow, 1993; Yan *et al.*, 1993; Kimura *et al.*, 1994; Chong *et al.*, 1995; Todorov *et al.*, 1995; Coue *et al.*, 1996). Therefore, these origin-associated proteins could be the targets of phosphorylation events essential for G_1 to S transition (Bell, 1995; Carpenter *et al.*, 1996; Leatherwood *et al.*, 1996). The Cdc7–Dbf4 kinase complex in budding yeast is likely to be bound at the origins due to the origin-binding activity of Dbf4 protein (Dowell *et al.*, 1994), suggesting the possibility that ORC and MCM may be phosphorylated by Cdc7 kinase.

We examined whether MCM components can be phosphorylated by huCdc7 kinase. Extracts were prepared from

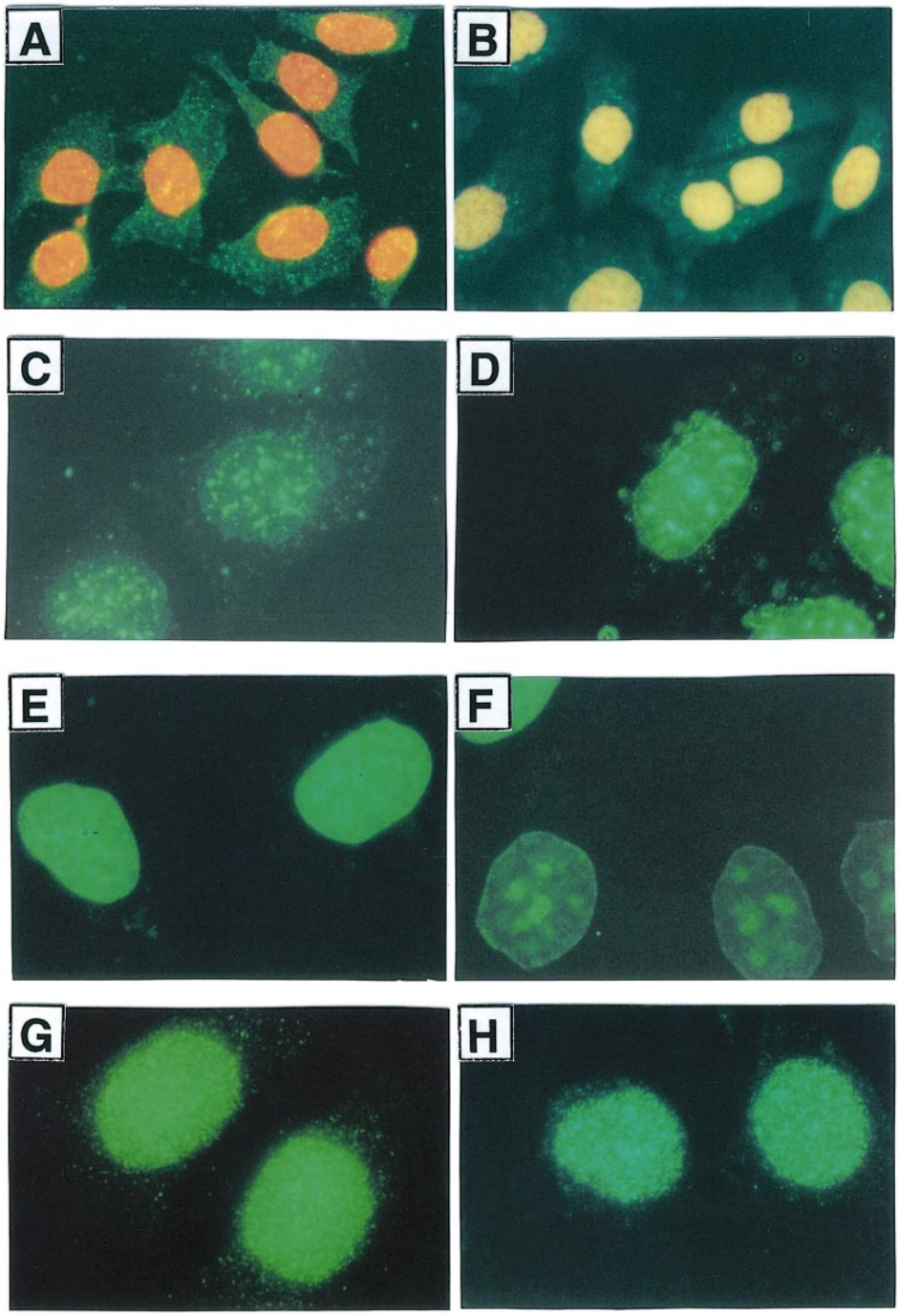

Fig. 7. Nuclear localization of huCdc7 does not alter before and after G_1/S transition. HeLa cells were synchronized at the G_1/S boundary by double thymidine block (**A**, **C**, **E** and **G**) and then released for 4 h to proceed into the S phase (**B**, **D**, **F** and **H**). (A) and (B) are superimposed images of propidium iodide staining of DNA (red) and BrdU staining (green). PCNA (C and D), MCM4 (E and F) and huCdc7 (G and H) are immunostained with specific antibodies. Magnification; 200× (A and B), 400× (C–H).

COS7 cells transfected with a vector, haemagglutinin (HA)-tagged wild-type huCDC7-expressing plasmid, or HA-tagged K90R kinase-negative huCDC7-expressing plasmid. Roughly equal amounts of transiently expressed huCdc7 protein were immunoprecipitated from the wild-type and K90R transfectants by anti-HA antibody, while huCdc7 was not detected in the immunoprecipitate from vector-transfected COS7 cells (Figure 8C). Upon incubation of the immunoprecipitates with [γ-^{32}P]ATP in the presence of purified GST fusion protein containing *Xenopus* MCM2N (amino acid residues 1–559) or human MCM3 (amino acid residues 1–808), the MCM proteins were phosphorylated efficiently by the wild-type huCdc7 immunoprecipitate, while the level of phosphorylation by K90R huCdc7 was no more higher than the vector control (Figure 8A and B). The results indicate that MCM2 and MCM3 proteins can be phosphorylated by huCdc7 *in vitro* and suggest the possibility that functions of the MCM

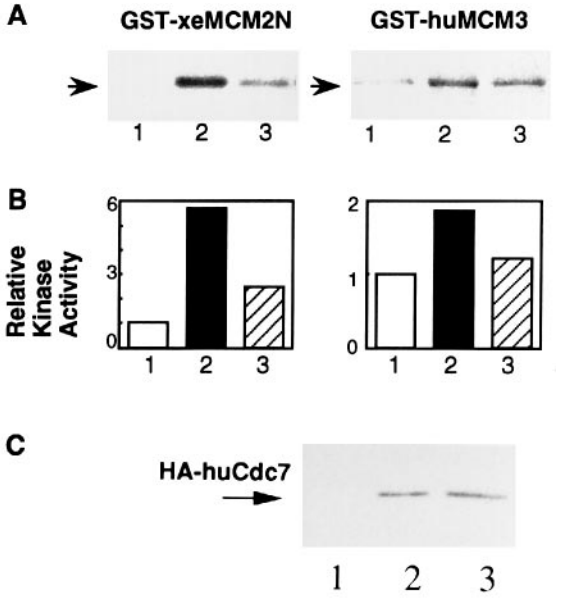

Fig. 8. *In vitro* phosphorylation of GST–MCM proteins by huCdc7 kinase. COS7 cells were transfected with 1 μg of either pKU3 vector (lane 1), pKU-HA-tagged WT huCDC7 (wild-type, lane 2) or pKU-HA-tagged K90R huCDC7 (kinase-negative form, lane 3). Two days after the transfection, cells were lysed and transiently expressed huCdc7 protein was immunoprecipitated with anti-HA antibody. (**A**) The immunoprecipitates were incubated with [γ-^{32}P]ATP in the presence of either GST-fused *Xenopus* MCM2N (amino acid residues 1–559) or GST-fused human MCM3 (amino acid residues 1–808). The reaction mixtures were resolved by 10% SDS–PAGE, and the gel was dried and autoradiographed. (**B**) ^{32}P incorporation into the MCM protein bands was quantified by Image Analyzer (Fuji Film) and the values relative to the vector control, which is taken as 1, are shown. (**C**) Immunoprecipitates used for the kinase assays were blotted with the anti-huCdc7 polyclonal antibody #1.

complex are regulated by phosphorylation by the Cdc7-related kinase.

Discussion

Presence of CDC7-related kinases in higher eukaryotes

Replication of eukaryotic cells is controlled precisely during the cell cycle. In spite of apparent diversity in structures of replication origins in higher eukaryotes in comparison with those of a lower eukaryote such as *S.cerevisiae*, there appears to be striking conservation in proteins required for the processes of assembly of replication machinery (Gavin *et al.*, 1995; Hamlin and Dijkwel, 1995; Donovan and Diffley, 1996). Proteins related to components of ORC, a protein complex bound specifically to the budding yeast replication origins, were discovered in *Drosophila*, *Xenopus* and human. Furthermore, they also form a multi-protein complex containing protein components similar to those of budding yeast (Gavin *et al.*, 1995; Gossen *et al.*, 1995; Carpenter *et al.*, 1996). MCM proteins, originally discovered in *S.cerevisiae*, were identified as components for the licensing factor essential for DNA replication in *Xenopus* egg extracts (Chong *et al.*, 1995; Kubota *et al.*, 1995). Proteins related to all the six MCM components have been identified in human as well (Hu *et al.*, 1993). Thus, basic mechanisms of initiation of chromosomal replication as well as its regulation may be conserved from yeasts to human. We previously reported *hsk1*$^+$, a putative homologue of CDC7 from a distantly related yeast, *S.pombe*, and suggested the possibility that the S phase initiation in eukaryotes may be regulated in a conserved manner involving Cdc7-related kinases (Masai *et al.*, 1995). Isolation of Cdc7-related kinases from human and *Xenopus*, reported in this study, further strengthens our proposal that Cdc7-related kinases are the key regulators for initiation of DNA replication conserved in eukaryotes.

The Cdc7-related kinases from higher eukaryotes share structural similarity to the yeast counterparts, exhibiting 42–44% identity in the conserved domains for serine-threonine kinases in addition to the presence of two kinase insert sequences at the conserved locations (Figure 3A). The Cdc7-related kinases were grouped into a subset distantly related to other kinases in a phylogenetic tree (Figure 3B), indicating that Cdc7-related kinases belong to a distinct kinase subfamily. Unexpectedly, huCdc7 and xeCdc7 did not carry a C-terminal acidic region, which was present and essential for the activity in the yeast genes (our unpublished data). In budding yeast Cdc7, the C-terminal tail is involved in interaction with Dbf4 protein (Patteron *et al.*, 1986). We recently discovered that efficient interaction with Dbf4 protein requires the kinase insert II and III sequences of Cdc7 (our unpublished data). Therefore, a putative 'activator' for huCdc7 may well interact with the two kinase insert sequences in the absence of the C-terminal tail. An alternative possibility is that an as yet identified variant of Cdc7-related kinases, which does contain a C-terminal region, may be present in higher eukaryotes.

Expression of huCdc7 in various tissues and in the cell cycle

mRNAs for huCdc7 are expressed in most tissues examined (Figure 5), as expected from its essential function for cell proliferation, although the highest expression was detected in testis. This may be interesting in the light of a previous report (Sclafani *et al.*, 1988) and our unpublished observations that mRNAs for budding yeast *CDC7* and fission yeast *hsk1*$^+$ are induced during the course of meiosis, suggesting that Cdc7-related kinases may play additional roles during meiosis. Characterization of budding yeast Cdc7 mutants indicated that Cdc7 is required for synaptonemal complex formation during meiosis (Sclafani *et al.*, 1988). A huCdc7 transcript was also detected in brain. The functions, if any, of huCdc7 in mature neuronal cells which do not proliferate remain to be investigated.

Among the three transcripts detected, the 3.5 kb transcript was ubiquitously present in all the tissues, whereas the 4.4 kb transcript was seen in subsets of tissues, such as testis, peripheral blood leukocytes (PBL), thymus, spleen, small intestine, brain and placenta. In various human cell lines, the former was the major transcript and the latter was expressed at a lower level (data not shown). The 2.4 kb transcript was detected only in testis, suggesting that it may be specific to this tissue. Northern analysis using the C-terminal region of huCDC7 containing only the kinase domain XI as a probe (probe B) showed that the 2.4 kb transcript did not hybridize with this DNA segment (Figure 5). In accordance with this observation,

we have obtained from a testis library a variant cDNA whose coding frame is truncated at amino acid position 519. A mouse cDNA for Cdc7-related kinase that we have isolated from a spermatocyte library contained the kinase subdomains I–VII, but its coding region was truncated in kinase insert II and continued into unrelated sequences which were identified on the mouse genomic DNA upstream of the remaining kinase domains (VII–XI), suggesting that these cDNAs are products of alternative splicing (our unpublished data). On the other hand, clone #8 contained a 91 bp insertion at the N-terminal coding region, thus resulting in frameshifting in translation. This insertion occurs at an exon–intron junction of the murine CDC7 gene (our unpublished data). At present, we do not know the functions of these apparently kinase-inactive derivatives of Cdc7-related proteins.

In lysates from human cell lines such as HeLa, TF-1 and K562, huCdc7 protein was identified as a 68 kDa protein that co-migrated with the polypeptide expressed from an expression vector carrying the 574 amino acid long huCdc7 cDNA. The level of expression of the 68 kDa huCdc7 protein did not vary significantly during the course of the mitotic cell cycle (Figure 6B). Transcription of budding yeast *CDC7* and fission yeast *hsk1*$^+$ was also previously reported to be relatively constant during the cell cycle (Yoon *et al.*, 1993; our unpublished data)

huCdc7 is a nuclear protein

Indirect immunofluorescence staining using anti-huCdc7 monoclonal antibody (4A8) showed that fluorescence was confined mostly to the nucleus during the interphase. huCdc7 was localized in nuclei before and after DNA replication is initiated, and continued to stay in nuclei during the entire interphase (Figure 7). Localization of huCdc7 in nuclei did not coincide precisely with that of PCNA, which is known to co-localize at the replication foci. Further analysis is needed to determine the precise subnuclear localization of huCdc7. huCdc7 protein could be extracted by high salt, but not by DNase I (Figure 6C), suggesting that it may be associated with nuclear structures.

Cdc7 may regulate MCM function by phosphorylation

MCM appears to be phosphorylated at various stages of the cell cycle. The newly synthesized P1 (mouse MCM3) is phosphorylated in the G_1 phase, and the level of its phosphorylation increases during the S phase (Kimura *et al.*, 1994). Phosphorylation of *Xenopus* MCM4 (Cdc21) in early S phase was also reported (Coue *et al.*, 1996). Thus, phosphorylation of MCM may activate its functions for S phase initiation. Alternatively, phosphorylation of MCM may block re-replication by dissociating from the chromatin after the initiation of S phase. Specific phosphorylation of MCM subunits by huCdc7 (Figure 8) supports the idea that this kinase regulates DNA replication. huCdc7 may regulate both activation of the S phase and 'once and only once' replication through phosphorylation of MCM subunits as well as that of as yet identified substrates. In budding yeast, it was shown recently that MCM2, 3, 4 and 6 could be phosphorylated by the Cdc7–Dbf4 kinase complex *in vitro* (A.Sugino and B.K.Tye, personal communication). Experiments are in progress to determine whether other MCM subunits are phosphorylated by huCdc7 and to locate more precisely the phosphorylation sites on MCM proteins. MCM functions could be regulated through sequential phosphorylation and dephosphorylation by multiple kinases and phosphatases. It would also be important to understand how phosphorylation of MCM by a Cdc7-related kinase is coordinated with that by other kinases, which may include Cdks and DNA-dependent protein kinase, to achieve precise regulation of MCM functions for progression of the cell cycle.

The *in vitro* kinase activity of *S.cerevisiae* Cdc7 is strictly dependent on the presence of Dbf4 protein, and Cdc7 alone expressed in insect cells is inactive. Similarly, huCdc7 alone expressed in insect cells did not show any phosphorylation activity (our unpublished data), suggesting the requirement for an activator for kinase activity of huCdc7. Although we were able to measure huCdc7-dependent phosphorylation after overexpression, we had difficulties in measuring the kinase activity of endogenous huCdc7 protein. This may reflect scarcity and/or instability of the active huCdc7 kinase complex in the cells. Identification of the putative activator for huCdc7 will help to understand the precise roles of huCdc7-dependent phosphorylation in mammalian cell cycle progression.

In summary, we report here the presence of Cdc7-related kinases in higher eukaryotes and present data implicating this kinase in regulation of mammalian chromosomal replication. Further characterization of these Cdc7-related kinases should provide important insights into molecular mechanisms of cell cycle regulation of chromosomal replication in higher eukaryotes.

Materials and methods

Cells

Mouse ES cells were cultured on a 0.1% porcine skin gelatin-coated culture dish in high glucose Dulbecco's modified Eagle's medium (DMEM) containing 0.1 mM non-essential amino acids (Gibco), 2 mM L-glutamine (Irvine Scientific), 1× nucleotide mix (3 mM each of dATP, dCTP, UTP, dGTP and 1 mM dTTP), 50 mM 2-mercaptoethanol (Sigma), the supernatant from human leukaemia-inhibiting factor (hLIF)-expressing COS7 cells and 20% fetal calf serum (FCS). COS7 and HeLa cells were cultured in DMEM containing 10% FCS. TF-1 cells were cultured in RPMI 1640 containing 2 ng/ml hGM-CSF and 10% FCS. K562 cells were cultured in RPMI 1640 containing 10% FCS.

Oligonucleotides

The oligonucleotides used for degenerate PCR amplification for cloning of CDC7-related kinases were designed on the basis of the amino acid sequences conserved between the products of *S.cerevisiae CDC7* and *S.pombe hsk1*$^+$. The combination of the oligonucleotide primers that led to the isolation of a mammalian Cdc7-related kinase was 5′-CGGAATT-CAA(AG)AT(TCA)AA(AG)GA(TC)AA(AG)AT-3′ and 5′-CGGAATT-CIGCIA(GA)ICC(AG)AA(AG)TC(ATGC)AC-3′, corresponding to the amino acid stretches from 34 to 39 and from 186 to 181, respectively, of Cdc7 protein. The other nested combination was 5′-CGGAA-TTCAA(AG)AT(TCA)GG(TCGA)GA(AG)GG(TCGA)AC-3′ and 5′-CGGGATCCIGG(TC)TT(AGT)AT(AG)TC(TCGA)C(TG)(AG)TG - 3′, corresponding to the amino acid stretches from 38 to 43 and from 166 to 161 (Patteron *et al.*, 1986).

Antibodies

Portions of the huCDC7 coding frame (amino acid residues 128–276 and 128–433), isolated as *Sau*3A fragments, were subcloned at the *Bam*HI site of pGEX-3X to generate GST fusion proteins #1 and #2, respectively, which were purified as previously described (Ikeda *et al.*, 1996a,b). Polyclonal antibodies #1 was developed in rabbit against the purified fusion protein #1 and the antibodies reacting to the GST portion

of the fusion protein were depleted by glutathione–Sepharose 4B resin to which non-fused GST protein was attached. Mouse monoclonal antibody (4A8) was developed against the GST–huCDC7 fusion protein #2. Anti-rat PCNA antibody was purchased from MBL (Nagoya, Japan) and anti-mouse Cdc21 rabbit serum was kindly provided by Dr H.Kimura (Hokkaido University, Japan). Anti-BrdU antibody containing DNase I was purchased from Amersham. Anti-HA antibody 12CA5 was purchased from Babco (CA).

GST–MCM fusion proteins

GST–human P1 (MCM3) (amino acid residues 1–808) and GST–*Xenopus* MCM2 (amino acid residues 1–559) were gifts from Dr H.Takisawa (Osaka University, Japan).

mRNA isolation and reverse transcription

Poly(A) RNA was isolated from mouse ES cells using a FAST TRACK mRNA isolation kit (Invitrogen). Reverse transcription was performed with Superscript II (Gibco) as suggested by the manufacturer. One μl of the 20 μl reaction mixture was used for subsequent PCR amplification.

PCR screening and subcloning of amplified fragments

PCR, isolation and subcloning of amplified DNA fragments were performed as described earlier (Masai *et al.*, 1995). The isolated fragments were subcloned into the T vector prepared from KS (pBluescript) vector (Marchuk *et al.*, 1991). Plasmid DNAs containing insert DNAs were recovered from white colonies of DH5α on LB plates containing ampicillin (50 μg/ml) and Xgal (40 μg/ml), and the nucleotide sequences of the inserts were determined. One of the clones carrying an insert of ~420 bp contained a coding frame that resembled a part of Cdc7 and Hsk1, which was designated pKS-420.

Screening of cDNA libraries

A human fetal liver cDNA lambda library was kindly provided by Dr Soma (Kirin Brewery Co., Japan) and a human testis cDNA lambda library was purchased from Clontech. *Xenopus* oocyte cDNA lambda libraries were kindly provided by Dr Douglas Melton and Dr Tim Hunt. A total of 10^6 plaques from each library were screened with the ^{32}P-labelled 414 bp PCR-amplified DNA fragment isolated from pKS-420. Clones #8 and #13 from the fetal liver library and #1 from the testis library carried 1.8, 2.9 and 0.8 kb cDNA inserts, respectively, and were analyzed further. Similarly, #25, #28 and #23, which carried a 1.3, 1.3 and 1.35 kb cDNA insert, respectively, were isolated from the *Xenopus* libraries. These inserts were subcloned into KS vector and the entire nucleotide sequences were determined.

Northern and genomic Southern analysis

Human multiple tissue Northern blots and zoo blot (*Eco*RI digestion) were purchased from Clontech. ^{32}P-labelled DNA probes were prepared by random priming reactions on the mixture of the 0.7 kb and 1.1 kb *Eco*RI fragments derived from the huCDC7 #8 clone containing amino acids 1–511. Hybridization was carried out in a buffer containing 6× SSPE, 10% formamide, 5× Denhardt's, 0.1% SDS and 100 μg/ ml heat-denatured sonicated salmon sperm DNA (Sigma) at 42°C for 12 h. For cross-species hybridization, the filters were washed at 42°C in 6× SSC followed by washing in 2× SSC at the same temperature. For more stringent hybridization, they were washed further in 0.2× SSC at 42°C and finally at 50°C in 0.1× SSC. The washing buffer contained 0.1% SDS.

Construction of vectors that express full-length wild-type and mutant forms of huCDC7

The 0.7 (N-terminal) and 1.1 kb (C-terminal) *Eco*RI fragments constituting the 1.8 kb insert of #8 cDNA (lacking a portion of the C-terminus coding region) were subcloned into KS vector, resulting in KS(0.7) and KS(1.1), respectively. The N-terminal coding region missing in the #13 cDNA was reconstructed by replacing the 0.25 kb *Sac*I–*Bcl*I fragment of #13 with that (0.6 kb) of KS(0.7), generating KS-huCDC7-#6 which contained the entire coding frame of huCDC7 with a 91 nucleotide insertion at amino acid position 39 as well as the 3′-untranslated region. The spliced form containing the entire coding region in-frame was constructed by replacing the *Age*I–*Kas*I fragment of KS-huCDC7-#6 (containing the 91 nucleotide insert) with that of testis-derived #1 cDNA (without the insert), resulting in KS-full huCDC7. The N-terminal *Not*I–*Age*I fragment of the KS-full huCDC7 was replaced by the fragment generated by *Not*I–*Age*I digestion of a PCR-amplified DNA, resulting in the HA-tagged 'short' huCdc7 coding frame (starting from amino acid position 13; KS-short huCDC7). This PCR was conducted by using an oligonucleotide containing *Not*I–*Nde*I sites followed by the sequences encoding the 10 amino acid HA peptide (MYPYDVPDYA) and the huCdc7 coding region (amino acids 13–17) in combination with an internal primer 5′-TTT GTC CTC AAT CTT AAT CTT-3′ present downstream of the *Age*I site. The 2.3 kb *Not*I–*Xba*I fragment of KS-short huCDC7 containing the HA-tagged short form was cloned into pKU3 (a gift from Dr Muto of our laboratory), a neomycin-resistant derivative of pME18S, resulting in pKU3-HA-short huCDC7 which expressed HA-tagged 562 amino acid huCDC7 under the SRα promoter. For construction of a plasmid expressing the full-length huCDC7, the 180 bp *Eco*RI–*Age*I fragment of KS-full huCDC7 containing the N-terminal huCDC7 coding frame (from amino acid position 1 to 22) was further digested by *Alu*I (at 31 nucleotides upstream of the first ATG), and a *Not*I linker was attached to this *Alu*I site. This *Not*I–*Age*I fragment replaced the same fragment of pKU3-HA-short huCDC7, resulting in pKU3-long huCDC7 containing the 574 amino acid full-length huCDC7 coding frame. To generate pKU3-short huCDC7, the same *Not*I–*Age*I fragment of pKU3-HA-short huCDC7 was replaced by an oligonucleotide containing a *Not*I site followed by the sequence encoding 10 amino acids from position 13 to 22 of huCDC7.

A mutant form of huCDC7 in which the lysine at position 90 was replaced by arginine was constructed by the PCR method. Oligonucleotides 5′-AAAATTGCCTTAAGACACTTGATTCCAACA-3′ and 5′-TCAAGTGTCTTAAGGCAATTTTCTCTTCAG-3′ were used to create the mutation.

Transfection of plasmid DNA into mammalian cells

Plasmid DNAs were introduced into COS7 cells by electroporation as previously described (Kitamura *et al.*, 1991).

Cell synchronization and preparation of cell lysates

HeLa cells were synchronized at the G_1/S boundary by double thymidine block as described (O'Connor and Jackman, 1995). Factor-depleted TF-1 cells were prepared by deprivation of hGM-CSF for 16 h and the G_1 phase TF-1 cells were obtained by mimosine treatment as described (O'Connor and Jackman, 1995). Cells were synchronized in metaphase by nocodazole treatment (O'Connor and Jackman, 1995). Synchronization of the cell cycle was monitored by flow cytometry (Giunta and Pucillo, 1995). Cells were washed with ice-cold phosphate-buffered saline (PBS) and were resuspended in IP buffer [50 mM HEPES/KOH (pH 7.6), 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1% Tween-20, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 100 μM β-glycerophosphate, 10 mM NaF and 1 mM Na_3VO_4] at 2×10^7 cells/ml. After sonication of the cell suspension, lysates were cleared by centrifugation. Preparation of nuclear extracts and salt/DNase I extraction were conducted as described by Kimura (1994).

Immunoprecipitaion, immunoblot and in vitro kinase assays

Anti-huCDC7 antibody #1 or anti-HA antibody (12CA5) was added to the cell lysate at a final concentration of 1 μg/ml and incubated for 2 h on ice. Immunoprecipitates were collected on protein A–agarose beads, washed three times with IP buffer. For immunoblot analysis, bound proteins were extracted by boiling with 20–40 μl of 2× Laemmli's sample buffer. Proteins were separated on an 8% SDS–PAGE and were blotted onto an Immobilon-P membrane (Millipore). The membranes were probed with anti-huCDC7 followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham). The immunoreactive proteins were detected by chemiluminiscence (ECL, Amersham, UK). For *in vitro* kinase assays, immunoprecipitates were washed further by pre-kinase buffer [40 mM HEPES/KOH (pH 8.0) and 40 mM potassium glutamate] and incubated in 24 μl of kinase reaction buffer [40 mM HEPES/KOH (pH 8.0), 40 mM potassium glutamate, 1 mM EGTA, 8 mM magnesium acetate, 2 mM DTT, 0.5 mM EDTA, 0.1 mM ATP and 2 μCi of [γ-^{32}P]ATP] in the presence or absence of 1 μg of GST-fused MCM protein at 30°C for 20 min. The reaction was stopped by addition of 6 μl of 5× Laemmli's sample buffer. Samples were heated at 95°C for 5 min and were separated on a 10% SDS–PAGE. The gels were dried and phosphorylated proteins were detected by autoradiography.

Indirect immunofluorescence

Cells grown on coverslips were washed twice with PBS containing 1 mM $CaCl_2$ and 1.5 mM $MgCl_2$. Cells were fixed for 5 min with 3.7% formaldehyde and permeabilized with 0.2% Triton X-100 in PBS for 5 min followed by incubation in blocking solution (0.2% gelatin, 5% FCS and 0.1% Tween-20 in PBS) when extraction of soluble proteins was not necessary. For BrdU staining, cells were incubated with 20 μM BrdU for 20 min prior to the staining. In case soluble proteins needed

to be extracted, cells were washed twice with PBS and once with CSK buffer [100 mM NaCl, 300 mM sucrose, 10 mM PIPES (pH 6.8) and 3 mM $MgCl_2$] (Fey *et al.*, 1984), permealized by 0.5% Triton X-100 in CSK buffer and fixed with 3.7% formaldehyde at room temperature for 5 min. The coverslips were incubated in blocking solution for 10 min and for a further 1 h after addition of the first antibody (5 μg/ml) and washed three times with the same solution. They were incubated further with a second antibody [rhodamine-conjugated goat anti-rabbit antibody or rhodamine-conjugated goat anti-mouse antibody (Immunotech, France) or fluorescein isothiocyanate-conjugated rabbit anti-mouse antibody (Zymed)] which had been diluted 1:250 in blocking solution. Finally, the coverslips were washed three times with PBS and then visualized under the immunofluorescence microscopy (Nikon Optiphot-2).

Accession numbers

The DDBJ/EMBL/GenBank accession numbers for huCDC7 and XeCDC7 reported here are AB003698 and AB003699, respectively.

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