The EMBO Journal vol.13 no.23 pp.5732–5744, 1994

A mutant form of the Ran/TC4 protein disrupts nuclear function in *Xenopus laevis* egg extracts by inhibiting the RCC1 protein, a regulator of chromosome condensation

M.Dasso, T.Seki[1], Y.Azuma[1], T.Ohba[1] and T.Nishimoto[1]

Laboratory of Molecular Embryology, NICHD, National Institutes of Health, Building 18, Room 101, Bethesda, MD 20892-5430, USA and [1]Department of Molecular Biology, Graduate School of Medical Science, Kyushu University, Fukuoka 812, Japan

Communicated by R.Laskey

The Ran protein is a small GTPase that has been implicated in a large number of nuclear processes including transport, RNA processing and cell cycle checkpoint control. A similar spectrum of nuclear activities has been shown to require RCC1, the guanine nucleotide exchange factor (GEF) for Ran. We have used the *Xenopus laevis* egg extract system and *in vitro* assays of purified proteins to examine how Ran or RCC1 could be involved in these numerous processes. In these studies, we employed mutant Ran proteins to perturb nuclear assembly and function. The addition of a bacterially expressed mutant form of Ran (T24N-Ran), which was predicted to be primarily in the GDP-bound state, profoundly disrupted nuclear assembly and DNA replication in extracts. We further examined the molecular mechanism by which T24N-Ran disrupts normal nuclear activity and found that T24N-Ran binds tightly to the RCC1 protein within the extract, resulting in its inactivation as a GEF. The capacity of T24N-Ran-blocked interphase extracts to assemble nuclei from de-membranated sperm chromatin and to replicate their DNA could be restored by supplementing the extract with excess RCC1 and thereby providing excess GEF activity. Conversely, nuclear assembly and DNA replication were both rescued in extracts lacking RCC1 by the addition of high levels of wild-type GTP-bound Ran protein, indicating that RCC1 does not have an essential function beyond its role as a GEF in interphase *Xenopus* extracts.

Key words: guanine nucleotide exchange factor/nuclear assembly/Ran/RCC1/*Xenopus* egg extracts

Introduction

The eukaryotic nucleus is a highly ordered structure that carries out an assortment of complex functions. In order for the nuclear tasks to be carried out properly, they must be temporally and spatially coordinated amongst themselves and with respect to the other functions of the cell. However, the essential organizing mechanisms used by the cell to achieve this coordination have largely yet to be discovered and characterized. Among the proteins that are thought to be important for maintaining nuclear integrity, two proteins that appear to be essential for the spatial and temporal order of the nucleus are Ran and RCC1. These two proteins interact enzymatically with each other and they are required for almost every nuclear process including RNA transcription and processing, nuclear transport, DNA replication and cell cycle control (reviewed by Dasso, 1993). Ran is a small, very abundant Ras-like GTPase which is mainly nuclear (reviewed by Moore and Blobel, 1994), while RCC1 is a chromatin-bound protein which acts as a guanine nucleotide exchange factor (GEF) for Ran (Bischoff and Ponstingl, 1991a,b). The manner in which these proteins are directly or indirectly involved in so many nuclear functions has yet to be clearly established. It is attractive to speculate that all of the nuclear defects observed in their absence result from either a loss of nuclear transport or an inability to rearrange internal nuclear structures, or both (Moore and Blobel, 1994). Since Ran and RCC1 play a critical role in the eukaryotic nucleus, it is of considerable interest to understand their interactions with other nuclear components and to discover the mechanism by which they act at a molecular level.

RCC1 was originally discovered in the tsBN2 cell line, a temperature-sensitive BHK21/13 cell line that carries a single point mutation in the *RCC1* gene (Nishimoto *et al.*, 1978; Uchida *et al.*, 1990). At the restrictive temperature, S phase-arrested tsBN2 cells degrade their RCC1 protein and enter mitosis prematurely, undergoing premature chromosome condensation (PCC) and nuclear envelope breakdown regardless of the replicative state of the DNA (Nishitani *et al.*, 1991). These observations demonstrated unambiguously that RCC1 is required for the checkpoint control mechanisms that ensure the correct temporal order of events in the cell cycle by detecting unreplicated DNA and blocking premature mitosis when it is present. Subsequently, a number of other groups isolated homologs of RCC1 independently by genetic screening for mutants defective in mRNA transcription, splicing and 3′-end formation (Aebi *et al.*, 1990; Forrester *et al.*, 1992) and in the regulation of the yeast mating pathway (Clark and Sprague, 1989). It has also been shown that mutants in RCC1 are defective in their capacity for RNA export and in their maintenance of the nuclear architecture (Aebi *et al.*, 1990; Kadowaki *et al.*, 1993). *In vitro* studies of RCC1-depleted *Xenopus* extracts have demonstrated that nuclear assembly from de-membranated sperm chromatin templates is aberrant in the absence of RCC1 and that nuclei thus assembled are completely unable to replicate their DNA (Dasso *et al.*, 1992). Taken together, these observations show that nuclei require RCC1 in order to maintain their integrity and functionality, as well as their temporal coordination to the cytoplasmic cell cycle.

Ran (originally named TC4) was discovered by virtue of its homology to Ras (Drivas *et al.*, 1990). It was subsequently demonstrated that RCC1 and Ran exist as a tight complex in the absence of magnesium and nucleotides

and that RCC1 acts as a guanine nucleotide release protein for Ran (Bischoff and Ponstingl, 1991a,b). The tsBN2 mutation presumably causes a decay of cellular Ran protein pools to the GDP-bound form after the loss of RCC1. Thus, the pleiotropic effects observed in these cells may result either from this decay or from the loss of the RCC1 protein itself. The genetic interaction between RCC1 and Ran homologs (named *pim*1 and *spi*1, respectively) has also been reported in *Saccharomyces pombe* along with evidence that Ran is important for the proper coordination of nuclear events in the cell cycle (Matsumoto and Beach, 1991). More recent observations re-confirmed the genetic relationship between the *S.pombe* homologs of RCC1 and Ran (renamed *dcd*1 and *fyt*1, respectively), but suggested that the primary defect in the cell cycle of these mutants occurs from their failure to properly reassemble their nuclei after mitosis rather than as a consequence of true PCC (Sazer and Nurse, 1994). These findings implicate Ran as an important factor for many of the same nuclear functions previously shown to require RCC1. However, the results in *S.pombe* also serve to highlight a difficulty in studying the Ran/RCC1 system. Deficiencies in this system appear to target multiple aspects of nuclear function, so that the defects observed may depend heavily upon the experimental protocol, organism and mutant allele employed.

Recent *in vitro* studies have further demonstrated that Ran is required for nuclear import in a digitonin-permeabilized cell assay (Melchior *et al.*, 1993; Moore and Blobel, 1993). These experiments were of particular interest, since the effect on transport *in vitro* would not be complicated by issues of nuclear assembly and cell cycle control, as would be the case for many of the *in vivo* observations. This result therefore shows a relatively direct requirement for Ran in transport, but this requirement is not yet understood at the molecular level nor is it by any means clear that all of the defects resulting from the loss of Ran/RCC1 function are solely the consequence of nuclear transport deficiencies. For instance, it has been suggested that Ran might be a 'cargo'-carrying factor, involved not only in nuclear transport but also in the dynamic rearrangement of nuclear components during the cell cycle (Moore and Blobel, 1994).

A series of mutations have been made in Ran which correspond to mutations known to have effects in Ras on nucleotide binding or turnover. These mutants have been assayed in different ways to determine their effects on the cell cycle and on nuclear functions. At present, the results from different systems appear to be somewhat contradictory. Ren *et al.* (1993, 1994) have reported the expression in tissue culture cells of a Ran mutant (G19V, Q69L double mutant) analogous to an activated form of Ras. This Ras allele causes the transformation of cells in which it is expressed, because the protein remains locked in an activated state through inability to hydrolyze GTP (reviewed by Bourne *et al.*, 1990). The expression of this double-mutant form of Ran inhibits DNA replication under some conditions and causes cell cycle abnormalities. Cells expressing this mutant form of Ran appear to be unable to progress through both the G_1/S and G_2/M phase transitions of the cell cycle (Ren *et al.*, 1994). These results have been taken as evidence that the GTP-bound form of Ran is able to halt cell cycle progression at the G_2/M boundary by invoking the checkpoint control mechanism that normally prevents the activation of mitotic factors before the completion of S phase. This interpretation is consistent with the earlier observations on tsBN2 cells cited above. In contrast to the observations in tissue culture cells, another GTPase-defective form of Ran (G19V-Ran) had little effect on nuclear assembly, DNA replication or the cell cycle in *Xenopus* extracts. However, the addition of T24N-Ran, which was predicted to be a predominantly GDP-bound form of Ran (Feig and Cooper, 1988), was able to disrupt cell cycle control and nuclear assembly in extracts (Kornbluth *et al.*, 1994). The results in *Xenopus* extracts are the exact opposite to those expected from tissue culture cells expressing mutant Ran and from tsBN2 cells.

We have been interested in understanding how T24N-Ran perturbs the Ran/RCC1 system and in discovering how it causes the observed phenotypes for nuclear assembly and the cell cycle in *Xenopus* extracts. We therefore set out to determine how this mutant acts in a detailed molecular manner, using both *Xenopus* egg extracts and *in vitro* assays with purified proteins. We found that T24N-Ran bound tightly to the RCC1 protein within the extract, resulting in its inactivation as a GEF for the endogenous wild-type Ran. The capacity of T24N-Ran blocked interphase extracts to assemble nuclei from sperm chromatin and to replicate their DNA could be restored by supplementing the extract with excess RCC1 and thereby providing excess GEF activity. In order to determine whether RCC1 had any role in nuclear assembly beyond its activity as a GEF, we examined the formation of nuclei in RCC1-depleted extracts supplemented with high levels of bacterially produced Ran protein. Nuclear assembly and DNA replication were both rescued by the addition of high levels of wild-type GTP-bound Ran protein, demonstrating that RCC1 is not essential for any function beyond its role as a GEF. Taken together, these results indicate that GTP-Ran is essential for nuclear assembly and DNA replication in *Xenopus* extracts and that these processes are blocked when nucleotide exchange is either inhibited by T24N-Ran mutant protein or absent because of RCC1 depletion.

Results

T24N mutant Ran protein associates tightly with nuclei and with RCC1 protein

The results of Kornbluth *et al.* (1994) suggested that the T24N-Ran protein was perturbing nuclear assembly and cell cycle control in some significant way, but the mechanism by which this occurred remained unclear. We therefore set out to determine how this mutant acts in a detailed molecular manner, using both *in vitro* assays with purified proteins and *Xenopus* egg extracts. *Xenopus* egg extracts are an extremely useful *in vitro* system for the analysis of nuclear assembly, DNA replication and the cell cycle (reviewed by Smythe and Newport, 1991). Nuclear assembly may be studied by observing nuclei formed around de-membranated sperm chromatin or phage λ DNA added to crude *Xenopus* egg extracts (Lohka and Masui 1984; Blow and Laskey, 1986; Newport, 1987). These nuclei can be directly assayed for a number of functions: they assemble nuclear envelopes, assemble a nuclear lamina,

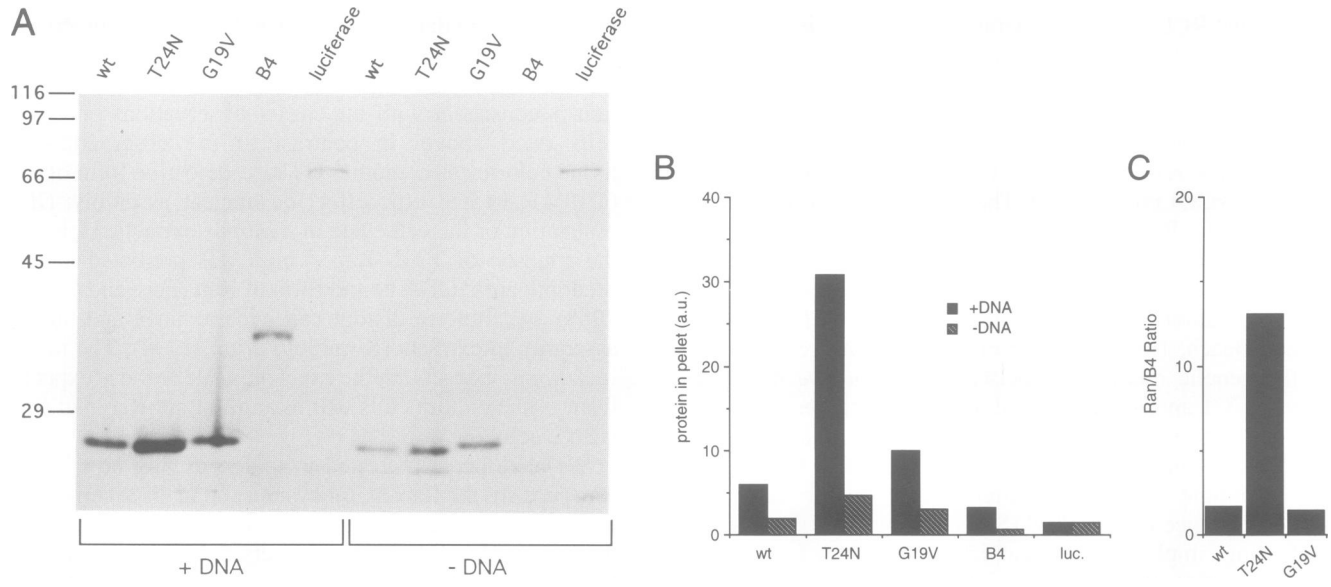


Fig. 1. Association of mutant Ran proteins with chromatin. (**A**) Affinity of mutant Ran proteins for nuclei. Crude extracts were incubated as described in Materials and methods, either with (+DNA) or without (−DNA) added sperm chromatin, as indicated. At the beginning of the incubations, ^{35}S-labelled wild-type Ran (wt), T24N-Ran (T24N) or G19V-Ran (G19V) was added to the incubation and allowed to associate with the assembling nuclei. After 2.5 h, nuclei and other large insoluble particles were remove by centrifugation, washed and repelleted. Equal amounts of the pellet proteins from each reaction were run on SDS−acrylamide gels and processed for direct autoradiography. As positive and negative controls, ^{35}S-labelled B4 protein and luciferase were subjected to similar treatment. B4 is thought to be a histone H1 homolog in the early *Xenopus* embryo (Dimitrov *et al.*, 1993; Hock *et al.*, 1993), while luciferase is a bacterial protein that was not anticipated to have any affinity for the eukaryotic nucleus. (**B**) Quantitation of association. The ^{35}S-labelled bands in (A) were quantified using a Molecular Dynamics PhosphorImager. Each set of columns shows the amount of protein found in the pellets from reactions containing nuclei (dark) or without DNA (hatched). (**C**) In order to compare the nuclear association of each of the different forms of Ran quantitatively, the three forms of Ran were co-translated with the B4 protein in reticulocyte lysates containing [^{35}S]methionine. The ratio of ^{35}S incorporation into each of the Ran proteins and into the B4 protein was approximately equal in the three translations. The translations were introduced into nuclear formation reactions in egg extracts, as in the previous experiment. The nuclei were pelleted by centrifugation, washed and repelleted. The pellets were resuspended and run on an SDS−polyacrylamide gel, which was processed for autoradiography. The amount of radioactivity in each band was quantitated by PhosphorImager analysis and the ratio of radioactivity in the Ran band to that in the B4 band is shown for each sample.

actively transport through their pores and undergo a single round of DNA synthesis per cell cycle. The replication of double-stranded DNA within these nuclei is entirely dependent on the formation of an enclosed nuclear envelope, but naked single-stranded DNAs can replicate to completion in membrane-free cytosol (Almouzni and Mechali, 1988; Sheehan *et al.*, 1988). High speed centrifugation separates crude extracts into several fractions, allowing the analysis of the sequential steps of nuclear formation and the further sub-fractionation of activities required for nuclear function (Newport, 1987). It has been demonstrated that a reconstituted extract made from the soluble cytoplasmic and particulate membrane fractions is competent to form nuclei from added sperm chromatin and to replicate the DNA within them (Newport, 1987; Wilson and Newport, 1988).

In order to understand the effect of T24N-Ran better, we wished to ascertain whether it was preferentially recruited to or excluded from the chromatin-bound subpopulation of Ran protein. To ask this question, we translated mRNA encoding wild-type, T24N- and G19V-Ran proteins in rabbit reticulocyte lysates containing [^{35}S]methionine. The translation reactions, which contained equal concentrations of the ^{35}S-labelled Ran proteins, were added to crude nuclear assembly extracts during the formation of nuclei from de-membranated sperm chromatin. The addition of reticulocyte lysate reactions provided ^{35}S-labelled Ran in amounts that were sufficient to detect the fate of the labelled proteins but insufficient to disrupt nuclear formation. Nuclear assembly was monitored visually, by phase microscopy and fluorescence microscopy with the DNA dye Hoechst 33258. The nuclei were indistinguishable in all of the reactions and there was no evidence that any of the translated proteins interfered with assembly (data not shown). DNA replication was also assayed by incorporation of labelled dNTPs to assure that normal nuclear function had not been disturbed. The incorporation of labelled nucleotides was equivalent in all reactions containing nuclei (data not shown).

After 2.5 h of incubation at room temperature, the extracts were diluted 10-fold with buffer, pelleted by centrifugation and washed once with buffer. The resulting pellets were resuspended in SDS sample buffer and run on polyacrylamide gels (Figure 1A and B). A significant pellet is obtained by this procedure whether or not sperm chromatin has been added, because the conditions of the centrifugation are sufficient to pellet membranes and organelles from the crude extract. We therefore prepared parallel reactions to which no DNA had been added as control for non-specific association of the labelled Ran protein to the membranes. As further positive and negative controls, *in vitro* translations of B4 protein and luciferase were subjected to similar treatment. B4 protein is a chromatin-associated protein, thought to serve as a histone H1 homolog in the early *Xenopus* embryo (Dimitrov *et al.*, 1993; Hock *et al.*, 1993), while luciferase is a bacterial protein that was not anticipated to have any affinity for the eukaryotic nucleus.

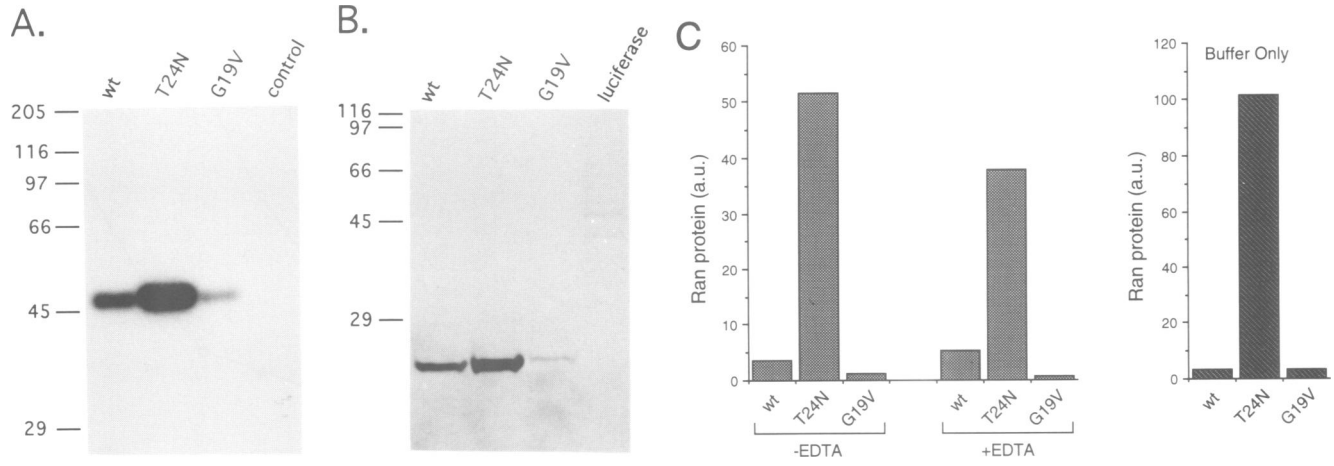


Fig. 2. Association of mutant Ran proteins with RCC1. (**A**) Association of RCC1 with GST−Ran fusion proteins. Fusion proteins consisting of an N-terminal glutathione-*S*-transferase (GST) moiety fused in-frame wild-type Ran (wt), T24N-Ran (T24N) or G19V-Ran (G19V) were incubated in *Xenopus* extracts for 80 min at room temperature. A similar reaction was made simultaneously with the addition of XB* buffer instead of a fusion protein preparation (control). The fusion proteins and any other extract components complexed with them were purified on glutathione−Sepharose beads. The proteins associated with the beads were eluted, electrophoresed on a SDS−10% acrylamide gel and Western blotted with affinity-purified anti-RCC1 antibodies. (**B**) Association of ^{35}S-labelled Ran proteins with a GST−RCC1 fusion protein. ^{35}S-labelled rabbit-reticulocyte translation products from each of the Ran cDNAs were incubated with *Xenopus* extracts and a GST−RCC1 fusion protein for 60 min. The proteins complexed with GST−RCC1 were purified on glutathione−Sepharose beads. They were eluted, electrophoresed on a SDS−12.5% acrylamide gel and processed for direct autoradiography. As a negative control, luciferase was also translated in the reticulocyte lysate; a roughly equal amount of labelled luciferase protein was added to a parallel reaction and treated similarly. (**C**) Bischoff and Ponstingl (1991a,b) demonstrated that the RCC1/Ran complex is very stable in the presence of EDTA, but that it can dissociate in the presence of magnesium and guanine nucleotides. We therefore examined whether the presence of EDTA in the binding buffer had a large effect on the stability of the GST−RCC1/Ran complexes. A comparison of samples that were diluted and washed with binding buffer (+EDTA) or XB* (−EDTA) were qualitatively similar (left histogram). We consistently saw a slightly lower relative recovery of the wild-type Ran protein in the absence of EDTA. A reasonable interpretation of these results might be that the GST-RCC−wild-type Ran complex dissociates more easily in the absence of EDTA than the complex containing T24N-Ran. A similar set of experiments in which the egg extract and ATP regenerating system were replaced by XB* buffer (right histogram, buffer only), gave results consistent with the interpretation that T24N-Ran binds more tightly to GST−RCC1 than wild-type Ran under these conditions. In this case, the recovery of T24N-Ran relative to the wild-type protein was even greater than when the egg extract was present in the initial incubation. This greater recovery may reflect the absence of completing Ran proteins from the egg extract.

In a number of independent experiments, we found that the level of accumulation of T24N-Ran protein was consistently higher than either wild-type or G19V-Ran. Typically, 4- to 7-fold more labelled Ran protein accumulated in the nuclei formed in the reactions containing the T24N-Ran than in nuclei with the other two forms of Ran (Figure 1C). Samples from the complete reactions were also run on SDS−acrylamide gels after nuclear formation to assure that there was no instability of the labelled Ran proteins and there appeared to be little or no degradation during the reaction (data not shown). Further, it is unlikely that the T24N-Ran protein became aggregated or preferentially associated with large, non-nuclear structures, since the amount of the three labelled proteins distributed to the pellets was essentially the same in the absence of DNA. Thus, this result suggests a significant preference for the association of T24N-Ran to nuclei. It was therefore likely that the T24N-Ran protein was perturbing nuclear assembly and cell cycle control by disrupting functions that normally occur in association with nuclear structures or with chromatin.

Ran binds to chromatin as part of a complex that also includes the RCC1 protein (Bischoff and Ponstingl, 1991b). We therefore wished to ascertain whether the T24N-Ran protein might become highly associated with nuclei by becoming preferentially bound to RCC1 and whether this could have some functional consequence for the Ran/RCC1 system that would explain the earlier observations regarding this mutant Ran protein. To ask this question, plasmid expression constructs were made encoding an N-terminal glutathione-*S*-transferase (GST) moiety fused in-frame to each of the Ran cDNAs and to the cDNA encoding RCC1. This was done to allow the rapid and clean purification of complexes containing Ran and RCC1 on glutathione−Sepharose beads. We then performed two experiments. In the first, equal amounts of the three purified bacterially expressed GST−Ran fusion proteins were incubated with clarified *Xenopus* cytosol for 80 min at room temperature. After this incubation, the samples were diluted with buffer and incubated with glutathione−Sepharose beads at 4°C for 60 min. The beads were removed by centrifugation and washed extensively with buffer. The GST−Ran proteins and cytosolic proteins that had been retained in association with the beads were then eluted with SDS sample buffer and boiling. Each of the samples was run on an SDS−polyacrylamide gel and Western blotted using affinity-purified anti-RCC1 antibodies. In a number of independent experiments, the T24N-Ran fusion protein consistently had a much higher affinity for cytosolic RCC1 than the wild-type Ran fusion protein (Figure 2A). Interestingly, the G19V-Ran fusion protein had much lower affinity for the cytosolic RCC1 than wild-type.

However, we did not have a convenient assay to determine whether the GST−Ran proteins were fully active biologically. Recent reports have suggested that GST−Ran fusion proteins are able to bind guanine nucleotides and to interact with RCC1 in mammalian extracts,

but that they might not interact normally with other proteins that associate with the endogenous Ran protein (Lounsbury *et al.*, 1994) Thus, there was some possibility that our results did not accurately reflect the behavior of the non-fusion Ran proteins, lacking the GST moiety. We therefore performed the inverse experiments using the GST−RCC1 fusion protein, because the activity of this protein could be easily confirmed by assaying its capacity to rescue nuclear formation and DNA replication in RCC1-depleted egg extracts. In fact, the extent of rescue and molar optimum concentration for this protein were very similar to that observed with preparations of bacterially expressed human RCC1 protein (data not shown). In the second experiment, we incubated equal amounts of ^{35}S-labelled Ran proteins produced by translation in reticulocyte lysates with the GST−RCC1 fusion protein and *Xenopus* cytosol. The samples were incubated with glutathione−Sepharose beads and extracted in a manner similar to the first experiment. The samples were electrophoresed on SDS−acrylamide gels and the amount of each of the ^{35}S-labelled Ran proteins was determined by direct autoradiography. Again, we found that ^{35}S-labelled T24N-Ran was more tightly associated with the GST−RCC1 fusion protein than the wild-type Ran, while the G19V-Ran protein was less tightly associated (Figure 2B). This tight association did not require the presence of EDTA in the dilution and wash buffers, nor did it require any components contributed by the egg extract (Figure 2C).

Thus, the results of these two different experiments are highly consistent with T24N-Ran becoming more tightly associated with RCC1 than either the wild-type or G19V mutant proteins and that it thereby becomes preferentially associated with chromatin. This suggests that T24N-Ran may act to alter nuclear formation and cell cycle control by either disrupting RCC1 function or by forming an inappropriate complex with RCC1. The idea that T24N-Ran blocks RCC1 function was particularly intriguing, given the similar defects in nuclear assembly in RCC1-depleted and T24N-Ran-containing extracts. We therefore wished to assay to discover whether T24N-Ran has a direct effect on the ability of RCC1 to act as a GEF for wild-type Ran.

T24N-Ran blocks the activity of RCC1 as a GEF for Ran

The capacity of T24N-Ran to block the function of RCC1 as a GEF was tested in two ways. First, we determined whether T24N-Ran inhibits guanine nucleotide exchange in *Xenopus* extracts and second, we determined whether T24N-Ran blocks the activity of RCC1 as a GEF in an assay using purified components. In order to test the effect of T24N-Ran protein on the nucleotide exchange capacity of *Xenopus* extracts, increasing amounts of either T24N-Ran or wild-type Ran were added to 25 μl of clarified cytosol. The nucleotide exchange capacity of the cytosol was determined using a nitrocellulose filter-binding assay for the release of [^{3}H]GDP that had been previously bound to bacterially expressed Ran protein (Figure 3; see Materials and methods). We found that the T24N-Ran protein was very effective in inhibiting nucleotide exchange activities in the *Xenopus* cytosol. The samples containing wild-type Ran showed a decrease in [^{3}H]GDP release only when the protein was added at a high

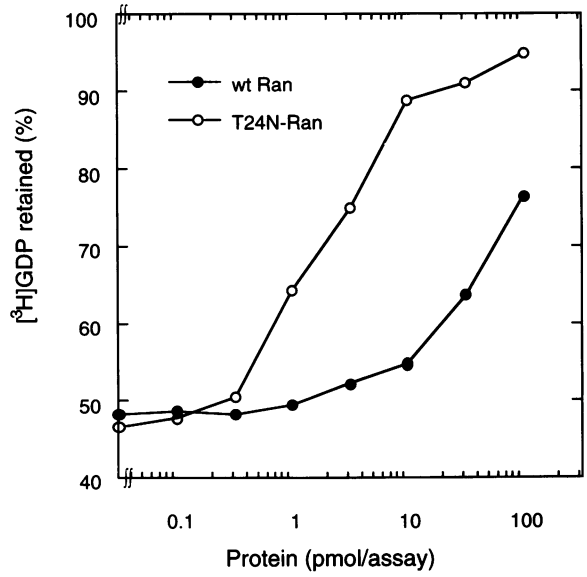


Fig. 3. T24N-Ran blocks guanine nucleotide exchange in *Xenopus* egg extracts. Wild-type Ran (●) or T24N-Ran (○) proteins were added to 25 μl of a reaction mixture containing 5 μl of clarified egg cytosol in the indicated amounts (see Materials and methods). In order to assay the exchange capacity of the cytosol containing added Ran proteins, it was mixed with an equal volume of [^{3}H]GDP-bound Ran protein and incubated at 27°C for 5 min. This incubation was terminated by the addition of ice-cold stop buffer and the samples were filtered through nitrocellulose. The filters were dried and the amount of [^{3}H]GDP retained in association with Ran was determined by liquid scintillation counting.

concentration. The inhibition by wild-type protein in this case could be attributed to a dilution of the [^{3}H]GDP-bound Ran by protein that was not in association with a labelled nucleotide. However, the T24N-Ran protein effectively blocked exchange activity in the extracts when it was present at much lower concentration (at least 30-fold less), such that the decrease in [^{3}H]GDP release could not be ascribed to a simple increase in the size of Ran protein pools. We also assayed a series of dilutions of egg cytosol which were preincubated with wild-type or mutant Ran proteins at a constant ratio of added Ran to cytosol (data not shown). Consistent with the prior observations, the cytosol pre-incubated with T24N-Ran showed significantly less GEF activity at all dilutions tested. In the course of these experiments we also observed that immunodepleted extracts of RCC1 have essentially no capacity to promote guanine nucleotide exchange, confirming that RCC1 is the major GEF for Ran in *Xenopus* extracts and arguing against the presence of other significant nucleotide exchange factors for Ran in *Xenopus* eggs (data not shown).

The previous observations clearly demonstrate that T24N-Ran blocks the activity of RCC1 as a GEF in the extract. In order to prove that T24N-Ran protein does this by directly inhibiting RCC1, we assayed the activity of purified bacterially expressed RCC1 in the presence of purified wild-type Ran or T24N-Ran (Figure 4). As a negative control, a similar reaction was conducted in the absence of RCC1 protein and as a positive control, buffer was added in the place of the Ran proteins. We found that the T24N-Ran protein was very effective in inhibiting RCC1 in this purified assay, while the wild-type Ran

protein had little effect on RCC1's GEF activity. This result demonstrated unambiguously that T24N-Ran blocks RCC1 through a direct mechanism rather than by an indirect interaction mediated by other components in the cytosol. Together, these experiments demonstrate that RCC1's activity as a GEF is blocked in the presence of mutant Ran and suggest that the endogenous Ran pools are driven into the GDP-bound state under these conditions. It was therefore important to examine whether nuclear assembly defects seen in the presence of T24N-Ran result solely from the depletion of GTP-bound Ran pools.

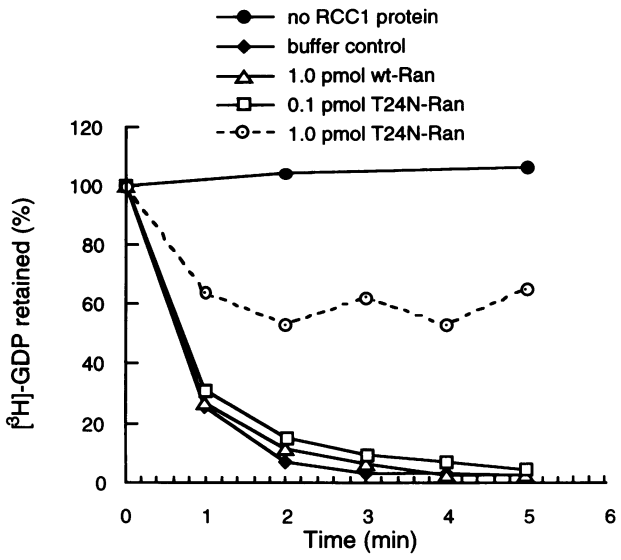


Fig. 4. T24N-Ran protein blocks RCC1 activity in an assay using purified proteins. Bacterially expressed, purified RCC1 protein was assayed for its capacity to catalyze the release of [^{3}H]GDP from purified wild-type Ran protein to which it had previously been bound. The conditions for this assay were essentially as those described for extracts, except that the final reaction mixture contained 40 fmol/assay of RCC1 protein rather than clarified egg cytosol. Wild-type Ran (1.0 pmol) that had previously been incubated with GDP (Δ), 1.0 pmol (○) or 0.1 pmol (□) of T24N-Ran that had not been incubated with guanine nucleotides, or buffer (◆) were also added from the start of the final reaction. As a control for spontaneous dissociation of [^{3}H]GDP from Ran, an incubation was also produced which contained neither bacterial RCC1 nor additional Ran (●). To determine the percentage of [^{3}H]GDP released from Ran, samples were taken at the indicated times and treated as described in Materials and methods.

Nuclear assembly and DNA replication defects caused by T24N-Ran can be reversed by the addition of excess RCC1

The previous experiments suggest that the addition of T24N-Ran protein perturbs the endogenous Ran/RCC1 functions of the extract by inhibiting the activity of RCC1 as a GEF. It was of interest to determine whether the effect of T24N-Ran can be reversed by supplementing the extract with excess RCC1. Alternatively, the presence of the T24N-Ran–RCC1 complex itself may disrupt endogenous functions, perhaps through recruitment of other important factors into blocked complexes. To discriminate between these possibilities, we added T24N-Ran protein to reconstituted assembly extracts in the minimum amount required for the inhibition of replication and nuclear growth. To separate aliquots of this reaction, we added a purified preparation of bacterially expressed RCC1 protein or buffer. The extent of nuclear assembly was determined visually and the amount of DNA replication was determined by examining the incorporation of [^{32}P]dCTP into high mol. wt DNA on agarose gels. In a number of independent experiments, the addition of RCC1 protein was fully able to restore nuclear assembly in extracts treated with T24N-Ran (Figure 5). These nuclei grew to a size comparable with that of nuclei incubated with buffer or wild-type Ran protein instead of T24N-Ran. The addition of RCC1 also restored DNA replication in extracts

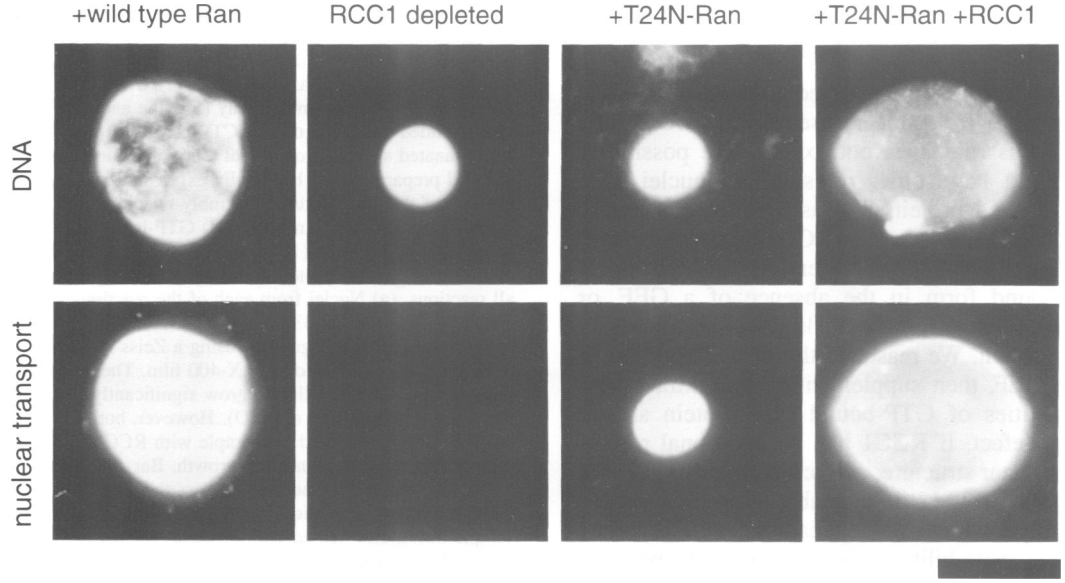


Fig. 5. The effect of T24N-Ran protein on nuclear assembly can be reversed by the addition of RCC1 protein. De-membranated sperm chromatin was added at 1000 nuclei/μl to extracts reconstituted with (from left) untreated cytosol plus 7.5 μg/ml wild-type Ran, RCC1-depleted cytosol, untreated cytosol plus 7.5 μg/ml T24N-Ran and untreated cytosol plus 7.5 μg/ml T24N-Ran and 10 μg/ml RCC1. The upper panels show nuclei stained with Hoechst 33258 DNA dye. The lower panels show equivalent exposures of nuclear uptake of a fluorescently labelled transport substrate. Bar = 5×10^{-6} m.

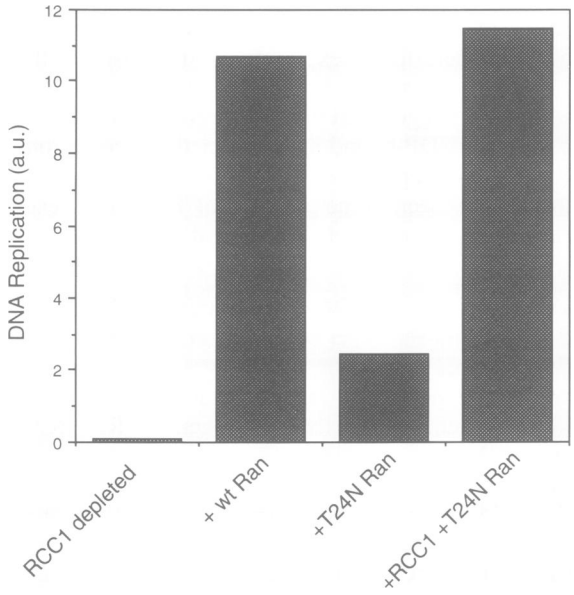
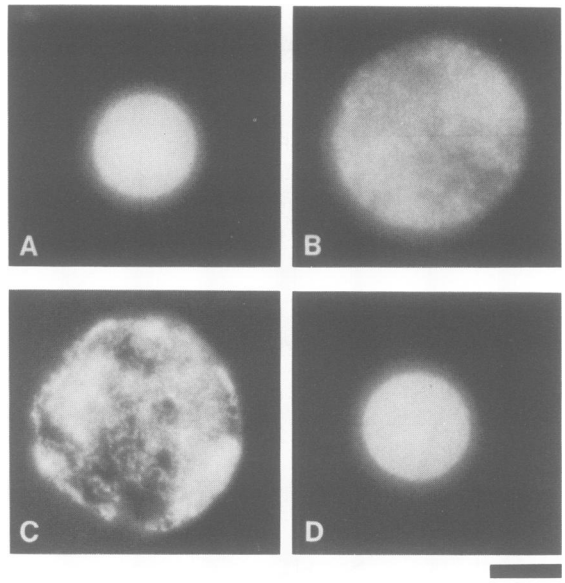


Fig. 6. The effect of T24N-Ran protein on DNA replication can be reversed by the addition of RCC1 protein. De-membranated sperm chromatin was added at 1000 nuclei/μl to extracts reconstituted in the presence of [α-^{32}P]dCTP using (from left) RCC1-depleted cytosol, untreated cytosol plus 5 μg/ml wild-type Ran, untreated cytosol plus 5 μg/ml T24N-Ran and untreated cytosol plus 5 μg/ml T24N-Ran and 10 μg/ml RCC1. After 4 h, the reaction was terminated by the addition of sample buffer and treated as described in Materials and methods. The incorporation of label into high mol. wt DNA was assayed by electrophoresis of the labelled replication products on a 0.8% agarose gel and quantitated using a Molecular Dynamics PhosphorImager.



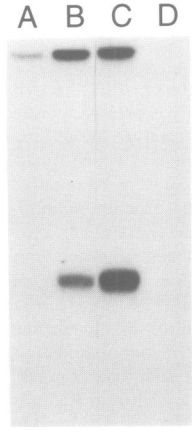


Fig. 7. Wild-type GTP-bound Ran protein can overcome the effects of RCC1 depletion in *Xenopus* extracts. Egg extracts were immunodepleted of the RCC1 protein by incubation with affinity-purified rabbit anti-RCC1 antibodies pre-bound to protein A−Sepharose beads (Dasso *et al.*, 1992). These extracts were used in a standard nuclear assembly assay (Smythe and Newport, 1991), which also included 1 μCi of [α-^{32}P]dCTP per 10 μl of the reaction and de-membranated sperm chromatin at a concentration of 1000 nuclei/μl. Purified preparations of bacterially expressed proteins were added to aliquots of this reconstituted assembly reaction as follows: (**A**) GDP-bound Ran protein (0.1 mg/ml), (**B**) GTP-bound Ran protein (0.1 mg/ml), (**C**) RCC1 protein (10 μg/ml). (**D**) XB* buffer only. The volume of buffer or purified protein made up one-tenth of the final mixture in all reactions. (**a**) Nuclei from each of the reactions were stained with the DNA dye Hoechst 33258 after 2 h of nuclear assembly. The nuclei were viewed and photographed using a Zeiss Axiophot microscope (63× objective) and Kodak TriX-400 film. The nuclei incubated with GDP-bound Ran (A) failed to grow significantly larger than those in the reaction with buffer only (D). However, both the sample with GTP-bound Ran (B) and the sample with RCC1(C) were restored in their ability to undergo nuclear growth. Bar = 2.5×10^{-6} m. (**b**) After 2.5 h, aliquots were removed to assess the amount of DNA replication in each reaction. The aliquots were added to an equal volume of sample buffer and treated with Pronase K (Smythe and Newport, 1991). These samples were run on a 0.8% agarose gel, which was dried and the amount of incorporation determined by direct autoradiography. DNA replication was extensively restored by the addition of GTP-bound Ran (B) or RCC1 (C). A very small amount of incorporation was seen with the addition of GDP-bound Ran (A) and essentially no [α-^{32}P]dCTP incorporation was observed when only buffer was added (D).

containing T24N-Ran to levels very close to the control extracts, indicating that the other functions of the nucleus had also been rescued (Figure 6). These results have two important implications. First, they confirm that T24N-Ran blocks nuclear assembly by inhibiting the GEF function required for the endogenous Ran and second, they show that the presence of stable Ran–RCC1 complexes does not inhibit nuclear assembly by sequestering other essential components of the system.

The previous result demonstrated that RCC1's GEF activity is essential for nuclear assembly. However, the importance of this role does not exclude the possibility that RCC1 might have other roles within nuclei as a chromatin-associated protein. It was therefore necessary to ascertain whether the defects of RCC1-depleted extracts result simply from the decay of the endogenous Ran pools to the GDP-bound form in the absence of a GEF, or whether these defects also reflect additional requirements of the RCC1 protein. We reasoned that if RCC1 is solely required as a GEF, then supplementing the extract with sufficient quantities of GTP-bound Ran protein should overcome any defect. If RCC1 had an additional role in chromatin or nuclear structure, extracts supplemented with high levels of Ran would still be unable to promote proper nuclear assembly and/or DNA replication. To differentiate between these possibilities, interphase cytosol was depleted of RCC1 by incubation with affinity-purified anti-RCC1 antibodies bound to protein A−Sepharose beads in the manner previously described (Dasso *et al.*, 1992). This RCC1-depleted cytosol was used to reconstitute nuclear assembly extracts with the addition of buffer,

or of bacterially expressed RCC1, GDP-bound Ran or GTP-bound Ran. We found that 0.1 mg/ml GTP-bound Ran was able to promote nuclear assembly (Figure 7A) and to restore DNA replication to 62% of the nucleotide incorporation obtained when extracts were rescued with RCC1 protein (Figure 7B). Preparations of GDP-bound Ran were much less active in rescuing nuclear assembly and DNA replication (11% of RCC1 control), demonstrating the specificity of this rescue and the requirement for the GTP-bound form of the protein. At very high concentrations of added Ran (>0.5 mg/ml), we observed complete rescue of DNA replication with GTP-bound Ran preparations and substantial rescue with the GDP-bound Ran preparations (data not shown). Rescue by GDP-bound Ran preparations may reflect the existence of residual GTP-Ran or some capacity of the GDP-Ran to restore RCC1-depleted extracts when added to high concentrations. Little RCC1 remained in RCC1-depleted extracts (1–2% of the RCC1 in control extracts), so we do not believe that this result reflects significant exchange of the GDP-bound Ran to the GTP-bound form. In any case, the much greater capacity of GTP-bound Ran to rescue nuclear formation and DNA replication indicates that this is the more active form of Ran for interphase functions in *Xenopus* extracts and confirms the essential role of RCC1 as a GEF for Ran in this system. Further, our findings demonstrate that any structural role of RCC1 can be accomplished by much less than the normal complement RCC1 within interphase nuclei and suggest that it is unlikely that RCC1 acts as a basic building block of the nucleus. Rather, RCC1's localization to the nucleus may be important for correctly directing the nucleotide exchange activity of Ran.

Discussion

We have used *Xenopus* extracts and *in vitro* analysis of purified proteins to examine the roles of the RCC1 and Ran proteins. We observed that T24N-Ran, a dominant-negative mutant that preferentially binds GDP (T.Ohba, manuscript in preparation), becomes tightly associated with the RCC1 and blocks its GEF activity in *Xenopus* extracts. The nuclear assembly and cell cycle defects caused by T24N-Ran may thus result from a failure to regenerate the endogenous Ran into its GTP-bound form or from a skewed ratio between GTP- to GDP-bound Ran pools. Consistent with this idea, the effect of the of T24N-Ran protein on nuclear assembly and DNA replication could be reversed by supplementing the extract with exogenous RCC1 and thereby providing excess GEF activity. In order to determine whether RCC1 had any function in nuclei beyond its activity as a GEF, we also examined the formation of nuclei in extracts lacking RCC1 but supplemented with high levels of bacterially produced Ran protein. Nuclear assembly and DNA replication were both restored by exogenous GTP-bound Ran, indicating that RCC1 itself probably does not play a large role in the nuclear assembly in this system. It therefore seems likely that RCC1's nuclear localization and association to chromatin may serve to facilitate its regulation as a GEF or to enhance its association with other components of the Ran/RCC1 system.

T24N-Ran blocks RCC1's activity as a guanine nucleotide exchange factor

The T24N mutation in Ran was made in analogy to the S17N-Ras mutant, which acts in a dominant-negative manner and inhibits cell proliferation when expressed in tissue culture cells (Feig and Cooper, 1988). Feig and Cooper suggested that this inhibition resulted because the mutant had an increased affinity for some component of the Ras pathway and thereby interfered with the activity of wild-type Ras protein. Subsequently, there has been indirect evidence to suggest that the S17N-Ras mutant acts by inhibiting the GEF for Ras *in vivo*, since overexpression of the Ras-GEF can reverse the growth-inhibitory effects of the S17N-Ras mutant (Schweighoffer *et al.*, 1993). An analogous T36N mutation in the Rab3A protein was directly demonstrated to increase its affinity for its GEF (Burstein *et al.*, 1992). In this report, we have demonstrated that the threonine to asparagine mutation within the nucleotide binding region of a Ran gives it an increased affinity for its nucleotide exchange factor, RCC1 (Figure 2). We have also demonstrated that this binding disrupts the normal nucleotide dynamics of the wild-type Ran (Figures 3 and 4). The ability of added RCC1 protein to reverse the effects of the mutant Ran protein (Figures 5 and 6) further confirmed the conclusion that RCC1 is the major target of T24N-Ran inhibition in *Xenopus* extracts.

In many ways, the addition of T24N-Ran may be functionally equivalent to a loss of RCC1. Nuclei formed under both of these conditions appeared very similar and failed to grow or to undergo DNA replication. The one exception to this generalization was the consistent observation that nuclei formed in RCC1-depleted extracts were more restricted in their capacity to carry out nuclear transport than those formed in the presence of mutant Ran (Figure 5, lower panels). Nuclei formed in the presence of T24N-Ran demonstrated a significant capacity for import of an artificial nuclear transport substrate (see Materials and methods). In contrast, nuclei formed in RCC1-depleted extracts typically had a much lower rate of substrate import. As would be predicted from their lower transport capacity, RCC1-depleted nuclei were also variable in the extent to which they formed a nuclear lamina. Nuclei formed in the presence of T24N-Ran typically had a well formed nuclear lamina, as judged by indirect immunofluorescence with monoclonal anti-*Xenopus* LIII antibodies (Stick and Hausen, 1985; M.Dasso, unpublished observations). The simplest explanation for the differences between RCC1-depleted and T24N-Ran treated nuclei may be that the T24N-Ran mutant was less effective in blocking GEF function than RCC1 depletion. However, the similar morphology of nuclei under these two conditions does show that the inhibition of nuclear growth in *Xenopus* extracts by T24N-Ran is not simply proportional to decreased transport capacity—nuclei formed with T24N-Ran have far more transport capacity than RCC1-depleted nuclei, yet they fail to grow any larger. We have also observed that T24N-Ran has little effect on nuclear structure or transport when it was added to extracts after nuclei are assembled (M.Dasso, unpublished observations). These results could suggest that nuclear assembly requires a larger amount of GTP-Ran than nuclear transport.

The nuclear role of RCC1 protein

The capacity of GTP-bound Ran protein to rescue RCC1-depleted nuclei demonstrates that RCC1 has little role as a structural protein in the nucleus, if any at all. Since RCC1 is a very abundant chromatin-associated protein, it was previously attractive to suggest that it might be directly involved in maintaining the structure of the chromosome or of the nucleus (Dasso *et al.*, 1992). Data presented here are inconsistent with this hypothesis, since GTP-bound Ran protein alone is sufficient to overcome the defects of nuclear assembly and DNA replication caused by RCC1 depletion (Figure 7). Approximately 98–99% of the RCC1 protein was removed from *Xenopus* extracts by the immuno-depletion protocol employed (Dasso *et al.*, 1992). While we cannot formally exclude the possibility that the small residual pool of protein performs some useful function in nuclear assembly, we consider it unlikely that RCC1 is itself required as a major building block in nuclear construction. Some previous *in vivo* observations may point toward a similar conclusion. Overexpression of Ran homologs in yeast can suppress some temperature sensitive alleles of RCC1 (Matsumoto and Beach, 1991; Belhumeur *et al.*, 1993; Kadowaki *et al.*, 1993; Sazer and Nurse, 1994). However, it was not stated in those reports whether the high levels of Ran suppressed defects in RCC1 by circumventing the requirement for RCC1 as in our experiments, or simply by stabilizing the mutant proteins. Together, these results imply that RCC1's primary activity in nuclear assembly is the generation of a substantial pool of GTP-Ran.

It seems probable that RCC1's nuclear localization might facilitate its association with other factors in the Ran/RCC1 system (Lee *et al.*, 1993) or might serve to regulate Ran by restricting its GEF to the nucleus. We have examined RCC1's activity as a GEF in *Xenopus* extracts in response to a number of factors, including the cell cycle state and the presence or absence of chromatin, but we have not seen a significant variation of GEF capacity (T.Seki and M.Dasso, unpublished observations). While these results were not definitive, they might bias the consideration of models in favor of the nuclear localization of RCC1 acting primarily to direct GEF activity to the appropriate location. It is interesting to note that the amount of RCC1 per nucleus varies in different organisms and cell types, with the protein being relatively abundant in rapidly dividing embryonic systems (reviewed in Dasso, 1993). In this regard, it is possible to speculate that the amount of Ran-GEF activity might be modulated to respond to the dynamic state of the nucleus, with nuclei undergoing more rapid rearrangements requiring more RCC1 protein.

Ran in the cell cycle

Bacterially expressed T24N-Ran blocks the normal oscillations of p34cdc2/cyclinB kinase (MPF) in *Xenopus* cycling extracts by activating a kinase that phosphorylates a tyrosine residue within p34cdc2 (Kornbluth *et al.*, 1994). This block occurs in the absence of DNA, so it is presumably a direct response to T24N-Ran rather than a checkpoint invoked by unreplicated DNA or abnormal nuclear structures. Results presented in this paper demonstrate the molecular nature of the defect that this mutant Ran causes in *Xenopus* extracts. T24N-Ran blocks the

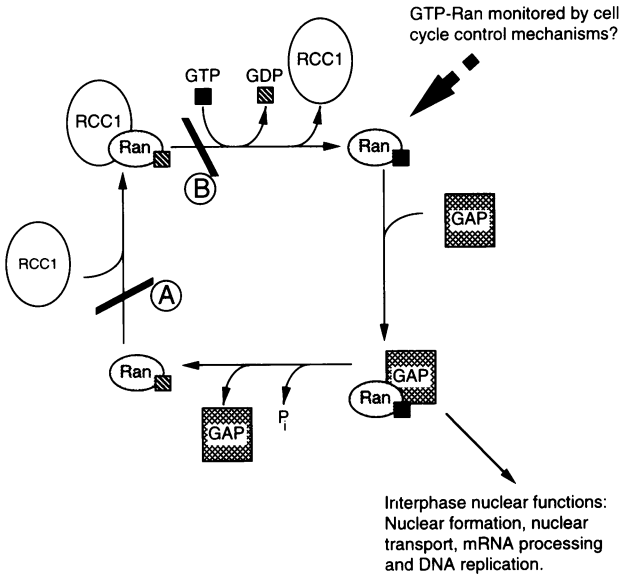


Fig. 8. The Ran GTPase cycle (see Discussion).

regeneration of the endogenous pool of GTP-Ran by preventing nucleotide exchange and/or RCC1 release (Figure 8, point B). This would be anticipated to produce a high level of GDP-bound Ran relative to the GTP-bound form. Taken together, these observations suggest that cell cycle control mechanisms are able to monitor the relative levels of GDP- and GTP-bound forms of Ran. Such monitoring could produce the observed cell cycle block in one of two ways. The GDP-bound form could inhibit mitosis, or the GTP-bound form could be a positive signal required for the activation of MPF. Although a cell cycle block by GDP-Ran is formally possible, it is unprecedented that a small Ras-like GTP-binding protein would facilitate signal transmission in its GDP- rather than GTP-bound state. The greater capacity of GTP-bound Ran protein to rescue RCC1-depleted extracts also shows that it is the more active form of Ran for interphase functions in *Xenopus* extracts. While these arguments do not directly disprove a possible role of GDP-Ran in cell cycle signalling, we consider the latter case a more likely scenario.

The loss of RCC1 in tsBN2 cells at the restrictive temperature should also block the regeneration of GTP-Ran (Figure 8, point A), but in this case the opposite outcome occurs and the cells enter mitosis prematurely (Nishitani *et al.*, 1991). Further, Ren *et al.* (1994) have recently shown that the expression of a GTP-bound mutant Ran causes a G_2 phase arrest in tissue culture cells. In order to reconcile this apparent conflict, it might be noted that the *Xenopus* cycling extracts have a much simpler cell cycle than tissue culture cells, which possess multiple checkpoints and a greater sensitivity to the state of the genome (Nishimoto *et al.*, 1981; Schlegel, *et al.*, 1987; Dasso and Newport, 1990). The observations in tissue culture cells may therefore represent not only the influence of the Ran/RCC1 system on the cell cycle but also the response of other checkpoint mechanisms to nuclear abnormalities that occur when Ran activity is perturbed. A much better understanding of both the Ran/RCC1 system and cell cycle controls will clearly be needed in order to reconcile these observations fully.

Our results also suggest that maintenance of an adequate pool of GTP-bound Ran is essential for the assembly of nuclei. This is consistent with the proposal that Ran protein serves to facilitate the internal rearrangement of the nucleus within the cell cycle (Moore and Blobel, 1994). If the rate at which Ran is utilized for these nuclear functions changes or if RCC1's activity alters during G_2 phase, it would effectively modulate the level of GTP-Ran in the cell and produce the mitosis-activating signal. For instance, if GTP-Ran is consumed at a high rate as long as DNA replication is occurring, then it might work well as an indicator of the cell cycle's state with respect to the completion of S phase. Since we still have no clear evidence of how GTP hydrolysis and nucleotide exchange are regulated for Ran, this mechanism remains very speculative. It will now be of interest to determine both how the activity of RCC1 is controlled and what other proteins interact with Ran to mediate its effects on nuclear assembly and the cell cycle.

Materials and methods

Preparation of Xenopus extracts

Crude interphase egg extracts were prepared and used for nuclear formation reactions as described in Smythe and Newport (1991). Fractionated extracts were also prepared according to the methods described in Smythe and Newport (1991) with the exception that HEPES was omitted from the egg lysis buffer. For the majority of experiments described in this paper, the high speed spins were performed in a Beckman SW50.1 swinging-bucket rotor. (This allowed us to process larger volumes of extract and our results with these extracts were indistinguishable from those found using extracts produced by centrifugation of extracts in a TLS-55 rotor under the conditions given by Smythe and Newport.) After the first 260 000 *g* centrifugation, the membrane and soluble fractions were collected and each subjected to an additional centrifugation. The soluble fraction was recentrifuged at 260 000 *g* for 30 min to remove any remaining membranes. The membrane fraction was diluted >5-fold in egg lysis buffer and the diluted membranes were layered upon a 1.0 ml cushion of egg lysis buffer containing additional sucrose to a final concentration of 0.5 M. The membranes were then pelleted through a 1 ml sucrose cushion for 35 min at 14 000 r.p.m. in a Beckman SW50.1 swinging-bucket rotor. The membrane pellet was resuspended in a minimal volume of egg lysis buffer/0.5 M sucrose. De-membranated sperm nuclei were prepared exactly according to Smythe and Newport (1991). Western blotting analysis reveals that these nuclei contain undetectable amounts of RCC1 (Dasso *et al.*, 1992) or Ran (data not shown) and thus they contributed very little to the pools of these proteins in our experiments.

Assays for nuclear assembly, nuclear transport and DNA replication

For the reactions shown in Figures 5 and 6, 25 μl of RCC1-depleted or untreated cytosol was mixed with 2.5 μl of 0.2 M creatine phosphate, 0.5 μl of 100 mM ATP, 0.25 μl of creatine kinase (5 mg/ml), 2.0 μl of 300 mg/ml glycogen and de-membranated sperm nuclei (final concentration ~1000 nuclei/μl). Bacterially expressed proteins were added simultaneously, at concentrations as indicated in the figure legends. If replication was also to be monitored in the experiment, 2.5 μCi of [α-^{32}P]dCTP was added. This mixture was incubated at room temperature for 1 h. To begin nuclear assembly, 4 μl of membranes were added and the incubation was continued at room temperature. Samples for DNA replication were taken after 3.5 h of incubation and treated exactly as described in Smythe and Newport (1991). For the reactions shown in Figure 7, 75 μl of RCC1-depleted cytosol was mixed with 7.5 μl of 0.2 M creatine phosphate, 7.5 μCi of [α-^{32}P]dCTP, 1.5 μl of 100 mM ATP, 0.75 μl of creatine kinase (5 mg/ml) and de-membranated sperm nuclei (final concentration ~2000 nuclei/μl). This mixture was incubated for 30 min at room temperature. The mixture was returned to ice and 15 μl of membranes were added with extensive mixing. The complete reaction was aliquoted into smaller samples and the bacterially expressed proteins were added to the concentrations indicated in the figure legends. The nuclear assembly reactions were begun by placing the samples at room temperature. The nuclei were examined by microscopy after 2 h of incubation and samples for DNA replication were taken after 2.5 h.

Nuclear assembly was monitored visually by both phase microscopy and fluorescent microscopy with the DNA dye Hoechst 33258 as described in Smythe and Newport (1991). In order to assay nuclear transport (Figure 5), a fluorescently labelled transport substrate, consisting of a rhodamine-labelled SV40 large T antigen signal sequence peptide coupled to human serum albumin (Newmeyer and Forbes, 1988), was added to nuclear assembly reactions at a concentration of ~10 μg/ml. The transport substrate was added 1.5 h after the addition of membranes; 2 h after the addition of membranes, the samples were mixed on slides with an equal volume of fix solution (50 mM sucrose, 100 mM KCl, 1 mM $MgCl_2$, 10 mM K–HEPES, pH 7.7, 3.7% formaldehyde, 10 μg/ml Hoechst 33258) and covered with a coverglass. Photographs were taken of the fixed samples with a Zeiss Axiophot microscope using Kodak TriX 400 film.

Construction of Ran mutants and production of expression vectors

Two mutants of the human TC4/Ran protein were constructed, one changing the 19th amino acid residue from a glycine to a valine, the other altering the 24th residue from a threonine to an asparagine (Kornbluth *et al.*, 1994). Mutagenesis was carried out on a human clone of Ran/TC4 cDNA (the kind gift of Dr Peter D'Eustachio) which had been sub-cloned into M13mp19, using an Amersham single-stranded DNA mutagenesis kit. RF form DNA was prepared from phage encoding the wild-type TC4 and from each of the mutants. This DNA was then restricted with *Nco*I and *Bam*HI (using the site from the m13mp19 polylinker) and subcloned into *Nco*I/*Bam*HI-cut pET8c for expression in *Escherichia coli*. Alternatively, the inserts were restricted with *Nco*I and *Pvu*II for subcloning into *Nco*I/*Ecl*136II-cut pGEX.KG (Guan and Dixon, 1991) for expression of GST fusion proteins in *E.coli*. For transcription of mRNA using the T7 phage RNA polymerase, the clones were restricted with *Eco*RI and *Dra*I for subcloning into *Eco*RI/*Eco*RV-cut pcDNA I (Invitrogen). The success of each subcloning was confirmed by sequencing the 5′ regions of each construct through the sites of amino acid substitutions. In order to produce *Xenopus* RCC1 as a GST fusion protein, the pGNO9 (Nishitani *et al.*, 1990) was restricted with *Eco*RI and subcloned directly into *Eco*RI-cut pGEX.KG. The orientation of the clone was determined both by restriction analysis and by sequencing though the site of fusion. All subcloning, DNA preparation and sequencing were performed by standard methods (Sambrook *et al.*, 1989). An expression vector for human RCC1 (pET3b-RCC1hs) was constructed by inserting the human RCC1 cDNA which had been restricted with *Nde*I and *Bam*HI into pET3b (Azuma *et al.*, manuscript in preparation).

Association of ^{35}S-labelled Ran proteins with nuclei formed in Xenopus extracts

CsCl-purified pcDNA-based plasmids containing each of the Ran clones or B4 protein were restricted for mRNA production with *Xho*I endonuclease, followed by phenol extraction and ethanol precipitation. Capped mRNAs were produced from the T7 phage RNA polymerase promoter in these templates using a mCAP mRNA capping kit (Stratagene) exactly according to the manufacturer's protocol. An aliquot of each of the mRNAs was checked on an agarose gel to assure that the template DNA had been completely removed after termination of the transcription reaction and to assure that the mRNA was intact. These mRNAs were then translated in a reticulocyte lysate system (Promega Biotech) exactly according to the manufacturer's protocol. To label the products of the translation reaction, [^{35}S]methionine (Amersham SJ204) was included to a specific activity of 1.0 mCi/ml. The lucifierase control mRNA provided within the reticulocyte lysate kit was translated simultaneously, to be used as a negative control. The products of this translation reaction were electrophoresed on 12.5% SDS–polyacrylamide gels to ensure that each of the Ran proteins was expressed equally.

In order to examine whether the labelled mutant Ran proteins associated preferentially with nuclei, the following experiment was performed. Crude extracts were supplemented with an ATP-regenerating system and divided into two aliquots. To one aliquot, de-membranated sperm nuclei were added to a concentration of 5000–10 000 nuclei/μl. To the other aliquot, water was added to give an equivalent dilution. Each of these mixes was then divided into five portions, of 7.5 μl each and 2.5 μl from each of the translation reactions was added to these portions and mixed thoroughly, giving a total of 10 assembly reactions. The reactions were incubated for 2.5 h at room temperature, after which they were checked microscopically to ensure that nuclear assembly was

equivalent in all five of the reactions containing DNA. An aliquot of 5 μl from each reaction was diluted with 45 μl of wash buffer (50 mM sucrose, 150 mM KCl, 10 mM EDTA, 1 mM $MgCl_2$, 10 mM K−HEPES, pH 7.7) and centrifuged for 5 min in an Eppendorf microfuge to the pellet nuclei and membranes. This pellet (3 μl) was re-washed with 100 μl of the same buffer and pelleted again by centrifugation. The pellets were then resuspended in 30 μl of SDS−sample buffer and 15 μl per reaction was electrophoresed on a 12.5% SDS−polyacrylamide gel. This gel was fixed, stained with Coomassie Blue and de-stained to assure equal loading of each reaction. The gel was then dried and exposed to film for direct autoradiography (Figure 1A). To determine the amount of label in each band, the gel was exposed to a storage phosphor screen and quantitated using a Molecular Dynamics Phosporimager (Figure 1B). The samples in Figure 1C were treated similarly, except that the Ran mRNAs were co-translated in the reticulocyte lysates with B4 mRNA.

Bacterial expression of GST fusion proteins

The pGEX.KG plasmids containing wild-type Ran, T24N-Ran, G19V-Ran or RCC1 inserts were transformed into *E.coli* (LysS strain) and the expression of the fusion proteins was induced by the addition of 0.5 mM IPTG (isopropylthiogalactoside) to exponentially growing 500 ml cultures at an OD_{600} of 0.4. All bacterial inductions of GST fusion proteins were carried out at 37°C. The bacteria expressing the fusion proteins were pelleted after 3 h of induction by centrifugation in a GSA rotor at 5000 r.p.m. for 10 min. The pellets were then stored at −80°C. After thawing, each culture was resuspended with 5 ml of buffer A [2.3 M sucrose, 50 mM Tris−HCl, pH 8.0, 10 mM EDTA, 100 μg/ml phenylmethylsulfonyl fluoride (PMSF)] and incubated on ice for 30 min. Buffer B was added [50 mM Tris−HCl, pH 7.5, 100 mM KCl, 1mM EDTA, 1 mM dithiothreitol (DTT), 100 μg/ml PMSF and 0.15 mg/ml lysozyme, 20 ml per culture] and the incubation on ice was continued for 1 h. Bacterial lysis was completed by adding sodium deoxycholate to 0.1%, $MgCl_2$ to 10 mM and DNase I to 20 μg/ml, followed by a final 15 min on ice. Cellular debris was removed by centrifugation of the lysates at 25 000 r.p.m. in a Beckman SW28 swinging-bucket rotor for 30 min. The supernatants from this centrifugation were incubated with rotation for 1 h at 4°C with 1 ml of glutathione−Sepharose beads (Pharmacia) that had previously been washed extensively with buffer C (10 mM HEPES, pH 8.0, 1 mM DTT, 75 μg/ml PMSF). After this incubation, the supernatant was removed and the beads were washed extensively with buffer C plus 150 mM NaCl. The fusion protein was eluted from the beads using buffer C plus 10 mM glutathione. The proteins were concentrated using Centricon 30 microconcentrator units (Amicon) and buffer-exchanged into XB* buffer (50 mM sucrose, 100 mM KCl, 1 mM $MgCl_2$, 10 mM K−HEPES, pH 7.7), according to the manufacturer's suggestions. The concentration of the fusion proteins was determined by electrophoresing different volumes of the final preparation in a 10% gel, staining with Coomassie Blue, destaining and quantitating the fusion protein bands relative to each other and to a series of molecular weight standards.

Association of GST−Ran fusion proteins with RCC1 and of the GST−RCC1 fusion protein with labelled Ran mutants

In order to determine the relative affinity of the RCC1 protein for each of the GST−Ran fusion proteins (Figure 2A), the following experiment was performed. Ten microliters of three different concentrations of the Ran fusion proteins (~1 mg/ml, 0.5 mg/ml and 0.25 mg/ml) were added to 100 μl of clarified *Xenopus* egg cytosol, along with 11 μl 0.2 M creatine phosphate, 2.2 μl 100 mM ATP and 1.1 μl creatine kinase (5 mg/ml). These reactions were incubated at room temperature for 80 min to allow binding of the fusion proteins to the endogenous RCC1. A volume of 120 μl was taken from each reaction and diluted with 350 μl of binding buffer (50 mM sucrose, 100 mM NaCl, 1 mM $MgCl_2$, 10 mM EDTA and 10 mM K−HEPES, pH 7.7). This diluted mixture was added to 45 μl of glutathione−Sepharose beads that had been prewashed in binding buffer and incubated with rotation for 1 h at 4°C. The beads were removed by centrifugation and washed four times with 350 μl of binding buffer. After the final wash, proteins bound to the beads were eluted with 80 μl of SDS sample buffer and boiling. Two equal volumes of the eluted proteins were run on identical SDS−10% polyacrylamide gels, one of which was stained with Coomassie blue and destained, while the other was blotted to a PVDF membrane. The stained gel was used to quantitate the recovery from each of the reactions by densitometry, in order to assure that the amount of fusion protein recovered was directly proportional to the input and to assure that both of the Ran mutants were retained on the glutathione−Sepharose beads equivalently to the fusion protein of wild-type Ran. The samples transferred to PVDF membrane were Western blotted with affinity-purified anti-RCC1 rabbit polyclonal antibodies using standard methods and visualized by autoradiography after incubation with [^{125}I]protein A (Dasso *et al.*, 1992). For each of the Ran fusion proteins, the amount of RCC1 eluted from the beads was proportional to the input of the fusion protein, indicating that the assay was roughly linear over the range employed. The samples shown in Figure 2A are from the incubation with 0.5 mg/ml input of the fusion proteins.

In order to determine the relative affinity of the GST−RCC1 fusion protein for each of the Ran mutant proteins (Figure 2B), ^{35}S-labelled proteins were produced in reticulocyte lysates from mRNAs encoding wild-type Ran, each of the mutants and luciferase as described above. GST−RCC1 was added at a concentration of 16 μg/ml to clarified cytosol containing a ATP regenerating system (20 mM creatine kinase, 2 mM ATP, 50 μg/ml creatine kinase). This mix was then divided into four portions. One volume from each of the translation reactions was added to 25 volumes of the cytosolic mixture containing GST−RCC1 and mixed thoroughly. These reactions were incubated for 1 h at room temperature. The reactions were diluted 3-fold with binding buffer and incubated with prewashed glutathione−Sepharose beads for 1 h at 4°C with rotation. The beads were washed extensively with binding buffer and the bound proteins were eluted with SDS sample buffer and boiling. Equal volumes from each elution were electrophoresed on a 12.5% SDS−polyacrylamide gel. This gel was fixed, stained with Coomassie brilliant blue and destained to assure equivalent recovery of the GST−RCC1 protein from each reaction. The gel was then dried and exposed to film for direct autoradiography. The '+EDTA' samples in Figure 2C (left graph) were produced identically to those in Figure 2B, while the dilutions and washes of the '−EDTA' samples were performed with XB*. The original incubation of the 'buffer only' samples (Figure 2C, right graph) substituted XB* for the egg extract and ATP-regenerating system. Dilutions and washes for these samples were performed using XB*. The recoveries of Ran proteins for all of the samples shown in Figure 2C were quantitated by phosphorimager analysis.

Bacterial expression of human RCC1 and Ran proteins

The pET3b-RCC1hs expression vector was introduced into *E.coli* [strain BL21(DE3)] and the expression of the RCC1 protein was induced by the addition of 0.5 mM IPTG to a culture that was exponentially growing at 30°C (OD_{600} = 0.8). The culture was induced for 12 h, after which the bacteria were pelleted by centrifugation at 3500 r.p.m. in a Beckman GH-3.7 rotor and frozen at −20°C. The cells were thawed in bacterial lysis buffer [50 mM Tris−HCl, pH 8.0, 2 mM EDTA, 1 mM DTT, 1 mM *p*-APMSF (*p*-amidinophenyl)-methanesulfonyl fluoride hydrochloride), 10% glycerol (v/v) and 50 mM NaCl]. All steps in the preparation of proteins were performed at 4°C, unless noted otherwise. The cells were suspended with a Potter-Elvehjem homogenizer and lysozyme was added to a final concentration of 0.4 mg/ml. After a 1 h incubation, CHAPS detergent was added to a final concentration of 1% and incubated for a further 5 min. This mixture was sonicated and then centrifuged at 10 000 *g* for 30 min.

The supernatant from this centrifugation was applied to a DEAE−Sephacel column (Pharmacia), which had been pre-equilibrated with buffer 1 [20 mM Tris−HCl, pH 7.8, 1 mM DTT and 10% glycerol (v/v)] plus 250 mM NaCl. The RCC1 protein was recovered in the flow-through fraction of this column. This fraction was diluted 2-fold with buffer 1 and applied to a SP Sepharose (Pharmacia) column that had been equilibrated with buffer 1 plus 150 mM NaCl. After washing the column with 10 volumes of buffer 1 plus 200 mM NaCl, the RCC1 protein was eluted with 1 volumes of buffer 1 plus 600 mM NaCl. The fractions from this step-elution that contained RCC1 were collected and pooled. The pooled fractions were diluted 5-fold with buffer 2 (25 mM HEPES and 1 mM DTT) and applied to a MonoS HR5/5 FPLC column (Pharmacia) which had previously been equilibrated with buffer 2 plus 150 mM NaCl. Proteins were eluted from the MonoS column using a linear 150–750 mM NaCl gradient in buffer 2 at a flow rate of 1 ml/min. Under these conditions, RCC1 protein is eluted at a NaCl concentration of 450–550 mM. These fractions were pooled and diluted 5-fold in buffer 3 (20 mM Tris−HCl, pH 8.0, 1 mM DTT). The RCC1-containing fractions were then subjected to FPLC on a MonoQ HR5/5 column that had been pre-equilibrated with buffer 3 plus 50 mM NaCl. Proteins were eluted from the MonoQ column using a linear 50–350 mM NaCl gradient in buffer 3 at a flow rate of 1 ml/min. Under these conditions, RCC1 protein is eluted at a NaCl concentration of 150–200 mM (Y.Azuma *et al.*, manuscript in preparation). During this purification procedure, RCC1 was detected by SDS−PAGE and immunoblotting.

Prior to use in *Xenopus* extracts, the RCC1 preparations were dialyzed against XB* buffer (50 mM sucrose, 100 mM KCl, 1 mM $MgCl_2$, 10 mM K-HEPES, pH 7.7) and concentrated in a Millipore centrifugal concentrator (Ultra Free, C3).

The pET8c expression constructs containing wild-type Ran, T24N-Ran and G19V-Ran were introduced into *E.coli* [strain BL21(DE3)]. Cultures of transformed bacteria were grown at 37°C (wild-type Ran) or 23°C (mutants). The expression of Ran proteins was induced by the addition of 1.0 mM IPTG to exponentially growing cultures (OD_{600} = 0.3). The cultures were induced for 6 h (wild-type) or 12 h (mutants), after which the bacteria were harvested by centrifugation and frozen at −20°C. The cells were thawed in Ran bacterial lysis buffer (50 mM Tris−HCl, pH 8.0, 1.0 mM EDTA, 0.5 mM DTT and 0.1 mM *p*-APMSF) and resuspended by homogenization, as above. Lysozyme was added at a final concentration of 0.4 mg/ml and the mixture was incubated for 30 min. $MgCl_2$, sodium deoxycholate and DNase I were added to give final concentrations of 10 mM, 0.02% and 40 μg/ml respectively. The incubation on ice was continued for an additional 30 min. After the addition of 1 mM DTT, the homogenate was centrifuged at 10 000 *g* for 30 min to remove cellular debris. NaCl was added to the supernatant to give a final concentration of 100 mM, after which the supernatant was applied to DEAE−Sephacel that had previously been equilibrated with buffer 4 (50 mM Tris−HCl, pH 8.0, 1 mM DTT, 10 mM $MgCl_2$, 100 mM NaCl). The Ran proteins were recovered in the flow-through fractions from this column. These fractions were pooled and brought to 45% saturation with ammonium sulfate, followed by centrifugation at 10 000 *g* for 30 min. The Ran protein was contained in the supernatant from this centrifugation, which was then brought to 60% saturation with ammonium sulfate to precipitate the Ran protein and re-centrifuged as above.

The pellet from the 60% ammonium sulfate precipitation was resuspended in buffer 5 (20 mM HEPES−NaOH, pH 7.6, 1 mM DTT, 1 mM $MgCl_2$, 50 mM NaCl) and applied to a Sephacryl S-200 (Pharmacia) column (2.6×60 cm) which had been equilibrated in buffer 5. The column was run at a flow rate of 2 ml/min in the same buffer. The fractions containing Ran were pooled and incubated with 1 mM GTP or GDP and 10 mM EDTA and 1 mM *p*-APMSF at 15°C for 90 min, depending upon the experiment. After this incubation, $MgCl_2$ was added to a final concentration of 20 mM and the mixture was applied to a MonoS HR5/5 column equilibrated with buffer 6 (20 mM HEPES−NaOH, pH 7.6, 1 mM DTT, 5 mM $MgCl_2$ and 1 mM CHAPS). The column was washed with buffer 6 and then a linear gradient of 0–600 mM NaCl in buffer 6 was applied at a flow rate of 1.0 ml/min. The fractions containing Ran were pooled and EDTA and DTT were added to final concentrations of 25 mM and 1 mM, respectively. GDP or GTP was added to the pooled fractions at 100 times the molar concentration of Ran. After a 40 min incubation on ice, $MgCl_2$ was added to a final concentration of 50 mM. The samples were dialyzed against buffer 7 (50 mM HEPES−NaOH, pH 7.3, 0.5 mM $MgCl_2$, 25 mM NaCl, 1 mM DTT), concentrated and frozen in liquid nitrogen. The samples were stored at −80°C until use (T.Ohba, T.Seki, Y.Azuma and T.Nishimoto, manuscript in preparation). During this purification procedure, Ran proteins were detected by SDS−PAGE, immunoblotting and by use of a GTPγS binding assay.

Ran proteins incubated with guanine nucleotides during their preparation were analyzed by high-performance liquid chromatography according to the method of Tucker *et al.* (1986). The Ran preparations were applied to a YMC-Pack ODS-AQ column equilibrated with a mobile phase [50 mM phosphate buffer, pH 6.5, 0.2 mM NaN_3, 0.2 mM tetra-*n*-butylammonium bromide and 3% (v/v) acetonitrile] and eluted under isocratic conditions at a flow rate of 1.5 ml/min at room temperature. We estimate that the GTP-bound preparations contained ~65% GTP-bound Ran and 35% GDP-bound Ran. There was very little GTP-bound Ran in the GDP-bound Ran preparation, the amount being below the detection limit of our assay (<10 pmol).

GEF assays

Ran protein with bound [^{3}H]GDP was made as follows: 10 pmol of Ran protein was incubated with 5×10^4 c.p.m. of [^{3}H]GDP (Amersham TRK 335) in a mixture that also contained 20 mM Tris−HCl, pH 7.5, 6 mM EDTA, 1 mM DTT, 1 mM $MgCl_2$, 100 mM NaCl and 0.1% lubrol (ICN). The final volume of this mixture was 23 μl. The mixture was incubated for 30 min at 30°C to allow the Ran protein to bind [^{3}H]GDP. The association of [^{3}H]GDP with Ran was stabilized by the addition of $MgCl_2$ to a final concentration of 20 mM and the sample was placed immediately on ice. Unlabelled GDP was then added to the mixture at a final concentration of 2 mM in a volume of 25 μl. In order to measure the GEF activity of samples, 25 μl of the [^{3}H]GDP−Ran mixture was incubated with an equal volume of a sample mixture. The sample mixture contained 20 mM Tris−HCl, pH 7.5, 1 mM DTT, 100 mM NaCl, 0.1% lubrol and the sample (as indicated in figure legends). After mixing, the combined reaction was incubated at 27°C for 5 min (Figure 3), or as indicated in Figure 4. The reaction was stopped by the addition of 2 ml of ice-cold stop buffer (20 mM Tris−HCl, pH 7.5, 25 mM $MgCl_2$ and 100 mM NaCl). The reactions were then filtered through nitrocellulose (BA85, Schleicher and Schuell). The filters were washed with 20 ml of stop buffer and dried under a heat lamp. The filters were placed in scintillation vials and 5 ml of scintillation fluid was added. ^{3}H retained on the filters was quantitated by liquid scintillation counting.

Acknowledgments

We would like to thank Alan Wolffe for critical reading of this manuscript. T.N. acknowledges the support of the Ministry of Education, Science and Culture of Japan for Scientific Research and for Cancer Research and of the HFSP. Correspondence should be addressed to M.D. at the National Institutes of Health.

References

Aebi,M., Clark,M.W., Vijayraghavan,U. and Abelson,J. (1990) *Mol. Gen. Genet.*, **224**, 72–80.
Almouzni,G. and Mechali,M. (1988) *EMBO J.*, **7**, 665–672.
Belhumeur,P., Lee,A., Tam,R., DiPaolo,T., Fortin,N. and Clark,M.W. (1993) *Mol. Cell. Biol.*, **13**, 2152–2161.
Bischoff,R.F. and Ponstingl,H. (1991a) *Nature*, **355**, 80–82.
Bischoff,R.F. and Ponstingl,H. (1991b) *Proc. Natl Acad. Sci. USA*, **88**, 10830–10834.
Blow,J.J. and Laskey,R.A. (1986) *Cell*, **47**, 577–587.
Bourne,H.R., Sanders,D.A. and McCormick,F. (1990) *Nature*, **348**, 125–132.
Burstein,E.S., Brondyk,W.H. and Macara,I.G. (1992) *J. Biol. Chem.*, **267**, 22715–22718.
Clark,K.L. and Sprague,G.F. (1989) *Mol. Cell. Biol.*, **9**, 2682–2694.
Dasso,M. (1993) *Trends Biochem. Sci.*, **18**, 96–101.
Dasso,M. and Newport,J.W. (1990) *Cell*, **61**, 811–823.
Dasso,M., Nishitani,H., Kornbluth,S., Nishimoto,T. and Newport,J.W. (1992) *Mol. Cell. Biol.*, **12**, 3337–3345.
Dimitrov,S., Almouzni,G., Dasso,M. and Wolffe,A.P. (1993) *Dev. Biol.*, **160**, 214–227.
Drivas,G.T., Shih,A., Coutavas,E., Rush,M.G. and D'Eustachio,P. (1990) *Mol. Cell. Biol.*, **10**, 1793–1798.
Feig,L.A. and Cooper,G.M. (1988) *Mol. Cell. Biol.*, **8**, 3235–3243.
Forrester,W., Stutz,F., Rosbash,M. and Wickens,M. (1992) *Genes Dev.*, **6**, 1914–1926.
Guan,K.L. and Dixon,J.E. (1991) *Anal. Biochem.*, **192**, 262–267.
Hock,R., Moorman,A., Fischer,D. and Scheer,U. (1993) *Dev. Biol.*, **158**, 510–522.
Kadowaki,T., Goldfarb,D., Spitz,L.M., Tartakoff,A.M. and Ohno,M. (1993) *EMBO J.*, **12**, 2929–2937.
Kornbluth,S., Dasso,M. and Newport,J. (1994) *J. Cell Biol.*, **125**, 705–720.
Lee,A., Tam,R., Belhumeur,P., DiPaulo,T. and Clark,M.W. (1993) *J. Cell Sci.*, **106**, 287–293.
Lounsbury,K.M., Beddow,A.L. and Macara,I.G. (1994) *J. Biol. Chem.*, **269**, 11285–1129.
Lohka,M.J. and Masui,Y. (1984) *J. Cell Biol.*, **98**, 1222–1230.
Matsumoto,T. and Beach,D. (1991) *Cell*, **66**, 347–360.
Melchior,F., Paschal,B., Evans,J. and Gerace,L. (1993) *J. Cell Biol.*, **123**, 1649–1660.
Moore,M.S. and Blobel,G. (1993) *Nature*, **365**, 661–663.
Moore,M.S. and Blobel,G. (1994) *Trends Biochem. Sci.*, **19**, 211–216.
Newmeyer,D.D. and Forbes,D.J. (1988) *Cell*, **52**, 641–653.
Newport,J. (1987) *Cell*, **48**, 205–217.
Nishimoto,T., Eilen,E. and Basilico,C. (1978) *Cell*, **15**, 475–483.
Nishimoto,T., Ishida,R., Ajiro,K., Yamamoto,S. and Takahashi,T. (1981) *J. Cell. Physiol.*, **109**, 299–308.
Nishitani,H., Kobayashi,H., Ohsubo,M. and Nishimoto,T. (1990) *J. Biochem.*, **107**, 228–235.
Nishitani,H., Ohtsubo,M., Yamashita,K., Iida,H., Pines,J., Yasuda,H., Shibata,Y., Hunter,T. and Nishimoto,T. (1991) *EMBO J.*, **10**, 1555–1564.

Ren,M., Drivas,G., D'Eustachio,P. and Rush,M.G. (1993) *J. Cell Biol.*, **120**, 313–323.
Ren,M., Coutavas,E., D'Eustachio,P. and Rush,M.G. (1994) *Mol. Cell Biol.*, **14**, 4216–4224.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sazer,S. and Nurse,P. (1994) *EMBO J.*, **13**, 606–615.
Schlegel,R., Croy,R.G. and Pardee,A.B. (1987) *J. Cell. Physiol.*, **131**, 85–91.
Schweighoffer,F., Cai,H., Chevallier-Multon,M.C., Fath,I., Cooper,G. and Tocque,T. (1993) *Mol. Cell. Biol.*, **13**, 39–43.
Sheehan,M.A., Mills,A.D., Sleeman,A.M., Laskey,R.A. and Blow,J.J. (1988) *J. Cell Biol.*, **106**, 1–12.
Smythe,C. and Newport,J.W. (1991) *Methods Cell Biol.*, **35**, 449–468.
Stick,R. and Hausen,P. (1985) *Cell*, **41**, 191–200.
Tucker,J., Sczakiel,G., Feuerstein,J., Goody,R.S. and Wittinghofer,A. (1986) *EMBO J.*, **5**, 1351–1358.
Uchida,S., Sekiguchi,T., Nishitani,H., Miyauchi,K., Ohtsubo,M. and Nishimoto,T. (1990) *Mol. Cell. Biol.*, **10**, 577–584.
Wilson,K.L. and Newport,J. (1988) *J. Cell Biol.*, **107**, 57–68.

Received on May 20, 1994; revised on September 2, 1994