Casein Kinase II Is a Predominantly Nuclear Enzyme

W. Krek, G. Maridor, and E. A. Nigg
Swiss Institute for Experimental Cancer Research (ISREC), CH-1066 Epalinges, Switzerland

Abstract. Casein kinase II (CK II) has been implicated in regulating multiple processes related to cell growth, proliferation, and differentiation. To better understand the function(s) and regulation of this ubiquitous kinase, it is important to know its subcellular distribution. However, this issue has been the subject of contradictory reports. In this study, we have used indirect immunofluorescence microscopy and cell fractionation to study the subcellular distribution of all three subunits of chicken CK II, α, α', and β. We examined primary chick embryo fibroblasts, virally transformed chicken hepatoma cells, as well as HeLa cells transiently transfected with cDNAs encoding chicken CK II subunits. We found that each of the three CK II subunits was located predominantly in the cell nucleus, irrespective of the cell type analyzed or the procedure used for cell fixation. No major differences were detected in the subcellular distributions of individual CK II subunits, and no evidence was obtained for subunit redistributions during interphase of the cell cycle. During mitosis, the bulk of the enzyme was dispersed throughout the cell, though a fraction of all three subunits was associated with the mitotic spindle. Biochemical studies based on mechanical enucleation of chicken cells confirmed the predominantly nuclear location of all three CK II subunits. Finally, immunoblotting experiments were carried out to study the expression of CK II subunits. A survey of different adult chicken tissues revealed substantial tissue-specific differences in the levels of CK II protein, but no evidence was obtained for pronounced tissue specificity in the expression of individual CK II subunits. These results strongly suggest that CK II functions primarily in regulating nuclear activities, and that the two catalytic subunits, α and α', may carry out overlapping functions.

Casein kinase II (CK II) is a cyclic nucleotide- and calcium-independent serine/threonine-specific protein kinase. While little is known about the regulation of this ubiquitous enzyme, multiple functions have been proposed (for reviews see Edelman et al., 1987; Pinna, 1990; Tuazon and Traugh, 1991). In particular, current evidence suggests that CK II may serve to integrate multiple cellular activities related to cell growth and differentiation (for reviews see Krebs et al., 1988; Carroll et al., 1988; Schneider and Issinger, 1989). In support of a pleiotropic role of CK II, its proposed physiological substrates include metabolic enzymes, cytoskeletal proteins, transcription factors, as well as the products of several oncogenes and tumor suppressor genes (for reviews see Pinna, 1990; Tuazon and Traugh, 1991). Furthermore, CK II activity is increased in neoplastically transformed cell lines (Prowald et al., 1984), as well as in tumors (Müntermann et al., 1990). In cultured cells, CK II activity was shown to change during cell differentiation (Sommercorn and Krebs, 1987), and it was reported to be stimulated in response to various mitogens and growth factors (Sommercorn et al., 1987; Klarlund and Czech, 1988; Carroll and Marshak, 1989; Ackerman and Osheroff, 1989). These findings raise the possibility that CK II may be part of a protein kinase cascade involved in relaying signals from the plasma membrane to the cell nucleus.

Biochemical studies on CK II isolated from several organisms have revealed heterotetramers of the structure αβαβ, or αα'β (for reviews see Hathaway and Traugh, 1982; Edelman et al., 1987; Pinna, 1990). The two larger subunits α and α' have molecular masses between 36 and 44 kD, while the β subunit is ~25 kD. It is well established that the α and α' subunits provide the catalytic activity but the role of the β subunit remains poorly defined. Current evidence indicates that the β subunit stimulates CK II activity, and thus presumably plays a regulatory role (Lin et al., 1991; Grankowski et al., 1991). The recent sequencing of CK II cDNA clones from different species revealed a very high degree of structural conservation of the α, α', and β subunits during evolution (Padmanabha and Glover, 1987; Saxena et al., 1987; Chen-Wu et al., 1988; Heller-Harrison et al., 1989; Meisner et al., 1989; Jakobi et al., 1989; Hu and Rubin, 1990; Kopatz et al., 1990; Lozeman et al., 1990; Boldyreff et al., 1991; Maridor et al., 1991). The α and α' subunits are closely related to each other, but differ in their carboxyl-termini. While it is certain that α and α' subunits are derived from separate genes, it remains unclear whether or not they carry out distinct functions. Gene disruption experiments in Saccharomyces cerevisiae demonstrate that expression of at least one catalytic subunit of CK II is essential for cell viability (Padmanabha et al., 1990).
Based on extensive studies with peptide substrates, the sequence specificity of CK II in vitro is well defined (Meggio et al., 1984; Kuenzel et al., 1985; Marchiori et al., 1988; Carroll et al., 1988; Pinna, 1990). However, primary sequence constraints are not the only parameters dictating kinase specificity in vivo. Another major factor determining sequence constraints are not the only parameters dictating kinetic parameters (Marchiori et al., 1988; Kuenze et al., 1985; Marchiori et al., 1984). The bacterial pellet was frozen at -70°C.

Induction of cell cultures was achieved by centrifugation at 12,000 g for 10 min. The resulting 831-bp fragment was treated with Klenow polymerase (partial) NarI and (complete) EcoRI digestion. The resulting 1,337-bp fragment was ligated into pET3b, which had been prepared as described by Nakagawa et al. (1989). Anti-peptide antibodies were affinity purified as follows: 1 g of CH-Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ), as described by the manufacturer. Following pre-equilibration of the affinity matrix in 0.2% Tween-20 in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.2), 3 ml of anti-CK II α or 8 ml of anti-CK II β serum were diluted with 2 vol of 0.2% Tween-20 in PBS and added to the corresponding affinity matrices. Samples were agitated gently overnight at 4°C. Then, the material was poured into a column (Bio-Rad Econocolumn; 1 x 13 cm), and washed twice with 20 ml of 0.2% Tween-20 in PBS followed by 40 ml of 0.2% Tween-20 in PBS. Subsequently, the matrix was washed extensively with 0.2 M glycine-HCI (pH 2.2). Each 0.75-ml fraction was immediately neutralized by the addition of 0.25 ml of 1 M K2HP04. Finally, antibodies were dialyzed against cold PBS containing 50% glycerol and stored at 4°C.

Antibodies against an 18 amino acid peptide corresponding to the carboxy-terminus of the chicken CK II α' subunit were prepared using the carrier-coupling and immunization procedures described in Krek and Nigg (1989). Anti-peptide antibodies were affinity purified as follows: 1 g of CH-Sepharose 4B (Pharmacia Fine Chemicals) was activated following the protocol of the manufacturer. Then, 20 mg of synthetic peptide was added to the matrix and coupled by bis-diazobenzidine cross-linking (Bassiri et al., 1989). Subsequently, the matrix was washed extensively with 0.2% Tween-20 in PBS; 6 ml of anti-CK II' α serum were applied to the affinity matrix, and specific IgGs were bound and eluted as described above. However, the resulting 831-bp fragment was treated with Klenow polymerase and then ligated into pET3-a, which had been prepared as described above. The resulting 1,337-bp fragment was ligated into pET3b, which had been prepared as described above. The protein expressed from this construct contains 12 residues encoded by the pET3-a vector, followed by one amino acid (alanine) arising from the untranslated leader sequence of the chicken CK II β cDNA, and the entire coding region of the β subunit. Expression plasmids were introduced into the E. coli strain DH5a. Overexpression of the CK II α and β fusion proteins was induced by infection of cells with the phage CB6 (i.e., a lambda derivative carrying the gene encoding the bacteriophage T7 RNA polymerase), using the conditions described in Krek and Nigg (1989). After induction, cells were harvested by centrifugation and the bacterial pellet was frozen at -70°C.

For purification of the CK II fusion proteins, frozen bacterial pellets from 50-ml cultures were thawed on ice and resuspended in 1.8 ml of lysis-buffer containing 100 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM PMSE, 1% aprotinin (Sigma Chemical Co., St. Louis, MO), 0.3% Triton X-100, and 1 mg/ml lysozyme. After incubation at room temperature for 30 min, cells were centrifuged at 12,000 g for 10 min. The pellet was resuspended in ice-cold 100 mM NaCl, 1 mM EDTA, 0.1% sodium deoxycholate, 50 mM Tris-HCl, pH 8.0, and incubated on ice for 10 min. MgCl2 and DNase I were added to final concentrations of 8 mM and 10 μg/ml, respectively, and the incubation on ice was continued for 30 min. Inclusion bodies were collected by centrifugation at 12,000 g for 15 min and solubilized in 3X gel sample buffer. Following boiling for 10 min, samples were loaded onto 10% preparative SDS-polyacrylamide gels. Relevant protein bands were visualized by staining of the gels with an ice-cold solution containing 250 mM KCl and 1 mM β-mercaptoethanol. They were excised and transferred to a dialysis bag containing 0.5% SDS-PAGE running buffer (10X stock: 10 g SDS, 30 g Tris-base, 144 g glycine) and proteins were electrophoresed at 3 h at 4°C using a minigel apparatus (Bio-Rad Laboratories, Cambridge, MA) at 100 V. Electrophoresis efficiency and purity of the corresponding proteins were determined by subsequent analyses on SDS-PAGE, followed by Coomassie blue staining.

Preparation of Anti-Chicken CK II α, α', and β Antibodies

For intramuscular injection into rabbits, gel-purified CK II α and β proteins were emulsified with either Freund's complete adjuvant (for the first injection) or Freund's incomplete adjuvant (for all subsequent injections). Approximately 0.3–1.0 mg of protein were administered at 2-wk intervals, for a total of 2 mo. Bleedings were done at 2-wk intervals.

For affinity purification of antibodies, 1 mg of bacterially expressed, purified α or β subunit protein was covalently coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ), as described by the manufacturer. Following pre-equilibration of the affinity matrices in 0.2% Tween-20 in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.2), 3 ml of anti-CK II α or 8 ml of anti-CK II β serum were diluted with 2 vol of 0.2% Tween-20 in PBS and added to the corresponding affinity matrices. Samples were agitated gently overnight at 4°C. Then, the material was poured into a column (Bio-Rad EconoColumn; 1 x 13 cm), and passed twice with 20 ml of 0.2% Tween-20 in PBS followed by 40 ml of 0.2% Tween-20 in PBS. Subsequently, the matrix was washed extensively with 0.2 M glycine-HCl (pH 2.2). Each 0.75-ml fraction was immediately neutralized by the addition of 0.25 ml of 1 M K2HP04. Finally, antibodies were dialyzed against cold PBS containing 50% glycerol and stored at 4°C.

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Preparation of Protein Extracts and Immunochemical Techniques

Protein extracts from different adult tissues were prepared for immunoblotting experiments exactly as described previously (Krek and Nigg, 1989). To solubilize tissue culture cells, 3X sample buffer was added directly to monolayer cultures. After boiling for 10 min, the cellular proteins were resolved by SDS-PAGE. Electrophoretic transfer to nitrocellulose membranes (Schleicher & Schüll, Inc., Keene, NH) was carried out for 3–4 h at 200 mA at room temperature, using 1X blotting buffer (10X stock: 30 g Tris-Base, 144 g glycine). Nitrocellulose membranes were blocked with 5% nonfat dry milk in PBS, either overnight at 4°C, or for 3 h at room temperature. Then, they were incubated for 3–4 h at room temperature (or overnight at 4°C) with affinity purified anti-CK II α (1:10,000 dilution of 0.3 mg/ml specific IgG), anti-CK II α' (1:300 dilution of 0.2 mg/ml specific IgG) or anti-CK II β IgGs (1:300 dilution of 0.2 mg/ml specific IgG), washed three times for 15 min with PBS, followed by 3–2 h at room temperature with 5 μCi of [125I]labeled donkey anti–rabbit IgG (Amersham Corp.) diluted in 10 ml of PBS containing 5% nonfat dry milk. Finally, membranes were washed three times for 15 min with PBS, dried, and exposed for autoradiography.

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For immunoprecipitation, cells grown on a 10-cm dish were washed twice in ice-cold PBS and lysed in 1 ml of phosphate-buffered RIPA (P-RIPA; 1% Triton X-100, 16 mM deoxycholate, 0.1% SDS, 20 mM sodium phosphate [pH 7.2], 100 mM NaCl, 20 mM NaF, 0.3 mM sodium orthovanadate, 0.2% NaN₃) containing 1% aprotinin, 1 mM PMSF, 30 μg/ml DNase I, and 30 μg/ml RNase A. After 30 min on ice, lysates were centrifuged for 10 min at 15,000 g, and supernatants were pre-incubated for 60 min at 4°C with 50 μl of a 30% (wt/vol in P-RIPA) suspension of protein A-Sepharose (Pharmacia Fine Chemicals), followed by centrifugation. Then, 2 μl of anti-CII α or 4 μl of anti-CII β antisera were added to the lysates. After incubation on ice for 1 h, 50 μl of protein A-Sepharose was added and incubation was continued for another hour, under continuous gentle shaking at 4°C. Immune complexes were collected by centrifugation and washed four times in cold PBS, and cells were harvested with a rubber policeman and taken up in phosphate-buffered RIPA (P-RIPA) and lysed in 1 ml of phosphatase assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM KCl, 10 mM MgCl₂, 1 mM DTT). Kinase reactions were carried out for 20 min at 30°C in assay buffer supplemented with 4 μM ATP and 20 μCi [γ-³²P]ATP (Amersham Corp.), in a total volume of 50 μl. Where indicated, dephosphorylated casein (Sigma Chemical Co., C-4032) was included at 0.5 mg/ml, to provide an exogenous substrate. In the case of these latter samples, reactions were stopped by addition of 50 μl of 3× gel sample buffer. In the other cases, samples were washed three times with P-RIPA and once with PBS before the addition of 3× gel sample buffer. Reaction products were visualized by SDS-PAGE and autoradiography.

**In Vitro Kinase Assays**

Immunoprecipitates of CII α or CII β were prepared as described above, except that immune complexes were washed three times in kinase assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM KCl, 10 mM MgCl₂, 1 mM DTT). Kinase reactions were carried out for 20 min at 30°C in assay buffer supplemented with 4 μM ATP and 20 μCi [γ-³²P]ATP (Amersham Corp.), in a total volume of 50 μl. Where indicated, dephosphorylated casein (Sigma Chemical Co., C-4032) was included at 0.5 mg/ml, to provide an exogenous substrate. In the case of these latter samples, reactions were stopped by addition of 50 μl of 3× gel sample buffer. In the other cases, samples were washed three times with P-RIPA and once with PBS before the addition of 3× gel sample buffer. Reaction products were visualized by SDS-PAGE and autoradiography.

**Immunofluorescent Experiments**

Cells were prepared and processed for indirect immunofluorescence microscopy as described previously (Krek and Nigg, 1991b). For pre-extraction, cells were treated for exactly 30 s with ice-cold Triton X-100 buffer, before being fixed with paraformaldehyde (Nigg et al., 1985). Affinity-purified primary antibodies were used at the following concentrations: 5 μg/ml of anti-CII α, 10 μg/ml of anti-CII β, and 15 μg/ml of anti-CII α or IgG. Mouse hybridoma supernatant CTR 2611, containing antibodies against a centrosomal antigen (Buendia et al., 1990), was used undiluted. Secondary reagents were affinity-purified rhodamine-conjugated goat anti-rabbit IgG (Pierce Chemical Co., Rockford, IL) and fluorescein-conjugated goat anti-rabbit IgG (Pierce Chemical Co., Rockford, IL). Hoechst dye 33258 (Sigma Chemical Co., St. Louis, MO) was used to stain the nuclei. Fluorescein-conjugated donkey anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO) was used as the secondary antibody. goat anti-rabbit IgG (Pierce Chemical Co., Rockford, IL) and fluorescein-conjugated goat anti-mouse IgG (Sigma Chemical Co.) were used as secondary antibodies. For immunoperoxidase staining, cells were fixed with 3% formaldehyde in PBS for 10 min, permeabilized with 0.1% Triton X-100 in PBS, for 30 min at room temperature, and incubated with the primary antibody for 1 h at room temperature. After three washes in PBS, the cells were incubated with the secondary antibody for 1 h at room temperature, followed by three washes in PBS. The cells were then mounted in a 1:1 mixture of PBS and 50% glycerol and examined under a Nikon Microphot F microscope equipped with epifluorescence illumination and appropriate filters for rhodamine, fluorescein, and 350-nm UV light. Controls included omission of primary antibody or substitution of normal rabbit serum for primary antibody. Immunofluorescent experiments were performed at least twice on different cell preparations.

**Production and Characterization of Antibodies Monospecific for Chicken CII Subunits α, α', and β**

To produce monospecific antisera against individual chicken CII subunits, α and β subunits were expressed as fusion proteins in E. coli (Studier and Moffat, 1986), and the purified proteins were used for immunization of rabbits. In the case of the α' subunit, rabbit antibodies were raised against an 18 amino acid synthetic peptide corresponding to the predicted carboxy-terminus (Maridor et al., 1991). Figs. 1 and 2 illustrate the specificity of the various anti-CII antibody-bodies. By immunoblotting on total cell lysates of either chicken DU249 hepatoma cells (lanes I) or chick embryo fibroblasts (lanes 2), affinity purified anti-α' (Fig. 1A), anti-α' (Fig. 1B) and two different anti-β antibodies (Fig. 1, C and D) recognized single proteins of the expected molecular weights, i.e., 42 (α'), 38 (α'), and 25 kD (β), respectively. All three CII subunits were found to be more abundant in the transformed DU249 cell line than in primary fibroblasts (Fig. 1, compare lanes 1 and 2).

The results of immunoprecipitation experiments are summarized in Fig. 2. From lysates of [³⁵S]methionine-labeled DU249 cells, anti-α antibodies specifically precipitated two polypeptides (Fig. 2A, lane I). These correspond to the α and β subunits of CII, respectively, as confirmed by immunoprecipitation experiments (not shown). To test for activity of the immunoprecipitated holoenzyme, we also carried out in vitro kinase assays, using immunoprecipitates prepared from unlabeled cells. In the absence of exogenous substrate, strong phosphorylation of the β subunit could be seen, while phosphorylation of the α subunit occurred to a much lesser extent (Fig. 2A, lane 3). High kinase activity could also be demonstrated using dephosphorylated casein as an exogenous substrate (Fig. 2A, lane 5). In similar experiments with anti-β antibodies, one major protein of the expected molecular weight was specifically immunoprecipitated from [³⁵S]methionine-labeled cells (Fig. 2B, lane 1, *arrowhead*). A second protein of lower molecular weight could also be seen (Fig. 2B, lane 1, *star*). We presume that this protein represents a proteolytic degradation product of the β subunit, although we cannot exclude that it may represent a tightly associated, as yet unidentified protein. Co-precipitation of CII catalytic subunits was not very prominent in the β immunoprecipitates from [³⁵S]methionine-labeled...
cells, but could be demonstrated by kinase assays, monitoring phosphorylation of either the \( \beta \) subunit (not shown) or exogenous casein (Fig. 2B, lane 3). No CK II subunits and no significant kinase activities were precipitated when using pre-immune sera for control immunoprecipitations (Fig. 2A, lanes 2, 4, and 6; Fig. 2B, lanes 2 and 4). From these experiments we conclude that active CK II could be specifically immunoprecipitated using either anti-\( \alpha \) or anti-\( \beta \) subunit antibodies. In contrast, the anti-\( \alpha' \) peptide antibody was not reactive in immunoprecipitation experiments (not shown).

The monospecificity of the anti-\( \alpha' \) antibody seen in immu-

Figure 1. Specificity of antibodies against individual chicken CK II subunits \( \alpha, \alpha' \) and \( \beta \). Whole-cell extracts were prepared from chicken DU249 cells (lanes 1) and chicken embryo fibroblasts (lanes 2). Proteins were resolved by SDS-PAGE on 10% (A and B) or 12% (C and D) gels and transferred to nitrocellulose. Filters were probed with affinity-purified antibodies directed against bacterially expressed chicken CK II (A), a carboxy-terminal peptide of CK II \( \alpha' \) (B), or bacterially expressed CK II (C and D), followed by \( ^{125} \) I-labeled secondary antibodies. The two preparations of anti CK II \( \beta \) antibodies were obtained from two rabbits. The position of the respective CK II subunits are indicated by arrowheads.

Figure 2. Anti-\( \alpha \) and anti-\( \beta \) antibodies immunoprecipitate active CK II holoenzyme. Immunoprecipitations of CK II subunits from chicken DU249 cell lysates were carried out as described in Materials and Methods, using antibodies specific for CK II \( \alpha \) (A) and \( \beta \) (B), or the corresponding pre-immune sera. CK II subunits were immunoprecipitated either from \( ^{35} \) S-methionine-labeled cells (A and B, lanes I and 2), or from unlabeled cells (A, lanes 3-5; B, lanes 3 and 4). The latter samples were then assayed for CK II kinase activity in the absence (A, lanes 3 and 4) or presence (A, lanes 5 and 6; B, lanes 3 and 4) of dephosphorylated casein as an exogenous substrate. All experiments were carried out using immune (I) and pre-immune sera (P) in parallel. The position of CKII \( \alpha \) is indicated by arrows, whereas CKII \( \beta \) is marked by arrowheads. The star next to lane I in B indicates an unidentified protein.
Immunofluorescent Localization of Casein Kinase II Subunits in Interphase and Mitotic Chicken Cells

To determine the subcellular distribution of individual CK II subunits, primary chick embryo fibroblasts and transformed DU249 hepatoma cells were examined by indirect immunofluorescence microscopy. Since previous immunocytochemical localization studies yielded conflicting data, great care was taken to control for the specificity of the observed staining. In particular, several different fixation–permeabilization procedures were used for sample preparation, and antibody specificity was controlled by blocking with antigen. In a first series of experiments (Fig. 3), cells were fixed with paraformaldehyde before being permeabilized with non-ionic detergent (left and center panels); alternatively, they were treated briefly with detergent before fixation (right panels). In additional experiments, cells were subjected to aldehyde fixation for different lengths of time (Fig.

**Figure 3.** Immunofluorescent localization of CK II subunits in chicken cells. Chicken embryo fibroblasts (a–c) or DU249 cells (d–i) were fixed and permeabilized using either formaldehyde/Triton X-100 (a, d, and g) or pre-extracted with Triton X-100 buffer (see Materials and Methods) before formaldehyde fixation (c, f, and i). They were then incubated with affinity-purified anti-chicken CK II α (a and c), anti-chicken CK II α' (d and f) or anti-chicken CK II β (g and i) antibodies, followed by rhodamine-conjugated goat anti-rabbit IgG. b, e, and h show differential interference contrast pictures corresponding to a, d, and g, respectively. Bar, 15 μm.

noblotting experiments was expected in view of the unique sequence of the α' carboxy-terminal peptide used for immunization. However, it is remarkable that the antibody raised against the bacterially expressed α subunit did not detectably cross-react with the α' subunit, irrespective of whether its specificity was assayed by immunoblotting (Fig. 1) or immunoprecipitation (Fig. 2). This observation indicates that the anti-α subunit antibodies were directed predominantly, if not exclusively, against regions where the α subunit differs from the α' subunit (see Maridor et al., 1991).
Figure 4. Distribution of CK II α subunit after prolonged fixation of DU249 cells with formaldehyde. Chicken DU249 cells were fixed with formaldehyde for either 5 min at room temperature (a) or for 60 min at 37°C (b). Then they were permeabilized with Triton X-100 and processed for indirect immunofluorescence microscopy, using affinity-purified anti-CK II α antibodies. Bar, 15 μm.

Figure 5. Nuclear staining by anti-CK II α and β antibodies is independent of fixation procedure, but abolished by antibody preabsorption. Chicken embryo fibroblasts (a, b, e, f, and g) or DU249 cells (c, d, h, i, and j) were fixed and permeabilized using either methanol-acetone (a–d) or formaldehyde/Triton X-100 (e–j). They were then incubated with affinity-purified antibodies against CK II α (a and e), or β (c and h), or with antibodies that had been preabsorbed on the corresponding bacterially expressed α (f) or β (i) subunits. Secondary antibodies were rhodamine-conjugated goat anti–rabbit IgG. b, d, g, and j show differential interference contrast pictures corresponding to a, c, f, and i, respectively. Bar, 15 μm.
Figure 6. The β subunit of CK II is associated with centrosomes. Double indirect immunofluorescent labeling of formaldehyde/Triton X-100-treated chicken DU249 cells by affinity-purified anti-CK II β antibodies (a) and a mouse mAb (CTR 2611) directed against a centrosomal protein (b). Arrowheads in a and b point to centrosomes. Bar, 15 μm.

Figure 7. Distribution of CK II α, α', and β subunits during mitosis. Asynchronously growing chicken embryo fibroblasts (a and b) or chicken DU249 cells (c–g) were either fixed with formaldehyde and subsequently permeabilized with Triton X-100 (a, c, e, f, and g) or pre-extracted with Triton X-100, and then fixed with formaldehyde (b and d). Cells were stained with affinity-purified antibodies against CK II α (a and b), α' (c and d), or β (e, f, and g). Appropriate mitotic stages were identified under the microscope: (a, b, c, d, and f) metaphase; (e) prophase; (g) telophase. (a'–g') Show differential interference contrast pictures corresponding to a–g, respectively. Bar, 10 μm.
Figure 8. Localization of ectopically expressed chicken CK II α and β subunits in HeLa cells. HeLa cells were transfected with either cDNAs encoding chicken CK II α (a and c) or β subunits (b and e). Expressed proteins were visualized using the appropriate affinity-purified anti-CK II α or β antibodies for indirect immunofluorescence microscopy. Transfected interphase cells are readily identified on the basis of bright nuclear fluorescence (a and b). In transfected mitotic cells, chicken α and β subunits are dispersed (c and e), consistent with their distribution in chicken cells. d and f show untransfected metaphase HeLa cells stained with affinity-purified anti-chicken CK II α and β antibodies, respectively. Bars: (b) 20 μm; (f) 10 μm.

Although all three CK II subunits were predominantly nuclear, we emphasize that some cytoplasmic staining could also be seen, particularly when prolonged fixation conditions were used to minimize extraction of soluble cytoplasmic protein (see Fig. 4 b for the α subunit; not shown for α' and β). Staining for CK II subunits was rather uniform among different cells in a population, suggesting that no major redistributions occurred during interphase stages of the cell cycle. In contrast, all three CK II subunits were distributed throughout the cell during mitosis (Fig. 7). In addition, CK II α and α' could be seen to associate with mitotic spindles, particularly in pre-extracted cells (Fig. 7, b and d, respectively). While anti-β antibodies produced a strong labeling of the nucleus during early prophase (Fig. 7 e), they stained spindle caps in metaphase (Fig. 7 f) and telophase (Fig. 7 g) cells. These observations suggest that a subpopulation of CK II may interact with mitotic microtubules.

Centrosomes (Fig. 6). We note that two different rabbits immunized with β subunits produced antibodies reacting strongly with centrosomes. In contrast, little or no staining of interphase cell centrosomes was detectable with anti-α and anti-α' antibodies. We do not know whether this reflects the existence of a pool of free β subunits at the centrosome, or alternatively, is because of technical limitations (e.g., steric hindrance of antigen accessibility). Biochemical studies with highly purified centrosomes will be required to resolve this issue.

Chicken CK II α and β Subunits Localize Predominantly to the Nucleus When Transiently Expressed in HeLa Cells

To provide a further control for the specificity of our anti-CK II antibodies, the subcellular distribution of chicken CK II subunits was examined following their transient expression in heterologous cells. The cDNAs coding for either the α or the β subunit of chicken CK II were cloned into the mammalian expression vector pCMVneo (Bender et al., 1989; Krek and Nigg, 1991b), and the constructs were transfected into HeLa cells. Although our anti-chicken CK II antibodies display some cross-reactivity with mammalian CK II subunits, they could readily be used to monitor the ectopic overexpression of chicken CK II subunits by indirect immunofluorescence microscopy (Fig. 8). Both chicken CK II α (Fig. 8 a) and β (Fig. 8 b) subunits were localized predominantly to the nucleus of transiently transfected HeLa cells. In the case of the anti-β antibodies, staining of nuclei was punctate rather than uniform, and labeling of a perinuclear area, probably corresponding to the region of the centrosome, could also be seen (Fig. 8 b). Note that non-
transfected HeLa cells also displayed weak staining of nuclei, because of cross-reactivity of the anti-α and anti-β antibodies with endogenous CK II subunits (Fig. 8, a and b). For the same reason, weak staining of spindle poles was visible in non-transfected mitotic cells (Fig. 8, d and f).

Interestingly, transient overexpression of CK II α and β subunits did not detectably influence HeLa cell morphology. Moreover, we note that the transfected HeLa cells proceeded normally through mitosis (see Fig. 8, c and e for metaphase cells transfected with chicken α and β subunits, respectively). As shown above for chicken cells, CK II α and β subunits were distributed throughout mitotic HeLa cells, and there was evidence for a partial association with mitotic spindle caps (Fig. 8, c and e).

### Biochemical Analysis of the Subcellular Distribution of CK II Subunits

Since immunocytochemical studies are prone to multiple types of artefacts (for a brief discussion see Nigg, 1988), we sought to corroborate the above localization data with independent biochemical evidence. Two different procedures were used for subcellular fractionation of chicken cells. In initial experiments, nuclei were separated from cytoplasm according to a conventional protocol based on cell homogenization and centrifugation (Scheidtmann, 1989). Under these conditions, up to 70% of the CK II subunits were found in the cytoplasm (not shown), consistent with the early literature describing CK II as a cytosolic enzyme (for review see Hathaway and Traugh, 1982). However, because many nuclear proteins are known to leak to the cytoplasm during cell homogenization (for discussion see Gordon et al., 1981; Bensch et al., 1982), we attribute this result to an artefactual redistribution of CK II subunits. To obtain more definitive biochemical evidence, we used an alternative procedure based on the rapid isolation of nuclei by cell enucleation (Gordon et al., 1981, Baeuerle and Baltimore, 1988). This technique yields intact nuclei with minimal contamination by cytoplasm, and cytoplasts with minimal contamination by intact cells. Following separation of nuclei and cytoplasts, the partitioning of CK II subunits was examined by immunoblotting. As controls, the distribution of a nuclear marker (lamin B2; Lehner et al., 1986) and a cytoplasmic marker (cytosolic aspartate aminotransferase; Behra et al., 1981) were determined in parallel. The efficiency of the enucleation procedure was monitored by DNA staining of cytoplasts with Hoechst dye 33258 (not shown). Fig. 9 summarizes the results of these experiments. Efficient separation of nuclear and cytoplasmic fractions is illustrated by Coomassie blue staining (Fig. 9 A), as well as by the almost complete segregation of the two marker proteins, i.e., nuclear lamin B2 (Fig. 9 E) and cytoplasmic aspartate aminotransferase (Fig. 9 F). As shown in Fig. 9, B–D, all three CK II subunits were located predominantly, though not exclusively, in the nuclear fraction. These results fully support and extend the immuno-cytocchemical data shown above.

### Expression of CK II Subunits α, α′, and β in Different Adult Tissues

The immunolocalization studies described above do not provide any evidence for major differences in the subcellular distribution of the α and α′ CK II subunits in primary fibroblasts or transformed hepatoma cells. However, we had previously observed that the two subunits differed dramatically in their expression in different tissues, when analyzed at the mRNA level (Maridor et al., 1991). In addition, we had found that in many tissues the mRNA levels for the β subunit were not balanced to match those of the transcripts for the two catalytic subunits (Maridor et al., 1991). With the availability of monospecific antibodies for the corresponding proteins, it became possible to examine whether or not the observed tissue-specific expression patterns of CK II transcripts were reflected at the protein level.

Protein extracts were prepared from those adult chicken tissues that we had studied previously by Northern analyses (Maridor et al., 1991), and similar amounts of total protein (see Coomassie blue staining in Fig. 10 A) were probed with affinity-purified antibodies specific for the individual CK II subunits (Fig. 10 B). As a control, cell extracts from DU249 cells were also analyzed (Fig. 10, A and B, lane 10). From this study, the following conclusions may be drawn: first, all three CK II subunits are expressed at widely different levels in different tissues. For instance, CK II protein is abundant in brain (lanes 2) but very scarce in liver (lane 1). Second, despite variations in absolute amounts, the three subunits α, α′, and β, are expressed at comparatively constant ratios in all tissues examined. These results are in striking contrast to the previously observed tissue specificity of the α and α′ mRNAs and the unbalanced expression of the β mRNA (Maridor et al., 1991). The fact that large tissue specific differences in mRNA levels are not reflected at the protein...
level suggests that translational or posttranslational mechanisms contribute to control the expression of CK II subunits.

Discussion

Based on a combination of immunocytochemistry and subcellular fractionation, we have shown that all three known subunits of chicken CK II, α, α' and β, are located predominantly in the cell nucleus. No major changes in the subcellular distribution of CK II were observed during interphase of the cell cycle. In contrast, all three subunits were redistributed throughout the cell during mitosis, with evidence for association of a fraction of CK II with elements of the mitotic spindle. Our studies did not reveal major differences in the subcellular distribution of individual CK II subunits. In particular, no evidence was obtained for differential localizations of the two catalytic subunits α and α'. These results suggest, first, that CK II may function primarily to control nuclear activities and possibly mitotic events, and second, that the two CK II isoforms may carry out overlapping functions.

In additional studies, we have examined the tissue distribution of individual CK II subunits. These studies were of particular interest in view of previous data demonstrating a striking tissue specificity in the pattern of expression of CK II transcripts (Maridor et al., 1991). Our present results show that most of the tissue-specific variations in mRNA levels are not reflected at the level of the corresponding proteins. Although the absolute amounts of CK II displayed considerable variations among different tissues, the three CK II subunits α, α', and β were found to be expressed at a fairly constant ratio in all tissues examined. Thus, our present results provide no evidence for tissue-specific expression of individual CK II subunits.

Definitive information about the subcellular distribution of the CK II subunits is indispensable for a better understanding of the function and regulation of this kinase. However, no consensus has been reached on this issue. Based on subcellular fractionation, some workers reported CK II activity to be predominantly cytoplasmic (Singh and Huang, 1985; Edelman et al., 1987; Kandor et al., 1989), while others emphasized its presence in nuclei (Thornburg et al., 1979; Hathaway and Traugh, 1982; see also Filhol et al., 1990). Since activity measurements do not necessarily reflect protein levels, several laboratories have applied immunocytochemistry to determine the subcellular distribution of CK II. Again, the reported results are confusing. According to some studies CK II is associated with the nucleolus (Raff and Anderer, 1988; Belenguer et al., 1989), while others describe it to be distributed between nucleus and cytoplasm (Filhol et al., 1990), or present almost exclusively in the cytoplasm (Yu et al., 1991).

It is difficult to definitively explain why different laboratories have obtained such widely different results. Although one could invoke differences between species and/or cell types, we consider this to be an unlikely explanation. Instead, we note that it has been notoriously difficult to raise high titer sera against mammalian CK II. As a consequence, these reagents were frequently used at rather low dilutions, and antibody specificity has rarely been documented. Most recently, antisera were raised against peptides synthesized according to the predicted sequences of CK II subunits (Yu et al., 1991). Based on the use of these tools, an almost exclusive cytoplasmic localization of CK II α and β subunits was reported. Only antibodies reacting with both α and α' subunits displayed some staining of nuclei, at least at certain stages of the cell cycle (Yu et al., 1991). However, although the anti-peptide antibodies used in these studies were shown to recognize denatured CK II subunits in immunoblotting experiments on total cell lysates, it is not clear to what extent the various peptide epitopes were actually accessible to antibodies during the in situ immunolabeling experiments. A rigorous interpretation of these data will have to await molecular information on protein interactions involving CK II subunits.
We are confident that the immunofluorescent staining patterns reported here correctly describe the distribution of CK II α, α', and β subunits. This confidence is based on the following evidence: first, all antibodies used here were shown to be monospecific for their respective antigens by immunoblotting experiments on total cell lysates. Second, immunocytochemistry was carried out using affinity-purified immunoglobulins at very low concentrations (5-15 ng/ml). Third, very similar results were obtained irrespective of the chemical procedure used for cell fixation and permeabilization, and virtually identical results were obtained when analyzing either primary fibroblasts or an established, virally transformed hepatoma cell line. Fourth, immunostaining could be completely blocked by preincubation of antibodies with the respective antigen. Fifth, our immunocytochemical findings are fully supported by biochemical data obtained from cell enucleation experiments. Moreover, the anti-α and anti-β antibodies recognized active (and thus presumably native) CK II in immunoprecipitation experiments. In the case of the anti-α'-peptide antibody, no evidence could be obtained for a positive reaction with native antigen. While one might argue therefore, that the immunofluorescence data obtained with this reagent should be interpreted with caution (see the above discussion of the data reported by Yu et al., 1991), we emphasize that the cell fractionation data shown in Fig. 9 are not affected by in situ epitope accessibility. Finally, chicken CK II subunits α and β were shown to localize predominately to the cell nucleus when overexpressed in HeLa cells. This result is consistent with the observations made with chicken cells, and it provides a striking demonstration of the specificity of the anti-CK II antibodies used here.

Remarkably, transient overexpression of CK II α and β subunits did not detectably influence the morphology of the recipient cells. Moreover, we have not detected any effects of CK II subunit expression on either the frequency or structure of transfected mitotic HeLa cells. Although this preliminary finding indicates that overexpression of CK II does not interfere with normal cell cycle progression, definitive conclusions will have to await the results of further experiments.

While our studies emphasize a predominantly nuclear localization of CK II, we emphasize that minor amounts of CK II could be detected in the cytoplasm. These cytoplasmic pools of CK II may well be functionally important, as indicated by the existence of several cytoplasmic substrates of this kinase (for review see Pinna, 1990; Tuazon and Traugh, 1991). It remains an attractive possibility also, that cytoplasmic and nuclear pools of CK II may exist in a dynamic equilibrium, and that shuttling of CK II subunits may play a role in relaying signals from the cytoplasm to the nucleus (for discussion see Nigg, 1990, 1991). In this context, it will be of considerable interest to determine what signals specify the nuclear accumulation of CK II.

The present analysis of mitotic cells also provides evidence for an association of all three CK II subunits with the mitotic spindle apparatus. This observation is in agreement with previous studies (Serrano et al., 1989; Yu et al., 1991). Its physiological significance remains to be elucidated, but it is interesting that several microtubule associated proteins have been described as substrates of CK II (Serrano et al., 1987; Diaz-Nido et al., 1988). Also, we note that other protein kinases, including the cAMP-dependent protein kinase (Nigg et al., 1985), Ca2+/calmodulin-dependent protein kinase II (Ohta et al., 1990), and p34<sup>cdc2</sup> kinase (Riabowol et al., 1989; Bailly et al., 1989; Krek and Nigg, 1991b), have been localized to centrosomes and mitotic spindle poles.

At the level of resolution provided by immunofluorescence microscopy, all three CK II subunits were found to colocalize. This observation is consistent with biochemical evidence indicating that CK II exists predominantly as a multisubunit enzyme with the structure αβ<sub>a</sub> (or ααβ<sub>b</sub>) (for review see Pinna, 1990). Since we obtained no evidence for major differences in the subcellular distribution of α and α' subunits, it is likely that CK II holoenzymes phosphorylate substrates in the same subcellular compartments, regardless of whether they contain α or α' catalytic subunits. Although it would be premature to exclude the possibility that α and α' subunits may carry out subtly different functions, our present results provide no evidence for functional specialization. Instead, they suggest that α and α' subunits may carry out overlapping, if not identical functions. This conclusion is also supported by the previous finding that the two catalytic subunits of CK II can complement each other in <i>S. cerevisiae</i> (Padmanabha et al., 1990).

In conclusion, our present results indicate that CK II is likely to function predominantly in regulating the activities of nuclear proteins. This conclusion is consistent with current evidence indicating that nuclear substrates of CK II comprise multiple factors involved in controlling transcription and replication. Of particular interest, these include the products of several oncogenes, i.e., myc (Lüscher et al., 1989), myb (Lüscher et al., 1990), erb A (Glineur et al., 1989), the human papillomavirus (types 6, 16, and 18) E7 protein (Firzlaff et al., 1989; Firzlaff et al., 1991; Barbosa et al., 1990), the SV-40 large T antigen (Grässer et al., 1988), and the tumor suppressor gene product p53 (Meeke et al., 1990). With the availability of both cDNA probes and monospecific antibodies for CK II subunits, the stage is set for future attempts to unravel the function(s) of this ubiquitous but enigmatic kinase. The finding that CK II is a nuclear kinase provides a provocative link between signal transduction pathways and nuclear proteins controlling cell proliferation and differentiation.

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