Intracellular redistribution of Ku immunoreactivity in response to cell-cell contact and growth modulating components in the medium

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SUMMARY

Ku is a heterodimeric protein first recognized as a human autoantigen but now known to be widely distributed in mammalian cells. Analysis of repair-deficient mutant cells has shown that Ku is required for DNA repair, and roles in DNA replication and transcription have also been suggested on the basis of in vitro observations. Ku is generally regarded as a nuclear component. However, in the present paper, we show that a quantitatively significant fraction (half or more) of Ku is located in the cytoplasm of cultured primate cells, and that major changes in epitope accessibility of both nuclear and cytoplasmic Ku components are associated with the transition from sparse to confluent cell densities. The same changes in immunoreactivity were seen in HeLa, 293, CV-1 (monkey) and HPV-transformed keratinocyte cell lines, and in primary cultures of human keratinocytes. The immunostaining pattern of sparsely grown cells could be converted to the "confluent" configuration by re-plating them at the same low density on a monolayer of mouse 3T3 cells. The confluent antigen pattern could also be induced in sparse cells within 15-30 minutes by exposure of the cells to serum- or Ca^{2+}-free medium or overnight with 2 mM hydroxyurea. Somatostatin at 0.12 mM blocked the effects of serum/Ca^{2+} deprivation on Ku p70 antigen distribution in sparse CV-1 cells, and in confluent cultures reversed the usual nuclear concentration of p70 immunoreactivity. However, somatostatin did not alter the expected immunostaining patterns of p86. Preliminary studies indicate that sparse CV-1 cells, but not HeLa cells, respond to as little as 1 pM of TGF-β1 in the culture medium by the rapid appearance of nuclear immunoreactivity. TGF-α had no apparent effect. These findings are consistent with the participation of Ku in a signal transduction system responsive to the inhibitory effect of cell-cell contact on the one hand and to cytokines and growth-supportive components of the culture medium on the other.

Key words: Ku, Intracellular distribution, Immunostaining, Enucleation, Cell contact, Serum and Ca^{2+} depletion, Somatostatin, G_{1} arrest

INTRODUCTION

The human autoantigen Ku is a heterodimeric protein with subunits of 70 and 86 kDa, both of which have been cloned and sequenced (reviewed by Reeves, 1992). In the presence of DNA, Ku can associate with a 350-400 kDa catalytic subunit DNA-PKcs to form the DNA-dependent serine/threonine protein kinase DNA-PK (Dvir et al., 1992, 1993; Gottlieb and Jackson, 1993; Suwa et al., 1994; the properties of DNA-PK are reviewed by Anderson and Lees-Miller, 1992). Ku binds tightly to the ends of duplex oligodeoxynucleotides and larger DNA fragments (Blier et al., 1993; Mimori and Hardin, 1986), to circular DNA containing single-strand nicks and longer single-strand gaps (Blier et al., 1993; Falzon et al., 1993), and to internal sites of single-to-double strand transition such as stem-loop structures and bubbles (Falzon et al., 1993). Binding to any of these configurations can mediate the activation of DNA-PK (Morozov et al., 1994). Recent studies using DNA-repair deficient CHO and human cell lines (Getts and Stomato, 1994; Smider et al., 1994; Tacciaioli et al., 1994; Lees-Miller et al., 1995) and cells from immunodeficient scid mice (Blunt et al., 1995; Kirschgessner et al., 1995) have shown that mutations affecting the 86 kDa Ku subunit or DNA-PKcs block repair of double-strand DNA breaks and V(D)J recombination. A reported Ku-associated ATP-dependent helicase activity (Tuteja et al., 1994;) may be relevant to these functions. Models for the function of DNA-PK in the repair of DNA strand breaks have been proposed (Anderson, 1994; Gottlieb and Jackson, 1993; Jeggo et al., 1995), but the actual mechanism is not yet known. Ku prepared from HeLa cells exhibits a DNA-dependent ATPase reaction (Cao et al., 1994; Vishwanatha and Baril, 1990) in addition to the helicase (Vishwanatha et al., 1995). DNA-PK is required for SV-40 replication in vitro (Brush et al., 1994).

In addition to their involvement in DNA repair, Ku and/or DNA-PK have been implicated by in vitro studies in the regulation of RNA polymerases I and II. Preparations that were similar or identical to Ku have been shown to bind to and modulate transcription from rRNA genes (Hoff et al., 1994; Hoff and Jacob, 1993; Kuhn et al., 1993, 1995; Labhart, 1995). DNA-PK represses transcription from rRNA by phosphorylating components of the polymerase I transcription complex
(Kuhn et al., 1995). A Ku-like preparation was also shown to bind to the proximal sequence element of the U1 promoter (Knuth et al., 1990), transcribed by polymerase III.

DNA-PK has been found associated with isolated polymerase II transcription complexes from HeLa cells (Divir et al., 1992, 1993). In vitro, the enzyme can phosphorylate the C-terminal domain of RNA polymerase II in a DNA-dependent fashion (Arias et al., 1991; Divir et al., 1993; Peterson et al., 1992), as well as a number of polymerase II regulatory factors (Anderson and Lees-Miller, 1992; Jackson et al., 1990; Finnie et al., 1993). Ku-like preparations have been shown to bind to cis-regulatory elements of several genes transcribed by polymerase II (references in Reeves, 1992; also Messier et al., 1993; Generesch et al., 1995). EBP-80, a preparation from 293 cells that is similar or identical to Ku, binds site-specifically to a mouse retroviral long terminal repeat (LTR) element and enhances promoter activity of the LTR in vitro (Falzon and Kuff, 1991).

Ku has been identified as a nuclear component by both cell fractionation and immunocytochemical techniques (Yaneva and Busch, 1986; Yaneva and Jhiang, 1991; Higashiura et al., 1992; Reeves, 1992; Reeves et al., 1994; Wang et al., 1993). Immunostaining with antibodies to Ku or to the individual subunits has shown the antigen to be dispersed in particulate form throughout the nucleus when viewed by conventional microscopy or, in a study using confocal microscopy, concentrated at the nuclear periphery in interphase cells and associated with metaphase chromosomes (Higashiura et al., 1992). Ku is also associated with nucleoli at certain periods in the cell cycle (Yaneva and Jhiang, 1991). In nuclear fractionation experiments, Ku has been isolated from nucleoli and active (DNase-sensitive) chromatin (Yaneva and Busch, 1986), and as a DNA-dependent ATPase from SV-40 replication complexes prepared from permissively infected monkey cells (Cao et al., 1994).

Prabakar et al. (1990) detected the 70 kDa subunit at the surface of viable HeLa and human lymphoblastoid cells and pointed out that the amino acid sequence of p70 exhibits multiple peaks of hydrophobicity, of which one at least is large enough to qualify as a membrane-spanning element. They speculated that surface-bound p70 protein might participate in a signal transduction system. The 86 kDa subunit has also been detected at the surface of an established line of human T-cells (Danziel et al., 1992). The deduced amino acid sequence of this protein indicates a large hydrophobic region near the N terminus (Yaneva and Busch, 1986). Recently, a somatostatin receptor isolated from a line of human gastric carcinoma cells has been cloned and identified as an 86 kDa subunit of Ku (Le Romancer et al., 1989). Somatostatin was purchased from Calbiochem (San Diego, CA), genistein and TGF-α from Sigma (St Louis, MO), and methyl methanesulfonate from Aldrich Chemical Co. (Milwaukee, WI). The 293, CV-1 and HeLa cells used in this study were known to be free of mycoplasma contamination, but no information was available for the other cell lines utilized.

Immunoreagents

Anti-Ku sera used in this study were the kind gifts of several investigators. Dr E. M. Tan (Scripps Clinic and Research Foundation, La Jolla, CA) provided an affinity-purified human autoimmune antibody preparation, AF (Francoeur et al., 1986), which reacted with both Ku subunits, but primarily with p70. Dr B. Prabhaker (University of Texas Medical Annex, Galveston, TX) provided two polyclonal rabbit antisera, one directed against the 70 kDa Ku subunit from HeLa cells purified by polyacylamide gel electrophoresis (PAGE), which we have designated Pra70, and the other made against the 70 kDa subunit produced in a baculovirus system (Allaway et al., 1990), designated Prov70a. Mouse monoclonal antibodies (mAbs) N3H10 against the human Ku 70 kDa subunit (Knuth et al., 1990) and 111 against the 86 kDa subunit (Reeves, 1985) were provided by Dr W. Reeves, University of North Carolina Medical School, Chapel Hill. mAbs were provided in ascites fluid. The specificities of all antibodies were verified by western blot analysis utilizing HeLa or CV-1 whole cell extracts. Mouse mAb against human TGF-β1 was purchased from Genzyme (Cambridge, MA).

Immunofluorescent staining

Unless otherwise specified, cells used for immunofluorescent staining were plated 18 hours prior to staining at the density specified. Cells were fixed directly on 60 mm culture dishes at room temperature with 80% acetone for 4 minutes, and air dried. Circular ‘wells’ were created on the fixed monolayer utilizing a Papi Pen (RPI Corp., Mount Pleasant, IL), which provided a hydrophobic barrier to the immunostaining reagents and permitted reaction of the same culture with up to three different antibodies. The fixed monolayers were rehydrated in phosphate-buffered saline, blocked for 15 minutes with normal horse serum at a 1:100 dilution, and reacted for 30 minutes with the specified antibody(ies) at a 1:100 dilution. The monolayers were then treated with FITC-labeled secondary antibody for 30 minutes. Glass coverslips were mounted on the immunostained cells using Vectashield (Vector Labs, Burlingame, California). Indirect immunofluorescence was visualized and photographed using a Leitz Orthoplan epifluorescence microscope.

Several fixation procedures were tested. We found that 1:1 acetone:methanol or 100% methanol fixation at room temperature gave essentially the same results as acetone alone. Cells fixed with 100% methanol at −20°C for 10 minutes (Reeves, 1992) showed a predominantly nuclear reaction at all growth stages; however, cytoplasmic p70 was still demonstrable in sparse cells (see Results).
Cell fractionation

Cell extracts for western blots were prepared by swelling washed cells on ice for 10 minutes in 10 mM Tris-HCl, pH 7.5, 10 μM EDTA, 5 mM MgCl2 and protease inhibitors (1 μg/ml aprotinin, 0.2 μg/ml AEBSF [2-aminoethyl]-benzene sulfonyl fluoride), 10 μM leupeptin, 1 μM pepstatin. Cells were disrupted by 10-15 strokes of a teflon homogenizer. Cell disruption was monitored by phase contrast microscopy. Nuclei were separated from the cytoplasmic fraction by centrifugation at 400 g for 10 minutes. The volumes of the nuclear and cytoplasmic fractions were equalized to maintain cell equivalence of the fractions. Whole cell extracts were prepared by vortexing the cells in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% NP-40 and freeze-thawing the suspension three times to disrupt the nuclei. Protein concentrations were determined by the Coomassie Protein Assay (Pierce, Rockford, IL).

Enucleation

Enucleation of HeLa and CV-1 cells was carried out essentially according to established methods. Briefly, cells grown to approximately 1/2 confluence on 60 mm culture dishes were inverted into 500 ml centrifuge bottles containing 100 ml of prewarmed complete culture medium supplemented with 10 μg/ml cytochalasin B (Sigma). Air bubbles were removed and cells were incubated at 37°C for 10 minutes. After incubation the culture bottles were centrifuged at 4,000 g for 40 minutes using a GSA rotor in a Sorvall RC-5B centrifuge at 37°C. In the most successful experiments, with CV-1 cells, enucleation was essentially complete with only one or two nuclei remaining per low power microscope field. Cytoplasts were cultured for 30 minutes to regain their normal morphology, fixed with 80% acetone and immunostained as described. For immunoblot assay of cytoplasmic Ku, cytoplasts were lysed and centrifuged for 10 minutes at 400 g to remove the occasional nuclei. Further treatment of nucleoplasts is described in the legend of Fig. 5.

PAGE and immunoblotting of proteins

Cellular proteins were fractionated by SDS-polyacrylamide gel electrophoresis and transferred by the recommended procedures to Immobilon membranes (Millipore Corp., Bedford, MA). Blots were blocked with 5% nonfat dry milk dissolved in PBST (phosphate-buffered saline containing 0.1% Tween-20) for 30 minutes and then reacted with the appropriate antibody at a 1:4,000 dilution in PBST. Second antibody reactions and subsequent processing utilized the Vectastain-ABC kit (Vector Laboratories, Burlingame, CA).

RESULTS

The subcellular distribution of Ku in isolated fractions from sparse and confluent cells

Cytoplasmic and nuclear fractions were prepared from sparse and confluent CV-1 and HeLa cells and analyzed by SDS-PAGE and immunoblotting with mAbs N3H10 and 111 (Fig. 1A and B). Comparable amounts of each Ku subunit were found in the fractions from both cell types (the heavy p70-reactive band seen in the cytoplasmic extract of sparse HeLa cells was not observed in other experiments). Similar results were obtained with 293 cells (not shown).

Immunostaining of Ku p70 and p86 antigens in sparse and confluent cells

CV1 cells

Cells were plated at 5x10^4 cells per 60 mm dish (for sparse cultures) or 1x10^6 per dish (for confluent cultures), fixed after 16 hours growth and immunostained with mAbs N3H10 vs Ku p70 and 111 vs p86 (Fig. 2). With N3H10, reactive p70 antigen was localized principally in the cytoplasm of sparsely grown cells (Fig. 2A). In dense or confluent monolayers, however, immunoreactivity was concentrated in the nuclei (Fig. 2C) while cytoplasmic p70 antigen was much reduced. The polyclonal rabbit antibody Pra70 and the affinity-purified human antibody AF1 gave the same results (see Fig. 3A-C for Pra70), indicating that the immunoreactivity of N3H10 was not due to contaminating antibodies in the ascites fluid (Satoh and Reeves, 1994). The specificity of mAbs N3H10 and 111 on immunoblot analysis will be shown below.

mAb 111 also showed a striking difference in p86 immunostaining between sparse and dense cells (Fig. 2B and D). The nuclei of sparse CV1 cells reacted only weakly with this antibody, but nuclei of confluent cells were uniformly reactive. mAb 111 did not react with cytoplasmic p86 on immunostaining at any cell density.

The insert in Fig. 2A shows the sparse CV-1 cells in another area of the same culture dish, immunostained with a rabbit antiserum Pra70B, which reacts only with nuclei and equally well at all growth stages. The positive immunostaining with Pra70B shows that the weak reaction of the sparse nuclei with N3H10 was not due to extraction of p70 during the experimental manipulations.

HeLa cells

Similar changes in immunoreactivity of Ku p70 were observed in HeLa cells as the cultures increased in density. In sparsely grown cells (Fig. 3A-C), reactive p70 antigen was localized primarily in the cytoplasm, while nuclear stain was relatively light and occasionally undetectable (Fig. 3C insert). mAb N3H10 and the polyclonal rabbit antiserum Pra70 gave essentially the same results. On the other hand, in confluent cultures

Ku responds to cell contact and growth factors

Fig. 1. Detection of Ku subunit proteins in immunoblots of cytoplasmic and nuclear fractions. Homogenates were prepared from sparse and confluent cultures and the cytoplasmic (C) and nuclear (N) fractions separated by differential centrifugation, as described in Materials and Methods. Protein samples representing equal cell equivalents were electrophoresed by SDS-PAGE, transferred to Immobilon membranes, and reacted with a mixture of mAb N3H10 against Ku p70 and mAb 111 against p86. The positions of marker proteins are shown.
reactivity with both antisera was predominantly or exclusively nuclear (Fig. 3E and F). In cultures of moderate density, some cells showed an intermediate distribution of p70 immunostaining, with reduced cytoplasmic reaction and immunostaining concentrated in the peripheral region of the nuclei (example in Fig. 3C). Nucleoli were usually not reactive.

Sparse and confluent HeLa cells showed the same patterns of immunostaining with mAb 111 as CV-1 (not shown).

The redistribution of immunoreactive p70 was not correlated with cessation of cell multiplication, since it could be observed in small groups of cells formed in actively growing cultures. Singly dispersed cells in these same cultures exhibited the sparse distribution of antigen. The measured doubling times for sparse and sub-confluent HeLa cells were 24 and 27 hours, respectively.

The immunostaining changes in CV-1 and HeLa cells contrast with the constant subcellular distribution of Ku observed on immunoblots prepared with the same antibodies (Fig. 1). Evidently, the immunostaining of both subunits in fixed cells is strongly influenced by epitope accessibility.

Normal and transformed keratinocytes

Pra70 was used to immunostain primary cultures of neonatal human keratinocytes (Fig. 4A and B) and an established line of HPV16-transformed keratinocytes (Fig. 4C and D). Sparse cells of each type exhibited a largely cytoplasmic distribution of reactive antigen, while the antigen in confluent cells was mostly (primary keratinocytes) or entirely (transformed cells) nuclear. Thus the cytoplasmic immunostaining of Ku in sparse cultures and the response to cell density can be observed in normal as well as transformed cells.

**Technical factors affecting immunostaining**

Acetone fixation was not uniquely required for demonstration of p70 cytoplasmic immunoreactivity with N3H10 or Pra70; however, it gave by far the most reproducible results. Fixation with absolute methanol at either −20°C or room temperature, with methanol:acetic acid (1:1), or with 3.7% formaldehyde, all gave cytoplasmic immunostaining which, however, varied in intensity from one experiment to another.

Antibody requirements were highly specific. Three other rabbit antisera against Ku p70 (provided by B. Prabhakar) reacted exclusively with nuclear antigen (Fig. 2A, insert), as did anti-Ku antibodies isolated from subsequent bleedings of normal and transformed keratinocytes.

**Fig. 2.** Immunofluorescent staining of CV-1 monkey kidney cells in sparse and confluent cultures. Cells were fixed in 80% acetone and immunostained as described in Materials and Methods. (A) Sparse and (C) confluent cells reacted with mAb N3H10 against Ku p70. Inset in A, sparse cells immunostained with Pra70, which reacts with nuclear Ku p70 in both sparse and confluent cells. (B and D) Other areas in the same sparse and dense culture dishes reacted with mAb 111 against the p86 Ku subunit. Bar, 20 μm.

**Fig. 3.** Immunofluorescent staining of Ku p70 in HeLa cells at various degrees of cell density, using either rabbit antibody Pra70 prepared against a baculovirus recombinant protein, or mAb N3H10. (A,B,C) Cells from sparse cultures, immunostained with Pra70 (A and B) and mAb N3H10 (C), both of which give a predominantly or exclusively (insert C) cytoplasmic reaction. (D) Part of a larger cell cluster at intermediate culture density, reacted with Pra70. (E and F) Confluent cultures immunostained with mAb N3H10 (E) and Pra70 (F). Reactivity is concentrated in the nucleus. N3H10 gives some cytoplasmic reaction but Pra70 does not. Inset in F, nucleus of confluent cell showing typical appearance when less intensely immunostained. Bar, 20 μm.
Ku responds to cell contact and growth factors

We tested ascites fluids from one pristane-treated BALB/c mouse and three mice with primary plasmacytomas (provided by M. Potter, NCI). One gave strong cytoplasmic staining of both sparse and confluent CV-1 cells; none reacted with Ku on immunoblots. Three samples of mAb N3H10 ascites gave the results illustrated in Figs 2-4; however, a reconstituted frozen-dried sample (from N. Thompson, McArdle Laboratory) reacted with Ku p70 on immunoblots but not on staining. A sample of culture medium from cells secreting N3H10 antibody (provided by Dr Wesley Reeves) reacted with p70 on immunoblots but failed to immunostain the cytoplasm of sparse cells. It did show the expected increase in nuclear reactivity in confluent vs sparse cells and in response to withdrawal of serum or Ca²⁺ from the medium (see below). We have no explanation for this behavior.

The intracellular distribution of Ku studied by enucleation

To test whether cytoplasmic Ku was an artifact of fixation and/or cell fractionation, CV-1 and HeLa cells in actively growing sub-confluent cultures were enucleated with the use of cytochalasin B, as described in Materials and Methods, and the cytoplasts immunostained for Ku p70 with Pra70. HeLa cell cytoplasts giving a positive reaction are shown in Fig. 5A. In an experiment with CV-1 cells, some regions of the treated plates contained intact cells among the cytoplasts, permitting direct comparison between the two. In Fig. 5B, Ku p70 antigen has the same appearance in the cytoplasts as it does in the cytoplasm of the intact cells (the latter identified by their bright nuclei), indicating that no major redistribution of immunoreactivity had occurred on enucleation.

CV-1 nucleoplasts and cytoplasts were recovered quantitatively in an experiment where enucleation was essentially complete, as judged by phase microscopy. The nucleoplasts were swollen in the standard hypotonic homogenization buffer (10 mM Tris-HCl, pH 7.5, 5 mM MgCl₂), producing a small visible space between the nuclei and surrounding cell membranes, and then homogenized. The freed nuclei were separated by centrifugation from the supernatant fluid (‘nuclear extract’), which should contain any material that might leak from the nuclei on homogenization. DNA (determined by DAPI fluorescence) was distributed in the following proportions: cytoplasm, 10% (includes mitochondrial DNA); nuclei, 90%; nuclear extract, undetectable. An immunoblot of total proteins from the cytoplasts, isolated nuclei and nuclear extract is shown in Fig. 5C. Comparable amounts of the Ku subunits were found in the cytoplast and nuclear fractions; however no Ku was detected in the nuclear extract. We consider this strong evidence that Ku does not leak from the nuclei on homogenization under our standard conditions.

Change in distribution of Ku immunoreactivity in response to cell contact

By medium shift experiments not illustrated here, we found
that neither exhaustion of the culture medium nor secretion of soluble factors appeared to be responsible for the shift in intracellular distribution of immunoreactive antigen with increasing cell density. The progressive formation of cell-cell contacts was then considered as a contributing factor. To test this possibility, HeLa, CV-1 and 293 cells were each collected from sparse cultures and replated at the same low densities on monolayers of mouse 3T3 fibroblasts. HeLa cells fixed 16 hours after replating and immunostained with two antibodies against the Ku 70 kDa subunit are shown in Fig. 6A and B. mAb N3H10 reacted to a similar extent with the cytoplasm of mouse and human cells (Fig. 6A); however, the HeLa cells are identified by their more brightly fluorescent nuclei, typical of dense rather than sparse HeLa in homotypic culture (see Fig 3E and F). Immunostaining with Pra70 (Fig. 6B) was localized exclusively to the HeLa nuclei (Ku in 3T3 cells does not react with this antibody). There was no change in antigen distribution when sparse HeLa cells were cultured in medium conditioned by confluent 3T3 cells.

The nuclei of sparse CV-1 cells growing on the 3T3 monolayer reacted strongly with the anti-p86 mAb 111 (Fig. 6C), in contrast to their minimal reactivity when grown alone at low density (Fig. 6D), but similar to their appearance in confluent homotypic monolayers (Fig. 2C and D). Sparsely cultured 293 cells immunostained for p70 with Pra70 were typically rounded, with reactive cytoplasm and poorly stained nuclei (Fig. 6F). When these same cells were replated sparsely on monolayers of mouse 3T3 fibroblasts, immunoreactive antigen began to accumulate in nuclei within 2-4 hours (not shown), and a total redistribution of reactivity was observed after overnight culture (Fig. 6E).

Sparse CV-1 or HeLa cells that were trypsinized and replated at high densities displayed the confluent distribution of p70 immunoreactivity at 4 hours, the earliest time at which the cells had spread to the point where they could be examined. These experiments support the concept that the changes in Ku immunoreactivity with increasing culture density are related to the development of cell-cell contacts. Heterotypic as well as homotypic contacts were effective.

Exposure of sparse cells to serum-free medium or Ca\(^{2+}\) deprivation induces the confluent antigen distribution

Sparse CV-1 cells were immunostained with mAbs N3H10 and 111 at various times after transfer to serum-free medium. As shown in Fig. 7B, the pattern of immunoreactivity for the Ku subunit was converted to the confluent configuration by 30 minutes. The same results were obtained for the 86 kDa subunit in CV-1 cells and for both subunits in HeLa cells (not shown). Genistein, an inhibitor of protein phosphokinase c, did not prevent the antigenic changes when added at 200 \(\mu\)g per ml. Exposure of cells to Ca\(^{2+}\)-free medium (or addition of EGTA to the standard culture medium) had the same effects on immunostaining (Fig. 7C). Conversion to the confluent distribution of immunoreactivity was essentially complete by 15 minutes after Ca\(^{2+}\) removal.

Somatostatin maintains or restores the sparse configuration of p70 immunoreactivity in cells exposed to Ca\(^{2+}\)-free medium or grown to confluence

Addition of somatostatin at 200 ng per ml to Ca\(^{2+}\)-free medium prevented the re-configuration of immunoreactivity induced by Ca\(^{2+}\) depletion (Fig. 8A and B). In confluent cultures, many cells showed a reversal of p70 immunoreactivity to the sparse configuration when treated with somatostatin for 24 hours (Fig. 8C and D). However, somatostatin did not reverse the strong nuclear immunoreactivity of p86 in confluent cells (not shown).
Effects of other treatments on Ku immunoreactivity (data not illustrated)

CV-1 and HeLa cells were cultured in complete medium containing 0.2 mM hydroxyurea to arrest the cells at the G1/S boundary. By 5 hours a portion of the cells, and at 16 hours nearly all of them, had acquired the confluent distribution of Ku antigens.

DNA damage induces cell cycle arrest at the G1/S or G2/M checkpoints (Gottlieb and Jackson, 1994). The alkylating agent methyl methanesulfonate (MMS) at 200 μM induced the confluent configuration of both subunits in sparse CV-1 cells within 30 minutes, the shortest time tested. On the other hand, 500 rads of X-irradiation had no apparent effect on antigen distribution in sparse CV-1 cells. Heat shock (42°C for 3 hours) did not affect the immunostaining of sparse or confluent cells, nor did TGF-α added to the culture medium at 50 ng per ml.

The effects of added TGF-β1 are under investigation. With antibody against either Ku subunit, the nuclei of sparse CV-1 cells exposed to TGF-β1 at 1 or 10 pM were converted within 15-30 minutes to the brightly immunostained state characteristic of confluent cells. However, the cytoplasmic response has not yet been evaluated due to the current depletion of cytoplasmic-reactive antibody stocks. TGF-β1 had no discernible effect on the immunostaining of sparse HeLa cells.

DISCUSSION

Ku has not previously been considered to have a major cytoplasmic component or to undergo the changes in distribution of immunoreactivity seen in this study. One reason may be that immunostaining observations have usually been made on cultures of fairly high cell density in which Ku immunoreactivity is largely restricted to the nuclei. Another is the fact that most antibodies against p70 do not react on immunostaining with the cytoplasmic epitopes detected in this study (for the known epitope specificities of the anti-Ku mAbs used here, see Wang et al., 1993), nor do different methods of fixation necessarily give equivalent results with different antibodies. It is important to emphasize that the immunostaining results reported here were obtained under specific conditions of fixation (see Materials and Methods) and choice of antibodies.

Given the novel immunostaining and cell fractionation results, it was necessary to establish the specificity of the immunostaining reactions and the reality of cytoplasmic Ku. For several reasons, we think it unlikely that the cytoplasmic reactivity of ascites N3H10 is due to antibodies of random specificity such as can arise in mineral oil-treated mice (Satoh and Reeves, 1994): (a) immunoblots confirm the antibody specificity of the monoclonal ascites samples; (b) the same patterns of p70 immunostaining were obtained with mouse mAb N3H10, a rabbit polyclonal antibody Pra70 and an affinity-purified human antibody AF against Ku; it seems highly unlikely that all three preparations contained a contaminating antibody with the same specificity. (c) Among four mouse ascites fluids tested, none reacted with Ku and none gave the immunostaining patterns seen with N3H10. The presence of cytoplasmic Ku in intact cells was established by the enucleation experiments, which also showed that Ku did not leak from nuclei when nucleoplasts (mini-cells) were swollen and disrupted by the same procedure used to disrupt whole cells.

However, the situation is complex, since the observed immunostaining patterns clearly reflected the accessibilities of specific epitopes in the fixed cells rather than the total amounts of the Ku subunit proteins, as observed by immunoblotting. The epitopes for mAbs N3H10 and 111 are both conformational even though they react with their respective antibodies on immunoblots (Wang et al., 1993). mAb N3H10 recognizes an epitope next to or overlapping the DNA binding site on p70 (Chou et al., 1992); immunoreactivity may therefore reflect the status of this site. The mAb 111 epitope has been localized in the C-terminal region of p86 (Reeves, 1992; Wang et al., 1993) but no functional correlation has been shown as far as we are aware. We propose as a working hypothesis that the acetone fixation preserves the two Ku subunit proteins in alternative conformational states, one of which is recognized by the specific antibodies and in the other of which this epitope is blocked.

The reactive conformation of p86 is essentially lacking in sparse cells and confined to the nucleus in contacting or serum-deprived cells. The mAb N3H10 epitope is accessible primarily on cytoplasmic p70 in sparse cells and in the nuclear compartment in contacting cells. Nuclear transport of p70 is suggested by this reciprocal change in immunostaining. However, immunoblots of isolated fractions from sparse and confluent cells, or from sparse cells deprived of serum or Ca2+ for 30 minutes, showed no consistent change in the actual concentration of either subunit protein in the cytoplasmic and nuclear compartments. If nuclear transport of a specifically reactive form of p70 does occur in response to cell contact or other stimuli, the transported component may represent a small fraction of the total cytoplasmic pool. Alternatively, the cytoplasmic pool might be replenished in contacting cells by newly synthesized p70 that does not react with mAb N3H10.

Wang et al. (1994) have shown that in cell cultures transfected with p70- or p86-expressing vaccinia virus recombinants, newly synthesized subunits are individually transported to the nucleus, where they can form the Ku heterodimer in the presence of DNA. We have observed that immunoreactive p70 dispersed in the cytoplasm during mitosis reappeared in the nuclear plaques of late G1/S.
with 32 P in intact cells was unsuccessful (Stuiver et al., 1991). Contact with heterologous cells was as effective as contact with self in evoking the Ku antigen reconfiguration. The fact that the cells continued to divide on underlying 3T3 monolayers is islands containing between some 10 to 100 contacting cells. Maintenance of the sparse configuration of antigen in the cultured cells required fetal calf serum and Ca^{2+}-containing medium. The essential serum components have not yet been characterized. In complete medium, the development of cell-cell contacts rather than inhibition of growth seemed to be the signal for redistribution of Ku immunoreactivity to the confluent pattern, since this change was evident in cultures that were still growing rapidly and consisted chiefly of separated islands containing between some 10 to 100 contacting cells. One-dimensional (1-D) electrophoretic analysis of cell extracts, this protein showed of p86 in different functional states. On two-dimensional (2-D) electrophoretic analysis of cell extracts, this protein showed multiple charge variants, which changed in proportion to TGF-β signal for redistribution of Ku immunoreactivity to the confluent pattern, since this change was evident in cultures that were still growing rapidly and consisted chiefly of separated islands containing between some 10 to 100 contacting cells. Contact with heterologous cells was as effective as contact with self in evoking the Ku antigen reconfiguration. The fact that the cells continued to divide on underlying 3T3 monolayers again indicates that cell contact, rather than cessation of growth, was the primary signal for antigen redistribution.

There is published evidence for alterations in the properties of p86 in different functional states. On two-dimensional (2-D) electrophoretic analysis of cell extracts, this protein showed multiple charge variants, which changed in proportion according to the proliferative state of HeLa cells (Stuiver et al., 1991) or in CV-1 cells in response to SV40 infection (Quinn et al., 1992). An attempt to label the electrophoretic variants with 32P in intact cells was unsuccessful (Stuiver et al., 1991). Immunochemical changes in Ku were, including cleavage of some molecules, were observed in extracts of SV40-infected CV-1 cells and could be induced in untreated cells by treatment with PMA, calcium phosphate or brief exposure to serum-free conditions (Quinn et al., 1992), leading the authors of the study to suggest that Ku can act as a sensor of the cellular environment. The present results are consistent with this hypothesis.

Both Ku subunits have been detected at the surface of human cells of several culture lines (Danziel et al., 1992; Prabhakar et al., 1990), and there are other studies showing that proteins similar or identical to the Ku subunits can participate in cellular responses to external stimuli. In one, a somatostatin receptor present in epithelial cells of gastrointestinal organs was isolated from gastric cancer cells, cloned, sequenced and identified as an 86 kDa Ku subunit (Le Romancer et al., 1994). P86 was shown to bind and inhibit the 36 kDa catalytic subunit of protein phosphatase-2A (PP2A) purified from the cytosol of rat gastric epithelium. Somatostatin bound to p86 with high affinity and relieved the p86-induced block in PP2A activity. Since the PP2A-mediated dephosphorylation of histone H1 is essential for chromosome condensation, the authors suggested that Ku p86 might participate with somatostatin in vivo in regulating the mitotic activity of the gastric cancer cells.

We found that somatostatin added to the culture medium at a concentration of 200 ng per ml did not affect the distribution of Ku antigens in sparse CV-1 cells. However, somatostatin did block the redistribution of p70 antigen to the confluent pattern in sparse cells deprived of Ca^{2+}, and partially reversed the characteristic nuclear localization of p70 in dense culture (Fig. 8). Somatostatin had no apparent effect on nuclear p86 immunoreactivity (not shown), an indication that changes in the two Ku subunits can be independently regulated.

Growth-related changes in the properties of the heterodimeric Ku-like enhancer 1 binding factor E1BF have recently been reported (Hoff et al., 1994). E1BF, a transcription factor for RNA polymerase I in vitro (Hoff and Jacob, 1993), was shown to exist in two forms depending on the growth status of the cells from which it was obtained: E1BF prepared from growing cells was stimulatory for polymerase activity while the factor from serum-deprived cells was inhibitory. This study is important in showing that Ku can participate in the regulation of ribosomal RNA synthesis. In addition, it provides evidence for a functional change in the Ku subunits under one of the growth-restricting conditions that also induces changes in Ku immunolocalization. Other studies have shown that phosphorylation by DNA-PK inhibits RNA polymerase I activity in vitro (Kuhn et al., 1995; Labhart, 1995). Modifications of the E1BF(Ku) subunits of DNA-PK in response to external stimuli might modulate the effects of the holoenzyme on polymerase I transcription. Inhibition of ribosomal RNA synthesis could be an important mechanism by which serum deprivation and other factors restrict cell multiplication.

Cell-cell contact, serum or Ca^{2+} deprivation, and exposure to TGF-β or hydroxyurea, are all known to cause prolongation of or arrest in G1 of the cell cycle. Each of these stimuli evoked the appearance of Ku immunoreactivity in the nuclei of sparse cells. The rapidity of the responses to removal from or additions to the medium was noteworthy and suggests that Ku may be an intrinsic component of signal transduction pathways, responsive to a variety of external growth-regulating stimuli. The serum-sensitive modulation of ribosomal RNA synthesis by alternative forms of Ku (E1BF) is one such example, and the role of p86 as a somatostatin receptor in gastrointestinal cells is another. At a speculative level, Ku might participate in the incompletely characterized pathway from TGF-β1 receptor protein kinase activity (Carcamo et al., 1995) at the cell surface to the nuclear cyclin-Cdk inhibitor p27 Kip1 (Polyak et al., 1994; Toyoshima and Hunter, 1994). Polyak et al. (1994) have shown that this pathway links TGF-β and contact inhibition to cell cycle arrest.

**Addendum**

Immunostained juxtanuclear bodies are seen in Fig. 2A inset, Fig. 3D and E, and Fig. 6B and C. These may represent the centrosome in its singlet form.

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Ku responds to cell contact and growth factors 1945


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