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Protein Kinase *CK2α′* Is Induced by Serum as a Delayed Early Gene and Cooperates with Ha-*ras* in Fibroblast Transformation*

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Protein kinase CK2 is an ubiquitous and pleiotropic Ser/Thr protein kinase composed of two catalytic (α and/or α′) and two noncatalytic (β) subunits forming a heterotetrameric holoenzyme involved in cell growth and differentiation. Here we report the identification, cloning, and oncogenic activity of the murine *CK2α′* subunit. Serum treatment of quiescent mouse fibroblasts induces *CK2α′* mRNA expression, which peaks at 4 h. The kinetics of *CK2α′* expression correlate with increased kinase activity toward a specific CK2 holoenzyme peptide substrate. The ectopic expression of *CK2α′* (or *CK2α*) cooperates with Ha-*ras* in foci formation of rat primary embryo fibroblasts. Moreover, we observed that BALB/c 3T3 fibroblasts transformed with Ha-*ras* and *CK2α′* show a faster growth rate than cells transformed with Ha-*ras* alone. In these cells the higher growth rate correlates with an increase in calmodulin phosphorylation, a protein substrate specifically affected by isolated CK2 catalytic subunits but not by CK2 holoenzyme, suggesting that unbalanced expression of a CK2 catalytic subunit synergizes with Ha-*ras* in cell transformation.

Protein kinase CK2 (previously known as casein kinase II) is an ubiquitous Ser/Thr kinase present in the cytoplasm and the nucleus of eukaryotic cells (for review, see Refs. 1–5). CK2 holoenzyme consists of two catalytic (α and/or α′) and two regulatory (β) subunits assembled as stable heterotetramers, which *in vitro* do not dissociate unless under denaturing conditions. CK2 is unique among Ser/Thr protein kinases for its ability to use GTP, besides ATP, as phosphate donor and for its unusual site specificity, which is determined by multiple acidic and/or previously phosphorylated residues downstream ($n+3$) from the phosphoacceptor amino acid, determining the minimum consensus (S/T-*X*-*X*-E/D/Yp/Sp) (6).

More than 160 cellular proteins have been reported to be phosphorylated by CK2, and several are implicated in signal transduction, transcriptional activation, cell cycle progression, and cell differentiation. The nuclear proteins that are CK2 substrates includes: c-Myc (7), Max (7), c-Myb (8), serum response factor (SRF) (9), DNA ligase I (10), DNA topoisomerase 2 (11), p53 (12), and c-Fos (13). In mammalian cells phosphorylation of nuclear factors dependent on CK2 could be relevant for cell growth regulation and the progression into the cell cycle. A direct role of CK2 activity in cell cycle progression has been demonstrated by antibody-mediated CK2 depletion and by gene inactivation in *Saccharomyces cerevisiae* (14, 15). Although hundreds of papers have been published on the subject, it is still unknown how the enzyme is regulated *in vivo* (4, 5, 16). CK2β undergoes stoichiometric autophosphorylation and both CK2β and CK2α (but not CK2α′) are phosphorylated *in vitro* and *in vivo* by $p34^{Cdc2}$ kinase (17). However, these phosphorylations do not correlate with any regulation of activity. Moreover, it is not clear whether the holoenzyme represents an up- or a down-regulated form of the kinase, because some substrates are preferentially phosphorylated by the tetramer, but others, like calmodulin, are phosphorylated only by the free catalytic subunits (6).

In transgenic mice it was possible to demonstrate that in T cells the overexpression of the catalytic *CK2α* subunit enhanced the onset of lymphomas induced by either c-*myc* or *tal-1* (18, 19). These results shed new light on the previous observations that cattle infected by the parasite *Theileria parva* developed T cell lymphomas, because parasite-infected cells show increased CK2 activity (20–22). Opposite results were obtained by the overexpression of *CK2α* in NIH 3T3 mouse fibroblasts. In these cells *CK2α* overexpression resulted in deactivation of the mitogen-activated protein kinase kinase and suppression of *ras*-dependent cell transformation (23).

To identify genes potentially involved in cell growth, we performed a differential screening for the isolation of transcripts induced by mitogenic stimuli within the G_1 phase. Here we report the identification and cloning of the murine lower molecular weight catalytic subunit, *CK2α′*. We observed that in mouse fibroblasts *CK2α′* is induced by serum treatment as a slow-early gene. Together with *CK2α′* also *CK2α* and *CK2β* are induced. Cotransfection of an expression vector containing *CK2α′* together with a vector expressing Ha-*ras* induced foci formation in rat primary embryo fibroblasts. Moreover, *ras*-transformed BALB/c 3T3 fibroblasts overexpressing *CK2α′* showed a faster growth rate than cells transformed with Ha-*ras* alone. *ras*-Transformed fibroblasts overexpressing *CK2α′* also exibited increased phosphorylating activity toward calmodulin, which is a specific substrate of CK2 catalytic subunits. These findings suggest that unbalanced expression of either *CK2α′* or *CK2α* plays a role in fibroblast cell transformation.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ001420.

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EXPERIMENTAL PROCEDURES

Cells and Cell Culture—NIH 3T3 and BALB/c 3T3 fibroblasts were grown at 37 °C in DMEM[1] supplemented with 10% heat-inactivated FCS, penicillin-streptomycin, and glutamine. The cells were expanded by trypsin-EDTA treatment and subcultured at a ratio of 1:3 every 2–3 days. Rat embryo fibroblasts were isolated as described previously (24). Briefly, 14-day CDF(F344) rat embryos were sacrificed, rinsed, and trypsinized for 30 min at 37 °C. DMEM containing 10% FCS was added, and the cells were centrifuged, dispersed, counted, and plated on 100-mm tissue culture dishes at a density of 2×10^6/dish. After 48 h the cells were trypsinized, and aliquots were frozen in liquid nitrogen.

Differential Display and Cloning of the Murine CK2α′ cDNA—To induce a relatively quiescent cell population, subconfluent NIH 3T3 fibroblasts were incubated for 48 h in DMEM plus 0.5% FCS. Cells were then treated for 2 and 4 h with DMEM supplemented with 10% FCS. Total cellular RNA was extracted using the guanidinium thiocyanate method (25) from quiescent and serum-treated fibroblasts and subjected to the differential display technique as described previously (26, 27). The amplified cDNA fragments were compared in nondenaturing polyacrylamide gels. A serum-induced cDNA fragment, named L-0401, was excised, recovered by boiling, reamplified, and cloned into pGEM-T vector (Promega). The L-0401 cDNA fragment (230 bp), whose corresponding mRNA was homologous to human *CK2α′*, was labeled with [^{32}P]dCTP by random primer labeling and used to screen a mouse fibroblast cDNA library (27). The positive clones, inserted into the pBluescript SK vector, were sequenced on both strands either automatically using a Perkin-Elmer model 373 DNA sequencer or manually using a Sequenase 2.0 kit (U. S. Biochemical Corp). A positive clone, named pBS38α′ (FS304), contained the full-length mouse *CK2α′* cDNA.

Northern Blot Analysis—Total RNA (10 μg) was run on denaturing formaldehyde-agarose gels and stained with ethidium bromide to verify that each lane contained similar amounts of undegraded rRNA. RNA was transferred onto nylon membranes and cross-linked by UV irradiation. Filters were hybridized with ^{32}P-labeled probes and washed as described (27). The mouse *CK2α′* probe was obtained from the full-length cDNA (pBS38α′). The cDNA fragments of murine *CK2α* (base pairs 421–841) and *CK2β* (base pairs 912–1321) were obtained by cDNA amplification of a mouse fibroblast cDNA library (27). The sequences of the primers used for amplification (5′-GCTTCGATATGACCGTCACG-3′ and 5′-GACTCAACTACTAAATCCG-3′ for *CK2α* and 5′-GTACCAGCAGGGAGACTTTGGCTAC-3′ and 5′-CATAGACTTCCTGAAAGGGTGGCAG-3′ for *CK2β*), were obtained from EBI Nucleotide Sequence Data Base under accession number U17112 for *CK2α* and X56502 for *CK2β*. The amplified cDNA fragments were sequenced, labeled with [^{32}P]dCTP, and used in Northern blot.

Construction of Expression Vectors—CK2α′ open reading frame (from P-2 to R-350) was amplified by PCR from pBS38α′ with a 5′ primer containing a *Bam*HI restriction site (5′-CGCGGATCCCGGCCCGGCCGCG-3′) and a 3′ primer containing a *Kpn*I restriction site (5′-CGGGGTACCTCATCGTGCTGCGGT-3′). The PCR product was digested with *Bam*HI and *Kpn*I and cloned into the *Bam*HI/*Kpn*I site of a bacterial expression vector (FS310) made by subcloning the *Xba*I/*Xho*I fragment of pQE-31 (Qiagen) into the *Xba*I/*Xho*I site of pBluescript SK to give pQEBSα′ (FS311). The construct expressing murine *CK2α′* under the control of the cytomegalovirus promoter, pcDNAα′, was made by subcloning a *Stu*I/partial *Xho*I-digested fragment from pBS38α′ into the *Eco*RV/*Xho*I site of pcDNA3 (Invitrogen). To obtain murine *CK2α* under the control of the cytomegalovirus promoter, the 5′ end of *CK2α* was amplified by PCR from pT7–7CKIIα (28) with a 5′ primer containing a *Hin*dIII restriction site and an optimal Kozak sequence (29) (5′-GAGAAAGCTTCCACCGCCATGTCGGGACCCGTGCC-3′) and a 3′ primer downstream from an *Xho*I restriction site (5′-CTTGATTTCCCCATTCCACC-3′). The PCR product was digested with *Hin*dIII and *Xho*I and cloned into the *Hin*dIII/*Xho*I site of pcDNA3 to give pcDNAα5′ (MO339). A *Xho*I/blunt-ended *Hin*dIII fragment, corresponding to the 3′ end of *CK2α*, was released from pT7–7CKIIα and subcloned into the *Xho*I/blunt-ended *Apa*I site of pcDNAα5′ to give pcDNAα (MO346). All constructs were sequenced on both strands.

Transfection of Cells and Foci Formation—Subconfluent cells were fed with culture medium 1–2 h before transfection. Cells were cotransfected with 5 μg of activated Ha-*ras* and 10 μg of each construct for 8 h by standard $CaPO_4$ precipitation procedures (24). Where necessary vector plasmid (pcDNA3) was added to reach the total amount of 15 μg of DNA per transfected plate. At least two different cesium chloride DNA preparations of each construct were independently transfected. The cells were rinsed with PBS and re-fed with culture medium 15 h posttransfection. The cells were trypsinized 24 h after transfection and split 1:3. When the cells reached confluence they were re-fed with DMEM containing 2% FCS, and the medium was changed every 2 days. Stable cell transformants were visible after 7–10 days. Individual foci from BALB/c 3T3 cells transformed by Ha-*ras* or by Ha-*ras* plus *CK2α′* were picked and examined for their growth properties. For growth rate analysis cells were plated in duplicate at 1×10^5 cells on 60-mm Petri dishes in DMEM plus 2% FCS. Cells were counted every day using a hemacytometer and the medium was changed every 3 days. For tumor growth assays, 10^6 cells in midlog-phase growth were harvested, washed with PBS, resuspended in 200 μl of PBS, and injected subcutaneously into the scapular region of BALB/c nude mice. After 2 weeks of growth, the mice were sacrificed, and the tumors were surgically removed and weighed.

Cell Extracts and Phosphorylation Assays—Whole-cell extracts were prepared by rinsing cultures grown on Petri dishes with PBS followed by harvesting with a rubber policeman. Cells were pelleted by brief centrifugation and resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EGTA, 0.05% Triton X-100) containing a protease inhibitor mixture (1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin A). The cell lysates were centrifuged at $40{,}000 \times g$ for 20 min at 4 °C, and the supernatant was used in phosphorylation assays. Phosphorylation experiments were performed by incubating the substrate (200 μM peptide RRRADDSDDDDD or 10 μM calmodulin) in a buffer containing 50 mM Tris-HCl, pH 7.5, 12 mM $MgCl_2$, 100 mM NaCl, and 20 μM [γ-^{32}P]dATP for 10 min at 37 °C. NaCl was omitted when calmodulin was used as substrate. The reaction was stopped by cooling in ice followed, in the case of calmodulin, by SDS-polyacrylamide gel electrophoresis, staining with Coomassie Blue and either autoradiographed or directly scanned on Instant Imager Apparatus (Canberra-Packard). ^{32}P incorporated into the peptide substrate was evaluated by the phosphocellulose paper procedure (30).

RESULTS

Isolation and Characterization of the Murine CK2α′ cDNA—In cultured mouse fibroblasts growth factor depletion leads the cell to exit from the cell cycle and become quiescent. Serum treatment induces re-entry into the cell cycle, which is likely because of the induction of early genes. To identify new serum-induced genes, we used the mRNA differential display technique (26, 27). NIH 3T3 fibroblasts were serum-starved for 48 h and the RNAs were collected either from starved cells or from cells treated with serum at different time points. Several cDNAs, obtained only from serum-induced cells, were amplified and sequenced. Comparison of the cDNA fragments with the EBI Nucleotide Sequence Data Base revealed that a cDNA induced at 4 h after serum treatment was highly similar to the human protein kinase *CK2α′*. To clone the full-length cDNA coding for *CK2α′*, a mouse fibroblast cDNA library (27) was screened. The few positive clones were sequenced using internal sequencing primers, and the nucleotide sequence of one clone of 1877 base pairs in length revealed a single open reading frame coding for a putative protein of 350 amino acid residues. The mouse and human predicted protein products shared 98.9% amino acid identity. The strong similarity of the murine CK2α′ deduced protein sequence with its corresponding human homologue suggests a highly conserved function of CK2α′. Murine CK2α′ protein shows a lower degree of identity with CK2α. The two proteins share 82.4% identity over 347 amino acids overlap (Fig. 1). The greatest difference is in the C-terminal domains, because the deduced CK2α′ protein sequence is 41 amino acids shorter and thus lacks the $p34^{Cdc2}$ sites phosphorylated during the cell cycle (17). CK2α′ also lacks the HEHRKL amino acid residues (166–171 of CK2α) that have been implicated in the interaction with protein phosphatase 2A (23).

To confirm that the cDNA encoded a biologically active CK2α′ enzyme we expressed the cDNA in *Escherichia coli*.

[1] The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PCR, polymerase chain reaction; PBS, phosphate-buffered saline.

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                10        20        30        40        50        60
CK2α'      MPGPAAGSRARVYAEVNSLRSREYWDYEAHVPSWGNQDDYQLVRKLGRGKYSEVFEAINI
           | ||:  |||||::||: | ||||||:|| ||||||||||||||||||||||||||||
CK2α       MSGPVP-SRARVYTDVNTHRPREYWDYESHVVEWGNQDDYQLVRKLGRGKYSEVFEAINI
                10        20        30        40        50

                70        80        90       100       110       120
CK2α'      TNNERVVVKILKPVKKKKIKREVKILENLRGGTNIIKLIDTVKDPVSKTPALVFEYINNT
           ||||:|||||||||||||||:|||||||||| | | |||||||:||||||||::|||
CK2α       TNNEKVVVKILKPVKKKKIKREIKILENLRGGPNIITLADIVKDPVSRTPALVFEHVNNT
          60        70        80        90       100       110

               130       140       150       160       170       180
CK2α'      DFKQLYQILTDFDIRFYMYELLKALDYCHSKGIMHRDVKPHNVMIDHQQKKLRLIDWGLA
           ||||||| |||:||||||||:|||||||| |||||||||||||||:::|||||||||||
CK2α       DFKQLYQTLTDYDIRFYMYEILKALDYCHSMGIMHRDVKPHNVMIDHEHRKLRLIDWGLA
          120       130       140       150       160       170

               190       200       210       220       230       240
CK2α'      EFYHPAQEYNVRVASRYFKGPELLVDYQMYDYSLDMWSLGCMLASMIFRKEPFFHGQDNY
           |||||:|||||||||||||||||||||||||||||||||||||||||||||||:|||
CK2α       EFYHPGQEYNVRVASRYFKGPELLVDYQMYDYSLDMWSLGCMLASMIFRKEPFFHGHDNY
          180       190       200       210       220       230

               250       260       270       280       290       300
CK2α'      DQLVRIAKVLGTDELYGYLKKYHIDLDPHFNDILGQHSRKRWENFIHSENRHLVSPEALD
           |||||||||||::|| |: ||:|:|||:|||||:|||||| |:||||:||||||||||
CK2α       DQLVRIAKVLGTEDLYDYIDKYNIELDPRFNDILGRHSRKRWERFVHSENQHLVSPEALD
          240       250       260       270       280       290

               310       320       330       340       350
CK2α'      LLDKLLRYDHQQRLTAKEAMEHPYFYPVVKEQSQPCAENTVLSSGLTAAR
           :|||||||||:|||::|||||:|| |||:|::    :| :::| |
CK2α       FLDKLLRYDHQSRLTVREAMEHPFFYTVVKNQAR--MSSTSMAGGSTPVSSANMMSGISS
          300       310       320       330       340       350

CK2α       VPSPSPLGPLAGSPVIAAANSLGIPVQAAAGAQQ
          360       370       380       390
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FIG. 1. **Comparison of the deduced amino acid sequence of mouse CK2α′ with the murine CK2α protein sequence (accession number U17112).** Mouse CK2α′ amino acid sequence was determined from the cDNA clone pBS38α′, obtained by screening a fibroblast cDNA library as described under "Experimental Procedures." *Vertical lines* indicate identical amino acids, *colons* indicate a conservative amino acid change, and *dashes* represent a gap introduced to maximize sequence alignment. The HEHRKL amino acid sequence of CK2α (residues 166–171) is *underlined*.

Recombinant CK2α′ showed a molecular mass of about 41 kDa consistent with the predicted size of the protein, and the nondenatured soluble bacterial extract containing the immunoreactive CK2α′ was able to phosphorylate the CK2 peptide substrate RRRADDSDDDDD *in vitro* (not shown).

CK2 is an ubiquitously expressed protein kinase essential for cell growth. Northern blot analysis with a *CK2α′* probe revealed two hybridizing transcripts of 2.2 and 4.2 kilobases, respectively. The expression of *CK2α′* mRNA is relatively constant in all tissues with the exception of testis where a much stronger *CK2α′* signal was detected (Fig. 2). These results contrast with *CK2α* expression, which is abundant in brain and barely expressed in testis (31, 32). Thus, *CK2α* and *CK2α′* are differentially regulated in these tissues.

CK2α′ Is Induced by Serum Treatment in Cultured Fibroblasts—The identification of *CK2α′* in a screening for mRNAs induced by serum treatment of quiescent fibroblasts suggested that this gene is induced by mitogenic stimuli. To measure the induction of the *CK2α′* transcript, we performed Northern blot analysis on *CK2α′* mRNA in fibroblasts before and after serum treatment. As can be observed in Fig. 3*A* the *CK2α′* mRNA was low in quiescent fibroblasts and increased about 50% in serum-induced fibroblasts, with a peak induction at 4 h. The quantitative analysis of the transcripts normalized to the glyceraldehyde-3-phosphate dehydrogenase mRNA levels is reported in Fig. 3*B*. The serum-induced increase in *CK2α′* transcripts was not blocked by the presence of the protein synthesis inhibitor, cycloheximide, as shown by the superinduction of *CK2α′* mRNA (Fig. 3, *A* and *B*). Thus, in mouse fibroblasts *CK2α′* is induced with slow kinetics by serum treatment, and this induction is independent of protein synthesis. Because protein kinase CK2 is a tetramer containing two catalytic and two regulatory subunits, we also tested whether the mRNA corresponding to *CK2α* and *CK2β* were induced by serum treatment of quiescent cells. Northern blot analysis of *CK2α* revealed three hybridizing transcripts of 1.6, 3.1, and 4.6 kilobases, respectively. The *CK2α* mRNA showed a less pronounced but detectable increase at 2 h after serum treatment. Similar to *CK2α′* the *CK2α* mRNA increase was not

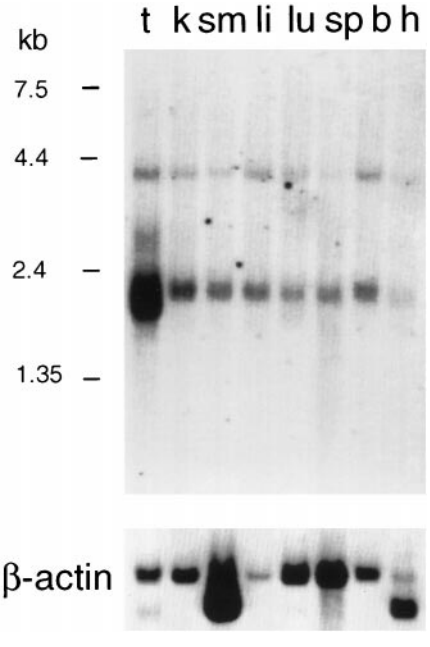

FIG. 2. **Expression of *CK2α′* mRNA in mouse adult tissues.** Multiple tissue Northern blot containing 2 μg of poly(A)$^+$ RNA (CLONTECH) was hybridized with *CK2α′* cDNA. *β-Actin* was used as a control for RNA loading. Tissues are indicated as follows: *t*, testis; *k*, kidney; *sm*, skeletal muscle; *li*, liver; *lu*, lung; *sp*; spleen; *b*, brain; *h*, heart.

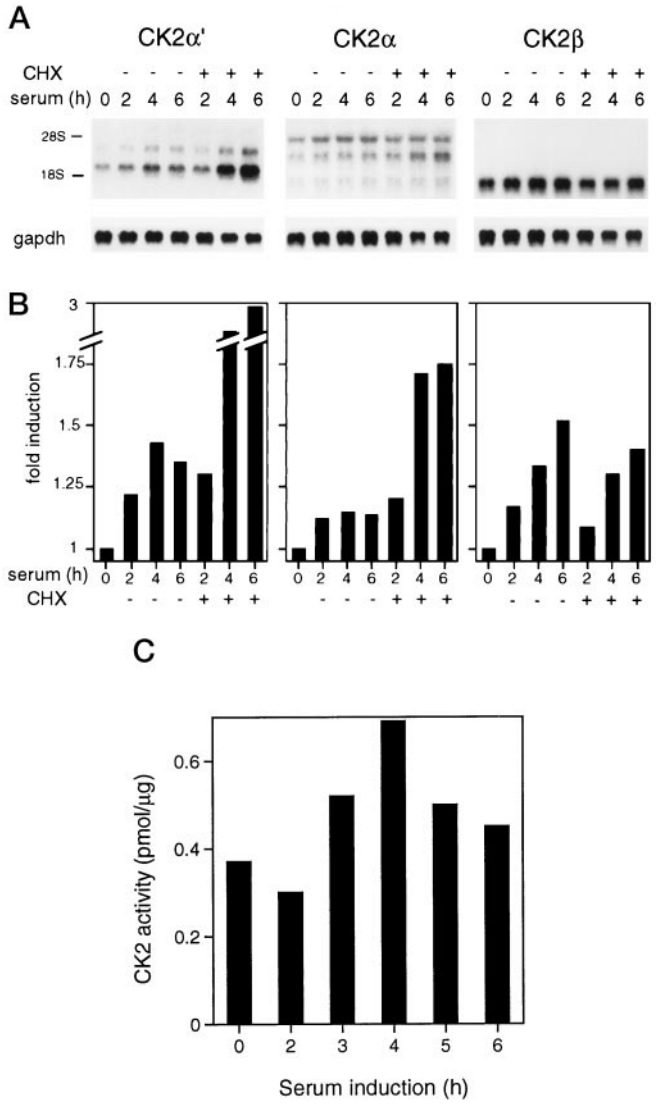

FIG. 3. **Expression of *CK2* mRNAs and CK2 kinase activity from cell extracts of quiescent and serum-induced fibroblasts.** *A*, Northern blots using the probes *CK2α′*, *CK2α*, and *CK2β* as indicated. Confluent NIH 3T3 fibroblasts were serum-starved for 48 h prior to the addition of medium plus 10% FCS alone or containing 10 μg/ml of cycloheximide as indicated (*CHX*). At various time points, total cellular RNA was extracted and Northern blot analysis was performed. *Numbers* indicate the hours of serum induction of quiescent fibroblasts. The relative positions of 18 and 28 S rRNA are shown. Glyceraldheyde-3-phosphate dehydrogenase (*gapdh)* was used as a control for RNA loading. *B*, quantitative analysis of *CK2* mRNA levels in cultured fibroblasts. The blots were analyzed using a PhosphorImager (Molecular Dynamics), and the values obtained were normalized to the glyceraldheyde-3-phosphate dehydrogenase mRNA levels. *C*, catalytic activity of the whole-cell extracts were determined using a specific CK2 substrate peptide. Confluent NIH 3T3 fibroblasts were serum-starved for 48 h and then induced with medium containing 10% FCS at the indicated time points. Cells were lysed, and whole-cell extracts were used in the phosphorylation assay. The CK2 activity is reported as picomoles of ^{32}P incorporated for μg of protein. The values shown are the mean of at least three experiments with a S.E. not exceeding 16%.

blocked by the inhibition of protein synthesis. Northern blot analysis with the probe for *CK2β* revealed a single transcript of 1.2 kilobases that increased with the same kinetic of *CK2α′*, and cycloheximide treatment did not influence its induction.

The mRNA analysis showed a moderate but measurable induction of the CK2 subunits. To confirm the functional significance of this induction, we determined whether the increased mRNA levels corresponded to an increase of CK2 by testing the kinase activity of the whole-cell extracts from quiescent and serum-induced cells, using the phosphorylation assay with the CK2 peptide substrate. The time course of the serum-induced CK2 activity showed a maximal increase of about 1.8-fold at 4 h compared with quiescent cells (Fig. 3*C*). To confirm that the activity monitored with the peptide substrate is because of CK2, the effect of heparin, a specific CK2 inhibitor, was examined. The phosphorylation of the peptide was entirely suppressed by 1 μg/ml heparin as expected for CK2-catalyzed phosphorylation (not shown).

TABLE I
Transformation of primary rat embryo fibroblasts

A standard focus assay was used to assess the ability of various constructs to transform primary rat embryo fibroblasts. The number of foci were counted after crystal violet staining. The experiments were repeated four times, and the average foci number with their S.E. are listed.

Transfected construct	Number of foci/100-mm dish
Ha-*ras*	0
CK2α′	0
CK2α	0
c-*fos*	0
Ha-*ras* + *CK2α′*	68 ± 9
Ha-*ras* + *CK2α*	75 ± 10
Ha-*ras* + c-*fos*	95 ± 10

CK2α′ Cooperates with Ha-ras in Rat Embryo Fibroblast Transformation—The above results showed that mouse fibroblasts respond to mitogenic stimuli with an increase of CK2 transcripts and kinase activity at the G_0/G_1 phase transition. Although the induction observed is not dramatic, we measured a reproducible increase of CK2 activity of about 80%. Previous experiments showed that as little as a 10% increase in *CK2α* expression in lymphoid organs of transgenic mice accelerated the onset of lymphomas induced by either c-*myc* or *tal-1* oncogenes (18, 19). To test whether *CK2α′* or *CK2α* play a direct role in tumor induction, we performed standard focus formation assay transfecting primary rat embryo fibroblasts with Ha-*ras*, *CK2α′*, or *CK2α* alone; the combination of Ha-*ras* with each catalytic subunit. Neither Ha-*ras* alone nor the *CK2* catalytic subunits transfected independently induced foci formation in primary cells (Table I). However, transformed foci were visible within 10 days in the plates cotransfected with Ha-*ras* and either *CK2α′* or *CK2α*. We therefore conclude that either *CK2α′* or *CK2α* cooperate with oncogenic *ras* in primary cell transformation.

The Expression Level of CK2α′ Correlates with Increased Growth Rate of Transformed Clones—To study further the effect of *CK2α′* expression on the growth rate of *ras*-transformed cells, we compared the growth behavior of mouse fibroblasts transformed either with Ha-*ras* alone or with Ha-*ras* and *CK2α′*. For this experiment we chose immortalized mouse fibroblasts, because these cells can be transformed with Ha-*ras* alone, and therefore it is possible to obtain transformed clones either expressing or not ectopic *CK2α′*. BALB/c 3T3 were used for this set of experiments, because the efficiency of NIH 3T3 transformation with Ha-*ras* alone was too high, making it difficult to quantitate the effects of *CK2α′*. As shown in Fig. 4*A* in BALB/c 3T3 cells the cotransfection of *CK2α′* and Ha-*ras* resulted in approximately a 3-fold higher number of foci compared with the number of foci induced by Ha-*ras* alone. Moreover, we observed that foci generated with Ha-*ras* alone were smaller than those from clones transformed with Ha-*ras* and *CK2α′*, suggesting that overexpression of *CK2α′* contributed to the cell growth of transformed cells (Fig. 4*B*). In parallel sets of experiments we observed a similar effect following cotransfection of Ha-*ras* with the *CK2α* catalytic subunit. The transfection of *CK2α′*, or *CK2α*, alone did not induce foci formation (not shown).

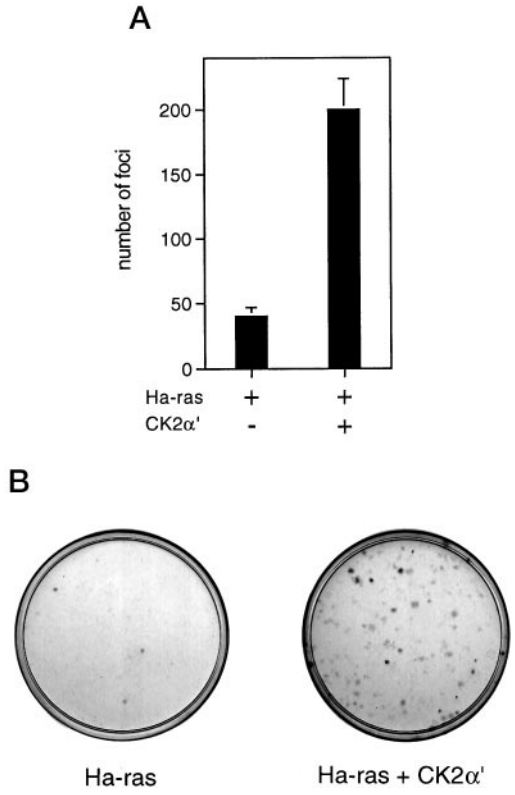

FIG. 4. **Analysis of foci formation in BALB/c 3T3 fibroblasts transformed either with Ha-*ras* or with Ha-*ras* and *CK2α′*.** BALB/c 3T3 fibroblasts were cotransfected with Ha-*ras* and either the vector plasmid (pcDNA3) or pcDNA*α′* (a plasmid expressing *CK2α′* under the control of the cytomegalovirus promoter). Cells were transfected, and foci formation was monitored at day 10. *A*, number of foci measured in four different transfection experiments, with *error bars* representing the S.D. of the measurements. *B*, a representative experiment showing foci formation of BALB/c 3T3 fibroblasts transfected with Ha-*ras* or with Ha-*ras* and *CK2α′* as indicated. Cells were stained with crystal violet.

To analyze in more detail the growth rate of the transformed clones, four *ras*-transformed clones (R-1 to R-4) and four *ras-CK2α′*-transformed clones (R*α′*-1 to R*α′*-4) were chosen for further analysis. Fig. 5*A* shows the growth curves obtained by counting cells over a period of 5 days. The clones obtained by cotransfection of Ha-*ras* and *CK2α′* showed a marked increase in their growth rates compared with *ras*-transformed clones. Eight other clones obtained from different transfections were analyzed, and those cotransfected with *CK2α′* and Ha-*ras* also exhibited increased growth rates (not shown).

To examine whether the growth differences correlated with the increased expression of *CK2α′* in the transformed clones, we tested the kinase activity mediated by *CK2α′* by measuring the phosphorylation of calmodulin. Calmodulin is an ideal substrate for examining the contributions of the CK2 catalytic subunits, because in reconstitution experiments with recombinant subunits its phosphorylation is suppressed completely by adding CK2*β* (5, 33–35). Clones, transformed with both Ha-*ras* and *CK2α′*, showed higher levels of calmodulin phosphorylation when compared with *ras*-transformed clones (Fig. 5*B*). This growth-related phosphorylation of calmodulin was inhibited (>90%) by addition of either a molar excess (0.5 mM) of the specific peptide substrate, or 1 *μ*g/ml heparin (not shown). These findings, in conjunction with the alkalilability of the phosphate incorporated into calmodulin, which rules out the possibility of tyrosine phosphorylation, show that calmodulin phosphorylation is entirely because of CK2 rather than to any other protein kinase(s). Thus, although we observed clonal variability, the enhanced growth correlated with increased *CK2α′*-dependent calmodulin phosphorylation (Fig. 5*C*). Finally, in mice we tested the growth of Ha-*ras versus* Ha-*ras* plus *CK2α′*-transformed clones. Exponentially growing transformed clones were collected and injected subcutaneously into the scapular region of nude mice, and the resultant tumors were removed surgically after 2 weeks of growth and weighed. Consistent with the results from the cell growth *in vitro*, *CK2α′* was found to produce a significant enhancement of tumor growth (Fig. 5*D*).

DISCUSSION

Here we report the cloning of the murine *CK2α′* subunit and show that its mRNA and kinase activity are induced in response to serum stimulation of quiescent fibroblasts. Furthermore, we show that expression of *CK2α′* under the control of a constitutive promoter cooperates with Ha-*ras* in transformation of rat primary fibroblasts and increases cell growth of transformed cells both *in vitro* and *in vivo*.

Our study originated from a screening for serum-induced messages in quiescent mouse fibroblasts, which allowed us to identify the *CK2α′* as an induced gene. We therefore cloned the murine full-length *CK2α′* cDNA, analyzed its expression pattern *in vivo*, *in vitro,* and activity in cultured cells. Northern blot analysis showed a *CK2α′* peak of induction at 4 h after serum treatment and that this induction does not require new protein synthesis. Therefore *CK2α′*, like c-*myc* and *MCP-1* (Refs. 36–38 and references therein), belongs to a subset of early genes induced with a slow kinetic. The analysis of induction revealed a lower but still measurable increase of both *CK2α* and *CK2β* at the same time points, suggesting that newly assembled CK2 tetramers can be formed following serum induction. In accordance with this prediction we observed an increased CK2-dependent phosphorylation activity in protein extracts from serum-induced cells. An active role of CK2 in cell cycle progression has already been suggested both in mammalian cells and yeast (14, 15, 39). Our data show, for the first time, that CK2 activity is indeed increased at the boundary between G_0 and G_1, suggesting that new CK2 synthesis is required at this stage of the cell cycle. Its specific induction could be necessary for several reasons. It is possible that the kinase already present in the cells is in a form which is not able to phosphorylate some critical substrates. Alternatively, because we observed a higher *CK2α′* induction compared with the other catalytic subunit, a higher proportion of $\alpha'_2\beta_2$ or $\alpha'\alpha\beta_2$ tetramers could be formed. The formation of these two types of tetramer may lead to different substrate specificity. Despite the fact that the catalytic properties of isolated recombinant CK2*α* and CK2*α′* are very similar (28, 34), which is consistent with their high sequence homology in their catalytic domains, significant structural differences suggest divergent functional commitments. Thus, the C-terminal segment of vertebrate CK2*α*, which lies outside the catalytic core and includes several phosphorylation sites affected both *in vitro* and *in vivo* by cyclin-dependent kinases, is absent in CK2*α′* (17). Likewise a motif (HEHRKL) responsible for association of CK2*α* with protein phosphatase 2A (23) is substantially altered in CK2*α′* (HQQKKL). This difference is especially remarkable as it occurs inside a region that is otherwise highly conserved between CK2*α* and CK2*α′*. Suggestive of specific function(s) of CK2*α′* in higher organisms is the observation that CK2*α′* could not be detected in *Drosophila*, *Xenopus,* and *Schizosaccharomyces pombe*. In *S. cerevisiae,* however, a somewhat atypical CK2*α′* subunit is found that exhibits functional differences from CK2*α* (39, 40).

CK2α′ cooperates with oncogenic *ras* in the transformation of primary fibroblasts. Our data show that neither CK2 catalytic

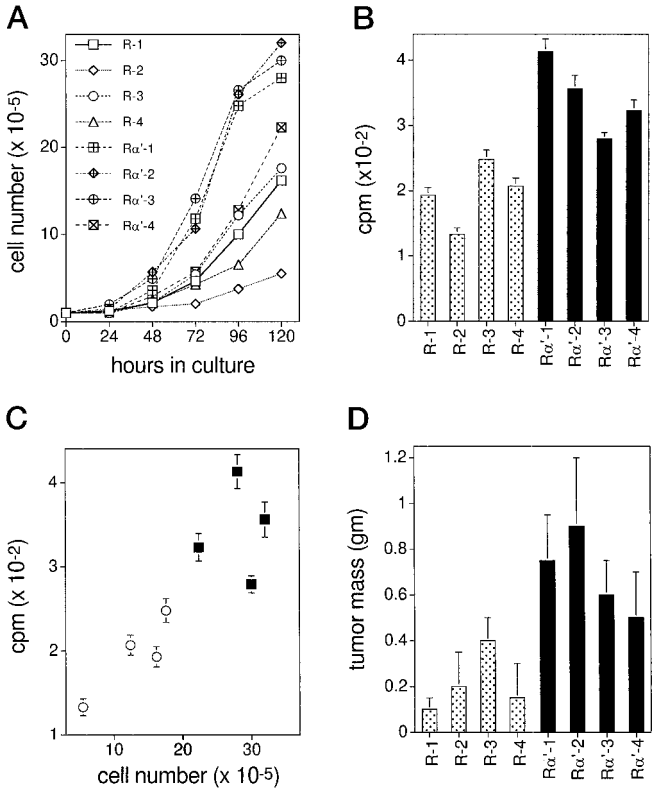

FIG. 5. ***CK2α′* overexpression accelerates proliferation of transformed cells.** Individual foci from BALB/c 3T3 cells transformed with Ha-*ras* (R-1, R-2, R-3, R-4) or with Ha-*ras* plus *CK2α′* (Rα′-1, Rα′-2, Rα′-3, Rα′-4) were picked and analyzed for their characteristics. *A*, growth curves of isolated foci. Cells were plated at a density of 10^5 cells/6-cm culture dish in medium containing 2% FCS and counted at daily intervals. The mean values of duplicate cultures are shown plotted against time. *B*, calmodulin phosphorylation by isolated foci. Confluent cells were lysed and total cellular extracts were used in phosphorylation assay using calmodulin as specific CK2 catalytic subunit substrate. The reaction products were run on SDS-polyacrylamide gel electrophoresis, and the blots were autoradiographed or directly scanned. All data are the mean of at least three separate determinations with a S.E. of less than 12%. *C*, correlation between calmodulin phosphorylation and growth curve rates. The cell number corresponds to the number of cells described in *A* at day 5 of growth. The values of calmodulin phosphorylation are the same of the experiment described in *B*. Clones transformed with Ha-*ras* are represented with *open circles*, whereas clones transformed with Ha-*ras* plus *CK2α′* are represented with *black squares*. *D*, tumor formation in nude mice. Mice were injected with 10^6 log-phase cells from the *ras*-transformed cells (R-1 to R-4) and from the Ha-*ras* with *CK2α′*-transformed cells (Rα′-1 to Rα′-4). Tumors were harvested after 2 weeks of growth and weighed. The *error bars* represent the S.D. for three mice used for each clone.

subunits nor Ha-*ras* alone induce foci formation when transfected in primary cells, whereas transformed foci become evident upon cotransfection with Ha-*ras* and either *CK2α′* or *CK2α*. Therefore, we can conclude that although the structural differences between CK2α and CK2α′ may reflect distinct functional roles, at least with respect to the cooperation with *ras* in cell transformation, these differences are not critical.

Recently, it was observed that exogenous expression of *CK2α* suppressed cell growth and inhibited foci formation induced by activated *ras* (23). Our results diverge from this observation. The reasons for such a discrepancy are presently unclear. It is possible that the inhibitory effect of *CK2α* previously observed (23) was dependent on the genetic background of the NIH 3T3 cells used in those experiments. Alternatively, the reduction of foci observed by Hériché and collaborators (23) may have resulted from a *CK2α* poisoning effect because of a too high expression of *CK2α*. In a standard focus formation assay we observed cooperation between *CK2* catalytic subunits and oncogenic *ras* both in primary and immortalized fibroblasts, suggesting we are observing a general phenomenon. Moreover, our results are in agreement with experiments in transgenic mice where the constitutive expression of *CK2α* accelerated the formation of lymphomas induced by c-*myc* or *tal-1* (18, 19). Comparison of the growth curves of fibroblasts transformed with Ha-*ras* alone *versus* fibroblasts transformed with Ha-*ras* and *CK2α′* demonstrated that transformed clones, expressing constitutively *CK2α′*, grew faster. The enhanced growth of Ha-*ras* and *CK2α′*-transformed clones correlates with increased catalytic activity when monitored using calmodulin as a phosphoacceptor substrate, symptomatic of the presence of free catalytic subunit (33, 34). Thus, these data suggest that unbalanced expression of *CK2α′* or *CK2α* leads to phosphorylation of some critical target(s) necessary to accelerate the cell cycle progression of *ras*-transformed cells. Therefore, it seems likely that the transforming potential of CK2 in each experimental model is because of a fraction of catalytic subunits not combined with CK2β to form the canonical holoenzyme. This hypothesis is supported by the phosphorylation of calmodulin, because this protein is unaffected by the CK2 holoenzyme.

In contrast the hypothesis that following serum treatment CK2α and CK2α′ not combined with CK2β could be transiently present in untransformed dividing cells is not consistent with available experimental data. Indeed, we observed that after serum treatment of fibroblast, *CK2β* was induced with the same kinetics of the catalytic subunits. In addition, by using the specific substrate calmodulin or by titrating in with recombinant CK2β, we did not detect free catalytic subunits in cell extracts (not shown). Therefore, it is likely that during the G_0/G_1 progression of the cell cycle the newly synthesized CK2α′ and CK2α are rapidly assembled into tetrameric CK2. Thus, the assembly of newly synthesized CK2 subunits into a tetrameric enzyme could represent a mechanism for the modulation of the too reactive free catalytic subunits necessary to reprogram the CK2 kinase activity during the progression of the cell cycle. Future studies aimed at the identification of specific target(s) of the CK2 catalytic subunits may unveil some target(s) critical for cell transformation.

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