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Effects of Mutant Ran/TC4 Proteins on Cell Cycle Progression

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***Ran/TC4*, a member of the *RAS* gene superfamily, encodes an abundant nuclear protein that binds and hydrolyzes GTP. Transient expression of a Ran/TC4 mutant protein deficient in GTP hydrolysis blocked DNA replication, suggesting a role for Ran/TC4 in the regulation of cell cycle progression. To test this possibility, we exploited an efficient transfection system, involving the introduction of cDNAs in the pMT2 vector into 293/Tag cells, to analyze phenotypes associated with mutant and wild-type Ran/TC4 expression. Expression of a Ran/TC4 mutant protein deficient in GTP hydrolysis inhibited proliferation of transfected cells by arresting them predominantly in the G_2, but also in the G_1, phase of the cell cycle. Deletion of an acidic carboxy-terminal hexapeptide from the Ran/TC4 mutant did not alter its nuclear localization but did block its inhibitory effect on cell cycle progression. These data suggest that normal progression of the cell cycle is coupled to the operation of a Ran/TC4 GTPase cycle. Mediators of this coupling are likely to include the nuclear regulator of chromosome condensation 1 protein and the mitosis-promoting factor complex.**

Ran/TC4 (Ras-related nuclear protein, cloned from a human teratocarcinoma cDNA library) defines a new subfamily of RAS-related proteins. The four stretches of sequence that specify the guanine nucleotide binding site of HRAS and define the *RAS* superfamily are conserved in Ran/TC4, but its predicted amino acid sequence is otherwise divergent (Fig. 1). Comparison of the predicted amino acid sequences of Ran proteins from diverse eukaryotes reveals several motifs specific to the Ran family, including an acidic carboxy-terminal sequence (DEDDDL in Ran/TC4) (5, 7, 34).

Like other proteins in the RAS superfamily, Ran/TC4 is a GTPase whose activity depends on the integrity of its GTP binding site and the presence of a GTPase-activating protein (GAP). Wild-type Ran/TC4 protein binds GTP but catalyzes only very slow hydrolysis of the bound nucleotide (6). Hydrolysis is sharply stimulated by incubation of the Ran/TC4 · GTP complex with a Ran-specific GAP from HeLa cells (7, 13). A Ran/TC4 protein altered in residues 19 and 69 (Fig. 1) binds GTP indistinguishably from wild-type protein but catalyzes no detectable hydrolysis of the GTP even in the presence of Ran-specific GAP (7, 13).

Ran/TC4 also interacts with a chromatin-associated DNA-binding protein, RCC1 (regulator of chromosome condensation 1). RCC1 was first identified as the product of a gene mutated in the tsBN2 temperature-sensitive baby hamster kidney cell line. Under nonpermissive conditions, tsBN2 cells in S or G_2 proceed directly to chromosome condensation and mitosis and arrest in G_1 (32). Bischoff and colleagues (5, 6, 8) have purified Ran/TC4 and RCC1 from HeLa cell nuclei and have shown that RCC1 interacts with Ran/TC4 · GDP complexes to promote guanine nucleotide exchange by the latter. That is, RCC1 is a Ran/TC4-specific guanine nucleotide release protein (GNRP) (5, 6, 8).

Although RCC1 is not a component of the basal complex required for initiation of eukaryotic DNA replication in vitro (24), it is required for replication of added sperm chromatin DNA in *Xenopus* egg extracts. Removal of RCC1 by immunoprecipitation blocked sperm chromatin DNA replication, and reconstitution of the depleted extract with *Escherichia coli*-expressed RCC1 protein largely restored it. RCC1 protein was present in these extracts in amounts sufficient to provide one molecule per nucleosome (15).

Inactivation of RCC1 in vivo leads to premature activation of MPF (mitosis/maturation-promoting factor) (32, 37). MPF is a complex of a mitotic cyclin and a cyclin-dependent serine-threonine protein kinase, $p34^{CDC2}$ (21, 23, 33, 38). MPF plays a key role in cell cycle control in all eukaryotes. In most cell types, a precursor of MPF (pre-MPF) is formed late in G_1 or in S, when newly synthesized cyclin polypeptide associates with $p34^{CDC2}$, and normally it is immediately inactivated by phosphorylation. Conversion of pre-MPF to active MPF in prophase requires partial dephosphorylation of $p34^{CDC2}$, while MPF inactivation and passage of cells through anaphase requires destruction of cyclin. The effects of RCC1 and Ran/TC4 on MPF can thus regulate cell cycle progression.

A plausible model that accommodates these data is that Ran/TC4, like other members of the RAS superfamily, functions as a GTPase switch. The Ran/TC4 switch would monitor and regulate cell cycle progression. RCC1 would be one of the sensor molecules that modulates the operation of the switch and links it to a downstream effector, MPF (Fig. 2) (34, 35).

This model derives from previous analyses of the role of small GTPases in protein synthesis, signal transduction, vesicle sorting, and cytoskeletal structuring. In these other systems, GTP- and GDP-bound forms of the GTPases interact differentially with effectors and regulators (9–11, 16). Most GTP-binding proteins, like Ran/TC4, have low intrinsic rates of GTP hydrolysis and of guanine nucleotide exchange, so the position of the switch is regulated by accessory proteins that interact with the GTP-binding protein. GAPs, by increasing the rate of GTP hydrolysis, promote the GDP-bound form of the switch, and GNRPs, by promoting exchange of bound for free guanine nucleotide in the high-GTP milieu of the cell, promote the GTP-bound form of the switch. Alteration in the functional state of these accessory proteins, in turn, can link a GTPase switch to other pathways in the cell (17, 28).

We propose (Fig. 2) that Ran/TC4 switches from a GTP-bound state during S phase to a GDP-bound state during the

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                                        *
Ran/TC4    1  MAAQGEPQVQ FKLVLVGDGG TGKTTFVKRH LTGEFEKKYV ATLGVEVHPL
HRAS       1         MTE Y...V..A.. V..SALTIQL IQNH.VDEYD P.IEDSYRKQ
                             ~~~~ ~~

                                        *
Ran/TC4   51  VFHTNRGPIK FNVWDTAGQE KFGGLRDGYY IQAQCAIIMF DVTSRVTYKN
HRAS      44  .-VIDGETCL LDIL..... EYSAM..Q.M RTGEGFLCV. AINNTKSFED
                          ~~~~~~

Ran/TC4  139  VPNWHRDLVR VCE--NIPIV LCGNKVDIKD RKVKAKSIVF H-RKKNLQYY
HRAS     133  IHQYREQIK. .KDSDDV.M. .V...C.LAA .T.ESRQAQD LA.SYGIP.I
                                   ~~~~~~~~          ~

Ran/TC4  148  DISAKSNYNF EKPFLWLARK LIGDPNLEFV AMPALAPPEV VMDPALAAQY
HRAS     143  ET...TRQGV .DA.YT.V.E IRQHKLRKLN PPDESG.GCM SCKCV.S    189
              ~~~~~

                                    ******
Ran/TC4  198  EHDLEVAQTT ALPDEDDDL  216
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FIG. 1. Ran/TC4 amino acid sequence. The complete sequence predicted for human Ran/TC4 (GenBank accession number M31469) (34) is aligned with the sequence of human HRAS (3). Dots indicate identical residues; dashes indicate gaps introduced to maximize homology. The sequences that form the HRAS guanine nucleotide binding pocket are marked with wavy underlines. The sequence used to generate anti-Ran/TC4 peptide antibody is underlined, and the amino acid residues altered for the expression studies described here are marked with asterisks (substitution of Val for Gly at residue 19, substitution of Leu for Gln at residue 69, and deletion of residues 211 to 216).

remainder of the cell cycle. In this model, START, the commitment of the cell to DNA replication, stimulates the GNRP function of chromatin-associated RCC1, converting Ran/TC4 to its GTP-bound form. Ran/TC4 · GTP then inhibits the conversion of pre-MPF to active MPF. RCC1 monitors DNA synthesis initiation and/or progression. FINISH inactivates RCC1 and activates the Ran/TC4-specific GAP, restoring Ran/TC4 to its GDP-bound form. Plausible FINISH signals include the elimination of single-stranded DNA at replication forks and/or the disassembly of replication complexes. According to this model, Ran/TC4 function would not be required during the pre-START G_1 phase of the cell cycle since pre-MPF levels are insignificant during this period. This model predicts that accumulation of a stable Ran/TC4 · GTP complex will result in arrest at the G_2/M boundary.

To test this model, we have determined the points in the cell cycle at which arrest occurs in the presence of Ran/TC4 mutant protein defective in GTP hydrolysis (and thus predicted to accumulate in a GTP-bound state). We have also determined that these effects on cell cycle progression require the presence of the acidic carboxy-terminal hexapeptide of Ran/TC4.

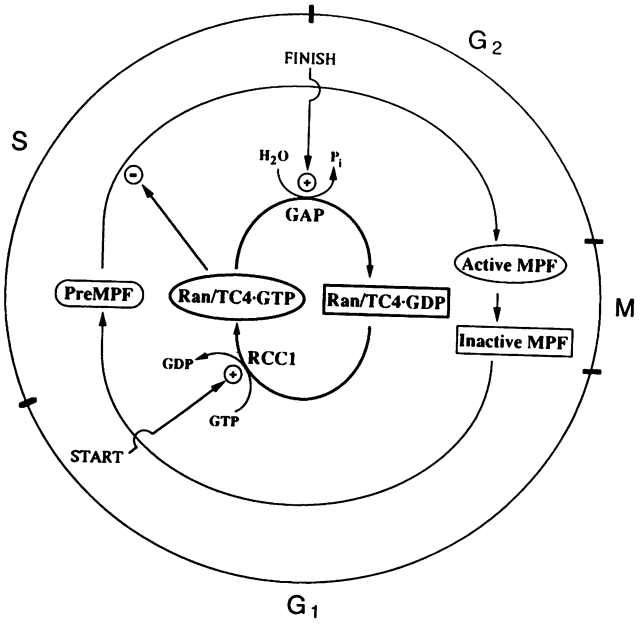

FIG. 2. A model for coupling the cell cycle and a GTPase cycle through the nuclear proteins Ran/TC4 and RCC1. The inner circle represents the Ran/TC4 GTPase cycle, the second circle represents the MPF cycle, and the outer circle represents the cell cycle. START is the point of commitment to DNA synthesis, as distinguished from the point at which replication begins. FINISH is the end of DNA replication. Key links between the cycles in the model are (i) the stimulation, at START, of the guanine nucleotide exchange activity of RCC1 and (ii) the role of Ran/TC4 · GTP as an inhibitor of the conversion of pre-MPF to active MPF.

MATERIALS AND METHODS

Materials and general procedures. 293/Tag cells, a human embryonic kidney cell line that expresses simian virus 40 large T antigen constitutively, were obtained from Garry Nolan, The Rockefeller University. Plasmid pMT2 (26) was obtained from Milton Adesnik, NYU Medical Center. Hydroxyurea and nocodazole were obtained from Sigma Chemical Company.

DNA sequencing, immunoblotting and immunostaining with anti-Ran/TC4 peptide antibody, and PhosphorImager analysis were performed as described previously (19, 34).

Ran/TC4 constructs. The insertion of wild-type and GTPase-defective mutant (dm) forms of the Ran/TC4 cDNA into the pMT2 plasmid has been described previously (34). Carboxy-terminal deletions of the wild-type and dm Ran/TC4 genes were generated by PCR using the pMT2 constructs as templates and *Pfu* DNA polymerase (containing 3′→5′ proofreading exonuclease activity) from Stratagene. Both downstream (GCGAATTCTCACGGGAGAGCACTTGTC) and upstream (GCGAATTCCGCGGAGACGCT) primers contained *Eco*RI restriction endonuclease cleavage sites, allowing the PCR fragments to be recloned into the pMT2 vector. Mutant constructs were confirmed by DNA sequencing.

DNA transfection. 293/Tag cells grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, in an atmosphere of 10% CO_2 at 37°C, were plated in 10-cm-diameter dishes at 10% of confluence 20 h before transfection. Cells were transfected with DNA-calcium phosphate coprecipitates (22). Ten micrograms of plasmid DNA in 0.5 ml of 244 mM $CaCl_2$ was mixed with an equal volume of 280 mM NaCl–1.5 mM sodium phosphate–55 mM *N*-2-hydroxyethyl-

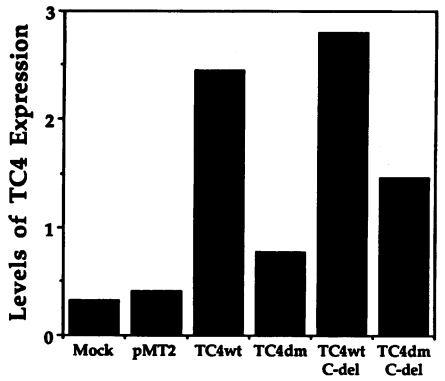

FIG. 3. Quantitation of Ran/TC4 levels in 293/Tag cells transfected with pMT2-Ran/TC4 constructs. Untransfected (mock) 293/Tag cells or cells transfected with 10 μg of pMT2 vector alone or with 10 μg of pMT2-Ran/TC4 constructs were incubated for 48 h, and extracts containing 50 μg of protein were analyzed for Ran/TC4 levels by immunoblotting and PhosphorImager analysis (34). Levels of Ran/TC4 expression (abbreviated here as TC4 expression) are in arbitrary units. Identities of constructs: wt, wild type; dm, GTPase-defective mutant; C-del, carboxy-terminal deletion; dm+C-del, combination of GTPase-defective mutant and carboxy-terminal deletion.

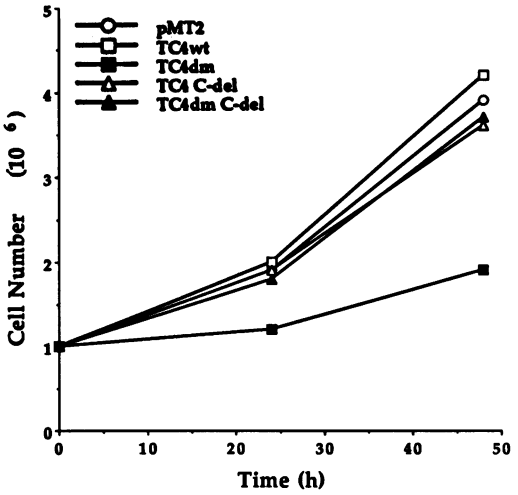

FIG. 4. Inhibition of cell division in 293/Tag cells transfected with pMT2-Ran/TC4 constructs. Subconfluent cultures of 293/Tag cells in 10-cm-diameter dishes were transfected with 10 μg of pMT2 vector alone or with 10 μg of pMT2 constructs containing Ran/TC4 genes. At the indicated times after transfection, cell cultures were harvested by trypsinization and counted. Designations are as in Fig. 3.

piperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.0) at room temperature and added immediately to one 10-cm-diameter culture dish containing 10 ml of Dulbecco modified Eagle medium supplemented with 10% fetal calf serum.

Cell proliferation. At various times after transfection, duplicate plates of cells were used to determine cell counts with a hemocytometer and to determine Ran/TC4 levels by immunoblotting and PhosphorImager analysis.

Flow cytometry. At 48 or 49 h after transfection, cells in 10-cm-diameter plates were trypsinized and washed twice in ice-cold Hanks balanced salt solution. A sample of cells was removed for analysis of Ran/TC4 levels by immunoblotting and PhosphorImager analysis. The remaining cells were fixed for at least 1 h in 70% ethanol, harvested by centrifugation, resuspended in Hanks balanced salt solution containing 100 μg of RNase (Sigma) per ml plus 50 μg of propidium iodide (Sigma) per ml, incubated for 30 min at 37°C, and analyzed for DNA content by flow cytometry with a Becton Dickinson FACScan in the NYU FACS Facility by John Hirst. For each cell population, 10,000 cells were analyzed and the proportions in G_1 and in G_2 and/or M (G_2+M) were estimated by using Modfit cell cycle analysis software (Verity Software, Topsham, Maine). Separate aliquots of stained cell populations were sedimented onto microscope slides and photographed by fluorescence microscopy, using an Axiophot microscope with a 40× Neofluar objective lens. Photographs were scored visually to determine mitotic indices.

RESULTS

293/Tag cells are an efficient transfection system for analysis of wild-type and mutant Ran/TC4 expression. We previously showed that in individual COS cells transiently expressing a GTPase-defective Ran/TC4 mutant protein, chromosomal DNA synthesis and extrachromosomal DNA synthesis were inhibited (34). This inhibition of DNA synthesis need not be due to blockage of the cell cycle in S phase, however. To determine the point(s) in the cell cycle at which cells expressing mutant Ran/TC4 protein are blocked, we carried out transient expression assays under conditions in which populations of transfected cells could readily be studied. We cloned wild-type and mutated forms of Ran/TC4 into the eukaryotic expression vector pMT2, which contains strong promoters of transcription and translation (26), and transfected these constructs into 293/Tag cells. These cells express simian virus 40 large T antigen, allowing extensive replication of the pMT2 vector which contains a simian virus 40 origin of replication. At least 80% of 293/Tag cells are transfected per experiment, as determined by cell sorting analyses identical to those described previously for transfected COS cells (34), so the 293/Tag transient transfection system is well suited for a population study of mutant gene expression.

We analyzed Ran/TC4 dm proteins made defective in GTP hydrolysis by the substitution of Val for Gly at position 19 and Leu for Gln at position 69 (Ran/TC4 dm) (13) and Ran/TC4 proteins lacking the six carboxy-terminal amino acids (Ran/TC4 C-del) (Fig. 1). Immunoblotting studies of 293/Tag cells transfected with wild-type or mutant Ran/TC4 expression constructs revealed less than twofold increases in levels of Ran/TC4 proteins 12 h after transfection (data not shown) but substantial increases in the amounts of Ran/TC4 protein above endogenous background levels 48 h after transfection (Fig. 3). The extent of Ran/TC4 overexpression varied between constructs but for each construct was approximately proportional to the amount of transfected DNA (data not shown). Consistent with previous observations (34), transfection of the Ran/TC4 dm construct led to substantially lower levels of Ran/TC4 protein synthesis than did transfection of the wild-type or C-del construct.

Expression of Ran/TC4 dm protein inhibits cell division. To extend our previous results indicating that DNA synthesis is blocked in cells expressing Ran/TC4 dm protein, both dm and wild-type Ran/TC4 were expressed in 293/Tag cells. DNA synthesis was measured by [^{3}H]thymidine incorporation and cell proliferation by cell counting. Over a 48-h time period, cell numbers increased about fourfold (two doublings) in cultures transfected with wild-type Ran/TC4 and no more than twofold (no more than one doubling) in cultures transfected with Ran/TC4 dm. Results of a typical experiment are shown in Fig.

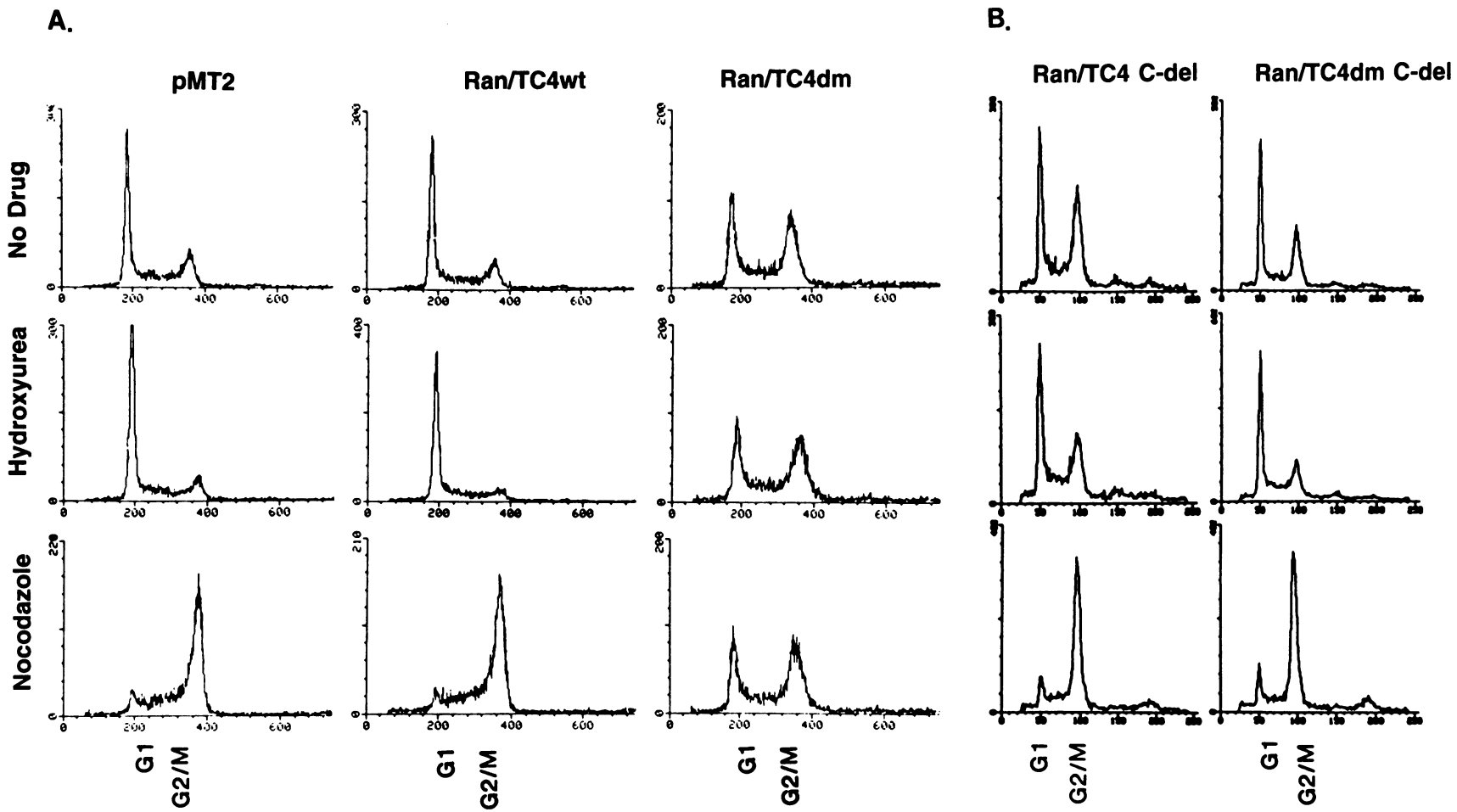

FIG. 5. Flow cytometry of 293/Tag cells transfected with Ran/TC4 wild-type, GTPase-defective, and carboxy-terminal deletion constructs. (A) Cultures were transfected with 10 μg of DNA (pMT2 [mock] or Ran/TC4 wild-type [wt] or dm construct in pMT2) and incubated for 38 h. Replicate cultures were then treated with no drug, 10 mM hydroxyurea, or 0.2 μg of nocodazole per ml, incubated for an additional 10 to 11 h, collected, stained with propidium iodide, and subjected to flow cytometry. (B) Cultures were transfected with 10 μg of DNA (Ran/TC4 C-del or Ran/TC4 dm+C-del construct in pMT2), incubated for 36 h, treated with hydroxyurea or nocodazole for 12 h, collected, stained with propidium iodide, and subjected to flow cytometry. Histograms are presented as cell number (ordinate) versus fluorescence intensity (abscissa) in arbitrary units. Each panel represents the analysis of 10,000 cells.

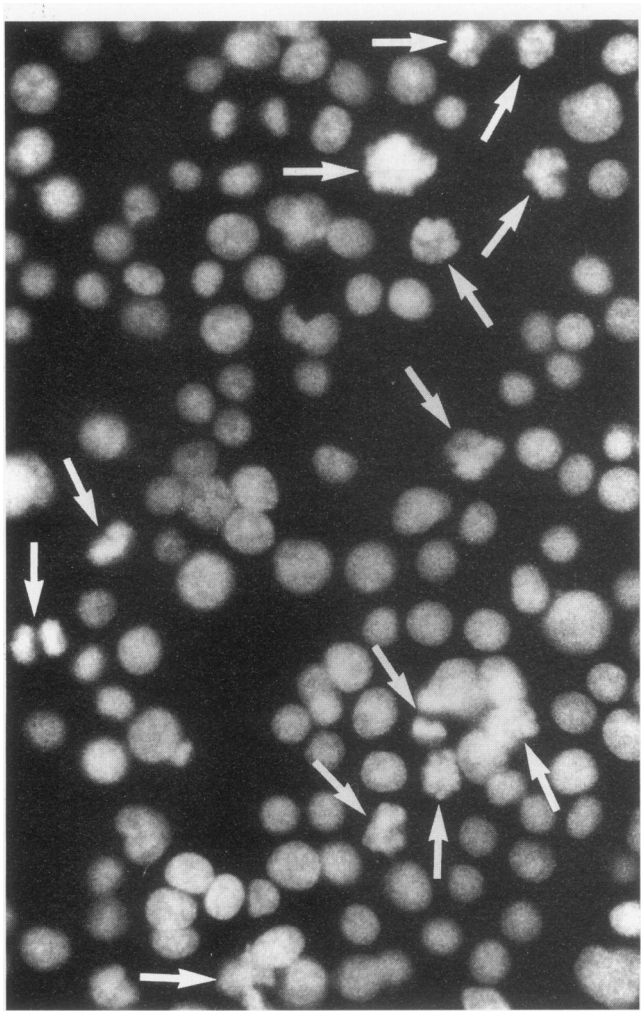

FIG. 6. Fluorescence photomicrograph of 293/Tag cells expressing Ran/TC4 dm protein. 293/Tag cells transfected with 10 μg of Ran/TC4 dm plasmid were incubated for 48 h, stained with propidium iodide, sedimented onto microscope slides, and photographed as described in Materials and Methods. Arrows indicate mitotic cells, which show intense granular fluorescence.

4. At 36 h after transfection, a 1-h pulse of [^{3}H]thymidine yielded no incorporation above background into acid-precipitable material in cultures transfected with Ran/TC4 dm; Ran/TC4 wild-type cultures showed levels of incorporation indistinguishable from those of mock-transfected controls (data not shown). These results confirm the inhibitory effect of Ran/TC4 dm on cellular DNA synthesis (34) and show that this effect is correlated with an inhibition of cell division.

Expression of Ran/TC4 dm+C-del protein fails to inhibit cell division. Since a rapid assay for Ran/TC4 function in vivo is inhibition of cell cycle progression by Ran/TC4 dm protein, we examined whether deletion of the carboxy-terminal DED DDL sequence characteristic of Ran proteins from the Ran/TC4 dm construct (dm+C-del) would affect this function. As a control, the same deletion was introduced into otherwise wild-type Ran/TC4 (C-del). These constructs, in the pMT2 vector, were transfected into 293/Tag cells. As shown in Fig. 4, the behavior of cells following transfection with either construct was similar to that of cells transfected with wild-type Ran/TC4, a result confirmed by [^{3}H]thymidine incorporation (data not shown).

Expression of Ran/TC4 dm protein induces arrest in both the G_2 and G_1 phases of the cell cycle. To identify the point(s) in the cell cycle at which 293/Tag cells expressing Ran/TC4 dm protein were arrested, transfected cells were analyzed by flow cytometry (Fig. 5A).

Analysis of cultures transfected with the Ran/TC4 wild-type construct or the vector alone revealed well-defined populations of cells in G_1 and G_2+M 48 to 49 h after transfection, with approximately four times as many cells in G_1 as in G_2+M (ratio of G_2+M to G_1 cells, approximately 1:4). If such cells were incubated with hydroxyurea, an inhibitor of DNA synthesis, for 10 h before flow cytometry, the G_2+M population was significantly depleted, while if they were incubated for 11 h with nocodazole, an inhibitor of mitosis, the G_1 population was significantly depleted. In cultures transfected with the Ran/TC4 dm construct, the proportion of G_2+M cells 48 to 49 h after transfection was substantially increased over that found in cells transfected with the wild-type construct or the vector alone (ratio of G_2+M to G_1 cells, approximately 1:1). These proportions were only slightly affected by treatment with hydroxyurea or nocodazole, consistent with the hypothesis that both the G_2+M and the G_1 cells were stably arrested, not continuing to progress through the cell cycle at a reduced rate. That is, expression of Ran/TC4 dm protein arrested a large fraction of transfected cells in G_2+M and a smaller fraction in G_1.

Expression of Ran/TC4 dm+C-del protein fails to induce cell cycle arrest. Under conditions in which populations transfected with the full-length Ran/TC4 dm construct exhibited cell cycle arrest (Fig. 5A), cells transfected with either the C-del or the dm+C-del construct showed no evidence of cell cycle arrest (Fig. 5B). These results demonstrate that the cell cycle arrest induced by expression of Ran/TC4 dm protein requires not only the point mutations in its GTP binding site but also the presence of its carboxy-terminal hexapeptide. The results also demonstrate the recessive phenotype of this carboxy-terminal deletion in an otherwise wild-type protein.

Cell populations arrested by expression of Ran/TC4 dm protein contain few mitotic cells. Flow cytometry as performed here resolves cell populations on the basis of DNA content and thus cannot distinguish G_2 cells from cells in M phase. To demonstrate that the large numbers of G_2+M cells accumulated in cultures expressing Ran/TC4 dm protein were mostly trapped in G_2, aliquots of some of the propidium iodide-stained cells used for the cytometry study shown in Fig. 5 were sedimented onto slides and examined by fluorescence microscopy. A photomicrograph of cells expressing Ran/TC4 dm is shown in Fig. 6. At least 300 cells in each treated cell population were scored visually to estimate the fraction in M. Half of the G_2+M population identified in cultures transfected with wild-type Ran/TC4 was mitotic, but only one-fifth of the G_2+M population in cultures treated with Ran/TC4 dm was mitotic, a 2.5-fold reduction.

Ran/TC4 dm+C-del protein localizes to the cell nucleus. Wild-type Ran/TC4 protein localizes predominantly to the cell nucleus (5, 34). To determine if the wild-type phenotype of Ran/TC4 dm+C-del protein was correlated with a defect in nuclear localization of this protein, transfected 293/Tag cells were analyzed by immunostaining (34). Cells were incubated in medium containing pMT2 construct DNA (vector alone, wild type, dm, C-del, or dm+C-del) for 12 h, replated onto polylysine-treated coverslips, and incubated for 22 h in medium lacking DNA. To allow newly synthesized proteins to reach their mature subcellular compartments, the cells were incu-

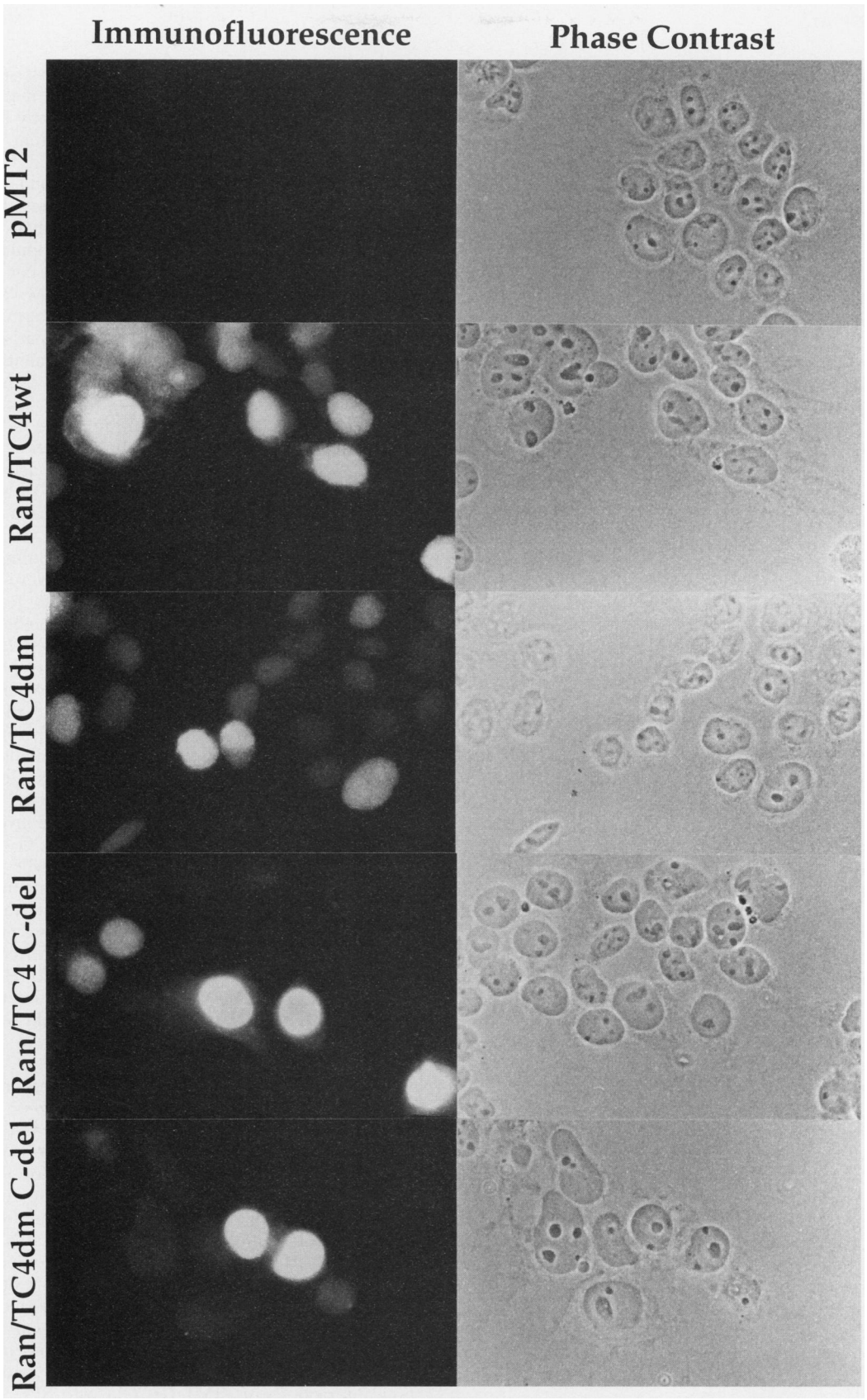

FIG. 7. Immunostaining of 293/Tag cells transfected with Ran/TC4 wild-type, GTPase-defective mutant, and carboxy-terminal deletion constructs. Cultures were transfected with 10 μg of DNA (pMT2 [mock] or Ran/TC4 wild-type [wt], dm, C-del, or dm+C-del construct in pMT2), incubated for 12 h, then replated onto polylysine-treated coverslips, and incubated for 24 h (the last two of which were in the presence of 10 μg of cycloheximide per ml). Cells were fixed and immunostained with a Ran/TC4-specific antipeptide antibody (34). The same exposure time was used to photograph each stained cell population.

bated finally for 2 h in medium plus 10 μg of cycloheximide per ml. Duplicate samples of replated cells then either were analyzed for total Ran/TC4 protein by PhosphorImager analysis of Western blots (immunoblots) or were fixed and immunostained.

Western blotting showed that the level of total Ran/TC4 protein in cell populations treated with the dm construct was approximately double that in mock-transfected cells; levels in cells transfected with all other constructs were increased four- to eightfold, consistent with previous results (Fig. 3). Immunostaining (Fig. 7) revealed cell-to-cell variation in the extent of Ran/TC4 overexpression in all transfected cell populations but consistent localization of Ran/TC4 protein to cell nuclei, even in cells expressing high levels of Ran/TC4 C-del or dm+C-del protein. The fact that nearly all staining above background was confined to cell nuclei, independent of total staining intensity, reinforces the conclusion that nuclear targeting is not affected by the absence of the six carboxy-terminal amino acids of Ran/TC4.

The staining obtained for cells transfected with the pMT2 vector alone, which represents endogenous Ran/TC4 (top panel of Fig. 7), was equivalent in intensity to that observed in similarly stained human (HeLa and 293/Tag), monkey (COS), and mouse (3T3) cells (reference 34 and data not shown). Exposure times were adjusted so that fluorescence due to endogenous protein was barely detectable, and most of the immunostaining shown in Fig. 7 was thus due to exogenously expressed Ran/TC4.

DISCUSSION

Expression of Ran/TC4 dm protein in proliferating 293/Tag human embryonal kidney cells blocked cell division (Fig. 4) by inducing arrest predominantly in the G_2, and secondarily in the G_1, phase of the cell cycle (Fig. 5). Deletion of the carboxy-terminal hexapeptide sequence, DEDDDL, from the mutant protein blocked this inhibitory activity. The same deletion in an otherwise wild-type protein had no detectable effect on cell proliferation (Fig. 4 and 5). The silencing of the cell cycle arrest phenotype of the dm construct by deletion of six carboxy-terminal amino acids suggests that these Ran subfamily-specific residues might be required, for example, for interaction with an effector.

The cell cycle arrest induced by the Ran/TC4 dm construct also explains the low levels of protein expression programmed by this construct in transiently transfected 293/Tag cells (Fig. 3). High levels of expression require replication of the construct DNA in the transfected cells (26), which is blocked even by low levels of Ran/TC4 dm protein (34). All other constructs, conversely, had no inhibitory effect on cell cycle progression, no blockage of construct DNA replication, and hence higher levels of expression of construct-encoded Ran/TC4 protein.

We have proposed that Ran/TC4 is a GTPase switch whose GTP-bound form generates a signal that blocks entry into mitosis. Cells expressing the Ran/TC4 dm protein should therefore be arrested late in G_2 (Fig. 2). Flow cytometry of cell populations arrested by expression of the Ran/TC4 dm construct revealed a substantial accumulation of cells with a fully replicated DNA complement (Fig. 5), and visual analysis indicated that most of these cells were in G_2 rather than M (Fig. 6).

How nearly complete is the barrier to entry into M in cells expressing Ran/TC4 dm protein? Nocodazole treatment of cells transfected with wild-type Ran/TC4 yielded a population 70% of which was in G_2+M by flow cytometry and 60% of which appeared mitotic by visual inspection (data not shown). That is, in a cell population blocked in M, six-sevenths of the blocked cells appeared mitotic. Therefore, if the cells transfected with Ran/TC4 dm were predominantly blocked in M, six-sevenths of cells with a G_2+M DNA content, or 43% of the total cell population ($6/7 \times 50\%$), should have appeared mitotic. Only 10% of the total cell population in fact appeared mitotic. Indeed, 10% probably overestimates the fraction of mitotic cells in the population expressing Ran/TC4 dm protein, inasmuch as 15 to 20% of the total cell population failed to undergo transfection with the Ran/TC4 dm construct.

The fractions of untreated cells in G_2+M varied between flow cytometry experiments, falling somewhat in cultures close to confluence at the time of analysis. Comparison of Fig. 5A (nearly confluent cells) and 5B (subconfluent cells) shows, however, that the effects of nocodazole and hydroxyurea were essentially unaffected by this variation.

The ability of the Ran/TC4 dm protein to induce G_2 arrest is consistent with our model of Ran/TC4 action (Fig. 2), since Ran/TC4 · GTP is predicted to inhibit the activation of pre-MPF. Its ability to induce G_1 arrest (Fig. 5) was unexpected. Two possible explanations for the G_1 block lead to distinct and testable predictions.

First, progression through G_1 may be regulated by another GTPase switch whose normal operation is overwhelmed by the presence of high levels of Ran/TC4 · GTP. Ran/TC4 shows only limited sequence similarity to other members of the RAS superfamily (Fig. 1) and does not interact detectably with GAPs of other RAS superfamily members (13). However, multiple transcriptionally active Ran loci have been identified in the mouse (12, 18). It is thus plausible to imagine that different Ran GTPase switches monitor progression past different points in the cell cycle and that abnormally high levels of Ran/TC4 · GTP could perturb more than one of these switches.

Second, a single Ran/TC4 switch could be involved not only in an S-to-M cell cycle control but also in a G_1-to-S cell cycle control, with accumulation of Ran/TC4 · GTP at more than one position of the cell cycle rather than only in S (Fig. 2). In analogy with the observation that MPF inactivation occurs at the metaphase-to-anaphase transition and is a prerequisite for mitotic exit (31), Ran/TC4 might be converted to its GTP-bound form in both S and metaphase. If so, Ran/TC4 · GTP would be present in G_1, where it could function as an inhibitor of START. Each turn of the cell cycle would require two full turns of the Ran/TC4 GTPase cycle instead of the single turn shown in Fig. 2.

In support of this more complex model, recent experiments in the fission yeast *Schizosaccharomyces pombe* suggest that Ran/TC4 is involved in controlling both entry into and exit from mitosis (27, 36). The *S. pombe pim1* (premature initiation of mitosis 1) gene product is a homolog of RCC1. One *S. pombe pim1* mutant allele allows entry into mitosis without completion of chromosomal DNA replication. In this regard, it is analogous to the RCC1 mutation in the tsBN2 hamster cell line (27, 32). A second *S. pombe pim1* allele does not perturb normal control of mitotic entry but blocks exit from mitosis (36). The phenotypes of both mutant alleles can be suppressed by overexpression of the wild-type *S. pombe* homolog of Ran/TC4, SPI1. The differences in the phenotypes of the two *pim1* mutant alleles provide strong genetic evidence for multiple interactions of PIM1 (RCC1) with SPI1 (Ran/TC4) in yeast cells, consistent with the notion that mammalian Ran/TC4 might also interact with RCC1 to generate different signals at different points in the mammalian cell cycle.

Recent results from other systems suggest that the roles of Ran/TC4 and RCC1 may be more general and more complex

than the foregoing discussion suggests. Ran and RCC1 are abundant proteins in all eukaryotes examined (7, 14). A mutant allele of the *S. cerevisiae* RCC1 homolog, *prp20*, causes accumulation of precursor mRNAs in the nucleus, aberrant transcription termination, and disruption of nuclear and nucleolar morphologies (1, 20). Two *S. cerevisiae* Ran homologs, GSP1 and GSP2, have been identified. Each has over 80% amino acid sequence identity with Ran/TC4, and overexpression of either GSP1 or GSP2 in the *prp20-1* mutant strain restores its viability and restores normal nucleoplasmic morphology (4, 25). These results suggest that Ran GTPases may play a role in nuclear organization and nuclear transport, a suggestion supported by the observation that the hamster tsBN2 RCC1 mutant cell line, which exhibits premature entry into mitosis, is also defective in mRNA transport at the nonpermissive temperature (2). Finally, in one in vitro model for protein import into nuclei that requires specific factors isolated from *Xenopus* oocyte cytosol, purified recombinant Ran/TC4 substitutes for purified *Xenopus* Ran protein (29, 30).

All of these functions may be interconnected. Wild-type Ran/TC4 and RCC1 might modulate the production of cell cycle control proteins, as part of a role in the general regulation of nuclear transport, by affecting traffic between the nucleus and cytosol of the relevant mRNAs or newly synthesized proteins. The diversity of systems now available should facilitate a rigorous dissection of the specific functions of Ran variant proteins and the systematic identification of the other cellular components with which they interact to regulate cell function.

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