Ran/TC4: A Small Nuclear GTP-binding Protein That Regulates DNA Synthesis

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Abstract. *Ran/TC4*, first identified as a well-conserved gene distantly related to *H-RAS*, encodes a protein which has recently been shown in yeast and mammalian systems to interact with RCC1, a protein whose function is required for the normal coupling of the completion of DNA synthesis and the initiation of mitosis. Here, we present data indicating that the nuclear localization of Ran/TC4 requires the presence of RCC1. Transient expression of a Ran/TC4 protein with mutations expected to perturb GTP hydrolysis disrupts host cell DNA synthesis. These results suggest that Ran/TC4 and RCC1 are components of a GTPase switch that monitors the progress of DNA synthesis and couples the completion of DNA synthesis to the onset of mitosis.

RAN/TC4, was initially described as a *RAS*-related transcript of unknown function. It was identified in a human teratocarcinoma cell line but is abundant in a variety of cultured cell lines, and is of interest because it defines a new, evolutionarily well-conserved branch of the *RAS* gene superfamily (Drivas et al., 1990, 1991*a*). Recent genetic and biochemical analyses (Matsumoto and Beach, 1991; Bischoff and Ponstingl, 1991*a*) suggest that *Ran/TC4* also plays a key role in the regulation of cell cycle progression in eukaryotes, and that this role depends on its nuclear localization and interaction with the product of a second gene, *RCC1*, defined by the tsBN2 mutation of BHK cells (Nishimoto et al., 1978; Uchida et al., 1990) and the *piml* mutation of the fission yeast *Schizosaccharomyces pombe* (Matsumoto and Beach, 1991). The compound name used here reflects this: a teratocarcinoma-derived cDNA clone that encodes a Ras-related nuclear (Ran) protein.

S. pombe piml (premature initiation of mitosis) mutants enter mitosis without completing chromosomal DNA replication. Overexpression of the wild-type allele of a second gene, *spil* (suppressor of *piml*), suppresses the piml mutant phenotype. The predicted amino acid sequences of *spil* (yeast) and *Ran/TC4* (human) are 80% identical. The fact that *spil* overexpression cannot rescue null mutants and the existence of a cold-sensitive mutation in *spil* suggest direct interaction between Piml and Spil proteins (Matsumoto and Beach, 1991).

The mammalian homolog of *piml* is *RCC1* (regulator of chromosomal condensation), a gene originally defined by the tsBN2 mutation of BHK cells (Uchida et al., 1990; Nishitani et al., 1991). The wild-type activity of *RCC1* is required both to initiate DNA synthesis (Dasso et al., 1992) and to prevent chromosome condensation until the completion of S phase (Uchida et al., 1990; Nishitani et al., 1991; Enoch and Nurse, 1991; Dasso and Newport, 1990). *piml* is predicted to encode a larger protein (539 aa) than *RCC1* (421 aa), but over the region shared by the two proteins, their sequences are 30% identical and share a sequence motif repeated seven times in each protein (Matsumoto and Beach, 1991).

RCC1 protein can bind DNA and is associated with chromatin (Ohtsubo et al., 1989). It is present in Xenopus egg extracts in amounts sufficient to provide one molecule per nucleosome (Dasso et al., 1992), and can be purified from HeLa cell chromatin in the form of a complex with a 25-kD protein. Partial amino acid sequence data indicate that the latter is Ran/TC4 (Bischoff et al., 1990; Bischoff and Ponstingl, 1991*a*).

To further characterize the function of Ran/TC4 and its interaction with RCC1 in mammalian cells, we have now prepared Ran/TC4-specific antibodies and used them to demonstrate that nuclear localization of Ran/TC4 is dependent on expression of RCC1. We have also shown that expression of a mutant *Ran/TC4* allele blocks cellular DNA replication.

Materials and Methods

DNA Sequencing and In Vitro Mutagenesis

DNA sequencing was performed as described previously (Drivas et al., 1990, 1991*b*) using double-stranded pMT2 or lambda phage templates, with Ran/TC4-specific oligonucleotides as primers.

In vitro mutagenesis was performed by oligonucleotide priming, using the 1.5-kb Ran/TC4 cDNA EcoRI fragment (Drivas et al., 1990) cloned in M13 as a template. Mutants were identified by differential hybridization with wild-type and variant oligonucleotide probes, and the mutated EcoRI fragments were isolated and cloned into the EcoRI site of pMT2. Mutant identifications were confirmed by DNA sequence analysis of both the initial M13 isolate and the pMT2 subclones.

Ran/TC4 and RCC1 Antibodies

The dodecapeptide underlined in Fig. 1 was synthesized, coupled with glutaraldehyde to Keyhole Limpet hemocyanin, emulsified in complete Freund's adjuvant, and injected intradermally into two rabbits (Pocono Rabbit Farms). One yielded a high-titer polyclonal antiserum used in all experiments described here. RCC1 antibody (Nishitani et al., 1991) was a generous gift of Dr. Takeharo Nishimoto.

Cell Lines

tsBN2 cells were a generous gift from Dr. Claudio Basilico. HeLa, COS, 3T3, and wild-type BHK21 hamster cells were from laboratory stocks derived ultimately from American Type Culture Collection, Rockville, MD.

Immunoblotting

Total Cell Lysates. Cells at 50–75% confluence in 10-cm dishes were lysed in 0.5 ml of 2% SDS, 50 mM Tris, pH 7.5, boiled for 10 min, sonicated, and added to an equal volume of 4% SDS, 0.05% Bromophenol blue, 40% glycerol, 10% β-mercapteothanol, 200 mM Tris, pH 6.8 (2× SDS-PAGE sample buffer). Aliquots corresponding to 50 μg total protein were then electrophoresed through 0.1% SDS, 15% polyacrylamide gels, and electroblotted onto nitrocellulose membranes. The membranes were incubated for 2 h at room temperature with a 1:400 dilution of rabbit anti-Ran/TC4 antiserum in PBS, pH 7.4, containing 5% (wt/vol) nonfat dry milk, and 0.3% Tween 20. Filters were washed with the same solution (minus antiserum), incubated with ^{125}I-protein A (ICN Biomedicals Inc., Costa Mesa, CA) for 2 h at room temperature, washed with 0.3% Tween 20 in PBS, and autoradiographed.

Digitonin-treated Cell Lysates. Cells at 50–75% confluence in 10-cm dishes were treated with digitonin in order to selectively permeabilize plasma membranes and release cytosolic components (Adam et al., 1990; Shi and Thomas, 1992; Walton et al., 1992). Specifically, cells were washed twice in 5 ml ice cold PBS and then incubated for 10 min in ice cold permeabilization buffer containing 25 μg/ml digitonin (Sigma Chem. Co., St. Louis, MO), 140 mM KCl, 1 mM $MgCl_2$, and 10 mM sodium phosphate, pH 7.0. Permeabilization buffer (containing cytosolic components) was removed, and cellular material remaining on the plate (including nuclei and other major membrane organelles) was solubilized in 1 ml 2% SDS, 50 mM Tris, pH 7.5. The proteins solubilized in the permeabilization buffer were precipitated in 10% TCA and redissolved in 1 ml 2% SDS, 50 mM Tris, pH 7.5. Solubilized proteins from each fraction were then boiled for 10 min, sonicated, added to equal volumes of 2× SDS-PAGE sample buffer, and analyzed for Ran/TC4 content by the immunoblotting procedures described above for total cell lysates.

Immunoprecipitation

Cells at 50–75% confluence in 3.5-cm dishes were washed with methionine-free DME, and incubated in 1 ml of the same supplemented with 125 μCi [^{35}S]methionine (1,130 Ci/mmol) for 6 h at 37°C. Cells were washed twice in PBS, lysed in 0.1 ml 2% SDS, 50 mM Tris, pH 7.5, boiled 10 min, sonicated, and diluted 1:10 in 1.25% Triton X-100, 150 mM NaCl, 50 mM Tris, pH 7.5, 6 mM EDTA (solution A). Lysates were precleared with protein A-Sepharose beads, incubated for 1 h at room temperature with 1:200 rabbit anti-Ran/TC4 antiserum, and then for 1 h at room temperature with protein A-Sepharose. Precipitated beads were washed four times with solution A, once with solution A containing 0.5 M NaCl, and once with PBS. Beads were then boiled in 50 μl SDS-PAGE sample buffer, and the eluate was electrophoresed through a 0.1% SDS, 15% polyacrylamide gel. The gel was fixed for 3 h in 25% methanol, 10% acetic acid, incubated 1 h in autoradiography enhancer (EN^{3}HANCE; DuPont Co., Wilmington, DE) and 1 h in water (all at room temperature), and then dried and autoradiographed (XAR film, Eastman Kodak Co., Rochester, NY).

Densitometry

Autoradiograms from Southern and Western blotting experiments were analyzed with a GS300 transmittance/reflectance scanning densitometer (Hoefer Sci. Instrs., San Francisco, CA) coupled to a computer. Integrated absorbances for each band were recorded as bar graphs.

Phosphorimaging

Quantitation of Western blots to obtain the data shown in Fig. 7 was carried out using a PhosphorImager unit (Molecular Dynamics, Sunnyvale, CA). Data are recorded as bar graphs, in arbitrary units.

Immunofluorescence Staining

Cells on coverslips were washed in PBS, fixed in 3% paraformaldehyde in PBS for 10 min, washed three times in PBS, permeabilized in 0.2% Triton X-100 in PBS for 5 min, washed three times in 3% BSA in PBS, incubated with 1:300 anti-Ran/TC4 antiserum or 1:500 anti-RCC1 antiserum (diluted in PBS + 3% BSA) for 1 h, washed three times in PBS + 3% BSA, incubated with FITC-conjugated goat anti-rabbit Ig antibody (Cappel Laboratories, Malvern, PA; 1:200 in PBS + 3% BSA) for 1 h, washed five times with PBS, and then mounted with an antibleaching agent (FITC-Guard; Testog, Inc.) and examined using an Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with 40× (low magnification) or 63× (high magnification) Plan-Neofluar objective lenses.

To determine the effects of digitonin treatment, cells were incubated for 10 min in ice cold permeabilization buffer, and then fixed and stained as described above.

Transfection Assays

The plasmids pMT2 (Kaufman, 1990), pRSV-TH (Rindler et al., 1990), and pRSV-CAT (Gorman et al., 1982) were gifts from Drs. M. Adesnik, M. Rindler, and G. Kreibich, respectively. pMT2 plasmid constructs were propagated in *E. coli* strain DH5α.

Transfections were performed essentially as described by Kaufman (1990): 10-cm dishes of COS cells at 50–70% confluence were incubated with a total of 8 μg plasmid DNA in 4 ml DEAE-dextran (500 μg/ml in serum-free DME) for 8 h at 37°C, washed once with serum-free DME, incubated 90 s in serum-free DME + 10% DMSO, washed, incubated in DME + 10% FCS + 100 μg/ml chloroquine for 3 h at 37°C, washed, and incubated for at least 60 h in DME +10% FCS at 37°C.

Cellular proteins were then examined by SDS-PAGE and stained with Coomassie blue.

Hirt (1967) extracts were prepared by lysing cells for 20 min at 25°C in 1 ml 0.6% SDS, 10 mM EDTA, 10 mM Tris, pH 7.5, adding 0.25 ml 5 M NaCl, and incubating for 16 h at 4°C. Supernatants collected by centrifugation (30 min at 17,000 rpm in a microfuge at 4°C (Eppendorf Inc., Fremont, CA) were made 10 μg/ml in RNase A, incubated 1 h at 37°C, made 20 μg/ml in Proteinase K, incubated another hour at 37°C, extracted twice with 1:1 phenol/chloroform and twice with chloroform, and ethanol precipitated. Precipitates were resuspended and digested for 2 h at 37°C with 20 U EcoRI, ethanol precipitated, resuspended, and digested for 2 h at 37°C with 20 U DpnI (65 μl total volume in each case). One fifth of this material was electrophoresed through a 1% agarose gel, transferred to a GeneScreen membrane filter (DuPont Co.), and probed with nick-translated ^{32}P-labeled pMT2 DNA as described by DuPont Co.

Chloramphenicol acetyltransferase assays were performed as described by Ausubel et al. (1989).

Chromosomal DNA Synthesis Assays

COS cells were cotransfected with 4 μg of a pMT2 construct encoding Ran/TC4 protein and 4 μg of plasmid pRSV-TH, encoding the Tamm-Horsfall membrane glycoprotein (TH)[1] by the modified DEAE-dextran procedure described above. Cells were then cultured for 72 h, the last 30 of which were in the presence of bromodeoxyuridine (DNA replication kit, Amersham Corp., Arlington Heights, IL). Cells were washed in calcium-free HBSS (cHBSS), detached from culture dishes by a 15-min incubation at 37°C in cHBSS + 10 mM EDTA, collected by centrifugation and washed twice in ice-cold cHBSS + 7% FCS, incubated with 1:100 goat anti-TH protein (Cappel Laboratories) for 1 h on ice, washed three times with cHBSS + 7% FCS, incubated with 1:500 FITC-conjugated donkey anti-goat Ig (Jackson Immuno Research Labs., Inc., West Grove, PA) for 1 h on ice, washed three times as before, and passed through a 60-μm nylon mesh. Approximately one million filtered cells were subjected to flow sorting (by Dr. John Hirst, Kaplan Cancer Center, New York University), and the brightest and dullest 5% of the distribution were recovered as separate fractions and sedimented onto slides. The cells were fixed in 95% ethanol, 5% acetic acid, and stained with a monoclonal antibromodeoxyuridine antibody (Amersham Corp.) followed by a phycoerythrin-conjugated donkey anti-mouse Ig antibody (Jackson Immuno Research Labs., Inc.).

1. *Abbreviations used in this paper*: GAP, GTPase activating protein; GDI, guanine nucleotide dissociation inhibitor; GNRP, guanine nucleotide release protein; MPF, mitosis/maturation promoting factor; TH, Tamm-Horsfall membrane glycoprotein.

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Ran/TC4   1  MAAQGEPQVQ FKLVLVGDGG TGKTTFVKRH LTGEFEKKYV ATLGVEVHPL
Spi1      1   M..PQNVPT ..........  ..........  .........I  ..........
HRAS      1         MTE Y...V..A..  V..SALTIQL  IQNH.VDE.D  P.IEDSYRKQ

Ran/TC4  51  VFHTNRGPIK FNVWDTAGQE KFGGLRDGYY IQAQCAIIMF DVTSRVTYKN
Spi1     50  H....F.E.C ..........  .L........  ..G..G....  .....I....
HRAS     44  .-VIDGETCL LDIL......  EYSAM..Q.M  RTGEGFLCV.  AINNTKSFED

Ran/TC4 101  VPNWHRDLVR VCE--NIPIV LCGNKVDIKD RKVKAKSIVF H-RKKNLQYY
Spi1    100  ..H.W.....  ...--.....  .......V.E  ......A.T.  .-........
HRAS     93  IHQYREQIK.  .KDSDDV.M.  .V...C.LAA  .T.ESRQAQD  LA.SYGIP.I

Ran/TC4 148  DISAKSNYNF EKPFLWLARK LIGDPNLEFV AMPALAPPEV VMDPALAAQY
Spi1    147  ..........  ..........  .V.N......  .S........  QV.QQ.L...
HRAS    143  ET...TRQGV  .DA.YT.V.E  IRQHKLRKLN  PPDESG.GCM  SCKCV.S  189

Ran/TC4 198  EHDLEVAQTT ALPDEDD-DL  216
Spi1    197  QQEMNE.AMP -......A..  215
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Figure 1. Ran/TC4 amino acid sequences. The complete sequence predicted for human and mouse Ran/TC4 is aligned with those predicted for *S. pombe* Spi1 (Matsumoto and Beach, 1991) and human H-RAS (Barbacid, 1987). Dots indicate residues identical to corresponding ones of Ran/TC4; dashes indicate gaps introduced to maximize homology. The sequence used to generate an anti-Ran/TC4 peptide antibody is underlined. The Ran/TC4 residues altered in the "double-activated" mutant construct are indicated with asterisks. The corrected human Ran/TC4 cDNA sequence has been deposited with GenBank (accession number M31469).

Results

Predicted Amino Acid Sequence of Human and Mouse Ran/TC4

We have resequenced our human Ran/TC4 clone (Drivas et al., 1990) on both strands using internal primers and have obtained a sequence that agrees well with those proposed from studies of the human protein (Bischoff and Ponstingl, 1991*a*) and of the homologous *S. pombe* gene (Matsumoto and Beach, 1991), as shown in Fig. 1. Of the 125 amino acid residues determined for the purified human protein, 124 agree with the sequence predicted for Ran/TC4 (Fig. 1). The exception is the nonconservative substitution of Ser (found at position 129) for Arg (predicted). Additional indirect support for the corrected sequence was provided by the isolation and analysis of a mouse testis cDNA clone whose nucleotide sequence was 90% identical to that of Ran/TC4 throughout its open reading frame (Hsieh, C.-M., V. Clarke, and M. G. Rush, data not shown), and which predicted an amino acid sequence identical to the corrected human Ran/TC4 sequence.

Ran/TC4 defines a novel subfamily of Ras-related proteins. It shares with H-RAS the four sequence domains that make up the guanine nucleotide binding pocket of the latter, including residues corresponding to H-RAS codons 12 and 61, whose mutations disrupt H-RAS GTPase activity. The Ran/TC4 sequence otherwise diverges considerably from that of H-RAS. Its distinctive features include a highly acidic carboxyterminal region that lacks the motifs associated with isoprenylation and membrane targeting characteristic of other RAS-related proteins (Barbacid, 1987; Drivas et al., 1991*a*; Der and Cox, 1991).

A Ran/TC4-specific Antipeptide Antibody

Previously, antibodies specific for Ran/TC4 have been obtained only in limited amounts by adsorption from complex antisera (Bischoff and Ponstingl, 1991*a*). To generate monospecific antibodies directly, we took advantage of the divergence of the carboxy-terminal sequence of Ran/TC4 from those of other RAS-related proteins to generate polyclonal rabbit antibodies specific for Ran/TC4. A hydrophilic dodecapeptide, indicated by underlining in Fig. 1, was synthesized, coupled to Keyhole Limpet hemocyanin, and used to immunize rabbits.

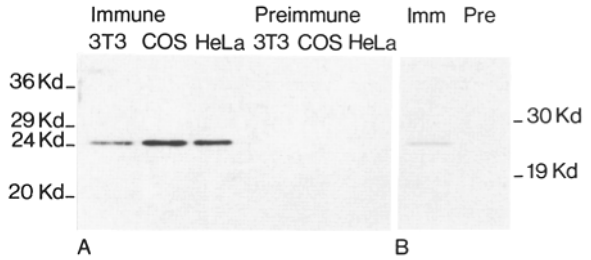

Figure 2. Cellular proteins recognized by anti-Ran/TC4 peptide antibodies. (*A*) Western blotting. Total cell lysates (50 μg total protein) prepared from 3T3, COS, and HeLa cells were fractionated by SDS-PAGE and analyzed by Western blotting with rabbit anti-Ran/TC4, or preimmune serum, and ^{125}I-protein A. Mobilities of size standards are indicated. (*B*) Immunoprecipitation. COS cells were metabolically labeled with [^{35}S]methionine and lysates were incubated with anti-Ran/TC4 antibodies, or preimmune serum, plus protein A–Sepharose. Precipitates were analyzed by SDS-PAGE and fluorography.

One of two rabbits tested yielded a high-titer, monospecific anti-Ran/TC4 antiserum. The antiserum reacted with a single 25-kD band in Western blots of extracts of bacteria and monkey cells harboring human Ran/TC4 cDNA in expression vectors. (The predicted molecular weight of Ran/TC4 is 24,423). It also reacted with a single 25-kD band in Western blots of total cell lysates of untreated mouse (3T3), monkey (COS), and human (HeLa) cell lines (Fig. 2 *A*). The same band was visualized in immunoprecipitates of [^{35}S]-methionine-labeled COS cell extracts resolved by SDS-PAGE (Fig. 2 *B*).

When COS and HeLa cells were reacted with anti-Ran/TC4 antiserum plus fluorescently labeled goat anti-rabbit Ig, staining was restricted to the nuclei of interphase cells. Mitotic cells were diffusely stained except that the metaphase chromosomes themselves were unstained (Fig. 3). Staining of 3T3 cells gave similar results (data not shown).

The absence of Ran/TC4 from metaphase chromosomes was confirmed using digitonin-permeabilized COS cells, in which most of the cytosol and background Ran/TC4 was removed (Fig. 4). Permeabilized mitotic cells were essentially unstained with anti-Ran/TC4, while metaphase chromosomes continued to be stained with anti-RCC1. Approximately 10 metaphases were examined with each antibody.

Dependence of the Nuclear Localization of Ran/TC4 on RCC1 Expression

Because of the genetic and in vitro biochemical evidence of interaction between Ran/TC4 and RCC1/Pim1 (Bischoff and Ponstingl, 1991*a*,*b*; Matsumoto and Beach, 1991), we asked whether the nuclear localization of Ran/TC4 is dependent on the presence of RCC1 in vivo. In the BHK cell line BHK21, Ran/TC4 and RCC1 proteins, as detected by immunofluorescence microscopy, were localized to cell nuclei at both 33.5 and 39.5°C (data not shown). The cell line tsBN2, a mutant derived from BHK21, synthesizes a temperature-sensitive RCC1 protein. Within 3 h of transfer from 33.5°C to the nonpermissive temperature of 39.5°C, immunologically detect-

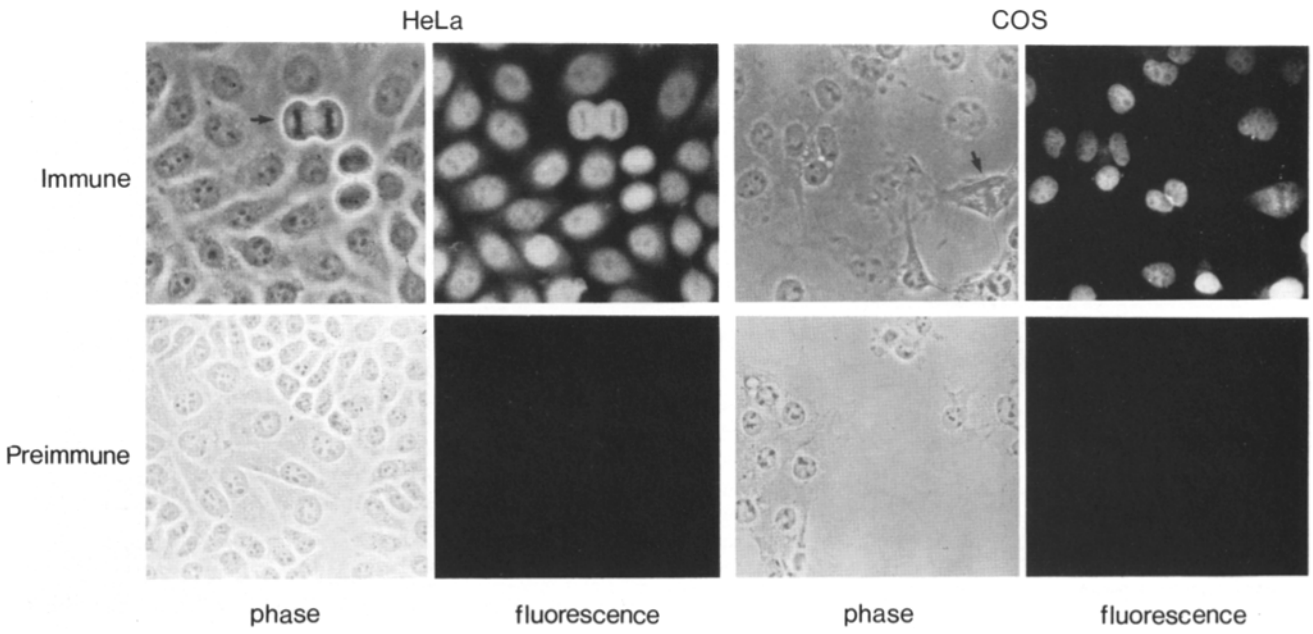

Figure 3. Nuclear localization of Ran/TC4. COS and HeLa cells were fixed, permeabilized, stained with rabbit anti-Ran/TC4, or preimmune serum, plus FITC-conjugated goat anti-rabbit Ig, and photographed under phase contrast or epiillumination. Arrows indicate mitotic cells.

able RCC1 in tsBN2 cells was greatly reduced (Figs. 5 and 7), a change which can be reversed only by returning the cells to the lower temperature and allowing new protein synthesis (Uchida et al., 1990; Nishitani et al., 1991). When we stained tsBN2 cells grown at 33.5°C with anti-Ran/TC4 antibody or with anti-RCC1 antibody, fluorescence was localized to cell nuclei (Figs. 5 and 6–0 h). When tsBN2 cells were incubated for 3–6 h at 39.5°C, however, RCC1 was no longer

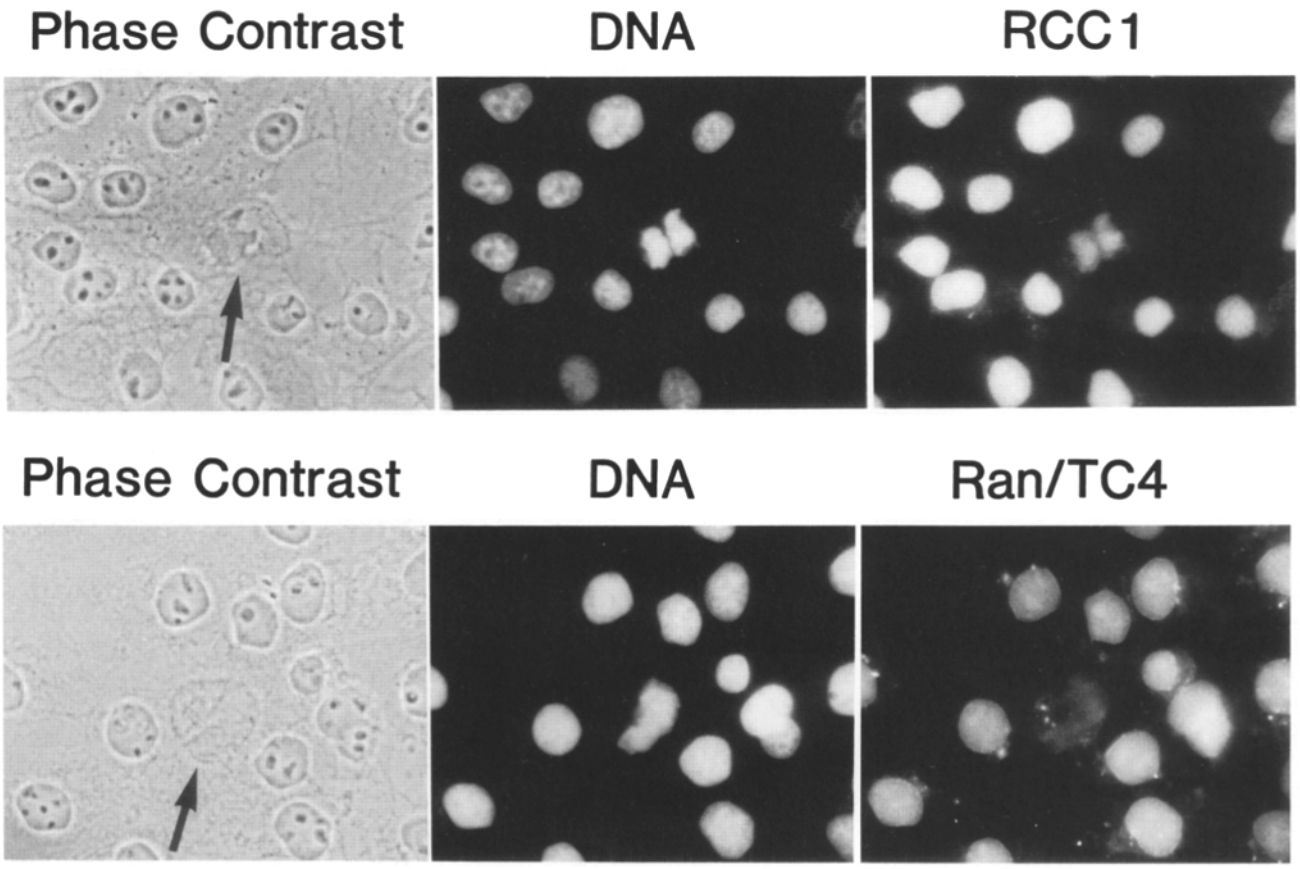

Figure 4. Localization of Ran/TC4 and RCC1 in digitonin-permeabilized COS cells. Cells were permeabilized, fixed, and stained with anti-Ran/TC4 or anti-RCC1. Arrows indicate mitotic cells.

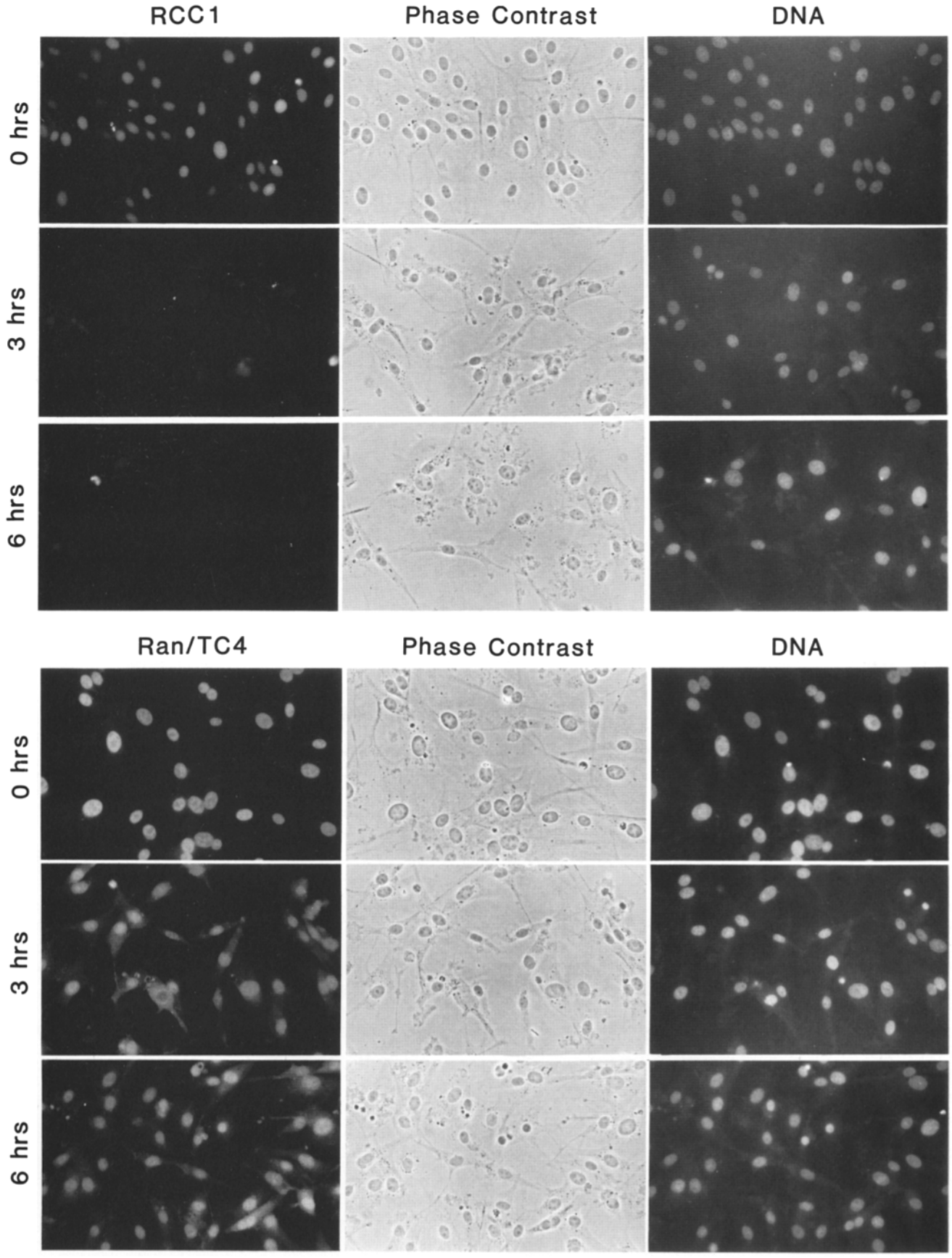

Figure 5. Subcellular localization of Ran/TC4 and RCC1 in tsBN2 cells—low magnification. Cells incubated for 0, 3, or 6 h at 39.5°C were fixed, permeabilized, and scored for Ran/TC4 and RCC1 localization as described in the legend to Fig. 3. DNA was localized by staining with HOECHST 33258 (Flow Labs., McLean, VA).

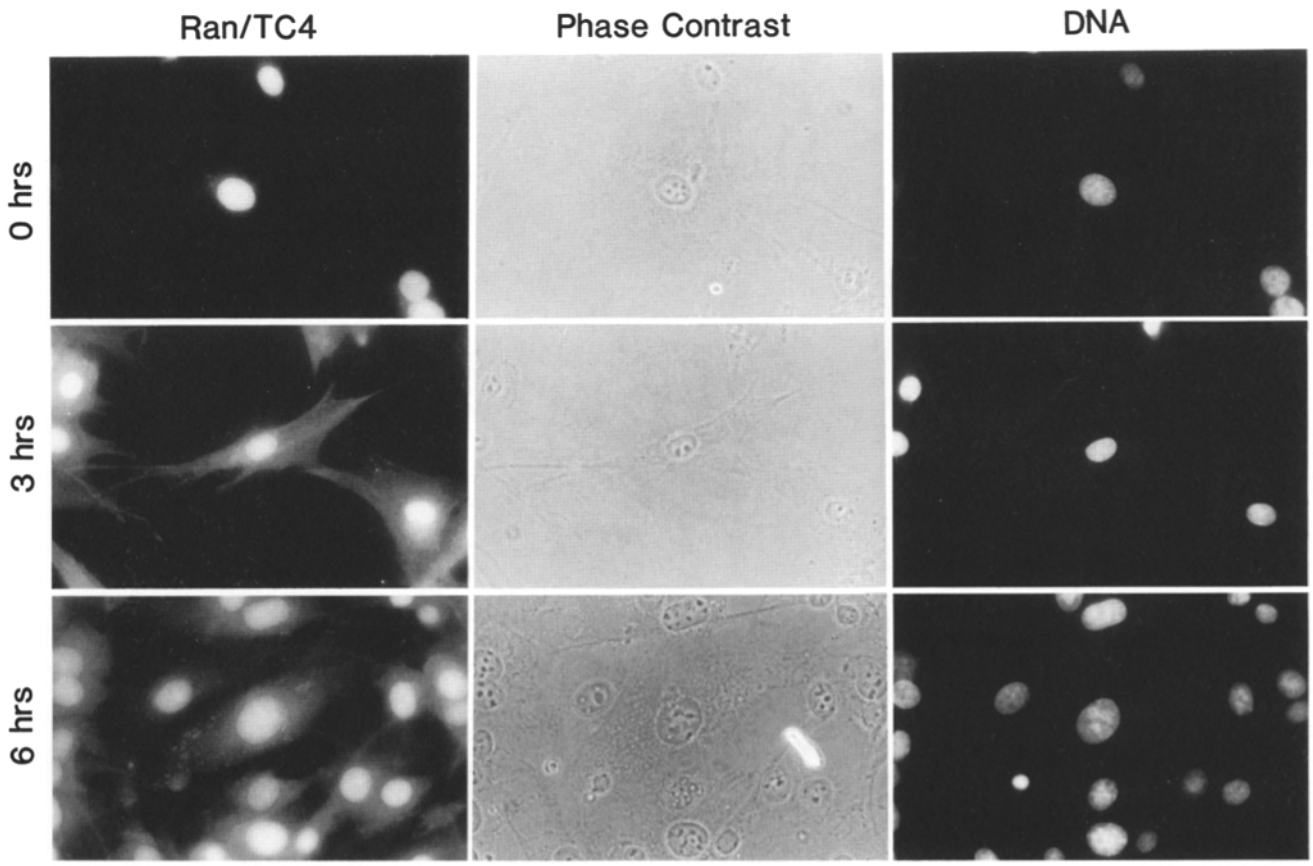

Figure 6. Subcellular localization of Ran/TC4 in tsBN2 Cells—high magnification. Cells incubated for 0, 3, or 6 h at 39.5°C were fixed, permeabilized, and scored for Ran/TC4 localization as described in the legend to Fig. 5.

detectable and substantial Ran/TC4 staining of cytoplasm was detected (Figs. 5 and 6). Visual scoring of photographs such as those shown in Fig. 5 indicated that at least 90% of cells exhibited Ran/TC4 cytoplasmic staining after a 3-h incubation at 39.5°C. Total cellular levels of Ran/TC4 protein were essentially unchanged over the course of these experiments in both tsBN2 and BHK21 cells (Fig. 7).

The release of Ran/TC4 from the nuclei of tsBN2 cells at 39.5°C was analyzed more quantitatively by using immunoblotting to measure the fraction of Ran/TC4 present in cytosolic extracts of digitonin-permeabilized cells. As shown in Fig. 7, the total amount of Ran/TC4 remained constant over 6 h at 39.5°C, but the fraction of this protein extracted by digitonin treatment increased from 19 to 55%. This redistribution of Ran/TC4 was specific to tsBN2 cells, as the fraction of Ran/TC4 released from normal BHK21 ranged between 10 and 15% over 6 h at 39.5°C. Both immunostaining and cellular fractionation procedures thus demonstrate that a significant fraction of Ran/TC4 protein is released from the nuclei of tsBN2 cells at 39.5°C.

Neither cell death nor increased entry into mitosis at the nonpermissive temperature is sufficient to explain the redistribution of Ran/TC4 into the cytoplasm. tsBN2 cells exhibit normal RNA and protein synthesis for at least 12 h at 39.5°C (Nishimoto et al., 1978). Also, after 6 h at 39.5°C, no more than 5% of unsynchronized tsBN2 cells exhibit a mitotic appearance (Fig. 5), consistent with the previous observation that after 6 h at 39.5°C, tsBN2 cells are arrested in G1 (Nishimoto et al., 1978; Nishitani et al., 1991). Most of these cells would have been in G1 at the time of the temperature shift and would have arrested there; the minority in S or G2 would be expected to progress through M to arrest in G1.

Effect of a Putative GTPase-deficient Mutant Ran/TC4 on Cellular DNA Synthesis

Members of the GTPase superfamily, including translation factors, heterotrimeric G proteins, and RAS and RAS-related proteins, function as switches, changing state according to the nucleotide, GTP or GDP, bound to the GTPase. Ran/TC4 is known to bind and hydrolyze GTP, and exchange of bound GDP for GTP is accelerated by interaction with RCC1 (Bischoff and Ponstingl, 1991*b*). We have therefore examined the functional consequences in vivo of transient expression of wild-type and mutated forms of the gene in COS cells.

The human Ran/TC4 cDNA was mutated in vitro to specify Val at codon 19 (equivalent to *H-RAS* codon 12) and Leu at codon 69 (equivalent to *H-RAS* codon 61). In previously studied RAS and RAS-related proteins, these substitutions alone or in combination cause GTPase deficiency (Bourne et al., 1991; Kaziro et al., 1991). The double-mutant and wild-type cDNAs were cloned into the pMT2 expression vector, which contains the SV-40 origin of replication. These constructs were transfected into COS cells, and protein products in cell extracts prepared 48–70 h after transfection were analyzed by SDS-PAGE. This system should allow transient expression of large amounts of any protein en-

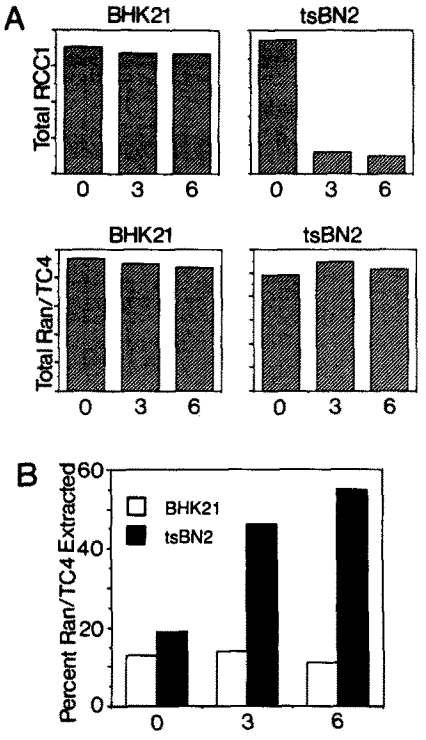

Figure 7. Quantitation of Ran/TC4 and RCC1 in tsBN2 and BHK21 Cells. (*A*) BHK21 and tsBN2 cells incubated for 0, 3, or 6 h at 39.5°C were analyzed for total Ran/TC4 and RCC1 proteins by immunoblotting and phosphorimaging. The bar graphs show the results of phosphorimaging in arbitrary units. (*B*) BHK21 and tsBN2 cells incubated for 0, 3, or 6 h at 39.5°C were treated with digitonin to generate cytosolic and residual fractions and the percentage of Ran/TC4 extracted in the cytosolic fraction was determined by immunoblotting and phosphorimaging.

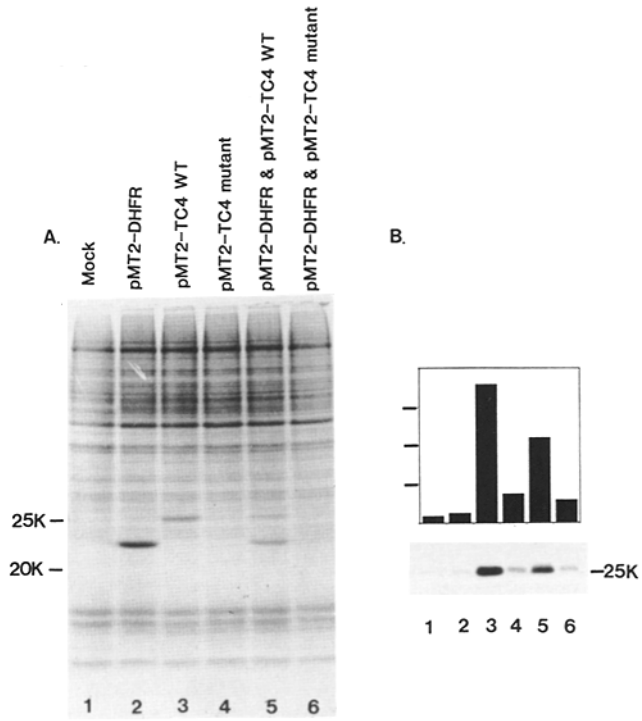

Figure 8. Proteins made in COS cells transfected with Ran/TC4 gene constructs. (*A*) SDS-PAGE. COS cells were transfected with (1) nothing (mock); (2) pMT2-DHFR, which specifies an ~22 kD protein; (3) pMT2-wild type Ran/TC4, which specifies an ~25 kD protein; (4) pMT2-mutant Ran/TC4; (5) equal amounts of pMT2-DHFR and pMT2-wild-type Ran/TC4; and (6) equal amounts of pMT2-DHFR and pMT2-mutant Ran/TC4. Cultures were harvested 62 h after transfection and analyzed by SDS-PAGE and Coomassie blue staining. (*B*) Western blotting. Total cell lysates (50 μg protein) prepared from each of the transfected cell populations (lanes 1–6) were analyzed by Western blotting as described in the legend to Fig. 2 *A*. The bar graph shows the results of densitometric analysis of the Western blot, in arbitrary units.

coded by the construct. Vector copy number should be high because the SV-40 replication origin in the vector interacts with T antigen produced by the COS cell host to generate hundreds of thousands of plasmid copies over the period of the experiment (Mellon et al., 1981), and gene expression should be efficient because the vector contains strong transcription promoters and translation enhancers (Kaufman, 1990).

COS cells transfected with the wild-type Ran/TC4 cDNA insert produced an abundant 25-kD polypeptide, visualized by Coomassie blue staining and measured by densitometric analysis of Western blots. Cells transfected with the Ran/TC4 double-mutant construct likewise yielded novel protein, but at 10% of the wild-type level (Fig. 8). This inhibition was also observed "in trans": when COS cells were cotransfected with pMT2-Ran/TC4 mutant constructs plus pMT2 constructs harboring dihydrofolate reductase, YL8 (a human homolog of the *S. pombe* YPT-3 RAS-related gene; Drivas et al., 1991*b*), or cytochrome P450, the latter proteins were produced at 10% of the levels observed in cotransfection experiments with wild-type Ran/TC4 constructs (Fig. 8 and data not shown).

Inhibition was a specific property of Ran/TC4 mutant constructs. COS cells transfected with pMT2 constructs containing the RAS-related cDNA clones TC10 or TC21 (Drivas et al., 1990), YL8, and five YL8 mutants (including GTPase⁻ ones) all produced high levels of the expected proteins (Zeng, J., and M. Ren, unpublished observations).

This reduction was due to failure of the transfected plasmids to replicate. Specifically, to demonstrate that transcription and translation of plasmid genes was normal, pMT2-Ran/TC4 mutant was cotransfected into COS cells with plasmid constructs whose product could be detected in the absence of plasmid replication. pRSV constructs encoding TH, β-galactosidase, or chloramphenicol acetyltransferase programmed the synthesis of proteins that could be detected even at low levels by immunofluorescence, β-galactosidase enzymatic activity, or chloramphenicol acetyltransferase assay, respectively. Equal levels of the products, as determined by densitometric analysis, were detected in COS cells transfected with the pRSV construct alone or with the pRSV construct plus pMT2-Ran/TC4 mutant (data not shown).

To show directly that the pMT2-Ran/TC4 mutant product blocked plasmid DNA replication, parallel cultures of COS cells were transfected with bacterially methylated pMT2-Ran/TC4 wild-type and mutant constructs. Supernatants prepared 60 h after transfection (Hirt, 1967) were digested with EcoRI (to linearize all plasmid molecules) plus DpnI (to digest methylated, hence unreplicated, DNA into multi-

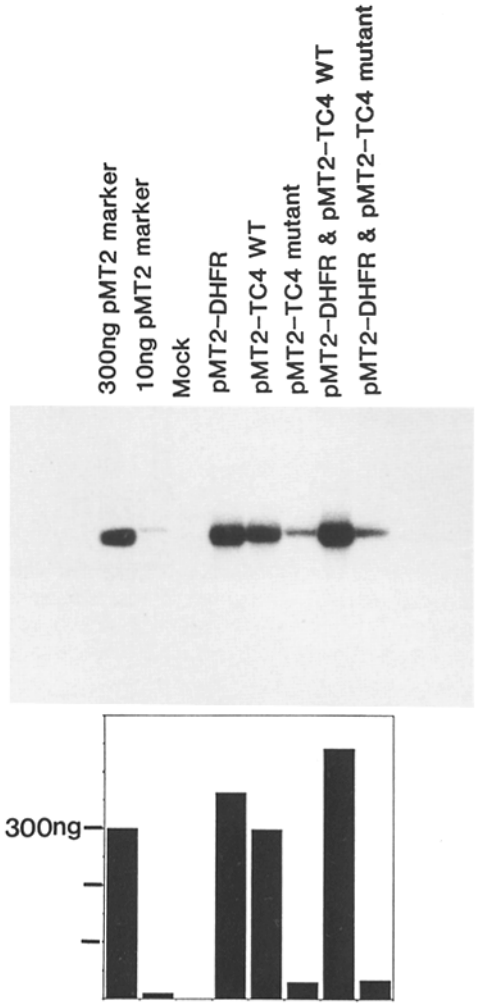

Figure 9. Correlation of transfection with mutant Ran/TC4 and cessation of DNA replication: cell populations. Supernatants (Hirt, 1967) from COS cells transfected with pMT2 constructs as described in Fig. 8 were analyzed by Southern blotting. Cells were transfected with nothing (mock), pMT2-DHFR, pMT2-wild-type Ran/TC4, pMT2-mutant Ran/TC4, equal amounts of pMT2-DHFR and pMT2-wild-type Ran/TC4, and equal amounts of pMT2-DHFR and pMT2-mutant Ran/TC4. The two leftmost tracks on the gel are loaded with 300- and 10-ng quantities of pure methylated pMT2 plasmid processed in parallel with the Hirt supernatants but digested only with EcoRI. The bar graph shows the results of densitometric analysis of the blot. Amounts of fragment in each track were calculated relative to the intensity of the 300-ng control fragment.

ple small fragments) and analyzed by Southern blotting. pMT2 DNA recovered as a large fragment (hence replicated after transfection) was reduced ~10-fold in extracts of mutant-transfected cells relative to cells transfected with the wild-type construct (Fig. 9).

To ask whether the mutant Ran/TC4 gene product would suppress host chromosomal DNA replication, we devised a FACS® assay to correlate expression of transfected genes with levels of DNA synthesis on a cell-by-cell basis. COS cells were cotransfected with pMT2-Ran/TC4 mutant plus pRSV-TH membrane protein and cultured at 37°C for 72 h. To assay replication of host cell chromosomal DNA, bromodeoxyuridine was added to the growth medium for the last 30 h of the culture period. Single cell suspensions were then stained with anti-TH antibody. FACS® analysis yielded populations of TH^+ cells (transfected—~25% of the total population) and TH^- cells (untransfected—~75%). These were collected and stained with antibromodeoxyuridine (Fig. 10). Most TH^- cells had incorporated bromodeoxyuridine, while most TH^+ cells had not. That is, presence of the Ran/TC4 mutant construct in a COS cell blocked its DNA replication. Transfection of other plasmids into COS cells, including pMT2-Ran/TC4 wild type, had no effect on bromodeoxyuridine incorporation. This result demonstrates a role for Ran/TC4 protein in either mammalian chromosomal DNA replication, cell cycle progression, or both, and confirms that the plasmid results are not an artifact due to averaging or to the specific replication properties of our transfected plasmids.

Discussion

Ran/TC4 is a remarkably well-conserved member of the *RAS* supergene family, specifying proteins of identical sequence in humans and mice and 80% identical between these species and *S. pombe* (Fig. 1). Using a dodecapeptide from its carboxy-terminal region, high-titer, monospecific polyclonal antibodies to Ran/TC4 were readily obtained (Figs. 2 and 3). Immunostaining experiments showed that Ran/TC4 is primarily restricted to the nuclei of interphase cells, and is diffusely distributed in mitotic cells (but excluded from the chromosomes). These staining patterns are consistent with the ones reported by Bischoff and Ponstingl (1991*a*) for antibodies extracted from a complex antiserum by adsorption to purified Ran/TC4 protein.

Ran/TC4 protein has been suggested to interact with RCC1 protein. As a structural correlate of that interaction, we have shown that nuclear localization of Ran/TC4 requires the presence of functional RCC1 protein (Figs. 5 and 6). The nuclear localization of Ran/TC4 and RCC1 is intriguing in two respects. First, although the amino-terminal DNA binding domain of RCC1 functions as a nuclear localization signal (Seino et al., 1992), neither protein includes clearly identifiable nuclear localization signals such as the stretches of arginine and lysine residues found in many nuclear proteins (Fig. 1; Bischoff et al., 1990; Garcia-Bustos et al., 1991). Second, while RCC1 protein is required to maintain the nuclear localization of Ran/TC4, the cellular molar ratio of Ran/TC4 to RCC1 is at least 10:1 and Ran/TC4·RCC1 complexes in vitro contain a 1:1 protein ratio (Bischoff and Ponstingl, 1990, 1991*a*). RCC1 must therefore be modulating the nuclear localization of Ran/TC4 by mechanisms in addition to simple binding.

A *Ran/TC4* mutant homologous to GTPase-defective *H-RAS* (Fig. 1) was constructed. When plasmids containing the mutant construct were transfected into COS cells, replication of the plasmid was blocked, as was replication of any cotransfected plasmid and replication of the host cell chromosomes (Figs. 8–10). The blockade appears to be replication specific, as transcription and translation of plasmid genes were not detectably affected. A crucial problem that remains unsolved is to identify the point(s) in the cell cycle at which cells arrest in response to mutant Ran/TC4 protein. Data from the experiments reported here are consistent with arrest at the G1/S boundary or in S caused by a direct effect of the *Ran/TC4* mu-

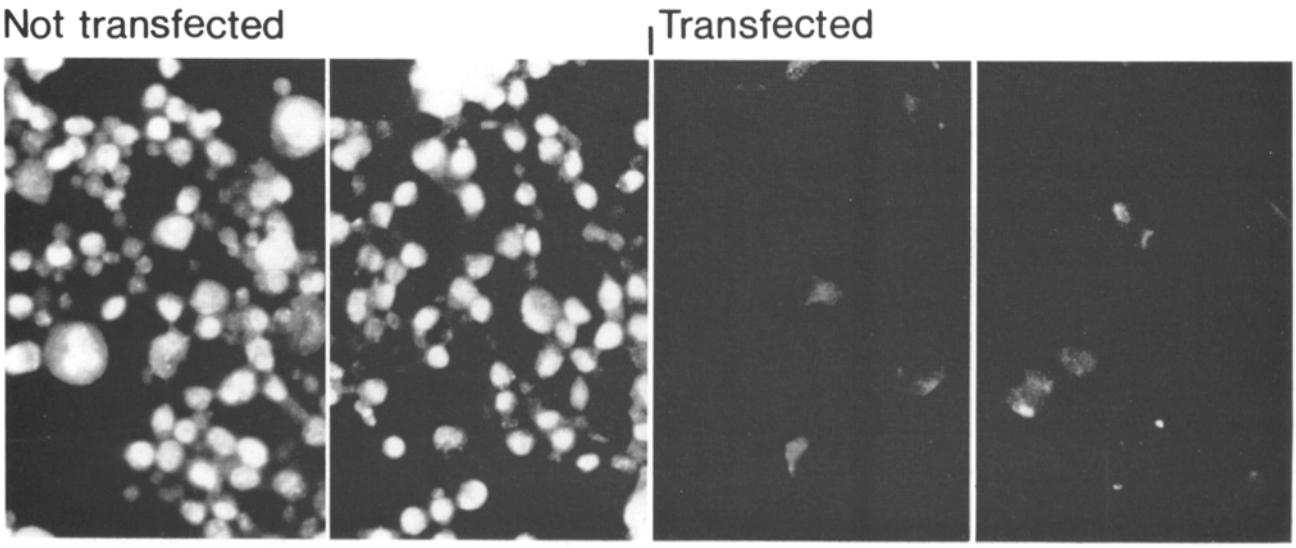

Figure 10. Correlation of transfection with mutant Ran/TC4 and cessation of DNA replication: single cells. COS cells cotransfected with pMT2-mutant Ran/TC4 plus plasmid pRSV-TH, encoding the TH membrane protein, were cultured for 72 h, the last 30 of which were in the presence of bromodeoxyuridine. Transfected and untransfected cells were resolved by flow sorting after staining with anti-TH antibodies, and DNA replication in each population was assayed by sedimenting cells onto slides, permeabilizing them, and staining with antibromodeoxyuridine antibodies. Two representative fields of each cell population are shown.

tant on DNA synthesis, but arrest elsewhere in the cell cycle cannot be excluded. Extension of the FACS® analysis of COS cells to measure DNA content was uninformative because the cells are heterogeneous and polyploid. Resolution of this issue will probably require studies of uniformly diploid cell populations.

A Hypothetical GTPase Switch Using Ran/TC4 and RCC1 to Monitor the Progress of DNA Replication

Guanine nucleotide binding proteins, whether they are true RAS proteins, members of the YPT/Rab subfamily involved in vesicle sorting, heterotrimeric G proteins, or protein synthesis initiation and elongation factors, function as switches whose GTP- and GDP-bound forms interact differentially with effectors and regulators (Bourne et al., 1990; Kaziro et al., 1991). Most guanine nucleotide binding proteins have low intrinsic rates of GTP hydrolysis and nucleotide exchange, so that the functional state of the switch is regulated by associated proteins that increase GTPase activity (GTPase activating proteins, or GAPs), and that inhibit or promote turnover of bound nucleotide (guanine nucleotide dissociation inhibitors, or GDIs, and guanine nucleotide release proteins, or GNRPs, respectively). For example, in the case of the bacterial protein synthesis elongation factor EF-TU, EF-TU·GTP binds to aminoacyl-tRNA, GAP and GDI activities are associated with the ribosome, and GNRP activity is provided by EF-TS. In the case of eukaryotic vesicle sorting, (G·GDP)·GDI complexes appear to be soluble, while GNRP and GAP activities may be associated with donor or acceptor membranes (for review see Pfeffer, 1992).

By analogy to these systems, models can be constructed in which Ran/TC4·RCC1 monitors the progress of DNA synthesis and couples the completion of DNA synthesis to the activation of mitosis/maturation promoting factor (MPF). MPF is a complex of two proteins, a cyclin and a serine-threonine protein kinase $p34^{CDC2}$ (for review see Freeman and Donoghue, 1991). MPF plays a key role in cell cycle control in all eukaryotes. Its regulation involves complex interactions among tyrosine and serine-threonine protein kinases, protein phosphatases, a cyclin protease, and other activators and inhibitors. MPF activation, required in prophase, involves at least cyclin–$p34^{CDC2}$ complex formation, cyclin phosphorylation, and activation of $p34^{CDC2}$ kinase via removal of esterified phosphate from a tyrosine residue. MPF inactivation, in anaphase, requires destruction of cyclin.

Two alternative models that both use RCC1 as a monitor of the completion of DNA synthesis, and that couple the cell cycle and a GTPase cycle involving Ran/TC4 and RCC1 are shown in Fig. 11. The models differ in the timing of GNRP and GAP activities and in the role of Ran/TC4·GTP as either an inhibitor or activator of MPF. Thus, in the upper model, START (the beginning of DNA synthesis or a signal that commits the cell to DNA synthesis) activates a GNRP activity, possibly RCC1, that stimulates the conversion of Ran/TC4 to its GTP-bound state, which in turn inhibits MPF activity. FINISH (a signal such as the elimination of single-stranded DNA or the disassembly of replication complexes that indicates the completion of DNA synthesis) attenuates GNRP activity, activates GAP, and thus sharply reduces the concentration of Ran/TC4·GTP. This in turn releases the inhibition of MPF. In the lower model, START activates GAP, FINISH activates GNRP, and Ran/TC4·GTP both stimulates MPF activation and directly inhibits DNA replication. A key feature of the lower model is the autoregulation of Ran/TC4·GTP function through the activation of a GDI that binds to Ran/TC4·GTP and prevents the further activation of MPF.

Our demonstration that expression of putative GTPase-defective Ran/TC4 protein inhibits DNA synthesis is consistent with either of these models.

In regard to the mechanism of MPF activation, our models predict that the unreplicated DNA present between START and FINISH alters levels of Ran/TC4·GTP. We speculate that this change is an essential step of a pathway that leads

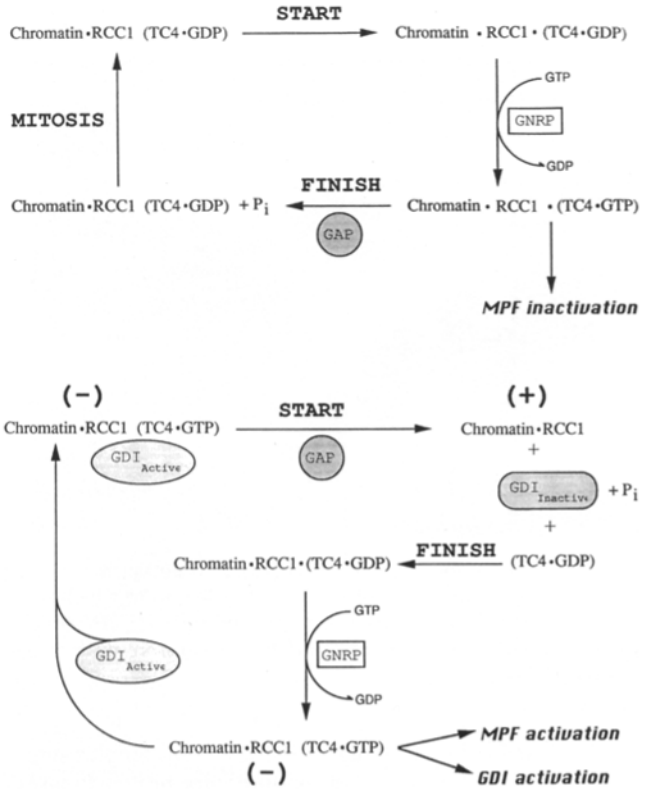

Figure 11. Two alternative models for coupling the cell cycle and a GTPase cycle through the nuclear proteins Ran/TC4 and RCC1. GAP and GDI are as-yet-unidentified proteins hypothesized to interact specifically with Ran/TC4 to promote GTP hydrolysis, and to inhibit effector interaction and nucleotide exchange, respectively. START and FINISH mark the beginning and end of DNA replication. GNRP activity appears to be provided by RCC1. In the lower model, (−) indicates the direct inhibitory effect of Ran/TC4·GTP on DNA synthesis and (+) indicates the release of this inhibition. Ran/TC4 is abbreviated simply as TC4 here.

to MPF inactivation, although there may be multiple intermediate steps. For example, considering the upper model of Fig. 11, Ran/TC4·GTP could stimulate, either directly or through regulatory kinases and phosphatases, the activation of the kinase that phosphorylates $p34^{CDC2}$ on tyrosine, thus inactivating MPF (Smythe and Newport, 1992). Ran/TC4·GTP could also inhibit, either directly or indirectly, the tyrosine phosphatase that dephosphorylates $p34^{CDC2}$ and is required for MPF activation (Millar and Russell, 1992). Finally, since the induction of premature chromosome condensation in tsBN2 cells at 39.5°C requires new protein synthesis (Uchida et al., 1990), the possibility that Ran/TC4 might regulate the synthesis of cell cycle control proteins cannot be excluded.

These models draw on results from both yeast and mammalian systems. There are important differences between these systems, however. *piml* ts mutants of yeast under nonpermissive conditions undergo chromosome condensation from any phase of the cell cycle (G1, G2, or S) and cells arrest with condensed chromosomes (Matsumoto and Beach, 1991). In hamster cell *RCC1* ts mutants under nonpermissive conditions, only cells in S and G2 proceed to chromosome condensation and mitosis, and cell arrest occurs at a point in G1 possibly analogous to the "start" point of the yeast cell cycle (Nishitani et al., 1991). The reasons for the yeast–hamster differences are unknown and may be specific to the mutant alleles studied in each species. The differences, however, do not alter the conclusions that *RCC1* and *Ran/TC4* play key roles in the orderly progression of the phases of the cell cycle.

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