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A Novel Growth- and Cell Cycle-Regulated Protein, ASK, Activates Human Cdc7-Related Kinase and Is Essential for G_1/S Transition in Mammalian Cells

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A novel human protein, ASK (activator of S phase kinase), was identified on the basis of its ability to bind to human Cdc7-related kinase (huCdc7). ASK forms an active kinase complex with huCdc7 that is capable of phosphorylating MCM2 protein. ASK appears to be the major activator of huCdc7, since immunodepletion of ASK protein from the extract is accompanied by the loss of huCdc7-dependent kinase activity. Expression of ASK is regulated by growth factor stimulation, and levels oscillate through the cell cycle, reaching a peak during S phase. Concomitantly, the huCdc7-dependent kinase activity significantly increases when cells are in S phase. Furthermore, we have demonstrated that ASK serves an essential function for entry into S phase by showing that microinjection of ASK-specific antibodies into mammalian cells inhibited DNA replication. Our data show that ASK is a novel cyclin-like regulatory subunit of the huCdc7 kinase complex and that it plays a pivotal role in G_1/S transition in mammalian cells.

The G_1/S transition in eukaryotic cells is strictly regulated so that DNA replication occurs only once during S phase. Studies with yeast as well as with higher eukaryotes have demonstrated a critical role for cyclin-dependent kinases in cell cycle progression (2, 7, 8, 25, 26, 32). Genetic studies with *Saccharomyces cerevisiae* have indicated an essential role for another class of serine-threonine kinase at the onset of S phase. Isolated as one of the cell division cycle mutants by Hartwell (12), *CDC7* has been shown to encode a protein which functions immediately prior to initiation of chromosomal replication and is required for activation of origins throughout S phase (1, 5, 29). The kinase activity of Cdc7 is dependent on the presence of a regulatory subunit, Dbf4 (17). Expression of Dbf4 is periodic and regulated at both the transcriptional and posttranslational levels (4). The increase in Cdc7 kinase activity at the G_1/S boundary is at least partly accounted for by the elevated expression of Dbf4 in late G_1 (17). Dbf4 interacts with replication origins in vivo (6), suggesting that Cdc7 may trigger S phase by directly activating the replication initiation complexes assembled at the origins.

We previously isolated kinases related to Cdc7 from *Schizosaccharomyces pombe*, *Xenopus*, mouse, and human (19, 22, 30), raising a possibility that eukaryotic chromosomal replication is regulated by a conserved mechanism involving this family of kinases. The putative human homologue of Cdc7, huCdc7, phosphorylates MCM2 and MCM3 proteins in vitro (30), suggesting that MCM functions may be regulated through phosphorylation by huCdc7 kinase. huCdc7 possesses only a low level of kinase activity when singly overexpressed in mammalian cells, while a baculovirus-expressed form of huCdc7 is inactive (our unpublished results), strongly suggesting the presence of a regulatory subunit for huCdc7.

Through interaction screenings, we have identified a novel molecule, ASK (activator of S phase kinase), which forms a complex with huCdc7 and activates its kinase activity. Expression of ASK is regulated by growth factor stimulation and fluctuates through the cell cycle, reaching a peak during S phase. Microinjection of ASK-specific antibodies into human cells inhibited DNA replication, suggesting that it plays an essential function for the entry into S phase. Our data show that ASK is a novel regulatory subunit of the huCdc7 kinase complex and that it plays a pivotal role in G_1/S transition in mammalian cells.

MATERIALS AND METHODS

Two-hybrid screening of huCdc7-interacting molecules. The yeast strain used was CG1945 (*MAT***a** *ura3-52 his3-200 lys2-801 trp1-901 ade2-101 leu2-3,112 gal4-542 gal80-538 LYS2*::*GAL1-HIS3 cyh*′*2 URA3*::[*GAL4* 17-mers]$_3$-*CYC1-lacZ*). pAS2-huCdc7 was constructed by cloning the *Nde*I-*Sal*I fragment of pKU3-HA-short huCdc7 (encoding amino acid residues 13 to 574) into the binding domain vector pAS2. The HeLa cDNA library in the activation domain vector pGAD-GH was purchased from Clontech. Yeast transformation was conducted by the lithium acetate method as previously described (9). Briefly, 5×10^7 cells of CG1945 harboring pAS2-huCdc7, grown in YPD medium containing adenine sulfate (50 μg/ml) to an optical density at 600 nm (OD_{600}) of 0.5, were incubated with 1 μg of plasmid DNA and 70 μg of heat-denatured salmon sperm DNA in 0.1 M lithium acetate–10 mM Tris · Cl (pH 7.5)–1 mM EDTA–40% polyethylene glycol 4000 for 60 min at 30°C and for 30 min at 42°C. The transformation efficiency ranged from 1.0×10^4 to 1.5×10^4 per μg of DNA. Transformants were initially selected on plates lacking histidine but containing 5 mM 3-aminotriazol. Growing colonies were assayed for β-galactosidase activity on nitrocellulose membranes (Schleicher & Schuell) by using X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). In quantitative β-galactosidase assays, colonies grown to an OD_{600} of 0.5 to 1.0 were frozen in liquid nitrogen and thawed to permeabilize cell walls. The cells were then incubated in the presence of 0.64 mg of ONPG (*o*-nitrophenyl-β-D-galactopyranoside) per ml at 30°C for 30 min, and the reactions were terminated by addition of calcium bicarbonate. The OD_{420} was measured, and β-galactosidase activity was normalized to the amount of the cells estimated from the OD_{600} values.

Plasmid DNAs. pME18S-myc vector containing a myc epitope tag under control of the SRα promoter was constructed by inserting a pair of oligonucleotides, 5′-AATTGATGGAGCAAAAGCTGATTTCTGAGGAGGATCTG-3′ and 5′-

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AATTCAGATCCTCCTCAGAAATCAGCTTTTGCTCCATC-3′, at the *Eco*RI site of pME18S (33). The *Eco*RI-*Xho*I fragments of the isolated pGAD clones were inserted at the same sites of pME18S-myc, resulting in the expression of a myc-tagged polypeptide encoded by each clone.

Antibodies. Anti-ASK-Cpep antisera were raised against a synthetic polypeptide derived from the C-terminal 19 amino acids (NVLDIWEEENSDNLLT AFF) of ASK protein. The peptide was coupled through the additional N-terminal cysteine to keyhole limpet hemocyanin for immunization. Antibodies were affinity purified against the antigenic peptide by standard protocols (11). Anti-ASK-N or anti-ASK-C antibody was developed against glutathione *S*-transferase (GST) fusion proteins containing, respectively, the N-terminal 305 amino acids or the C-terminal 369 amino acids of ASK protein expressed on pGEX-5X-3 and was affinity purified to remove antibodies reacting with the GST portion. Anti-huCdc7Cpep antibody was developed against a synthetic polypeptide derived from the C-terminal 18 amino acids (RITAEEALLHPFFKDMSL) of huCdc7 and was purified as described above. Culturing of *Escherichia coli* cells containing the plasmid and preparation and purification of the fusion protein were performed as described previously (14). Antibodies were affinity purified against their respective antigens. Anti-huCdc7 antibodies #1 and 4A8 raised against recombinant huCdc7 polypeptides were previously described (30).

Preparation of extracts, immunoprecipitation, and immunodepletion. CEM cells were lysed in Nonidet P-40 lysis buffer (0.1% Nonidet P-40, 50 mM HEPES-KOH [pH 7.5], 300 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 5 mg of aprotinin per ml, 5 μg of leupeptin per ml, 5 μg of pepstatin per ml, 5 μg of Pefabloc per ml) for 30 min at 4°C. An extract from 2×10^8 CEM cells was immunoprecipitated with antibody to either huCdc7 or ASK. For peptide blocks, antibodies were preincubated with 1 mg of peptide per ml for 30 min at 30°C. Immunocomplexes were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with monoclonal huCdc7 antibody (4A8) (30). K562 cell extracts were prepared from 2×10^8 cells by sonication in 500 μl of lysis buffer (50 mM HEPES-KOH [pH 7.5], 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Tween 20, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride), followed by centrifugation at 15,000 rpm for 5 min in a microcentrifuge. Immunodepletion was conducted with anti-huCdc7 antibodies and protein A-Sepharose at 4°C for 2 h, followed by centrifugation.

Deletion derivatives of ASK. A series of C-terminal deletion derivatives (ΔV, ΔP1, ΔB, and ΔP2) were constructed by introducing internal deletions on pGAD-ASK by using the *Eco*RV, N-terminus-proximal *Pvu*II, *Bgl*II, and C-terminus-proximal *Pvu*II sites, respectively, within the ASK coding frame in combination with the unique *Xho*I site present at the C terminus of ASK. ΔC, ΔN1, ΔN2, ΔN3, and ΔN4 were produced on KS-ASK by PCR with the following sets of primers: ΔC, T7 primer and 5′-CCG CTC GAG CGG TGA ATT TTC CTC TTC CCA AAT-3′; and ΔN1, ΔN2, ΔN3, and ΔN4, 5′-CCG GAA TTC CCA TAT GGA AAA ATC CAA ATG TAA GCC A-3′, 5′-CCG GAA TTC CCA TAT GGA CAA GCC ATC TAG TAT GCA A-3′, 5′-CCG GAA TTC CCA TAT GGA CTT TGT GGA ATA TGA AAA G-3′, and 5′-CCG GAA TTC CCA TAT GAG TGG ATC TCA ACC AAA ACA G-3′, respectively, in combination with T3 primer. These ASK fragments were subsequently cloned into the *Eco*RI-*Xho*I sites of vector pGAD-GH.

Fractionation of mammalian cells by elutriation. CEM cells (2.5×10^9) were separated by centrifugal elutriation with the JE-5.0 elutriation system (Beckman Instruments, Inc.). Eight fractions were collected with a rotor speed of 1,500 rpm, a starting flow rate of 23 ml/min, and a final flow rate of 52 ml/min. At each flow rate, 800 ml was collected. An aliquot of cells was removed from each fraction for fluorescence-activated cell sorter (FACS) analysis, and RNA was prepared from the remainder of the cells by using RNAzol B (Tel-Test, Inc.).

Northern analysis of ASK in tissues and in various stages of the cell cycle. The tissue expression pattern of ASK mRNA was studied by using the Multiple Tissue Northern blot (Clontech) according to the manufacturer's recommendations. In the analysis of ASK mRNA after growth stimulation, 2 μg of total RNAs extracted from WI-38 cells as previously described (30) was run on 1% agarose–formaldehyde gels, blotted onto nylon membranes, and hybridized with ^{32}P-labeled DNA probes. WI-38 human diploid fibroblasts, obtained from the Japanese Cancer Research Resources Bank, were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (FCS) in a humidified atmosphere containing 5% CO_2 at 37°C. Cells were arrested in G_0 by incubation for 48 h in Dulbecco's modified Eagle medium containing 0.1% FCS. They were stimulated to reenter the cell cycle by addition of 10% FCS. For analyses in the proliferating cell cycle, HeLa cells were first arrested at the G_1/S boundary by subjecting them to two cycles of 24-h cultures in the presence of 2.5 mM thymidine, with an interval of 12 h without thymidine (double thymidine block). After the second thymidine block, cells were released into the cycle for 5 h and then arrested at the G_2/M boundary by treatment with 40 ng of nocodazole per ml for 5 h. Synchronized populations (G_1 through S) were obtained by releasing the G_2/M cells into the cell cycle. In this case, nocodazole was added at 3 h after the double thymidine block and kept present for 12 h. Cells were harvested at 6, 12, and 18 h after release from the nocodazole block. Cell cycle progression was monitored by analyzing the DNA content by flow cytometry. Cells (2×10^7) were harvested at the times indicated, and total RNA was prepared for Northern blot analysis.

SDS-PAGE of proteins. Proteins were normally separated on SDS-PAGE with a 29:1 ratio of acrylamide to bisacrylamide (regular gel), which was run at 50 V. In cases where phosphorylated forms needed to be separated, SDS-PAGE with a 59:1 ratio of acrylamide to bisacrylamide (low-bisacrylamide gel) was run at 250 V with cooling of the gel plates.

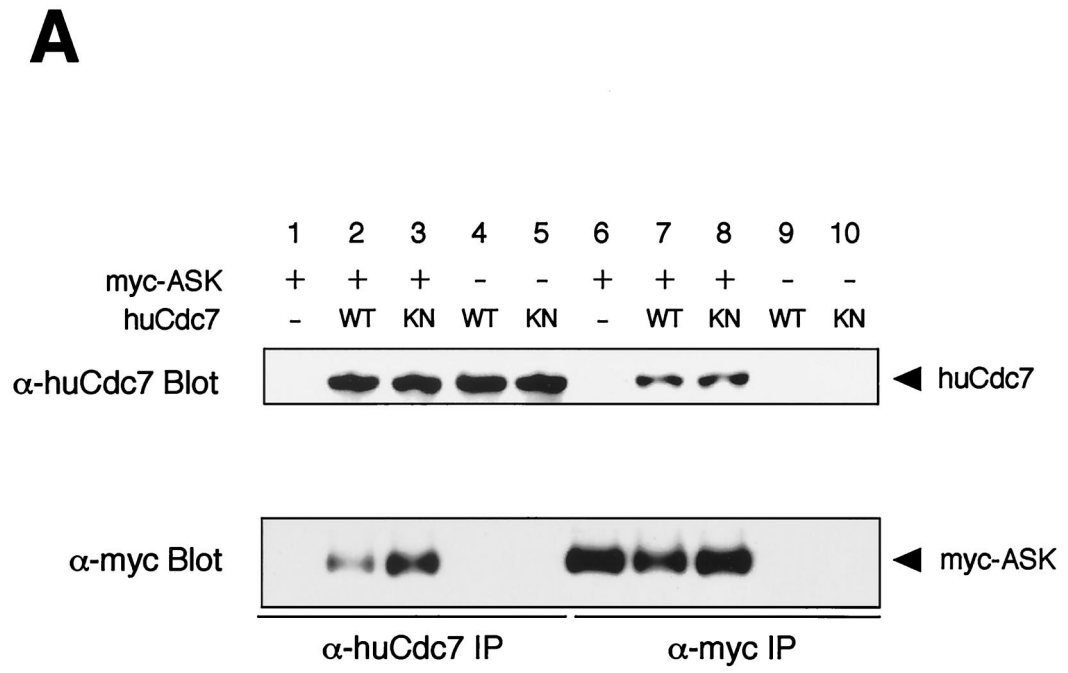
Indirect immunofluorescence analysis of ASK protein. WI-38 human fibroblasts and HeLa cells were grown to medium density on glass coverslips and rinsed twice with phosphate-buffered saline (PBS) before fixation in 2% formaldehyde–PBS–0.2% Triton X-100 on ice for 10 min, followed by incubation in cold acetone for 5 min at 4°C. Fixed cells were washed twice with PBS and incubated for 1 h in blocking solution (10% normal sheep serum, PBS, and 0.5% Tween 20). Primary antibody (anti-ASK-N or -C; 5 μg/ml) was then added to fixed cells in PBS–0.5% Tween 20 and left for 1 h. After three washes with PBS, the samples were incubated for 1 h in PBS–0.5% Tween 20 containing biotinylated sheep anti-rabbit antibody (Amersham; 1:200 dilution) and then washed three times with PBS, overlaid with PBS–0.2% Tween 20 containing streptavidin-fluorescein isothiocyanate (Amersham; 1:200 dilution), and incubated for 20 min. After three final washes in PBS, samples were mounted with DAPI (4′,6-diamidino-2-phenylindole)-containing mounting medium (Vectashield; Vector Laboratories), and signals were detected on a fluorescence microscope (Axioskop; Zeiss) at magnification of ×40 with Kodak Ektapress 1600 film. For neutralization of the antibody, primary antibody was preincubated with recombinant GST–ASK-N (0.1 μg/μl) for 30 min before incubation with the fixed cells. All of the antibody reactions were conducted at room temperature.

Microinjection of antibodies and analysis of DNA synthesis. Human primary fibroblast KD cells, obtained from the American Type Culture Collection, were serum starved for 2 days as described above except that the medium contained 0.5% FCS during starvation; the cells were then stimulated with 10% FCS and cultured in the presence of 5′-bromodeoxyuridine (BrdU) thereafter. At 12 h after stimulation, affinity-purified ASK antibody (anti-ASK-N or anti-ASK-Cpep; 200 μg/ml each), control antibody, or a mixture of anti-ASK-Cpep and the antigen peptide (500 μg/ml) was microinjected into the cytoplasm of KD cells by using a 5242 microinjector (Eppendorf). At 26 h after serum addition, the cells were prepared for immunostaining as follows. After fixation with 70% ethanol, they were treated with 4 N HCl for 20 min, washed twice with 1 M sodium tetraborate, and permeabilized with 0.5% Triton X-100 in PBS for 5 min. Fixed and permeabilized cells were then incubated for 1 h with monoclonal mouse anti-BrdU (Sigma; dilution, 1:1,000) in 5% FCS. Finally, injected antibodies and anti-BrdU antibody were separately stained with fluorescein-linked anti-rabbit antibody (Amersham; dilution, 1:50) and with Texas Red-linked anti-mouse immunoglobulin G antibody (Amersham; dilution, 1:100), respectively, in 5% FCS. After three washes with PBS, the cells were examined by fluorescence microscopy. All procedures were performed at room temperature. Experiments were performed three times (with about 200 injected cells each time), and mean values are presented.

RESULTS

Cloning of H37, encoding ASK, an huCdc7 binding protein. Yeast two-hybrid screening was conducted to isolate huCdc7 binding proteins. A HeLa cDNA library constructed with the activation domain vector pGAD-GH was introduced into yeast strain CG1945, expressing a hemagglutinin-tagged form of huCdc7 fused to the Gal4 DNA binding domain. Approximately 3×10^5 transformants were initially screened, yielding five β-galactosidase-positive clones. DNA sequence analysis indicated that these were all novel cDNAs, three of which were identical, and a representative clone, H37, was further investigated. For reasons that are explained below, we named the protein encoded by H37 ASK, for the activator of S phase kinase.

The interaction between ASK and huCdc7 was reexamined in mammalian cells (Fig. 1A). The initial clone isolated from two-hybrid screening turned out to contain a 189-bp 5′ noncoding region in addition to the full-length coding frame encoding 674 amino acids, resulting in a 741-amino-acid ASK-derived polypeptide. This extended polypeptide was fused to a myc epitope and was subcloned into a mammalian expression vector. myc epitope-tagged ASK was transiently coexpressed in COS7 cells with either the wild-type or kinase-negative form of huCdc7. Anti-huCdc7 antibody coprecipitated ASK, as detected by immunoblotting with anti-myc antibody (Fig. 1A, lower panel, lanes 2 and 3). Reciprocally, anti-myc antibody efficiently coprecipitated huCdc7 (Fig. 1A, upper panel, lanes



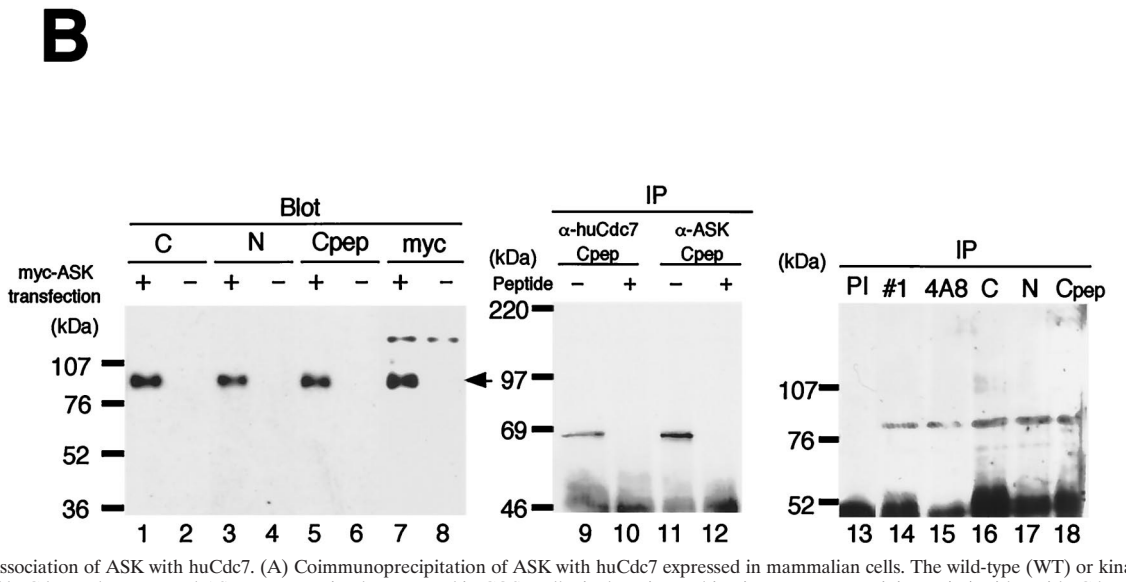


FIG. 1. Association of ASK with huCdc7. (A) Coimmunoprecipitation of ASK with huCdc7 expressed in mammalian cells. The wild-type (WT) or kinase-negative (KN) form of huCdc7 and myc-tagged ASK were transiently expressed in COS7 cells singly or in combination. Immunoprecipitates (IP) with anti-huCdc7 antibody #1 (lanes 1 to 5) (30) and with anti-myc antibody (lanes 6 to 10) were run on SDS–8% PAGE, and Western blotting was conducted with anti-huCdc7 monoclonal antibody 4A8 (upper panel) or anti-myc antibody (lower panel). A lower level of myc-ASK in the anti-huCdc7 immunoprecipitate containing the wild-type huCdc7 (lane 2) than in that containing the kinase-negative huCdc7 (lane 3) is due to the lower level of the total myc-ASK protein expressed in the former transfection in this particular experiment. (B) Characterization of antibodies against ASK protein and association of huCdc7 and ASK in vivo. Lanes 1 to 8, whole-cell extracts prepared from COS7 cells transfected with myc-tagged ASK (lanes 1, 3, 5, and 7) or mock transfected (lanes 2, 4, 6, and 8) were blotted with anti-ASK-C (lanes 1 and 2), anti-ASK-N (lanes 3 and 4), anti-ASK-Cpep (lanes 5 and 6), or anti-myc (lanes 7 and 8) antibody. The arrow indicates the myc-tagged ASK protein, which carries 63 amino acids derived from the 5′ noncoding region in addition to the myc tag at the N terminus. Lanes 9 to 12, immunoprecipitates from CEM extracts with either anti-huCdc7Cpep (lanes 9 and 10) or anti-ASK-Cpep (lanes 11 and 12) were separated by SDS-PAGE and blotted with huCdc7 monoclonal antibody (4A8). Lanes 13 to 18, immunoprecipitates prepared from nuclear extracts of HeLa cells with control preimmune antibody (PI) (lane 13), anti-huCdc7 #1 (lane 14), anti-huCdc7 monoclonal antibody 4A8 (lane 15), anti-ASK-C (lane 16), anti-ASK-N (lane 17), or anti-ASK-Cpep (lane 18) were blotted with anti-ASK-Cpep. Samples were run on regular SDS–8% PAGE at a low voltage to minimize the effect of phosphorylation on migration of protein bands.

7 and 8). The anti-huCdc7 antibody did not precipitate myc-ASK (Fig. 1A, lower panel, lane 1), nor did the myc antibody precipitate huCdc7 (Fig. 1A, upper panel, lanes 9 and 10), when singly expressed. These results demonstrate that ASK interacts with huCdc7. Wild-type and kinase-negative huCdc7 were coprecipitated with myc-ASK with similar efficiencies, indicating that the interaction between the two proteins is not affected by the kinase activity of the catalytic subunit.

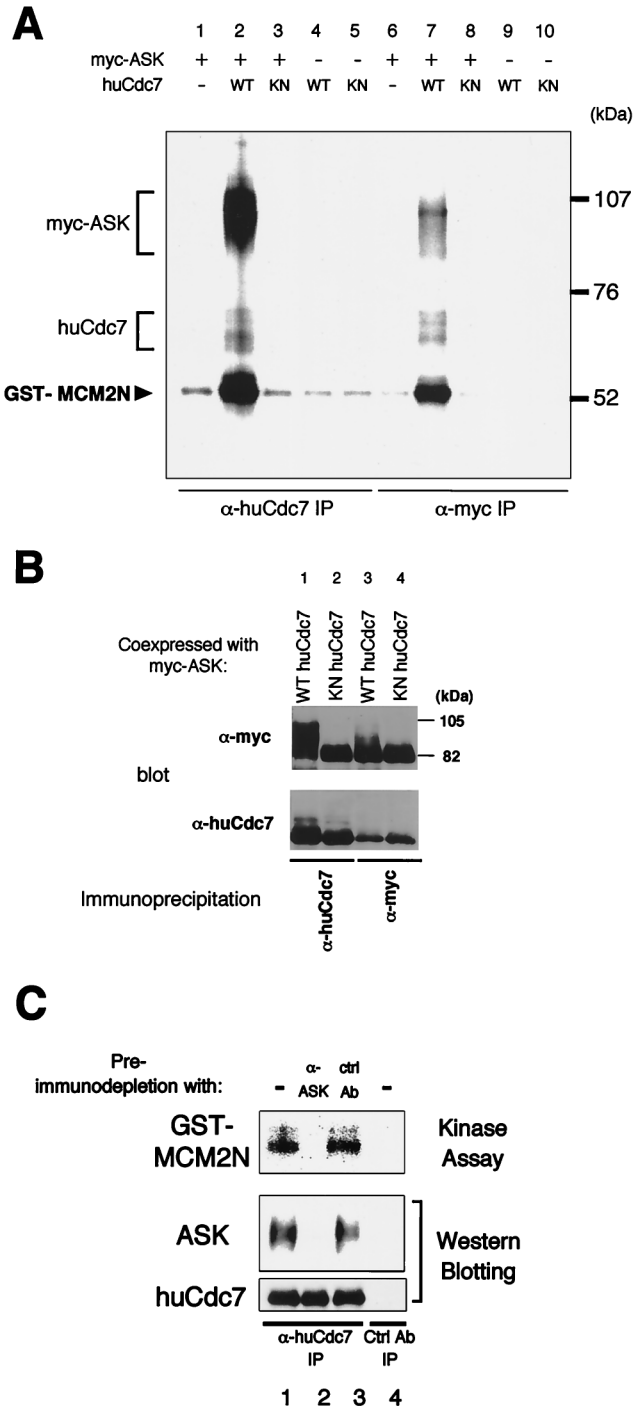


FIG. 2. ASK activates huCdc7 kinase activity. (A) The immunoprecipitates (IP) analyzed for Fig. 1A were used for kinase assays as previously described (30) with 0.5 μg of GST-huMCM2N protein as a substrate. The reaction mixtures were run on SDS–8% PAGE. WT, wild type; KN, kinase negative. (B) Mobility shift of ASK and huCdc7 induced by coexpression of wild-type huCdc7. Extracts were made from COS7 cells expressing either wild-type (lanes 1 and 3) or kinase-negative (lanes 2 and 4) huCdc7 together with myc-tagged ASK. Immunoprecipitates with anti-huCdc7 antibody 1 or with anti-myc antibody were blotted with anti-myc (upper panel) or with anti-huCdc7 (lower panel) antibody. For panels A and B, samples were run on SDS–8% PAGE with a low bisacrylamide content at a high voltage to obtain better separation of phosphorylated forms of proteins. (C) The K562 cell extract (lane 1) and that immunodepleted with anti-ASK-Cpep antibody (lane 2) or treated similarly with preimmune serum (lane 3) were immunoprecipitated with anti-huCdc7 antibody, and the precipitates were assayed for kinase activity as described in Materials and Methods. Phosphorylation of GST-MCM2N protein (upper panel) and the presence or absence of ASK (middle panel) and huCdc7 (lower panel) in the immunoprecipitates, as detected by Western blotting, are shown. Lane 4, assay with the immunoprecipitate with a control antibody (ctrl Ab). Samples were run on regular SDS–8% PAGE.

To investigate endogenous ASK protein, antibodies were raised against the N-terminal and C-terminal polypeptides of ASK (anti-ASK-N and anti-ASK-C, respectively) and against the C-terminal oligopeptides of ASK and huCdc7 (anti-ASK-Cpep and anti-huCdc7Cpep, respectively). Anti-ASK antibodies reacted specifically with COS7-expressed ASK protein (Fig. 1B, lanes 1, 3, and 5), which was not detected in the extract from mock-transfected cells (Fig. 1B, lanes 2, 4, and 6). Affinity purified antipeptide antibodies were used to specifically precipitate endogenous complexes from an extract prepared from human CEM cells. The presence of huCdc7 protein in both huCdc7 and ASK immunocomplexes could be demonstrated by immunoblotting with an huCdc7 monoclonal antibody (Fig. 1B, lanes 9 and 11). This interaction between ASK and huCdc7 was completely blocked by preincubation of the immunoprecipitating antibody with the antigenic peptide (Fig. 1B, lanes 10 and 12). In HeLa cell extracts, anti-Cdc7 antibodies and anti-ASK antibodies precipitated a single polypeptide of 80 kDa, which specifically reacted with an anti-ASK antibody (Fig. 1B, lanes 14 to 18). These results clearly demonstrate that huCdc7 and ASK exist together as a complex in vivo.

ASK is a putative regulatory subunit for huCdc7 kinase. To examine the ability of ASK to activate huCdc7, the huCdc7-ASK kinase complexes, immunoprecipitated with either anti-huCdc7 or anti-myc antibody, were tested for in vitro kinase activity by using the GST-MCM2N fusion protein, containing the N-terminal 209 amino acids of human MCM2 protein, as a substrate (Fig. 2A). In the presence of wild-type huCdc7, phosphorylation of MCM2 by both anti-huCdc7 and anti-myc immunoprecipitates was observed (Fig. 2A, lanes 2 and 7). Furthermore, two additional phosphorylated proteins were detected, which were identified as the transfected huCdc7 and myc-ASK (data not shown). They appear in the gel as smeared bands, since they are multiply phosphorylated (Fig. 2B). These phosphorylations were not detected with kinase-negative huCdc7, although it could form a complex with ASK (Fig. 1A and 2A, lanes 3 and 8, and 2B, lanes 2 and 4), indicating that huCdc7 is responsible for these activities. Furthermore, the mobility of ASK protein on SDS-PAGE was retarded when wild-type huCdc7 was coexpressed but not with kinase-negative huCdc7 (Fig. 2B). Mobility shift was also detected with huCdc7. The shifted bands could be eliminated by phosphatase treatment (data not shown), indicating that they are hyperphosphorylated forms of ASK and huCdc7. More phosphorylated ASK protein was detected when immunoprecipitation was with anti-huCdc7 antibody (Fig. 2B, lane 1) than when it was with anti-myc antibody (Fig. 2B, lane 3). This may indicate that not all of the expressed ASK protein is associated with huCdc7 protein. Alternatively, huCdc7 may not require persistent, stable interaction with ASK for activation of its kinase activity. These results show that ASK stimulates huCdc7 kinase and can also be phosphorylated by huCdc7. Kinase activity could not be detected when only the huCdc7 catalytic subunit was expressed, because the level of endogenous ASK protein was too low under these experimental conditions (Fig. 2A, lanes 4 and 9), even though the transfected huCdc7 was present in the immunoprecipitates (Fig. 1A, upper panel, lane 4).

In order to determine whether ASK is the major regulatory subunit for huCdc7, we immunodepleted ASK protein from

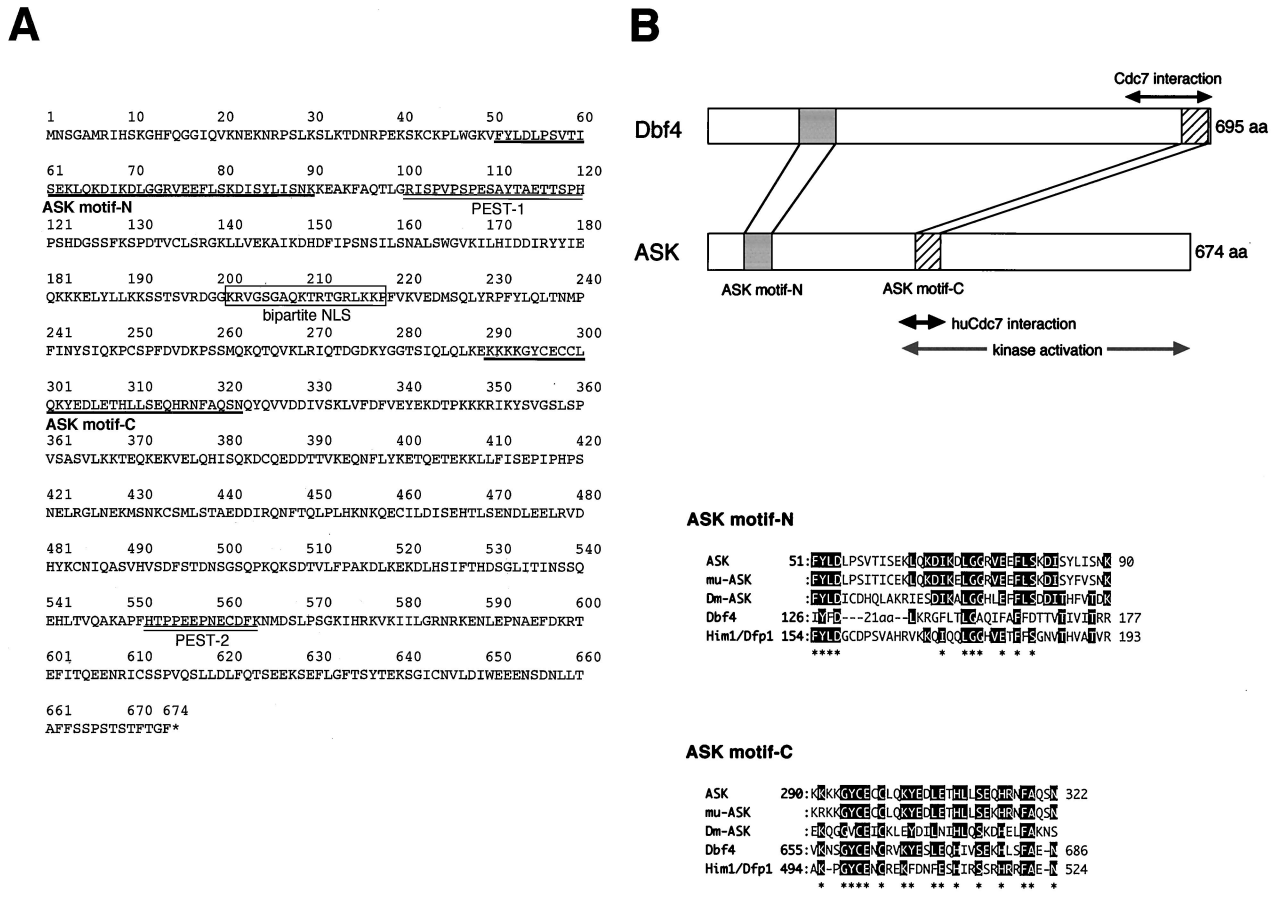


FIG. 3. Structure of ASK protein. (A) Amino acid sequence of the full-length ASK protein. The underlined, boxed, and double-underlined regions indicate the two conserved segments (ASK motif N and ASK motif C), a potential bipartite nuclear localization signal, and PEST-like sequences, respectively. (B) Schematic representation of comparison between Dbf4 and ASK and amino acid (aa) sequence alignments of the two conserved motifs, including sequences from ASK-related molecules from mouse (GenBank update accession no. AA624077) and *Drosophila* (GenBank accession no. AC000551) and the fission yeast Dbf4 homologue (Him1/Dfp1) (3, 33b). White letters and asterisks indicate those amino acid residues conserved in more than three and four members, respectively. The double-arrowheaded region in Dbf4 was reported to be sufficient for interaction with Cdc7 (10). Solid or dotted double-arrowheaded regions in ASK indicate the region essential (but not sufficient) for interaction with huCdc7 or that sufficient for activation of huCdc7 kinase activity (data not shown), respectively.

K562 cell extracts and examined the residual kinase activity in the supernatant associated with huCdc7 by immune complex kinase assays using antibodies to huCdc7. As shown in Fig. 2C, the removal of ASK largely abolished huCdc7 kinase activity, showing that ASK is associated with nearly all of the active huCdc7. huCdc7 is present in excess compared to ASK, and therefore, huCdc7 is still present in the ASK-immunodepleted extract. These results show that most of the huCdc7 kinase activity is associated with ASK and strongly suggest that ASK is the major regulatory subunit for huCdc7 which activates its kinase activity.

Structure of ASK: presence of two conserved domains. Analyses of the predicted amino acid sequence of ASK revealed a small stretch of amino acids possessing 55% identity with the C-terminal region of *S. cerevisiae* Dbf4 (Fig. 3A and B), a region known to be important for the interaction of Dbf4 with Cdc7 (10). This conserved domain (ASK motif C) is also present in ASK- and Dbf4-related proteins identified in mouse, *Drosophila*, and *S. pombe* (Fig. 3B) (3, 19a, 33a). Another stretch of amino acids of ASK (ASK motif N) was found to be conserved in the putative mouse and *Drosophila* ASK homologues as well as in a recently identified fission yeast homologue of Dbf4 (3, 33b), although this motif is only weakly conserved in the *S. cerevisiae* Dbf4 protein (Fig. 3B). Homology searches showed that no other proteins with significant similarity to ASK are present in the databases. A potential bipartite nuclear localization signal (amino acids 201 to 218) and two possible PEST sequences (amino acids 101 to 120 and 552 to 564) were identified in ASK, although their functions are presently unknown.

In order to determine the regions of ASK protein responsible for binding huCdc7, we constructed a series of N-terminal and C-terminal truncation mutants of ASK and expressed them as fusions with the Gal4 activation domain (on pGAD-GH) in yeast (Fig. 4A). The interaction of each deletion mutant with huCdc7 was first examined in two-hybrid assays. As shown in Fig. 4B, deletion of the N terminus (ΔN1 and ΔN2) demonstrated that the N-terminal 255 amino acids were dispensable for interaction with huCdc7. However, removal of ASK motif C, through deletion of an additional 80 amino acids (ΔN3), resulted in complete loss of interaction. A small deletion of just 20 amino acids from the C terminus (ΔC) resulted in a decrease of binding efficiency of about 60% as measured by the *lacZ* activity. Deletion of the C-terminal 234 or 369 amino acids (ΔP2 and ΔB, respectively) further decreased the interaction to about 10% of that observed with the full-length protein. ΔP1, containing only the N-terminal 235 amino acids, did not interact with huCdc7. Similar results were obtained

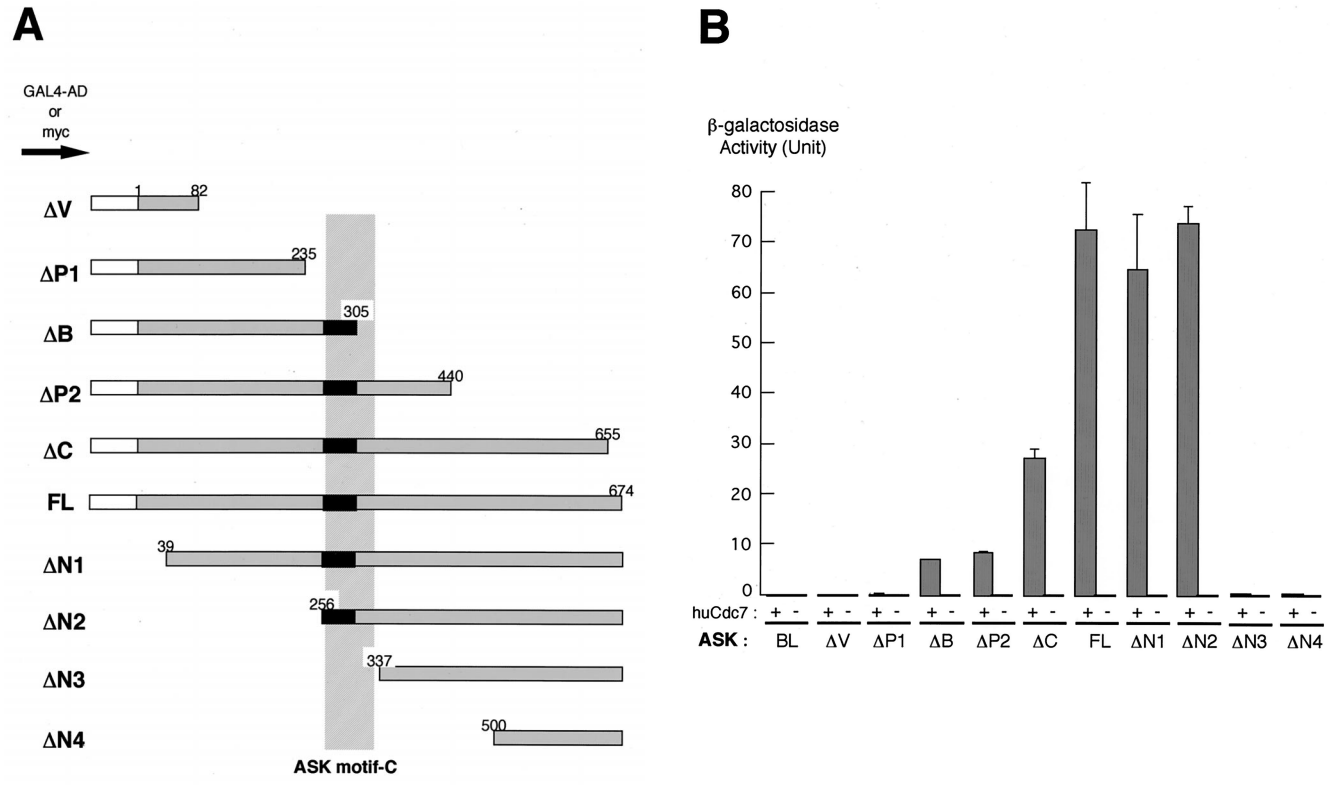


FIG. 4. Identification of the domain of ASK required for interaction with huCdc7. (A) Deletion derivatives of ASK protein. The N-terminal and C-terminal deletion derivatives of ASK are schematically represented. The number at the end of each bar indicates the position of the amino acid at the deletion endpoint. Gray and white boxes indicate the coding and 5′ noncoding regions, respectively. The hatched region indicates ASK motif C, and the solid boxes indicate the segment of ASK essential (but not sufficient) for binding huCdc7. AD, activation domain. (B) *lacZ* activity of ASK deletion derivatives in two-hybrid assays with pAS2-huCdc7. Error bars indicate standard deviations. (C) Immunoprecipitation assays of ASK deletion derivatives in mammalian cells. myc-tagged ASK deletion derivatives were transiently expressed in COS7 cells with (even-numbered lanes) or without (odd-numbered lanes) wild-type huCdc7. Lanes 1 and 2, mock; lanes 3 and 4, ΔP1; lanes 5 and 6, ΔB; lanes 7 and 8, full-length (FL) ASK; lanes 9 and 10, ΔN2; lanes 11 and 12; ΔN3. Immunoprecipitation (IP) was conducted with anti-huCdc7 antibody #1 (panels a and b) or with anti-myc antibody (panels c and d). Western blotting was conducted with anti-huCdc7 antibody (panels b and c) or with anti-myc antibody (panels a and d). All of the samples were run on regular SDS–8% PAGE.

with coimmunoprecipitation assays with mammalian cells. ASK and its deletion derivatives were tagged with a myc epitope at the N terminus and coexpressed with huCdc7 in COS7 cells. ΔB or ΔN2, containing the N-terminal 305 amino acids and the C-terminal 419 amino acids, respectively, was coimmunoprecipitated with anti-huCdc7 antibody (Fig. 4C, panel a), and reciprocally, huCdc7 was coprecipitated with these mutants when we conducted immunoprecipitation with anti-myc antibody (Fig. 4C, panel c). We next examined whether the region present in both ΔB and ΔN2 is sufficient for interaction with huCdc7. The polypeptide derived from residues 256 to 305, which contained a portion of ASK motif C, did not interact with huCdc7 in either two-hybrid or coimmunoprecipitation assays (data not shown). These results indicate that ASK motif C is essential but not sufficient for interaction with the huCdc7 catalytic subunit. The ASK region either N terminal or C terminal to this motif is also required for this interaction. In *S. cerevisiae*, the 123-amino-acid segment containing this conserved motif was sufficient for interaction with Cdc7 in two-hybrid assays (10).

Expression of ASK mRNA in tissues and cell lines. ASK mRNA expression patterns in various human tissues and in cancer cell lines were examined by Northern blot analyses (Fig. 5). The ASK cDNA probe detected a 2.5-kb transcript in all of the tissues and cell lines examined except for brain and kidney, in which relatively high-level expression of huCdc7 mRNA was detected (30). Among the tissues examined, the most abundant expression of ASK mRNA was detected in testis, followed by thymus, both of which showed abundant expression of huCdc7 as well. In testis, two additional RNA bands of 6 and 4 kb were also detected, although the nature of these transcripts is presently unknown (Fig. 5A). ASK mRNA is expressed at high levels in most cancer cell lines (Fig. 5B), consistent with its expected role in active proliferation.

Expression of ASK is growth and cell cycle regulated. To examine whether the expression of ASK is regulated during the cell cycle, WI-38 cells were synchronized in G_0 by serum starvation, and total RNA was extracted from cells harvested at various times after readdition of serum for Northern blotting analysis. FACS analysis indicated that the cells initiated S phase at between 16 and 20 h after serum stimulation, and most of the proliferating cells had completed S phase by 28 h (Fig. 6A). The relative levels of ASK mRNA (Fig. 6B) were obtained by normalizing the intensity of ASK transcripts to that of G3PDH (glyceraldehyde-3-phosphate dehydrogenase) transcripts, which is not affected by growth stimulation. The level of ASK mRNA was low in quiescent cells, rose steadily as the cells approached the G_1/S boundary, and continued to increase up to 28 h after stimulation (Fig. 6B). Consistent with a previous report (27), the cyclin E transcript level was repressed in quiescent cells, appeared at 6 h, and increased up to 20 h after stimulation, when most of the cells had just entered

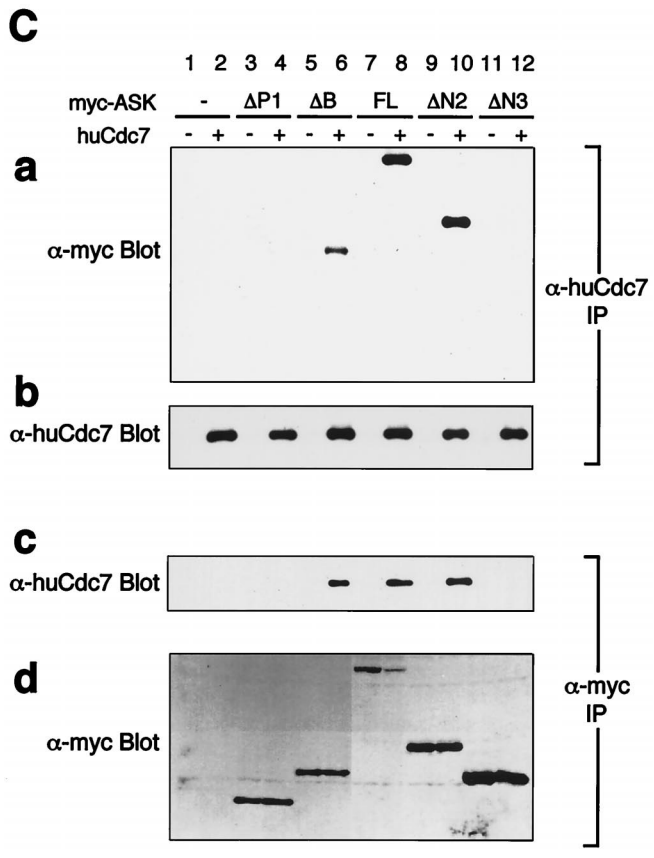


FIG. 4—*Continued.*

S phase. A similar pattern was also observed for huCdc6 (reference 34 and data not shown). This result indicates that ASK expression is regulated by growth stimulation in a manner similar to that for cyclin E and huCdc6. Cyclin E expression decreased at 24 h, reflecting the cell cycle oscillation of cyclin E transcription (7, 20). In contrast, the level of ASK mRNA stayed high thereafter, up to 28 h after serum stimulation (Fig. 6B).

Next, we analyzed ASK expression during the proliferating cell cycle. We fractionated proliferating CEM cells according to cell size by elutriation and examined the DNA content of each fraction by FACS analyses (Fig. 6C). Northern blot analyses of these cells (Fig. 6D) indicate that the ASK mRNA level is very low in fraction 1, which consists of over 95% very small G_1 cells. Surprisingly, larger G_1 cells collected in fraction 2 already showed a significant increase in ASK expression, which was sustained through fractions enriched for S phase cells (fractions 3, 4, 5, and 6). A significant level of ASK mRNA was still maintained in fraction 8, in which more than 50% of the cells are in G_2. In contrast, cyclin E mRNA, which is low in fraction 1, increases in fraction 2 and decreases sharply in later fractions containing more S phase cells, consistent with previous reports (Fig. 6D) (7, 20). We also obtained synchronized cell populations by using K562 cells arrested at the G_2/M boundary by nocodazole treatment. At 6 h after release from the nocodazole block, cells were mainly in G_1. At 12 h, approximately half of the cells were in S phase, while at 18 h, the majority of the cells were in S phase and some were completing S phase with a 4C DNA content (Fig. 6E). ASK mRNA was detected at G_2/M at a reduced level and decreased further in G_1. It rose steadily as cells progressed into S phase (Fig. 6F, upper panel). This profile is in contrast to that of cyclin E mRNA on the same blot, which was lowest in G_2/M, increased

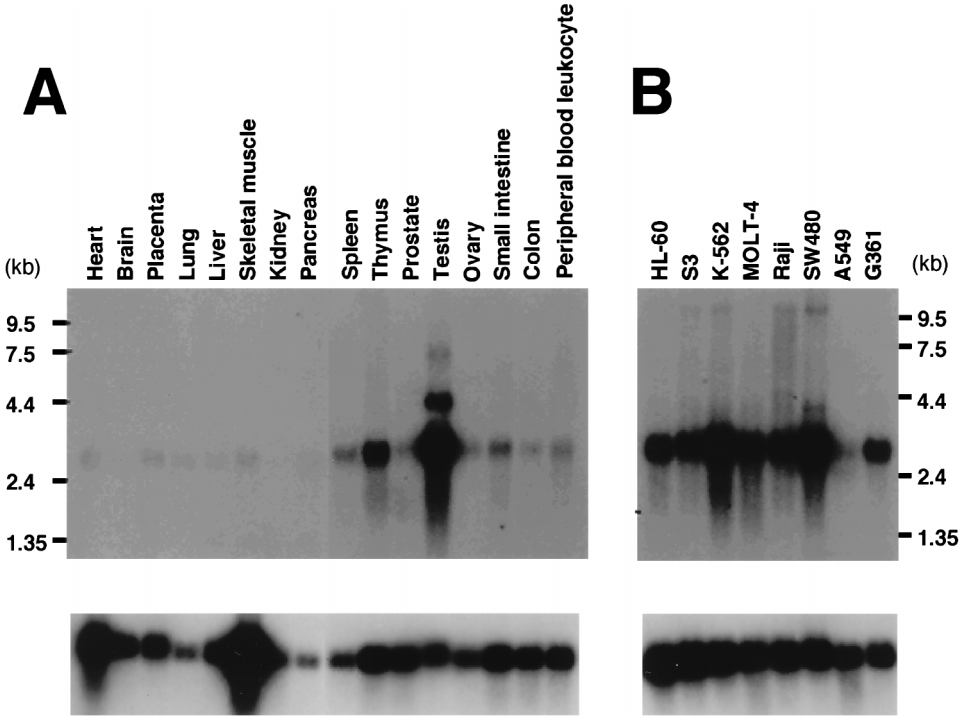


FIG. 5. Expression of ASK in human tissues and cancer cell lines. (A) Northern analyses of ASK mRNA in various human tissues. (B) Northern analyses of ASK mRNA in cancer cell lines. The cell lines are (from the left) promyelocytic leukemia HL-60, HeLa cell S3, CML K-562, lymphoblastic leukemia MOLT-4, Burkitts' lymphoma Raji, colon cancer SW480, lung cancer A549, and melanoma G361. In the lower panels, the same filters were blotted with a probe specific for G3PDH mRNA.

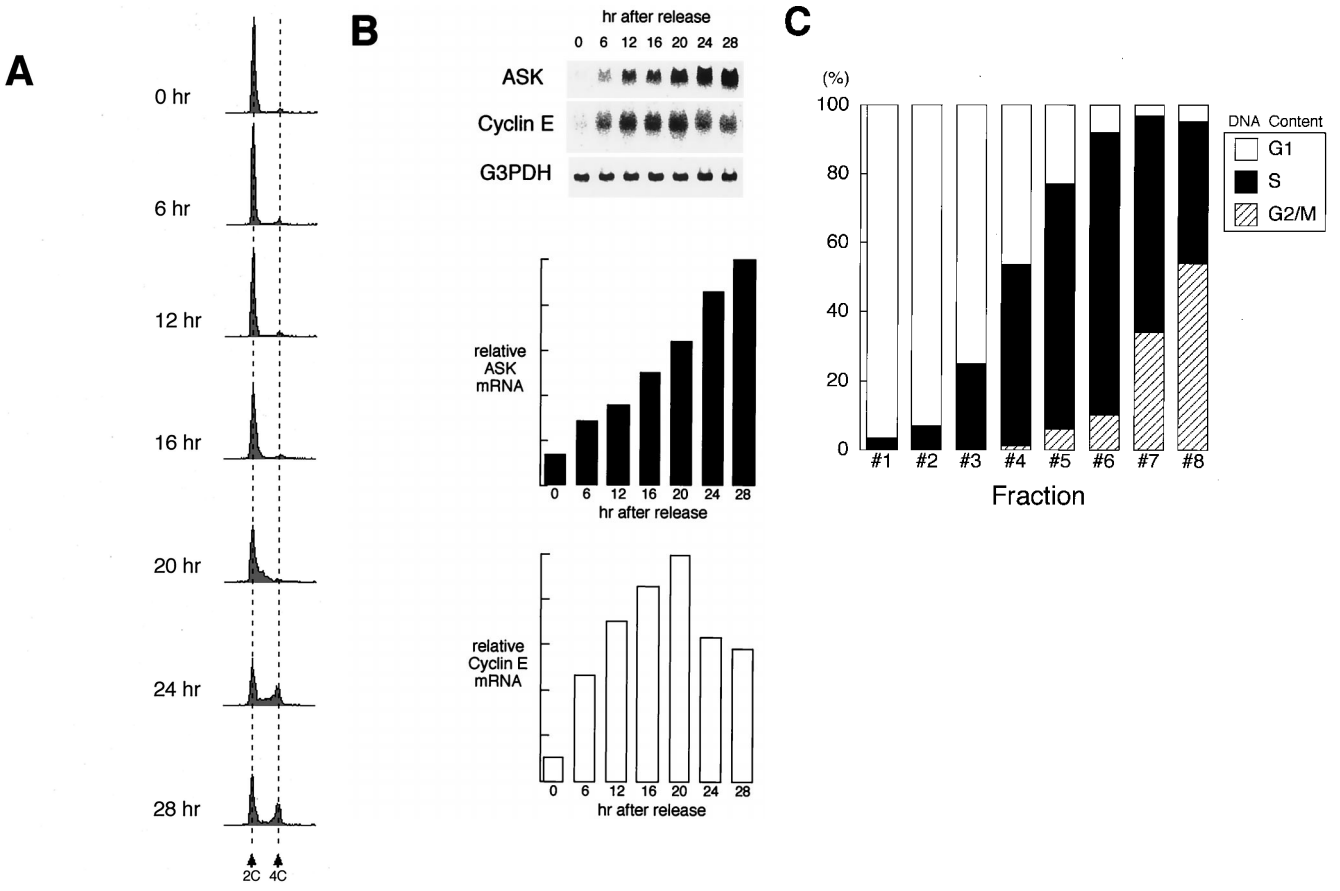
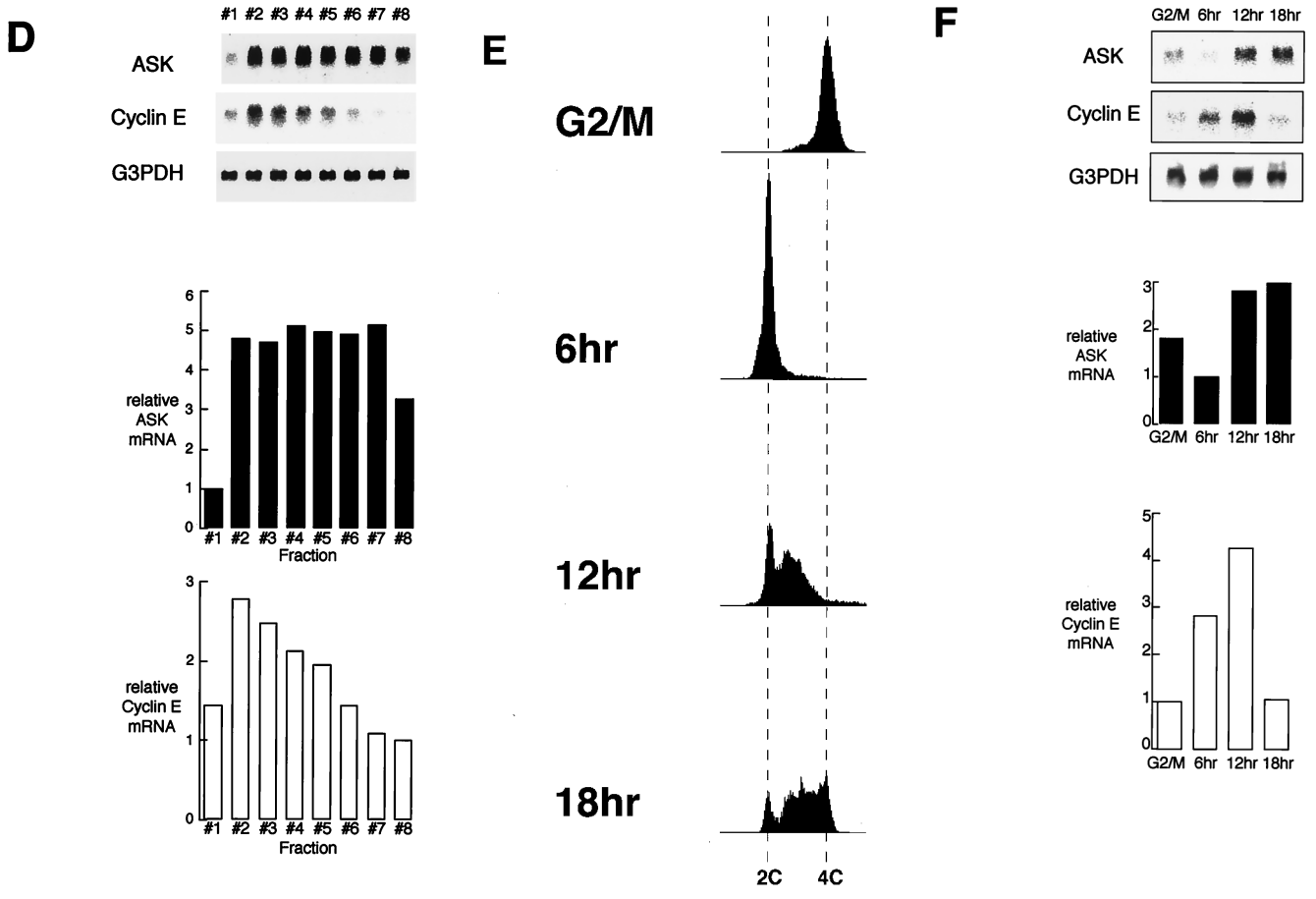


FIG. 6. Growth and cell cycle regulation of ASK mRNA and cellular localization of ASK protein. (A) Human primary fibroblast WI-38 cells were synchronously released from serum starvation by the addition of 10% serum. Cells were harvested and stained with propidium iodide at the times indicated, and DNA contents were estimated by FACS analyses. (B) Northern analyses of ASK mRNA after serum stimulation of quiescent WI-38 cells. Total RNA was prepared from the cells used for FACS analyses in panel A and was analyzed by Northern blotting with probes indicated to the left of each panel. (C) CEM cells were fractionated by elutriation into eight fractions, each of which was analyzed by FACS. The proportions of G_1, S, and G_2/M cells are indicated for each fraction. (D) Northern analyses of ASK mRNA in elutriated cell fractions. Total RNA was prepared from the elutriated CEM cells prepared as described above and was analyzed by Northern blotting with the probes indicated to the left. (E) K562 cells were arrested at G_2/M by nocodazole treatment and were synchronously released into the cell cycle, as described in Materials and Methods, and the DNA content at each time point was analyzed by FACS. (F) Northern analyses of synchronized cell populations. RNAs prepared from the cells used for panel E were analyzed by Northern blotting with the probes indicated to the left of each panel. In panels B, D, and F, the relative mRNA levels of ASK and cyclin E, normalized to the intensities of G3PDH bands, are shown. (G) Indirect immunofluorescence of ASK protein in WI-38 cells (panels a and c) and HeLa cells (panels e, g, and i). The antibodies used were anti-ASK-C (panel c), anti-ASK-N (panels a and e) and normal rabbit control antiserum (panel i). The ASK signal was completely blocked by preincubation of anti-ASK-N antibody with recombinant GST-ASK-N protein (panel g). Panels b, d, f, h, and j, DAPI staining of DNA in the respective fields located above each panel.

during G_1 phase, peaked in early S, and decreased as the cells progressed further into S phase (Fig. 6F, middle panel). The results from these two experiments are consistent and indicate that ASK expression is regulated during the proliferating cell cycle and that its expression is induced in G_1 at a time point later than that for cyclin E and is sustained through S phase, whereas expression of cyclin E mRNA is more transient. This pattern of ASK expression is consistent with the expected continuous requirement for huCdc7-ASK functions during S phase.

To further characterize the expression of ASK, we examined its subcellular distribution in human cells. Endogenous ASK protein was detected as bright speckles in the nuclei of both HeLa and WI-38 cells by indirect immunofluorescence with two different ASK-specific antibodies (Fig. 6G, panels a, c, and e). The staining by anti-ASK-C was completely blocked by preincubation of the antibody with the antigenic polypeptide (Fig. 6G, panel g). Similarly, huCdc7 was also detected as bright speckles under the same conditions (data not shown). These results suggest that the huCdc7 and ASK proteins are localized at particular subnuclear compartments in nuclei, although it is not known whether they are present at the same subnuclear localization.

huCdc7 kinase activity is activated in S phase when ASK expression increases. In order to examine the level of ASK protein during the cell cycle, cell extracts from synchronized culture were examined by Western blotting. Very little ASK protein was detected in cells in G_2/M or in G_1 phase (Fig. 7A, upper panel, lanes 2 and 3). ASK protein was detected in extracts from early- and late-S phase cells (Fig. 7A, upper panel, lanes 4 and 5), indicating that the ASK protein level also oscillates during the cell cycle and increases in S phase. In contrast, cyclin E increased through G_1, peaked in early S, and decreased by late S phase (Fig. 7A, middle panel). ASK protein appeared as a ladder of bands on SDS-PAGE, and bands with slow mobility were eliminated by phosphatase treatment (Fig. 7A, lane 1), indicating that ASK is extensively phosphorylated during S phase.



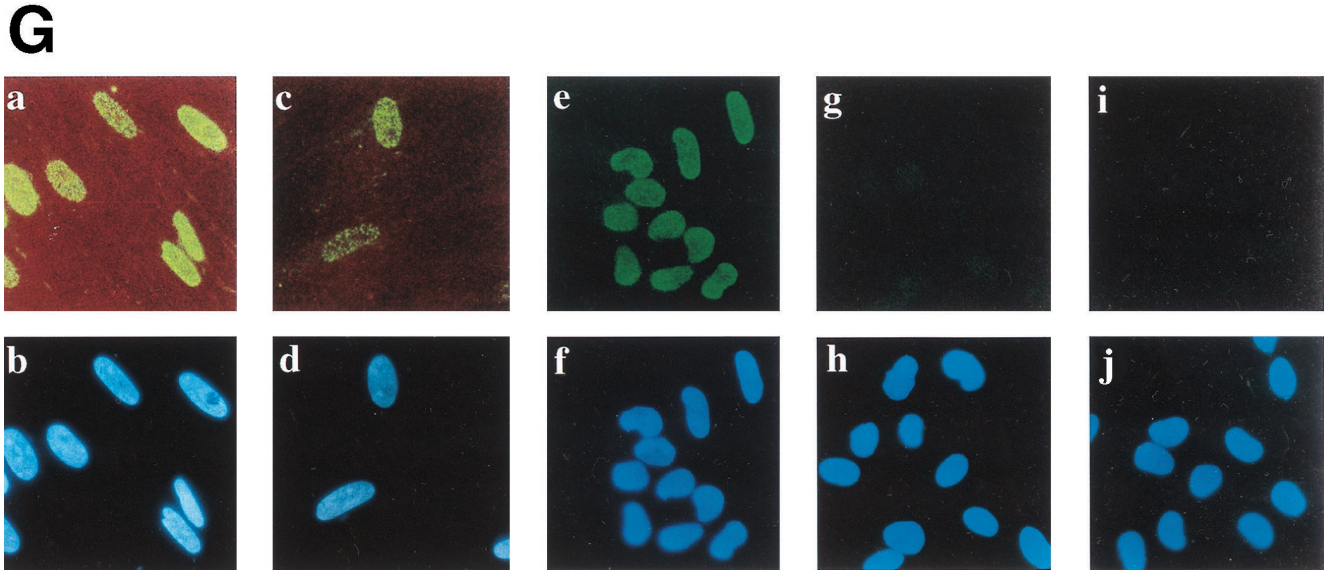


FIG. 6—*Continued.*

With the same set of extracts, kinase assays of the anti-huCdc7 immunoprecipitates were carried out. Only very low kinase activity was detected at G_2/M and in G_1 phase (Fig. 7B, upper panel, lanes 1 and 2), whereas vigorous kinase activity was detected when cells were in S phase (Fig. 7B, upper panel, lanes 3 and 4). ASK protein was present only in the immunoprecipitates from S phase cells (Fig. 7B, middle panel, lanes 3 and 4) consistent with the results of Western analyses, indicating that increased kinase activity is the result of elevated ASK protein, and consequently increased active huCdc7-ASK kinase complex, in S phase cells. This notion is further supported by the fact that the preimmunodepletion of ASK protein from

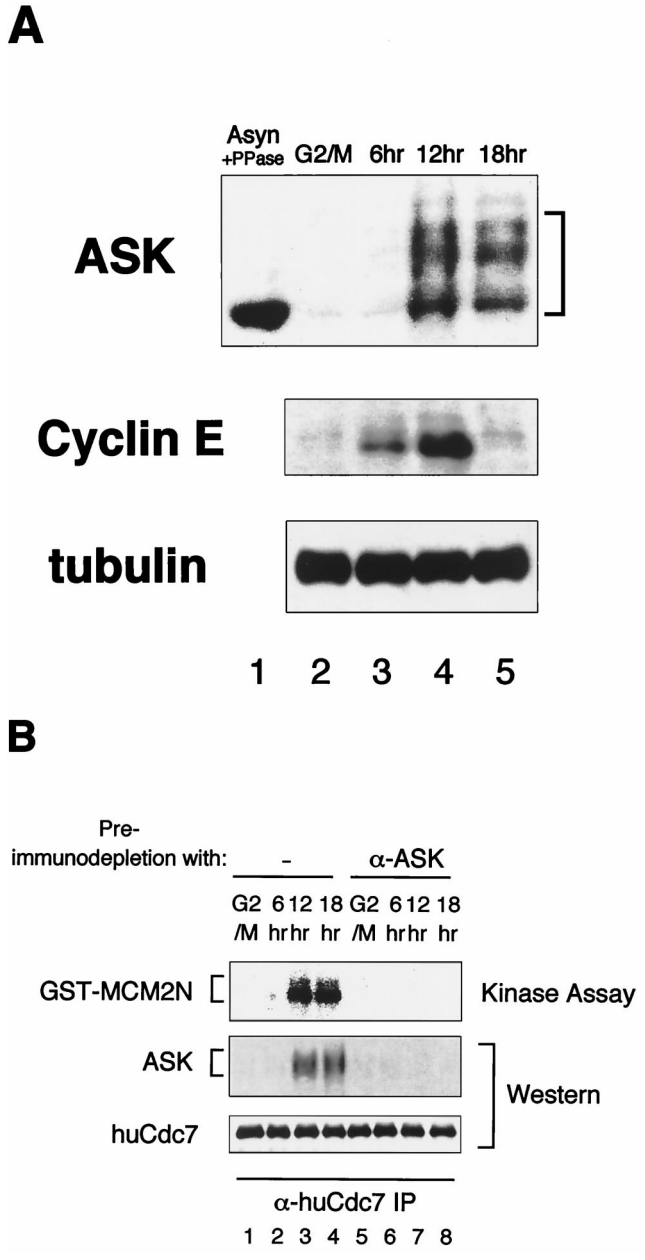


FIG. 7. Levels of ASK protein and huCdc7 kinase activity increase in S phase. (A) Extracts were prepared from the same K562 cells used for Fig. 6E (arrested with nocodazole and released into the cell cycle) and run on SDS–8% PAGE with a low bisacrylamide content at a high voltage to separate differentially phosphorylated proteins on the gel. The gel was blotted with anti-ASK-Cpep (upper panel). The same samples were run on regular SDS–10% PAGE, followed by blotting with anti-cyclin E (middle panel, lanes 2 to 5) or antitubulin antibody (lower panel, lanes 2 to 5). In lane 1 of the upper panel, the extract from asynchronous culture (Asyn) was immunoprecipitated with anti-ASK-Cpep antibody and then further treated with phosphatase (PPase) before being applied on the gel. (B) Anti-huCdc7 immunoprecipitates (IP) of each extract used for panel A were assayed for kinase activity (upper panel) or were examined for the presence of ASK (middle panel) and huCdc7 (lower panel) by Western blotting. In lanes 5 to 8, the extracts were immunodepleted with anti-ASK-Cpep antibody prior to immunoprecipitation with anti-huCdc7 antibody. Samples were run on regular SDS–8% PAGE at a low voltage. Hyperphosphorylated, slow-migrating ASK proteins appear to be more preferentially immunoprecipitated by anti-huCdc7 antibody (lanes 3 and 4), presumably reflecting efficient phosphorylation of the associated ASK subunit by huCdc7.

the extracts largely eliminated the kinase activity (Fig. 7B, middle panel, lanes 7 and 8). Similar levels of huCdc7 protein were detected at all of the cell cycle stages examined (Fig. 7B, lower panel), consistent with the constant huCdc7 mRNA level throughout the cell cycle (data not shown). The results indicate that huCdc7 kinase activity is cell cycle regulated, increasing in S phase, and that this is caused by an increased ASK protein level during S phase.

The functions of ASK are required for S phase entry in mammalian cells. To examine the function of ASK in the G_1/S transition of the cell cycle, we used an antibody microinjection strategy to inactivate endogenous ASK protein. Affinity-purified antibodies directed against either a GST-ASK fusion protein containing the N-terminal 305-amino-acid (anti-ASK-N) or the C-terminal peptide (anti-ASK-Cpep) of ASK were microinjected into the cytoplasm of normal human lip fibroblast (KD) cells which had been arrested in G_0 by serum starvation and released synchronously into the cell cycle. The fraction of cells in S phase, as measured by the percentage of BrdU-positive cells, at various times after serum readdition is shown in Fig. 8A. The cells started to synthesize DNA at 18 h, and approximately 90% of the cells had entered S phase by 24 h. Therefore, we microinjected the antibodies at 12 h after serum addition, when cells were in late G_1 phase, and fixed them at 26 h, by which time most of the cells should have entered S phase. Seventy percent of the cells injected with anti-ASK-N failed to enter S phase, whereas microinjection of a control antibody did not result in any significant inhibition (Fig. 8B, bars 2 and 4). Similarly, the ASK C-terminal peptide-specific antibody (anti-ASK-Cpep) inhibited S phase in more than 80% of the injected cells (Fig. 8B, bar 5). Furthermore, coinjection of the antigenic peptide with anti-ASK-Cpep antibody blocked the inhibition, resulting in 70% of the cells being in S phase (Fig. 8B, bar 6). Examples of BrdU and antibody staining of microinjected cells are shown in Fig. 8C. BrdU is not incorporated into those cells injected with the anti-ASK antibody (Fig. 8C, left panels), whereas it is incorporated into the cells when the antibody and the antigen peptide are coinjected (Fig. 8C, right panels). These results strongly indicate that the function of ASK, and therefore most likely that of the huCdc7-ASK kinase complex, is required for entry into S phase in mammalian cells.

DISCUSSION

We report here a novel cDNA, encoding ASK, a regulatory subunit for huCdc7 kinase (18, 30); huCdc7 is structurally related to Cdc7, which is known to be required for S phase initiation and progression in budding yeast. ASK is an intrinsic partner of huCdc7 kinase and is essential for mammalian DNA replication. The catalytic subunit of huCdc7 alone is inactive as a kinase in vitro, and complex formation with ASK is essential for activation of its phosphorylation activity. ASK, whose expression is growth and cell cycle regulated, appears to be a critical regulator for S phase in mammals which receives the upstream cell cycle signals and transmits them to machinery of DNA replication by activating the kinase activity of huCdc7. Depletion of ASK from cell extracts eliminated most of the huCdc7-dependent kinase activity, showing that ASK is the major activator of huCdc7 kinase. Thus, ASK may represent a human homologue of *S. cerevisiae* Dbf4. Recently, a putative regulatory subunit for Hsk1 kinase, a fission yeast homologue of Cdc7, was identified (3, 33b). While the kinase activities of huCdc7 and *S. cerevisiae* Cdc7 completely depend on the ASK and Dbf4 proteins, respectively (this paper and reference 22a), Hsk1 kinase possesses intrinsic autophosphorylation activity in

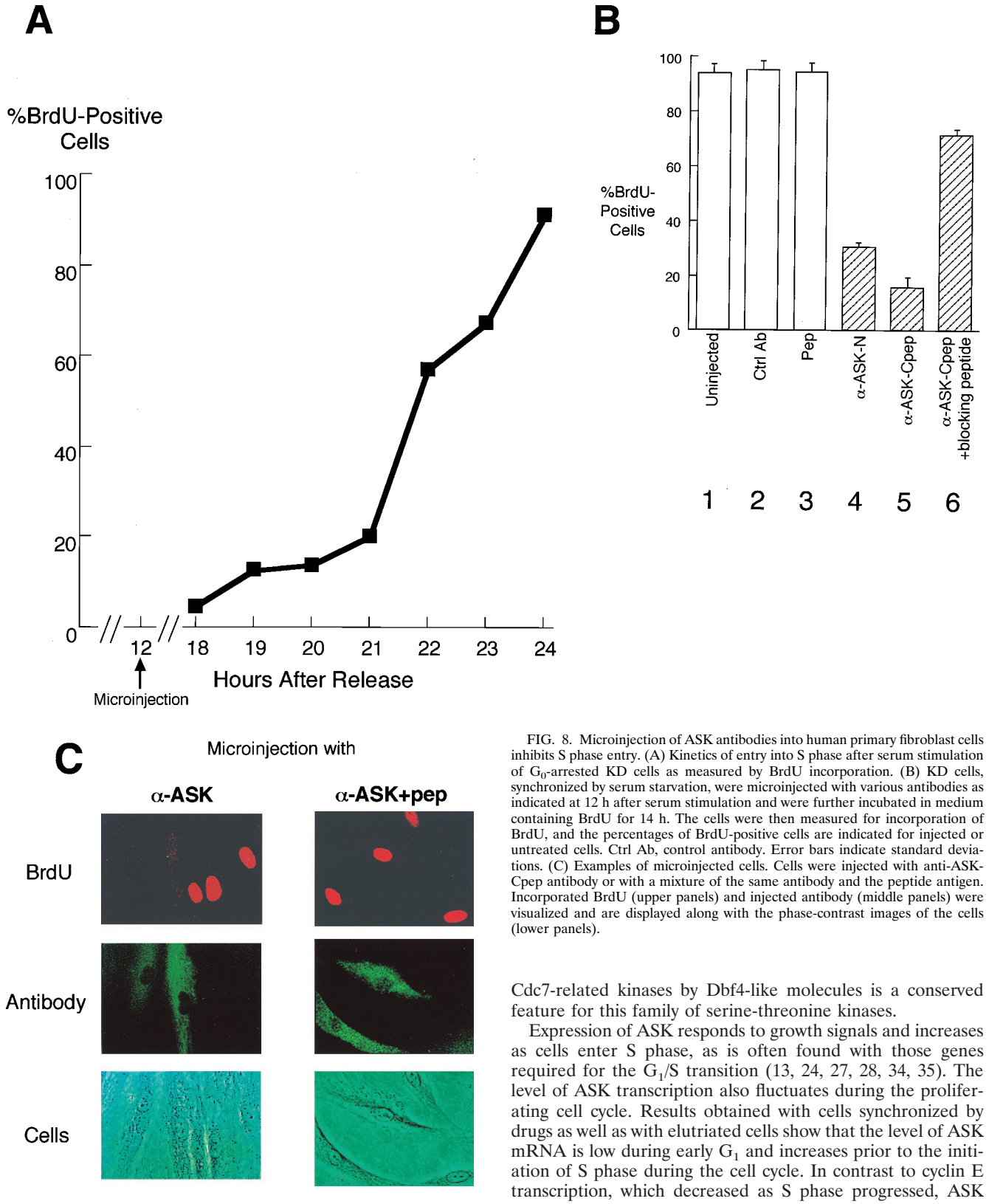


FIG. 8. Microinjection of ASK antibodies into human primary fibroblast cells inhibits S phase entry. (A) Kinetics of entry into S phase after serum stimulation of G_0-arrested KD cells as measured by BrdU incorporation. (B) KD cells, synchronized by serum starvation, were microinjected with various antibodies as indicated at 12 h after serum stimulation and were further incubated in medium containing BrdU for 14 h. The cells were then measured for incorporation of BrdU, and the percentages of BrdU-positive cells are indicated for injected or untreated cells. Ctrl Ab, control antibody. Error bars indicate standard deviations. (C) Examples of microinjected cells. Cells were injected with anti-ASK-Cpep antibody or with a mixture of the same antibody and the peptide antigen. Incorporated BrdU (upper panels) and injected antibody (middle panels) were visualized and are displayed along with the phase-contrast images of the cells (lower panels).

the absence of the regulatory subunit (3). In spite of this difference, the activity of Hsk1 is also significantly stimulated when associated with the regulatory subunit in an insect cell expression system (22a), suggesting that the regulation of Cdc7-related kinases by Dbf4-like molecules is a conserved feature for this family of serine-threonine kinases.

Expression of ASK responds to growth signals and increases as cells enter S phase, as is often found with those genes required for the G_1/S transition (13, 24, 27, 28, 34, 35). The level of ASK transcription also fluctuates during the proliferating cell cycle. Results obtained with cells synchronized by drugs as well as with elutriated cells show that the level of ASK mRNA is low during early G_1 and increases prior to the initiation of S phase during the cell cycle. In contrast to cyclin E transcription, which decreased as S phase progressed, ASK mRNA was maintained at a high level throughout S phase (Fig. 6D and 6F). In serum-stimulated cells, ASK mRNA keeps increasing as cells progress into S phase, whereas the transcripts of huCdc6 and cyclin E decrease at 24 h after stimulation, consistent with the results with elutriated cells

(Fig. 6B). The level of ASK protein, which is low during G_2/M and G_1, also increases in S phase, and ASK protein is maintained at a high level throughout S phase (Fig. 7A). We have identified two potential PEST sequences in ASK, whose role in selective degradation of ASK is being examined. ASK protein appears to be extensively phosphorylated during S phase, presumably by the kinase activity of huCdc7. Vigorous huCdc7 kinase activity which phosphorylates MCM2 was detected in the extracts from cells in early to middle S phase as well as from those in late S phase (Fig. 7B), suggesting that huCdc7 kinase activity is maintained at a high level throughout S phase. These results may indicate a continuous requirement for the functions of the huCdc7-ASK complex during S phase, as was shown for Cdc7 kinase of *S. cerevisiae* (1, 5).

ASK mRNA is expressed in most tissues examined, but it is by far most abundantly expressed in testis, and then in thymus. A high-level expression in testis was also observed with huCdc7 (30). Expression of *CDC7* and *hsk1*$^+$, a fission yeast homologue of *CDC7*, increases as cells enter meiosis, and *CDC7* and *hsk1*$^+$ are required for meiosis (23a, 31, 33a), indicating essential roles for yeast Cdc7 kinases in meiosis. The huCdc7-ASK kinase complex may also play additional roles in the processes of meiosis. ASK mRNA is generally expressed at high levels in cancer cell lines, except for lung cancer cell line A549, in keeping with its essential function for cell proliferation. It remains to be seen whether overexpression of ASK can cause accelerated growth. ASK is specifically localized in nuclei. The significance of the putative bipartite nuclear localization signal in selective localization of ASK in nuclei is under investigation.

Interaction of ASK with huCdc7 requires ASK motif C, the only stretch of amino acids which is strongly conserved in all of the Cdc7 kinase regulatory subunits, including *S. cerevisiae* Dbf4. The same motif, which is also conserved in an *S. pombe* homologue of Dbf4, is sufficient for interaction with Hsk1 in two-hybrid assays (33b). The C-terminal 419 amino acids containing ASK motif C are sufficient not only for interaction with but also for activation of huCdc7 kinase, although the efficiency of phosphorylation appears to be reduced compared to that of the wild type (data not shown). The N-terminal 305 amino acids, although capable of binding huCdc7, are not able to activate huCdc7 in vitro. ASK motif N, which is clearly conserved from fission yeast to human, is apparently dispensable for kinase activation. It will be of interest to investigate the functions of this conserved domain.

Two independent antibodies inhibited entry into S phase when injected into human fibroblast cells (Fig. 8). Furthermore, coinjection of the antigen peptide abrogated the inhibition, showing the specificity of the antibody action. The requirement of ASK function for mammalian DNA replication strongly argues for essential roles of the huCdc7 kinase complex in S phase initiation in mammals, although it is still formally possible that the function of ASK is required for the mammalian cell cycle in association with a protein(s) other than huCdc7 (10).

The huCdc7-ASK kinase complex phosphorylates MCM2 and MCM3 in vitro (30). Dissection of MCM2 into smaller polypeptides indicated that the N-terminal 209 amino acids contained strong phosphorylation sites (4a). Fission yeast Hsk1 kinase also efficiently phosphorylates the N-terminal 220-amino-acid polypeptide of *S. pombe* MCM2, and genetic and biochemical evidence indicates that MCM2 is an important target of the *S. cerevisiae* Cdc7-Dbf4 kinase complex and *S. pombe* Hsk1 kinase (3, 21, 33a). These results suggest that MCM2 may be a common target of Cdc7-related kinases. Recently, it was reported that a purified human MCM4-MCM6-MCM7 complex contains a helicase activity and that this helicase activity is inhibited by MCM2 (15, 16). It is an intriguing possibility that the huCdc7-ASK kinase complex activates the intrinsic helicase activity of MCM by counteracting the inhibitory effect of MCM2 protein or by directly activating the helicase component of MCM. Among other proteins examined, simian virus 40 T antigen (provided by Y. Ishimi) was phosphorylated by huCdc7-ASK in vitro. The significance of this phosphorylation is currently being investigated.

We demonstrated that ASK is a regulatory subunit for huCdc7 kinase. While it has no sequence homology to known cyclins, it shares with cyclins the properties of kinase regulation and periodic expression as well as periodic activation of its catalytic subunit. ASK is likely to be a critical target of growth factor-mediated signal transduction which ultimately induces S phase entry. Studies on how ASK expression and/or activity is regulated by G_1/S cell cycle signals and how phosphorylation by huCdc7-ASK kinase regulates initiation of DNA replication will shed a new light on the molecular mechanisms of cell cycle regulation of DNA replication in mammals.

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REFERENCES

1. **Bousset, K., and J. F. X. Diffley.** 1998. The Cdc7 protein kinase is required for origin firing during S phase. Genes Dev. **15:**480–490.
2. **Broek, D., R. Bartlett, K. Crawford, and P. Nurse.** 1991. Involvement of p34cdc2 in establishing the dependency of S phase on mitosis. Nature **349:** 338–393.
3. **Brown, G. W., and T. J. Kelly.** 1998. Purification of Hsk1, a minichromosome maintenance protein kinase from fission yeast. J. Biol. Chem. **273:**22083–22090.
4. **Chapman, J. W., and L. H. Johnston.** 1989. The yeast gene, DBF4, essential for entry into S phase is cell cycle regulated. Exp. Cell Res. **180:**419–428.

4a. **Cho, M. K., et al.** Unpublished results.

5. **Donaldson, A. D., W. L. Fangman, and B. J. Brewer.** 1998. Cdc7 is required throughout the yeast S phase to activate replication origins. Genes Dev. **15:**491–500.
6. **Dowell, S. J., P. Romanowski, and J. F. X. Diffley.** 1994. Interaction of Dbf4, the Cdc7 protein kinase regulatory subunit, with yeast replication origins in vivo. Science **265:**1243–1246.
7. **Dulic, V., E. M. Lees, and S. I. Reed.** 1992. Human cyclin E is associated with a periodic G1 protein kinase. Science **257:**1958–1961.
8. **Fang, F., and J. Newport.** 1991. Evidence that G1-S and G2-M are controlled by different *cdc2* proteins in higher eukaryotes. Cell **66:**731–742.
9. **Gietz, D., A. St. Jean, R. A. Woods, and R. H. Schiestl.** 1992. Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res. **20:**1425.
10. **Hardy, C. F. G., and A. Pautz.** 1996. A novel role for Cdc5p in DNA replication. Mol. Cell. Biol. **16:**6775–6782.
11. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
12. **Hartwell, L. H.** 1971. Genetic control of the cell cycle in yeast. II. Genes controlling DNA replication and its initiation. J. Mol. Biol. **59:**183.
13. **Hateboer, G., A. Wobst, B. O. Petersen, L. Le Cam, E. Vigo, C. Sardet, and K. Helin.** 1998. Cell cycle-regulated expression of mammalian CDC6 is dependent on E2F. Mol. Cell. Biol. **18:**6679–6697.
14. **Ikeda, M., K. Arai, and H. Masai.** 1996. A fusion protein library: an improved method for rapid screening and characterization of DNA binding or interacting proteins. Gene **181:**167–174.
15. **Ishimi, Y.** 1997. A DNA helicase activity is associated with an MCM4, -6, and -7 protein complex. J. Biol. Chem. **272:**24508–24513.
16. **Ishimi, Y., Y. Komamura, Z. You, and H. Kimura.** 1998. Biochemical func-

tion of mouse minichromosome maintenance 2 protein. J. Biol. Chem. **273:** 8369–8375.
17. **Jackson, A. L. P., M. B. Pahl, K. Harrison, J. Rosamond, and R. Sclafani.** 1993. Cell cycle regulation of the yeast Cdc7 protein kinase by association with the Dbf4 protein. Mol. Cell. Biol. **13:**2899–2908.
18. **Jiang, W., and T. Hunter.** 1997. Identification and characterization of a human protein kinase related to budding yeast Cdc7p. Proc. Natl. Acad. Sci. USA **94:**14320–14325.
19. **Kim, J. M., N. Sato, M. Yamada, K. Arai, and H. Masai.** 1998. Growth regulation of the expression of mouse cDNA and gene encoding a serine/threonine kinase related to Saccharomyces cerevisiae CDC7 essential for G1/S transition. Structure, chromosomal localization, and expression of mouse gene for S. cerevisiae Cdc7-related kinase. J. Biol. Chem. **273:**23248–23257.
19a. **Kim, J. M., et al.** Unpublished results.
20. **Koff, A., A. Giordano, D. Desai, K. Yamashita, J. W. Harper, S. Elledge, T. Nishimoto, D. O. Morgan, B. R. Franza, and J. M. Roberts.** 1992. Formation and activation of a cyclin E-cdk2 complex during the G1 phase of the human cell cycle. Science **257:**1689–1694.
21. **Lei, M., Y. Kawasaki, M. R. Young, M. Kihara, A. Sugino, and B. K. Tye.** 1997. MCM2 is a target of regulation by Cdc7-Dbf4 during the initiation of DNA synthesis. Genes Dev. **11:**3365–3374.
22. **Masai, H., T. Miyake, and K. Arai.** 1995. *hsk1*$^+$, a Schizosaccharomyces pombe gene related to Saccharomyces cerevisiae CDC7, is required for chromosomal replication. EMBO J. **14:**3094–3104.
22a. **Masai, H., et al.** Unpublished data.
23. **McGarry, T. J., and M. W. Kirschner.** 1998. Geminin, an inhibitor of DNA replication, is degraded during mitosis. Cell **93:**1043–1053.
23a. **Miyake, T.** Unpublished results.
24. **Nevins, J. R.** 1992. E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. Science **258:**424–429.
25. **Nigg, E. A.** 1995. Cyclin-dependent protein kinases: key regulators of the eukaryotic cell cycle. Bioessays **17:**471–476.
26. **Nurse, P., and Y. Bisset.** 1981. Gene required in G1 for commitment to cell cycle and in G2 for control of mitosis in fission yeast. Nature **292:**558–560.
27. **Ohtani, K., J. DeGregori, and J. R. Nevins.** 1995. Regulation of the cyclin E gene by transcription factor E2F1. Proc. Natl. Acad. Sci. USA **92:**12146–12150.
28. **Ohtani, K., J. DeGregori, G. Leone, D. R. Herendeen, T. J. Kelly, and J. R. Nevins.** 1996. Expression of the HsOrc1 gene, a human ORC1 homologue, is regulated by cell proliferation via the E2F transcription factor. Mol. Cell. Biol. **16:**6977–6984.
29. **Patterson, M. N. S., R. A. Sclafani, W. L. Fangman, and J. Rosamond.** 1986. Molecular characterization of cell cycle gene *CDC7* from *Saccharomyces cerevisiae*. Mol. Cell. Biol. **6:**1590–1598.
30. **Sato, N., K. Arai, and H. Masai.** 1997. Human and Xenopus cDNAs encoding budding yeast Cdc7-related kinases: in vitro phosphorylation of MCM subunits by a putative human homologue of Cdc7. EMBO J. **16:**4340–4351.
31. **Sclafani, R. A., M. Patterson, J. Rosamond, and W. L. Fangman.** 1988. Differential regulation of the yeast *CDC7* gene during mitosis and meiosis. Mol. Cell. Biol. **8:**293–300.
32. **Sherr, C., and J. M. Roberts.** 1995. Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev. **9:**1149–1163.
33. **Takebe, Y., M. Seiki, J. Fujisawa, P. Hoy, T. Yokota, K. Arai, M. Yoshida, and N. Arai.** 1988. SR alpha promoter: an efficient and versatile mammalian cDNA expression system composed of the simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat. Mol. Cell. Biol. **8:**466–474.
33a. **Takeda, T., et al.** Unpublished results.
33b. **Takeda, T., et al.** Submitted for publication.
34. **Williams, R. S., R. V. Shohet, and B. Stillman.** 1997. A human protein related to yeast Cdc6p. Proc. Natl. Acad. Sci. USA **94:**142–147.
35. **Yan, Z., J. DeGregori, R. Shohet, G. Leone, B. Stillman, J. R. Nevins, and R. S. Williams.** 1998. Cdc6 is regulated by E2F and is essential for DNA replication in mammalian cells. Proc. Natl. Acad. Sci. USA **95:**3603–3608.