Evidence for a Dual Role for TC4 Protein in Regulating Nuclear Structure and Cell Cycle Progression

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Abstract. TC4, a ras-like G protein, has been implicated in the feedback pathway linking the onset of mitosis to the completion of DNA replication. In this report we find distinct roles for TC4 in both nuclear assembly and cell cycle progression. Mutant and wild-type forms of TC4 were added to Xenopus egg extracts capable of assembling nuclei around chromatin templates in vitro. We found that a mutant TC4 protein defective in GTP binding (GDP-bound form) suppressed nuclear growth and prevented DNA replication. Nuclear transport under these conditions approximated normal levels. In a separate set of experiments using a cell-free extract of Xenopus eggs that cycles between S and M phases, the GDP-bound form of TC4 had dramatic effects, blocking entry into mitosis even in the complete absence of nuclei. The effect of this mutant TC4 protein on cell cycle progression is mediated by phosphorylation of $p34^{cdc2}$ on tyrosine and threonine residues, negatively regulating cdc2 kinase activity. Therefore, we provide direct biochemical evidence for a role of TC4 in both maintaining nuclear structure and in the signaling pathways that regulate entry into mitosis.

THE phases of the eukaryotic cell cycle (G1, S, G2, and M) must occur in a defined order if daughter cells are to receive a full complement of cellular components. In particular, DNA replication must be complete before the onset of mitosis if chromosome fragmentation and consequent unequal distribution of the genome are to be avoided. This temporal coordination of the cell cycle could potentially occur in one of two ways. If the products of completed DNA replication were essential substrates for the mitotic machinery, then mitosis would not be able to occur if replication were incomplete. Alternatively, regulatory controls could exist which monitor the state of replication of the chromosomes and prevent the onset of mitosis prior to completion of replication. There is considerable evidence for the existence of such checkpoint (or feedback) controls (Hartwell and Weinert, 1989). Fusion of cells in S phase to cells in G2 delays the G2 cell nuclei from entering mitosis until the S phase nuclei have completed replication (Rao and Johnson, 1970). Similarly, arrest of cells in S phase by addition of drugs which block complete replication, such as hydroxyurea or aphidicolin, inhibits entry into mitosis until the drugs are removed. Addition of caffeine (Schlegel and Pardee, 1986) or okadaic acid (Schlegel and Paradee, 1987; Smythe and Newport, 1992) can override such blocks, allowing entry into mitosis, suggesting that checkpoint controls are in effect despite the fact that the mitotic machinery is potentially completely functional.

Mutations that appear to disrupt the feedback pathway which links DNA replication to entry into mitosis have been identified in several organisms. In *Aspergillus nidulans*, a temperature-sensitive mutation of the BimE gene allows chromosome condensation and mitotic spindle formation even when cells are blocked in S phase by drug treatment or mutation of other cell cycle control genes (Osmani et al., 1988). Similarly, BHK cells carrying a temperature-sensitive mutation in the RCC1 gene (ts BN2 cells) prematurely enter mitosis when the temperature is raised, even if S phase has not yet been completed (Nishimoto et al., 1978; Ohtsubo et al., 1987; Uchida et al., 1990).

The coupling of the onset of mitosis to the completion of DNA replication can be viewed as a signaling pathway from the incompletely replicated DNA to the cellular machinery which controls the onset of mitosis. Entry into mitosis in eukaryotic cells is triggered by maturation-promoting factor (MPF)[1] composed of a 34-kD serine/threonine protein kinase (the product of the cdc2 gene) and cyclin B, which oscillates in abundance throughout the cell cycle, peaking at the G2-M transition (Nurse, 1990). Binding of cyclin is essential for the mitotic activation of the cdc2 protein kinase. Once cyclin is bound to $p34^{cdc2}$ this complex is held in an inactive state by inhibitory phosphorylation of tyrosine and threonine residues present in the ATP-binding site of $p34^{cdc2}$ (Dunphy

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1. *Abbreviations used in this paper*: GST, glutathione S transferase; MPF, maturation-promoting factor.

and Newport, 1989; Gould and Nurse, 1989; Solomon et al., 1990). In both *Schizosaccharomyces pombe* and *Xenopus laevis*, the control of entry into mitosis by incomplete DNA replication is largely exerted through modulation of this tyrosine phosphorylation of $p34^{cdc2}$/cyclin. In *S. Pombe*, the mik1 and wee1 kinases appear to carry out the tyrosine phosphorylation of $p34^{cdc2}$ (Featherstone and Russell, 1991; Lundgren et al., 1991; Parker et al., 1992). Cdc2 protein is not phosphorylated on tyrosine in *mik1 wee1* double mutants at the restrictive temperature, and these mutants can enter mitosis even when DNA replication is inhibited by hydroxyurea (Lundgren et al., 1991). The tyrosine dephosphorylation of $p34^{cdc2}$ is under the control of the tyrosine phosphatase cdc25 (Dunphy and Kumagai, 1991; Gautier et al., 1991; Millar et al., 1991). Overexpression of cdc25 protein can, like inhibition of wee1 and mik1, override a hydroxyurea arrest (Enoch and Nurse, 1990).

A critical role for tyrosine phosphorylation and dephosphorylation of $p34^{cdc2}$ in the regulation of entry into mitosis has been established in cell-free extracts of *Xenopus* eggs which can oscillate between S and M phases of the cell cycle even in the absence of nuclei (Murray and Kirschner, 1989; Dasso and Newport, 1990; Solomon et al., 1990). If chromatin is added to these "cycling extracts," nuclei are assembled during S phase, replicate their DNA, then become disassembled as the extract enters mitosis. Entry into mitosis can be monitored either microscopically by the observation of nuclear envelope breakdown and chromosome condensation or biochemically by assaying cdc2 kinase activity. Addition of aphidicolin to the extract causes interphase nuclei to be unable to replicate DNA and one observes a consequent inhibition of entry into mitosis (Dasso and Newport, 1990). Thus the feedback system linking completion of replication to entry into mitosis is active in this in vitro system. A similar feedback effect blocking entry into mitosis can be achieved by adding very large numbers of nuclei to the extract (greater than 2,000 nuclei/ul), presumably because the depletion of cellular components necessary for replication in the extract results in incomplete replication (Dasso and Newport, 1990). Overexpression of cdc25 protein in these extracts can override this feedback and promote entry into mitosis (Kumagai and Dunphy, 1991). With respect to the molecular target of feedback, we have found that the tyrosine kinase(s) which phosphorylates $p34^{cdc2}$ is activated when aphidicolin and nuclei are added to the extracts. This indicates that the feedback system linking replication and mitosis operates, at least in part, by increasing the rate of tyrosine phosphorylation of $p34^{cdc2}$, thereby suppressing its mitotic kinase activity and preventing entry into mitosis (Smythe and Newport, 1992).

A signaling system which inhibits entry into mitosis prior to completion of replication requires components which can monitor the replicative status of the DNA and propagate a signal to the factors such as wee1, mik1, and cdc25, which modulate $p34^{cdc2}$ activity. One attractive candidate for a participant in the monitoring system is the RCC1 protein mentioned above. In both mammalian cells and in *S. Pombe*, RCC1 mutants enter mitosis prematurely and can override a hydroxyurea arrest (Nishimoto et al., 1978, Matsumoto and Beach, 1991). In *Xenopus*, RCC1 has been shown to be an abundant chromatin-binding protein present at approximately one molecule per nucleosome (Dasso et al., 1992). Nuclei assembled in extracts depleted of RCC1 are altered in nuclear structure and defective for replication (Dasso et al., 1992). These data suggest that RCC1 acts in some manner to couple DNA replication to the regulation of entry into mitosis. In *S. Pombe*, RCC1 mutants (called *pim1*) are rescued by overexpression of a gene known as *spi1*, suggesting that spi1 and RCC1 may interact. Spi1 appears to be the *S. Pombe* homologue of a previously isolated *ras*-related G protein known as TC4/Ran (Drivas et al., 1990; Matsumoto and Beach, 1991). It has been recently demonstrated that RCC1 is a guanine nucleotide exchange protein for TC4/Ran in vitro (Bischoff and Ponstingl, 1991*a*), supporting the idea that these proteins functionally interact in vivo.

Given the demonstrated role of *ras*-like GTP-binding proteins in a variety of signaling pathways and the involvement of RCC1 in feedback control, it seemed possible that TC4 might be a component of the signaling system that modulates the activity of $p34^{cdc2}$. We now show, using mutant TC4 proteins, that TC4 has independent distinct effects on nuclear structure and cell cycle progression. Specifically, a TC4 mutant predicted to bind GDP with a much greater affinity than GTP disrupts nuclear structure and replication in interphase *Xenopus* egg extracts. Moreover, in cycling extracts without added nuclei (lacking the substrate for the signaling pathway) this mutant TC4 prevents $p34^{cdc2}$ activation. This inhibition occurs through phosphorylation of $p34^{cdc2}$. Therefore, we provide direct biochemical evidence for a role of TC4 in the regulation of $p34^{cdc2}$.

Materials and Methods

Preparation of Extracts from X. laevis Eggs

Cycling extracts were prepared as described by Murray and Kirschner (1989). All incubations were at room temperature. For visual monitoring of nuclear envelope breakdown and chromosome condensation, sufficient sperm chromatin prepared as described by Lohka and Masui (1983, 1984); (Wilson and Newport, 1988) was added to form 500 nuclei per μl of extract. Throughout the incubation, 2-μl samples were withdrawn at 10-min intervals and diluted 1:1 with 10 μg of Hoechst 33258 per ml in 37% formaldehyde and examined by phase contrast and fluorescence microscopy. In some experiments, aphidicolin (0.05 mg/ml) or caffeine (5 mM) were added at the start of the room temperature incubation. Okadaic acid was added to the extract 70 min after the start of the room temperature incubation to a final concentration of 1 μM.

Interphase extracts were prepared as described previously (Wilson and Newport, 1988). Samples were monitored by fluorescence microscopy as for the cycling extracts.

Production of Wild-Type and Mutant TC4 Proteins

Two mutants of human TC4 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. The human TC4 clone was provided to us as an EcoRI fragment in pUC19 (the kind gift of Dr. P. D'Eustachio, New York University Medical Center, NY). This EcoRI fragment was subcloned into M13mp19 for mutagenesis. Using the Amersham Corp. (Arlington Heights, IL) in vitro mutagenesis kit, we first introduced an NcoI site at the initiator codon of the TC4 clone with an oligonucleotide of sequence 5' AAC GCC GCC ATG GCT GCG 3'. Using phage which contained this NcoI site, we introduced mutations into the coding sequence with an additional step of mutagenesis using oligonucleotides of sequence 5' GTT GGT GAT GTT GGT ACT GGA 3' (for producing the G to V mutation) or 5' ACT GGA AAA AAC ACC TTC GTG 3' (for producing the T to N mutation). The altered bases are underlined. The success of each step of mutagenesis was confirmed by sequencing of the resultant phage. The mutagenized EcoRI fragments were excised from M13 and subcloned into pBluescript SK (Stratagene Inc., La Jolla, CA) at the EcoRI site. TC4-

containing bluescript plasmids were then digested with NcoI and HindIII (present in the polylinker of bluescript, 3′ of the TC4 termination codon). The insert was isolated and subcloned into pGEX KG (kindly provided by Jack Dixon, University of Michigan Medical School, Ann Arbor, MI) and digested with NcoI and HindIII for production of glutathione S transferase fusion protein. Alternatively, the RF DNA from the mutagenized phase was restricted with NcoI and BamHI and subcloned into the vector pET 8C for protein production.

Isolation of TC4 Protein and GST-sea Urchin Fusion Protein

The bacterial strain DH5α was transformed with the Gex-TC4 or Gex-sea urchin cyclin B constructs. The recombinant fusion proteins were expressed and purified as described (Solomon et al., 1990). For cleavage of the resultant GST-TC4 fusion proteins with thrombin, ~1 mg of fusion protein was incubated with 5 μg of thrombin at room temperature for 45 min in thrombin cleavage buffer (50 mM Tris 8.0, 150 mM NaCl, 2.5 mM $CaCl_2$, and 0.1% β-mercaptoethanol). These proteins were then diluted into XB buffer (100 mM KCL, 0.1 M $CaCl_2$, 1 mM $MgCl_2$, 10 mM K-Hepes, pH 7.7, and 50 mM sucrose) and reconcentrated by centrifugation in a centricon 10 microconcentrator. Proteins were aliquoted and stored at −70°C.

Preparation of Antibodies Against the Human TC4 Protein

In order to raise antibodies against the TC4 protein, a pET8C-derived plasmid containing the wild-type TC4 sequence was transformed into LysS strain *E. coli*. Expression of the protein was induced with IPTG for 3 h, after which the bacteria were pelleted by centrifugation, washed with 0.9% NaCl, repelleted, and frozen at −70°C. The TC4 protein expressed by induced bacteria fractionated to the inclusion bodies and it was purified as follows: the cells from 500 ml of bacterial culture were thawed and resuspended in 12.5 ml of buffer A (10 mM Tris HCl, pH 7.5, 50 mM NaCl, and 1 mM EDTA) plus 0.5% NP40 and allowed to sit on ice for 15 min. They were then sonicated for 2 min and the inclusion bodies were pelleted for 15 min at 12,000 rpm in a Sorvall SS34 rotor. The pellets were washed with 5 ml of buffer B (10 mM Tris HCl, pH 7.5, 500 mM NaCl, and 1 mM EDTA) and pelleted as above. The inclusion bodies were then resuspended in buffer A plus 6 M urea and incubated on ice for 5 min, after which insoluble material was pelleted for 15 min at 12,000 rpm in a Sorvall SS34 rotor. The supernatant containing the TC4 protein was then electrophoresed on a preparative SDS–polyacrylamide gel. The protein band containing TC4 was cut from the gel and used to generate antibodies by standard methods (Harlow and Lane, 1988).

GTP Blots

Proteins were resolved by SDS-PAGE and soaked in 50 mM Tris, pH 7.5, and 20% glycerol for 30 min. Transfer to nitrocellulose was done in 10 mM $NaHCO_3$, and 3 mM Na_2CO_3, pH 9.8. Filters were rinsed 2 times for 10 min in binding buffer (50 mM NaH_2PO_4, pH 7.5, 10 μM $MgCl_2$, 2 mM DTT, 0.3% Tween 20, and 4 μM ATP) and were incubated in binding buffer with 2 μCi/ml α[^{32}P]GTP (3,000 Ci/mM) for 2 h, rinsed three times for 10 min in binding buffer and exposed to x-ray film.

Replication Assays

For continuous labeling of DNA, extracts containing sperm nuclei were incubated at room temperature in the presence of 0.1 μCi of α[^{32}P]dCTP (3,000 Ci/mM) per μl. 8-μl samples were withdrawn into 8 μl of replication sample buffer containing 80 mM Tris-HCl, pH 8.0, 8 mM EDTA, 0.13% phosphoric acid, 10% ficoll, 5% SDS, and 0.2% bromophenol blue. These samples were digested with 1 mg/ml proteinase K for 2 h at room temperature and electrophoresed on 1% agarose gels. Gels were dried and exposed to x-ray film at −70°C with an intensifying screen.

Histone H1 Kinase Assays

For assay of histone H1 kinase activity, 2-μl aliquots of extracts were withdrawn at 10-min intervals into 2 μl of EB buffer (80 mM β-glycerophosphate, pH7.3, 20 mM EGTA, and 15 mM $MgCl_2$), frozen immediately in liquid nitrogen and stored at −70°C. Thawed samples were diluted 120-fold in EB buffer and were then added 1:1 to H1 assay cocktail (40 mM Hepes, pH 7.3, 10 mM EGTA, 20 mM $MgCl_2$, 0.2 mg of histone H1/ml, 0.2 mM ATP, 5 μCi γ[^{32}P]ATP, and 10 μM cyclin AMP-dependent protein kinase inhibitor peptide. [Sigma Chem. Co., St. Louis, MO]). After 10 min of incubation at room temperature, protein sample buffer for SDS-PAGE was added to each sample to stop the reactions. Samples were resolved by SDS-PAGE. Gels were dried and exposed to x-ray film for 1 h.

Translation Assays and Binding to p13 Sepharose

[^{35}S]methionine (0.04 Ci/ml) was added to extracts at the beginning of the incubation. Samples were removed, diluted 1:1 in protein sample buffer, boiled, and then electrophoresed on 10% SDS–polyacrylamide gels. After electrophoresis, gels soaked in 1 M sodium salicylate were exposed to x-ray film for autoradiography. For isolation of p34^{cdc2}–cyclin complexes on p13 Sepharose, p13 was purified as described by (Solomon et al., 1990) and coupled to Sepharose as described by (Dunphy and Newport, 1989). Extracts were incubated 45 min in the presence of [^{35}S]methionine and then diluted twofold with buffer I (80 mM β-glycerophosphate, 5mM EDTA, 2 mM sodium orthovanadate, 0.1% NP-40, and 0.5 M NaCl). p13 Sepharose was added to each sample and incubated at 4°C with agitation for 45 min. Beads were washed once in buffer I, once in buffer II (buffer I without NaCl or NP-40) and once in buffer III (10 mM Hepes, pH 7.5, 1 mM dithiothreitol, and 2 mM sodium orthovanadate). p13 beads were then boiled in an equal volume of sample buffer (5 mM EDTA, 5 mM sodium orthovanadate, 20 mM sodium pyrophosphate, 30 mM sodium fluoride, 1% SDS, 80 mM Tris, pH 6.8, 10% glycerol, and 0.1 M dithiothreitol) and pelleted. The supernatants were electrophoresed on 10% SDS–polyacrylamide gels for autoradiography.

For analysis of the state of phosphorylation of cdc2 protein on Tyr 15 and Thr 14, aliquots of extract were resolved by SDS-PAGE, transferred to polyvinyldifluoride membrane, and then immunoblotted with anti-cdc2 sera.

Chromatin Decondensation

Fractionated interphase extracts were prepared as follows. Eggs were dejellied in 2% cysteine and washed 3 times in MMR (100 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 0.1 mM EDTA, and 5 mM Hepes, pH 7.8), 3 times in egg lysis buffer (250 mM sucrose, 2.5 mM $MgCl_2$, 50 mM KCl, 50 ug/ml cycloheximide, 1 mM dithiothreitol, and 10 mM Hepes, pH 7.7), and lysed by centrifugation at 260,000 *g*. Demembranated sperm chromatin prepared as described in (Wilson and Newport, 1988) was added to the cytoplasmic fraction of the interphase extract in the absence of membranes and the presence of an ATP-regenerating system (20 mM phosphocreatine, 2 mM ATP, and 50 ug/ml creatine kinase). Samples were diluted 1:1 with 10 ug Hoechst 33258 per ml in 37% formaldehyde.

Nuclear Import

For nuclear import assays, a conjugate of TRITC-human serum albumin and a synthetic peptide containing the nuclear-localization signal of SV-40 T antigen was added to extracts (Newmeyer et al., 1986). Transport substrate/per nucleus was measured using a linearly calibrated charge-coupled device camera and National Institutes of Health Image 1.49 software.

Results

Mutations in Human TC4 Protein

The potential importance of RCC1 in the functioning of the feedback pathway linking completion of DNA replication to entry into mitosis and the interaction of RCC1 with TC4 in several systems suggested that TC4 might be an important component of the feedback signal in *Xenopus*. To test this, mutant forms of TC4 were produced. Oligonucleotide-directed mutagenesis was performed on a human TC4 cDNA clone (generously provided by P. D'Eustachio) (see Fig. 1 *A*). The amino acid residues we chose to alter (residues 19 and 24 of TC4) are conserved between *ras* and TC4 proteins and mutations at these residues in *ras* have been extensively studied. Mutagenesis of residue 19 from glycine to valine (hereafter referred to as the G19V mutant) was intended to

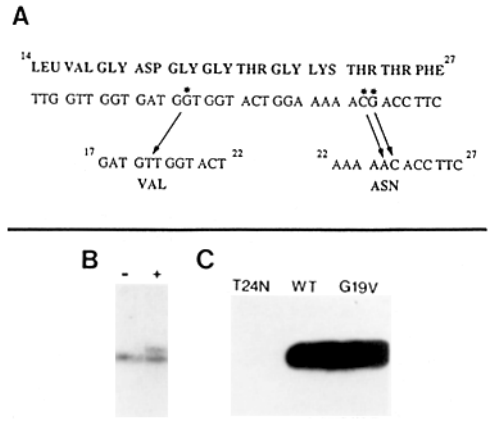

Figure 1. Mutagenesis of human TC4. (*A*) To produce the G19V mutant, the codon GGT encoding a valine was changed to GTT. Threonine 24 (ACG) was changed to an asparagine (AAC). Both mutants were made by oligonucleotide mutagenesis in M13 and were subcloned into vectors for expression of glutathione-S-transferase fusion proteins. (*B*) Aliquots of egg extract in the absence (−) or presence (+) of ~150 μg/ml of recombinant human TC4 protein (which had been cleaved with thrombin to separate the TC4 and GST portions of the fusion protein) were resolved by SDS-PAGE and immunoblotted with anti-TC4 sera. (*C*) GTP binding by bacterially synthesized TC4 proteins. T24N, G19V, and wild-type TC4 proteins were resolved by SDS-PAGE, blotted onto nitrocellulose and processed for binding to α[^{32}P]GTP as described in Materials and Methods. Samples were visualized by autoradiography with an intensifying screen. Exposure was for 1 h at −70°C.

provide a form of TC4 defective in GTP hydrolysis. The expression in tissue culture cells of human *ras* proteins which contain this mutation results in a transformed phenotype, presumably because this protein remains locked in an activated state through inability to hydrolyze GTP (McGrath et al., 1984). Similarly, mutation of residue 24 from threonine to asparagine (hereafter referred to as T24N) would allow production of a form of TC4 protein which would remain predominantly in a GDP-bound form since the corresponding mutants in *ras*^{H} have a 20–40-fold decreased affinity for GTP in vitro (Feig and Cooper, 1988). Expression of this *ras* mutant acts in a dominant negative fashion to inhibit cell growth, presumably by interfering with the normal functioning of the endogenous wild-type protein (Feig and Cooper, 1988).

To demonstrate that the bacterially produced TC4 proteins behaved as might be expected with respect to nucleotide binding, we resolved some of each of the bacterial protein preparations by SDS-PAGE, transferred them to nitrocellulose and blotted with α[^{32}P]GTP (Fig. 1 *C*). The wild-type and G19V proteins bounded GTP at similar levels, while the T24N protein had greatly reduced GTP binding (faintly visible on a 12-h exposure). This is similar to the ras Asn 17 mutant (Feig and Cooper, 1988). It is not yet clear whether GTP hydrolysis is affected by the G19V mutation.

To use these mutant proteins to examine TC4 functions in Xenopus egg extracts, we cloned the TC4-coding sequences into a vector (gex KG) capable of producing glutathione S transferase (GST) fusion proteins in *E. coli*. The fusion proteins produced were purified on glutathione Sepharose and cleaved with thrombin, removing the GST portion of the protein and leaving intact human TC4 proteins. Complete cleavage was confirmed by Western blotting with antibodies directed against human TC4 protein (data not shown).

We wished to add the recombinant TC4 proteins to Xenopus extracts at levels roughly equivalent to the levels of Xenopus TC4 found endogenously. Based on estimates of TC4 abundance in other cell types (Bishoff and Ponstingl, 1991*b*), we predicted that TC4 would be present at ~100 ng/ul in the extract. To determine if this was a correct estimate, we added between 100 and 200 ng/ul of *E. coli*-expressed TC4 protein (cleaved with thrombin to remove the GST portion of the fusion protein) to an extract. Aliquots of extract (with or without supplemented recombinant TC4) were resolved by SDS-PAGE, transferred to nitrocellulose, and then immunoblotted with anti-human TC4 sera which cross-reacts with Xenopus protein (Fig. 1 *B*). Since Xenopus and human TC4 are separable by SDS-PAGE, it is possible to see that extracts containing the recombinant TC4 protein had approximately equivalent amounts of Xenopus TC4 (Fig. 1 *B, lower band*) and bacterially produced human TC4 protein (Fig. 1 *B, upper band*). Since the antisera was prepared against human TC4, it seems unlikely that it would recognize Xenopus TC4 more efficiently. Therefore, if anything, we added less recombinant TC4 protein than that present endogenously. For all subsequent experiments, equal amounts of wild-type and mutant TC4 proteins were added to extracts.

Effects on Nuclear Morphology

When demembranated sperm chromatin is added to crude extracts of Xenopus eggs, the condensed chromatin serves as a template for the formation of nuclei. Previously, we have shown that depletion of RCC1 protein from such a nuclear assembly extract significantly limits the nuclear growth around sperm chromatin templates. In RCC1-depleted extracts, nuclear size, as measured by nuclear volume, is reduced ~10-fold relative to nuclei assembled in control extracts (Dasso et al., 1992; Dasso, M., unpublished observations). Because RCC1 acts as a nucleotide exchange factor for TC4 we investigated whether the addition of either the T24N or G19V mutants of TC4 caused any visible defect in nuclear assembly around sperm chromatin added to egg extracts. As shown in Fig. 2 *A*, addition of either wild-type or G19V mutant TC4 proteins had no effect on nuclear formation relative to controls. Under these conditions, an initial nuclear envelope formed around the sperm chromatin and then proceeded to grow rapidly (7–10-fold), coincident with decondensation of the initially highly condensed chromatin. In contrast to this, addition of the T24N mutant TC4 protein to assembly extracts severely restricted nuclear growth. In the presence of T24N protein, the initially elongated sperm chromatin rounded-up into a small sphere encapsulated within a nuclear envelope and then remained in this state for the duration of the experiment (3 h) (Fig. 2 *A*). This observation demonstrates that, like depletion of RCC1 protein, the addition of the T24N mutant TC4 protein to extracts inhibits a process required for nuclear formation and/or growth.

It has been shown that efficient replication of sperm chromatin added to Xenopus egg extracts occurs only after a nuclear envelope has assembled around the chromatin (Newport, 1987; Sheehan et al., 1988). Moreover, it has also been shown that depletion of RCC1 protein from extracts prevents replication of sperm chromatin which has been assembled

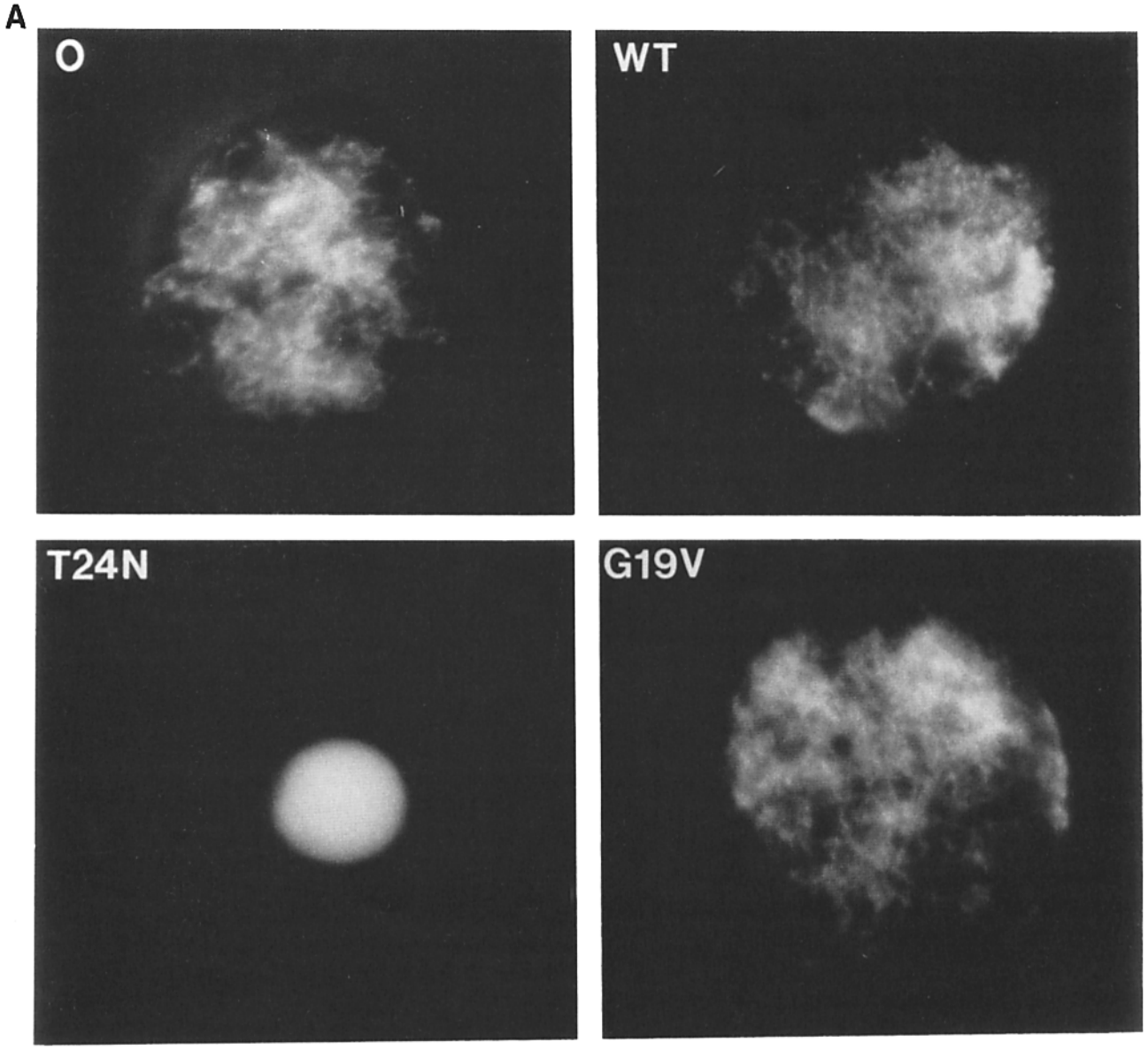

Figure 2. (*A*) Changes in nuclear morphology induced by the T24N protein. Interphase extracts were incubated at room temperature in the presence of sperm chromatin and the indicated TC4 protein variant. DNA was visualized by fluorescent microscopy following staining with Hoechst 33258. Photographs were taken at 60 min. (*B*) Interphase extracts were incubated in the presence of 1,000 sperm/μl and 0.2 μCi/μl α[^{32}P]dCTP. Continuously labeled samples were withdrawn at 30, 60, and 90 min, treated with proteinase K, and resolved by agarose gel electrophoresis on 1% gels. Gels were dried and visualized by autoradiography at −70°C with intensifying screen.

into nuclei (Dasso et al., 1992). Because TC4 interacts with RCC1 protein (Bischoff and Ponstingl, 1991*b*) and the T24N TC4 mutant inhibits proper nuclear assembly, we investigated how different TC4 mutations might effect DNA replication. To do this, we measured the incorporation of ^{32}P-labeled dCTP into sperm DNA incubated in egg extracts. In extracts containing either no addition or wild-type TC4 protein the rate of DNA replication was identical. When the G19V TC4 was added, there was a slight (twofold) decrease in replication rate. However, when the T24N TC4 protein was added to extracts, DNA replication was completely (>95%) inhibited over the entire 90-min period of the experiment (Fig. 2 *B*). These results show that addition of T24N TC4 blocks both nuclear growth and DNA replication.

T24N TC4 Does Not Block DNA Decondensation

The reassembly of an intact nuclear structure around a chromatin template following mitosis is a complex process in-

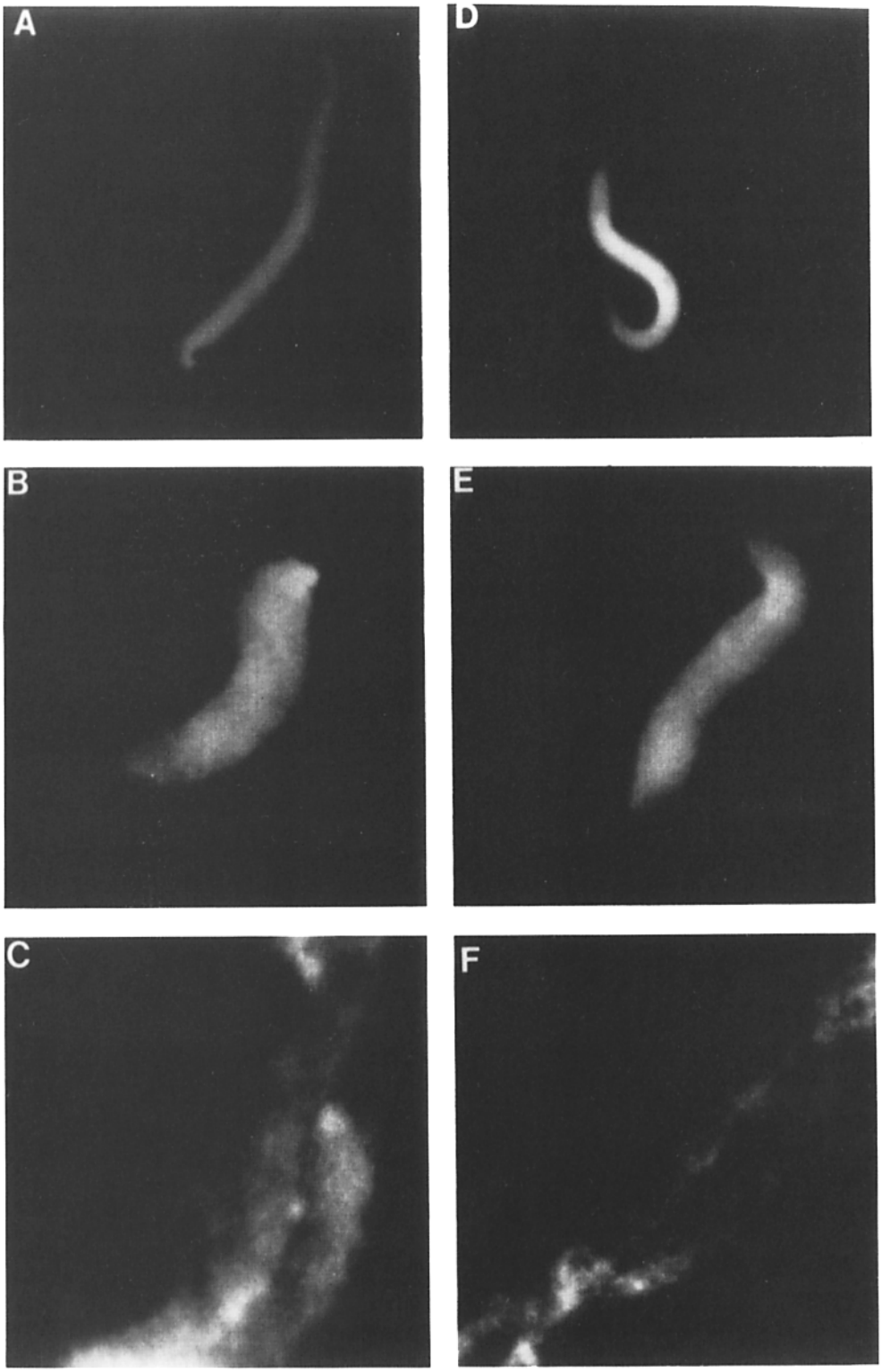

Figure 3. DNA decondensation in the presence of T24N protein. Interphase extracts of Xenopus eggs were fractionated into separate membrane and cytoplasmic components. Sperm chromatin and an ATP-regenerating system were incubated in the cytoplasmic fraction at room temperature in the presence (*D–F*) or absence (no addition) (*A–C*) of the T24N TC4 mutant protein. Samples were withdrawn, diluted with Hoechst 33258, and visualized by fluorescence microscopy at 5 (*A* and *D*), 15 (*B* and *E*), and 30 min (*C* and *F*) after the start of the room temperature incubation.

volving a large number of steps (Newport, 1987). Membrane vesicles bind to the surface of the chromatin and fuse to form an intact envelope (Wilson and Newport, 1988; Pfaller et al., 1991; Newport and Dunphy, 1992), nuclear pores are incorporated, (Newmeyer et al., 1986; Sheehan et al., 1988), and the initially condensed DNA begins to decondense. Although further growth of the nucleus beyond this point has not been well characterized, it is likely to require incorporation of additional membrane vesicles, selective transport of proteins into the nuclear interior (Newport et al., 1991), assembly of the transported proteins into macromolecular structures which can interact with both the envelope and DNA, and further decondensation of the DNA.

In order to address which processes might be blocked by the T24N mutant protein, the process of chromatin decondensation was examined first. When demembranated sperm

chromatin is added to egg extracts which have been depleted of membrane components by centrifugation, the sperm chromatin decondenses (Fig. 3, *A–C*). This decondensation process occurs in two stages. The initial stage is rapid (less than 5 min) and involves nucleoplasmin-dependent exchange of protamines for histones (Pfaller et al., 1991; Philpott et al., 1991; Newport and Dunphy, 1992). As a result of this exchange, the chromatin volume increases 10–20-fold compared to the size of the original sperm. Following this initial decondensation stage, the DNA continues to decondense slowly over the next 30 min (Fig. 3, *B* and *C*). This second stage of decondensation is dependent on ATP hydrolysis and is reversed if the extract is induced to enter mitosis by addition of cyclin B protein (Yan H., and J. Newport, unpublished data). Moreover, the kinetics of sperm chromatin decondensation observed in the absence of membrane appears similar, if not identical, to the rate of decondensation of sperm chromatin assembled into nuclei in complete extracts containing membranes. Based on these criteria, the decondensation observed in the absence of membranes appears to be mechanistically similar to the decondensation process which occurs within nuclei. When T24N TC4 protein was added to extracts which had been depleted of membrane (Fig. 3, *D–F*), both the rate and extent of sperm chromatin decondensation appeared to be identical to the decondensation observed in control extracts (no addition) (Fig. 3, *A–C*). Thus, the defect in nuclear growth observed with T24N TC4 is unlikely to be due to a failure of the DNA to decondense.

Effects of T24N TC4 on Nuclear Transport

Once an intact envelope containing nuclear pores has formed around chromatin, further assembly of the nucleus is dependent on selective transport of nuclear proteins through nuclear pores (Newport, 1987). Thus, inhibition of nuclear growth observed in the presence of the T24N TC4 mutant could be explained by a block in nuclear transport. To determine whether this was the case, a fluorescently labeled nuclear transport substrate consisting of rhodamine-labeled HSA coupled to the SV-40 nuclear localization signal peptide (Newmeyer and Forbes, 1986) was added to egg extracts containing nuclei assembled in the presence or absence of the T24N mutant TC4 protein. Nuclear transport was quantitated using a linearly calibrated CCD camera to determine the fluorescent intensity (luminance/pixel) of each nucleus, which is proportional to the substrate concentration. If the T24N mutant inhibited nuclear transport, we would expect nuclei assembled in the presence of this protein to accumulate significantly less fluorescent transport substrate than control nuclei. As shown in Fig. 4, this did not appear to be the case. Indeed, after a 2-h incubation, the concentration of fluorescently labeled transport substrate in nuclei assembled in the presence of the T24N mutant protein was significantly higher than the concentration of the same substrate in control nuclei (Fig. 4 *A*). This suggests that the failure of nuclei to grow in the presence of the T24N mutation is not due to a defect in nuclear transport.

Although nuclei formed in the presence of the T24N mutant accumulated higher concentrations of transport substrate than control nuclei, this cannot be interpreted as demonstrating that nuclear pores of the T24N nuclei trans-

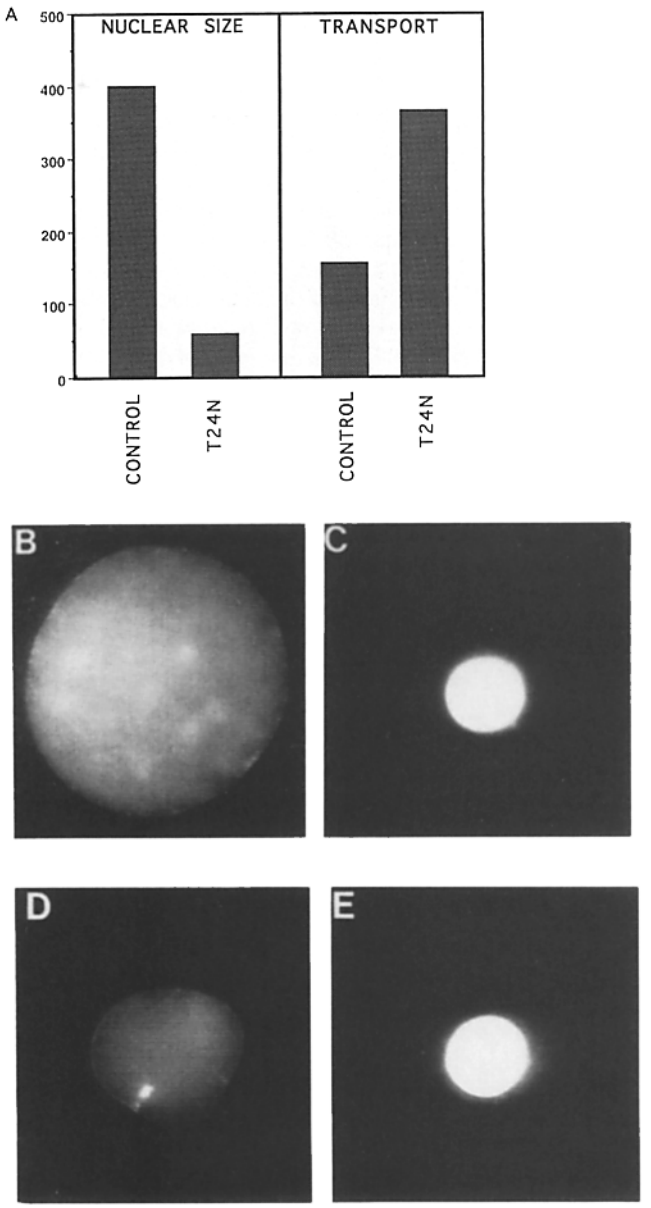

Figure 4. Protein import into nuclei. Sperm chromatin was added to crude interphase extracts of Xenopus eggs in the presence or absence (no addition) of T24N TC4 protein. TRITC-human serum albumin conjugated to a peptide containing the SV-40 T antigen nuclear import signal was added to the extracts at 30 min after the start of the room temperature incubation and samples were withdrawn at 120 min. Fluorescence was quantitated using a linearly calibrated CCD camera. Shown are relative values for nuclear volumes (calculated from microscopic measurements of nuclear radius) and relative values for luminance/pixel (intensity of fluorescence) which is proportional to the total substrate concentration. Values are the average from measurements of fluorescent intensity in 10–20 nuclei/sample. (*B–E*) Fluorescent micrographs showing the accumulation of rhodamine-labeled transport substrate. (*B*) Control nuclei. (*C* and *E*) Nuclei from extracts treated with T24N protein. (*D*) Nuclei grown in the presence of wheat germ agglutinin (0.25 mg/ml). T24N mutant TC4 protein was added to extracts along with sperm chromatin. After nuclear formation, rhodamine-labeled SV-40 T antigen–human serum albumin conjugate was added to the extracts and fluorescent micrographs were taken of the imported substrate.

port more efficiently than those of the control nuclei. This is because the volume of the T24N nuclei is much smaller than that of control nuclei. Therefore, a smaller absolute amount of substrate must be transported to achieve higher concentrations within the nuclei. In order to eliminate the effects of differences in nuclear size between control and T24N-treated extracts, we have modified the transport assay. In the above experiment, nuclei were assembled in the presence of wild-type or mutant TC4 and then tested for transport. We instead tested for an effect of mutant TC4 added after the assembly of nuclei. For this, chromatin was added to an extract and nuclei were allowed to grow for 40 min to a relatively large size. The T24N mutant TC4 protein was then added, the reaction incubated for 20 min, and fluorescent transport substrate was added. Quantitation of these assays at several time points revealed a very mild (less than twofold) defect in nuclear transport when T24N mutant TC4 protein was present (data not shown). In addition, the T24N mutant TC4 had less than a twofold effect on nuclear import when added to an in vitro assay system using digitonin-permeabilized tissue culture cells supplemented with Xenopus egg cytosol (similar to the assay used in Moore and Blobel, 1993 and Melchior et al., 1993) (Powers M., L. Strachan, and O. Forbes, unpublished observations).

The experiment described above indicates that addition of T24N to extracts might have a modest effect (twofold) on nuclear transport. However, because little is known about the relationship between nuclear transport and nuclear growth we could not, based on the experimental evidence presented above, rigorously eliminate the possibility that a twofold reduction in transport rate could account for the complete failure of nuclear growth in T24N-treated extracts. To address this possibility directly, we have used limiting concentrations of WGA, a known inhibitor of nuclear transport (Finlay et al., 1987), to investigate the relationship between nuclear transport and nuclear growth rates. By adding different concentrations of WGA to egg extracts we found that WGA concentrations below 0.1 mg/ml of extract had little effect on the rate of either nuclear growth or transport. Concentrations of WGA above 1.0 mg/ml of extract completely abolished both growth and transport. At this high concentration of WGA, small nuclei formed which were identical in size to nuclei formed in the presence of the T24N mutant TC4 protein (results not shown). However, unlike T24N nuclei, these WGA-treated nuclei did not accumulate measurable amounts of transport substrate. At concentrations of WGA between 0.1–1.0 mg/ml of extract, both nuclear transport and nuclear growth were slowed in a dose-dependent fashion. Therefore, using WGA we could reduce transport in a controlled fashion and determine how this reduction affected nuclear growth. Specifically, at a WGA concentration of 0.25 mg/ml we found that nuclear transport rates were decreased four- to fivefold and two- to threefold relative to nuclei in control and T24N-treated extracts, respectively. However, even though nuclear transport was reduced to levels significantly lower than that found in extracts treated with T24N protein, these nuclei continued to grow (Fig. 4, compare *D* and *E*). For example, at 70 min of incubation, nuclei assembled in the presence of WGA were 5.4 times larger in volume than nuclei formed in the presence of T24N despite the fact that the rate of transport by the WGA-treated nuclei was reduced approximately threefold relative to nuclei formed in the presence of the T24N mutant. Together these experiments demonstrate several points. First, the observation that limiting nuclear transport limits nuclear growth demonstrates that nuclear transport is, not surprisingly, required for nuclear growth beyond the initial phase of nuclear envelope assembly. Second, the observation that nuclear transport continues at high rates without nuclear growth in the presence of the T24N TC4 protein demonstrates that nuclear transport is necessary but not sufficient for nuclear growth (i.e., WGA-treated nuclei transported less protein but grew quite well). Third, their ability to accumulate the transport substrate demonstrates that nuclei formed in the presence of TC4 have an intact nuclear envelope. Thus, DNA replication is not blocked due to failure to form an intact envelope. Finally, the results indicate that T24N is not inhibiting nuclear growth as a result of inhibiting transport, since nuclear transport is almost unaltered.

TC4 T24N Protein Prevents Entry into Mitosis

To investigate a possible role for TC4 in the signaling pathway linking DNA replication and mitosis, sperm chromatin (500 nuclei/μl) was added to cycling extracts of *Xenopus* eggs which can perform multiple cell cycles in vitro, oscillating between S and M phases of the cell cycle (Murray and Kirschner, 1989; Dasso and Newport, 1990). Wild-type, T24N or G19V mutant TC4 protein was then added to these cycling extracts to test whether they affected the normal cycling of the extract. Additionally, we included a sample containing aphidicolin as a control for the functioning of the feedback system in the extract. Extracts were incubated at room temperature and samples were removed every 10 min, stained with Hoechst, and observed by fluorescence and phase contrast microscopy. Mitosis, as scored by nuclear envelope breakdown and chromatin condensation, occurred at 65–80 min in cycling extracts to which no TC4 was added. Addition of either wild-type or G19V protein delayed entry into mitosis slightly, if at all (Table I). This was true for several different preparations of proteins from *E. coli*. In contrast, addition of any of several preparations of mutant T24N protein significantly delayed or entirely arrested the cell cycle prior to mitosis (Table I). In several cases (see for example, Experiment 1, Table I) the arrest with the T24N mutant was even more enduring than that obtained with aphidicolin and nuclei (extract to extract variability in the permanency of the aphidicolin arrest may be due to the lability of some component of the DNA feedback system). These results indicate that the T24N mutant protein, like feedback from DNA replication, can prevent entry into mitosis.

Override of the T24N Arrest

In the experiment described above, it was essential to demonstrate that the T24N cell cycle arrest was due to a defect

Table I. Time of Mitosis

	Experiment 1	Experiment 2	Experiment 3
No addition	65	65	80
+Aphidicolin	95	ND	>140
Wild type	75	65	90
T24N	>155	>120	>140
G19V	ND	75	ND

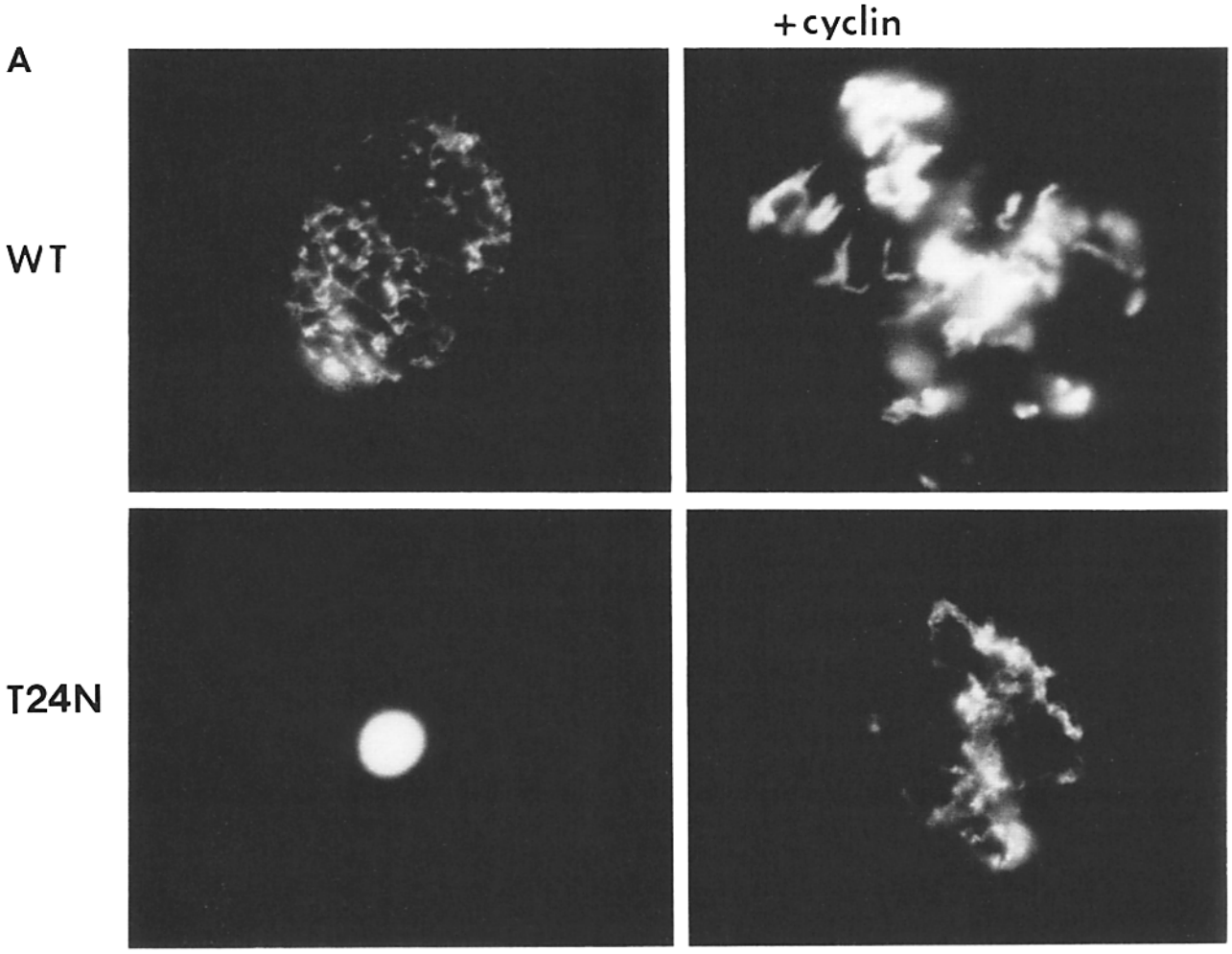

Figure 5. (*A*) Crude interphase extracts containing nuclei formed in the presence of wild-type T24N mutant TC4 protein were treated with recombinant GST-cyclin protein to promote entry into mitosis. Nuclei were fixed and stained with Hoechst dye for visualization by fluorescence microscopy. (*B*) Protein translation: cycling extracts containing wild-type or T24N mutant TC4 proteins were incubated in the presence of [^{35}S]methionine and samples were withdrawn and boiled in SDS loading buffer and resolved by SDS-PAGE to visualize total protein synthesis. Gels were soaked in 1 M sodium salicylate prior to autoradiography. (*C*) cdc2–cyclin complex formation: samples prepared as in *A* were withdrawn at 45 min. These samples were processed on p13 Sepharose beads. Proteins were eluted from these beads by boiling in SDS-containing sample buffer and resolved by SDS-PAGE. (*D*) cdc2 tyrosine-threonine phosphorylation: aliquots were withdrawn at 10-min intervals from cycling extracts containing either wild-type or T24N mutant protein and were resolved by SDS-PAGE. Proteins were transferred to PVDF-immobilon and detected with anti-cdc2 sera and HRP anti-rabbit sera in conjunction with the ECL kit from Amersham.

in regulatory pathways controlling entry into mitosis and not simply a nonspecific effect due to the destruction of some cellular component necessary for the execution of mitosis. Moreover, it was essential to establish that it would be possible to accurately identify the time of mitosis as monitored by nuclear envelope breakdown and mitotic chromatin condensation in the small aberrant nuclei produced in the presence of the T24N mutant. To do this, we have taken advantage of the fact that bacterially produced recombinant GST–sea urchin cyclin B induces mitosis when added to Xenopus egg extracts (Murray and Kirschner, 1989). In addition, it appears that p34^{cdc2}-cyclin complexes formed with this fusion cyclin protein are not sensitive to inhibition by signals from incompletely replicated DNA, since addition of

this protein to extracts arrested with aphidicolin drives these extracts into mitosis (Smythe and Newport, 1992). Using this fusion protein we found that addition of GST-cyclin B to cycling extracts arrested with the T24N mutant protein caused the extracts to enter mitosis within 20 min of addition. Furthermore, mitosis was easily identifiable in the T24N mutant-containing extract, since nuclear envelope disassembly was observed by phase contrast microscopy and the condensed mitotic chromosomes were quite similar to those seen in wild-type extracts (taking into account differences in fine structure resulting from the fact that the T24N nuclei did not replicate prior to condensation) (Fig. 5 *A*). The override of the T24N-induced arrest by the creation of active MPF in the extract indicates that the block to entry into mitosis is upstream of MPF, as has been shown previously for feedback generated by incompletely replicated DNA in *Xenopus* and other systems (Dasso and Newport, 1990; Enoch and Nurse, 1990).

Although creation of fresh MPF did override the T24N-induced cell cycle arrest, it remained possible that the failure to enter mitosis in the presence of T24N TC4 resulted from an inability of the extract to produce potentially functional endogenous MPF activity. Such a defect might result from a failure to synthesize adequate cyclin protein or an inability of newly translated cyclin to bind to $p34^{cdc2}$. To test these possibilities, we incubated cycling extracts containing either wild-type or T24N mutant TC4 proteins with [^{35}S]methionine and withdrew samples at 15-min intervals into sample buffer for SDS-PAGE, allowing us to look at both total and cyclin-specific protein synthesis in the extracts. The amount of protein synthesized by these two samples was approximately equivalent (Fig. 5 *B*). In the wild-type sample which went into mitosis at ~80 min, the cyclin protein was degraded and could not be seen at the 90-min time point. In contrast, in the T24N mutant-treated extracts, cyclin protein continued to accumulate during the entire 90-min time period. The sucl protein of *S. Pombe* (p13) binds to $p34^{cdc2}$ (and other members of the cdc2 protein family) and can, when coupled to Sepharose beads, be used to precipitate cdc2-containing protein complexes out of extracts. To determine if newly synthesized cyclin was associating with cdc2, extracts treated with either wild-type or T24N mutant TC4 protein were labeled with [^{35}S]methionine for 45 min and then the endogenous cdc2 was precipitated with p13 Sepharose beads. After several buffer washes, beads were boiled in protein sample buffer and the released labeled proteins were resolved by SDS-PAGE. Results from this experiment showed that the presence of the T24N TC4 protein had no adverse effect on the binding of $p34^{cdc2}$ to cyclin protein (Fig. 5 *C*). In addition, it is clear that similar amounts of $p34^{cdc2}$ and cyclin proteins were precipitable from the extracts at 45 min. From these experiments, it can be concluded that addition of the T24N mutant protein to extracts does not inhibit cyclin synthesis or its association with cdc2 protein.

Inhibition of $p34^{cdc2}$ by feedback from DNA replication operates largely through stimulation of the tyrosine kinase which phosphorylates and thereby inhibits $p34^{cdc2}$. In the presence of nuclei and aphidicolin, the activity of this kinase is stimulated approximately 5–10-fold over background levels (Smythe and Newport, 1992). In addition, phosphorylation of a threonine at residue 14 of cdc2, which also inhibits the kinase activity of $p34^{cdc2}$, is elevated when the feedback system is active (Kornbluth et al., 1994). Therefore, it is possible to monitor the presumed last step in the feedback pathway (phosphorylation of Tyr 15 and Thr 14 of $p34^{cdc2}$) by observing changes in the phosphorylation state of cdc2 protein throughout the cell cycle. It has been shown previously that cdc2 migrates as three bands on SDS–polyacrylamide gels. The fastest migrating form is not phosphorylated at either Tyr 15 or Thr 14 (both negative regulatory sites) (Solomon et al., 1992). The slowest migrating form is phosphorylated at both of these residues and the band between these two is singly phosphorylated at one or the other of these sites. When wild-type TC4 protein was added to a cycling extract, the extract entered mitosis at 75 min (monitored microscopically) and the cdc2 protein which had been phosphorylated prior to mitosis was returned to a predominantly dephosphorylated state (80 min; Fig. 5 *D*). The phosphorylated forms of $p34^{cdc2}$ began to accumulate again as the extract entered the next cell cycle (90–110 min). In contrast, addition of equivalent amounts of the T24N mutant protein to cycling extracts led to the progressive accumulation of the singly and double phosphorylated forms of cdc2 with an almost quantitative conversion to the doubly phosphorylated form by 110 min. This result demonstrates that T24N protein promoted the accumulation of the phosphorylated forms of cdc2 in a manner similar to that of feedback from DNA replication, thereby inhibiting MPF activation and preventing entry into mitosis.

In order to test whether the endogenous cdc2-cyclin complexes formed in the presence of the T24N mutant were capable of full activation, we added okadaic acid to the extracts. Okadaic acid can uncouple mitosis from the completion of S phase in BHK cells and in Xenopus egg extracts (Lorca et al., 1991; Schlegel and Pardee, 1987). Smythe and Newport (1992) have reported that the ability of okadaic acid to overcome cell cycle arrest is due, at least in part, to suppression of the tyrosine kinase which normally phosphorylates $p34^{cdc2}$, keeping it inactive during DNA replication. In addition, Kumagai and Dunphy (1992) have shown that okadaic acid can stimulate the cdc2-specific tyrosine phosphatase activity of cdc25 protein found in Xenopus extracts, thereby accelerating tyrosine dephosphorylation of $p34^{cdc2}$ and, consequently, MPF activation. In Experiment 2 shown in Table I, we added okadaic acid to the TC4 T24N protein-arrested extract at 73 min and these extracts entered mitosis by 80 min. This result demonstrates that there was endogenous MPF in the extract poised for activation but that this MPF was suppressed in activity by the added T24N mutant TC4 protein.

Interestingly, it has been shown in several systems that caffeine can override the cell cycle arrest induced by inhibition of DNA replication (Schlegel and Pardee, 1986; Dasso and Newport, 1990). In our experiments, caffeine was able to override the arrest induced by nuclei and aphidicolin but could not override the TC4 T24N protein-induced inhibition of entry into mitosis (data not shown). Therefore, if TC4 is a component of the DNA feedback system, it may well be that the site of action of caffeine is either upstream of or at the level of the TC4 protein.

The TC4 T24N Induced Inhibition of Mitosis Does Not Require DNA

In the experiments described above, the progress of the ex-

tracts through the cell cycle was monitored by microscopic observation of the nuclei formed in the extract. It might be argued that TC4 T24N protein could inhibit entry into mitosis under these conditions by blocking replication of the sperm nuclei and acting, in effect, like aphidicolin. In other words, these incompletely replicated nuclei would be responsible for generating the feedback signal. To test whether the TC4 mutant protein has an independent effect on the cell cycle, we added wild-type TC4 protein or T24N TC4 protein to cycling extracts in the absence of any sperm chromatin. Aliquots were withdrawn at 10-min intervals and cdc2/cyclin kinase activity was assayed by measuring in vitro phosphorylation of the substrate histone H1 (Arion et al., 1988; Dunphy and Newport, 1989; Labbe et al., 1989). In the presence of added wild-type TC4 protein, the histone H1 kinase activity peaked at 90 min and then dropped abruptly, indicating entry into mitosis and subsequent degradation of cyclin protein and inactivation of MPF (Fig. 6). In contrast, in the presence of added T24N mutant protein, the histone H1 kinase activity initially increased and then reached a steady level which was insufficient to induce mitosis (as shown by a visual observation of a parallel sample which contained nuclei and showed an identical suppression of histone H1 kinase activation in the presence of the T24N TC4 protein). This profile of histone H1 activity can also be seen in extracts where DNA replication feedback is created by addition of nuclei and aphidicolin (Dasso and Newport, 1990; Kornbluth, S., personal observation). The suppression of histone H1 kinase activity by the T24N protein in the absence of nuclei indicates that MPF activity is negatively regulated by this mutant TC4 protein even in the absence of any template DNA to generate a signal.

In support of this conclusion, we found that the phosphorylation of $p34^{cdc2}$ on Tyr 15 and Thr 14 promoted by the T24N mutant protein in the presence of nuclei (Fig. 5 *D*) was reproduced in the absence of any nuclei (Fig. 7). Samples of a cycling extract were incubated with either wild-type (Fig. 7, *B* and *D*) or T24N (Fig. 7, *A* and *C*) TC4 protein in the presence (Fig. 7, *C* and *D*) or absence (Fig. 7, *A* and *B*) of nuclei. Aliquots of the extracts were withdrawn at 10-min intervals, resolved by SDS-PAGE, and immunoblotted with anti-cdc2 sera. When T24N TC4 protein was added to the cycling extract, cdc2 became increasingly phosphorylated with time. This phosphorylation occurred even in the complete absence of nuclei (Fig. 7 *A*). In contrast, in the presence of wild-type TC4 protein, the phosphorylated forms of $p34^{cdc2}$ accumulated only transiently prior to the onset of mitosis both in the presence (Fig. 7 *D*) and absence (Fig. 7 *B*) of nuclei, showing that these extracts had entered mitosis. Taken together, these data show that TC4 protein has direct effects on the cell cycle which are distinct and separable from effects on nuclear structure and nuclear import.

Discussion

The mutant T24N TC4 protein described in this study had multiple effects when added to extracts of Xenopus eggs. Upon addition of demembranated sperm chromatin to interphase extracts, this mutant inhibited nuclear growth and DNA replication but did not interfere with nuclear envelope assembly or nuclear transport. In cycling extracts, the mutant protein prevented activation of MPF and hence entry into mitosis. This effect was completely independent of its effect on nuclear functions since activation of cdc2 kinase activity was inhibited even in the absence of nuclei. Together, these observations implicate TC4 as functioning both in an essential process for nuclear growth and in regulation of the initiation of mitosis.

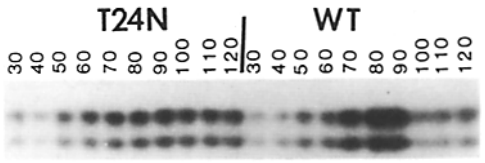

Figure 6. Suppression of histone H1 kinase activity by the T24N mutant of TC4. Cycling extracts were incubated at room temperature in the presence of wild-type TC4 protein or T24N protein. Samples were withdrawn every 10 min and frozen in liquid nitrogen. Frozen samples were rapidly thawed and assayed for histone H1 kinase activity. Assays were boiled in loading buffer for SDS-PAGE and resolved on 10% polyacrylamide gels. Dried gels were exposed to x-ray film for autoradiography for 1 h.

Effects of T24N Mutant on Nuclear Morphology

In this report, we found that addition of the TC4-T24N mutant protein to nuclear assembly extracts had profound effects on nuclear growth. Specifically, although sperm chromatin could act as a template for the initial formation of a nuclear envelope containing nuclear pores when mutant T24N TC4 was present, further growth of the nuclei beyond this minimal size did not occur. This observation suggests that TC4 may normally participate in a critical step in nuclear formation at the end of mitosis and/or in maintaining essential nuclear structures during interphase. Using Xenopus extracts, several steps in nuclear formation have been well characterized (Newport, 1987; Wilson and Newport, 1988; Pfaller et al., 1991; Boman et al., 1992; Newport and Dunphy, 1992). The first steps in nuclear formation involve binding of membrane vesicles to chromatin followed by fusion of these vesicles to each other to form an intact nuclear envelope immediately around the condensed chromatin.

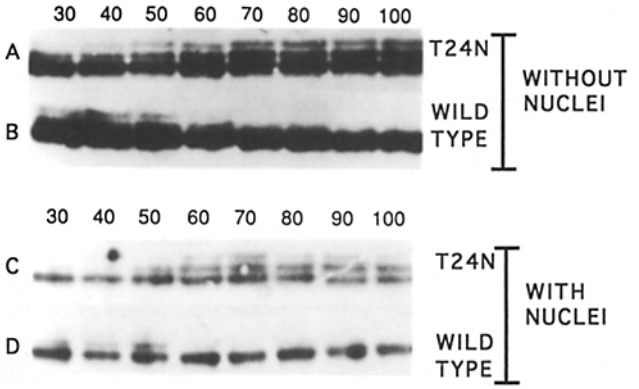

Figure 7. Phosphorylation of cdc2 induced by T24N TC4 protein. Cycling extracts were incubated in the presence of T24N protein (*A* and *C*) or wild-type TC4 protein (*B* and *D*). Samples in *C* and *D* contained nuclei formed from added sperm chromatin whereas *A* and *B* contained no nuclei. Aliquots of these extracts were processed as in Fig. 5 *D*.

Subsequent to or during this fusion step, nuclear pores assemble in the envelope. Our results demonstrate that the TC4-T24N mutation does not inhibit these initial stages of the assembly process. Growth of the nucleus beyond this initial stage is not well characterized but clearly must be contingent upon several processes including: (*a*) decondensation of chromatin, (*b*) continued fusion of new membrane to the existing envelope, (*c*) selective transport of proteins through the nuclear pores, and (*d*) assembly of these newly transported proteins into essential structural elements within the nucleus. Inhibition of one or more of these processes by the T24N mutant protein would inhibit further nuclear growth.

Our results strongly suggest that the T24N mutant protein does not block chromatin decondensation. In support of this conclusion, we have shown that when highly condensed sperm chromatin is added to egg extracts containing the T24N protein but lacking nuclear membrane components, the sperm chromatin decondenses to the same extent and at the same rate as chromatin added to identical extracts lacking the T24N protein (Fig. 3). Moreover, at the level of light microscopy the extent and rate of decondensation in these membrane-depleted extracts appears to be similar to the decondensation rate observed for chromatin assembled into normal nuclei. Therefore, the condensed appearance of chromatin within nuclei formed in the presence of the T24N mutant of TC4 is likely due to the fact that the chromatin is packaged at a very high density within the minimally sized nuclear envelope. We could find no evidence that the T24N mutant blocks fusion of membrane vesicles at the nuclear envelope. This conclusion is based on the observation that the initial binding and fusion of membrane vesicles to sperm chromatin is the same in control and T24N-treated extracts (data not shown) but, more importantly, on the finding that the nuclei formed accumulate transport substrate and thus clearly have an intact nuclear envelope. Once this initial minimal envelope is formed, further growth of the envelope would normally be dependent on the fusion of vesicles to the outer membrane of the nuclear envelope. This newly inserted membrane would then diffuse around the nuclear pores to distribute itself in both the inner and outer nuclear membrane. Although the T24N mutation could inhibit this later fusion event, preliminary studies indicate that the fusion of nonnuclear-associated endoplasmic reticulum vesicles (the major component of the outer nuclear envelope) is normal in the presence of T24N mutant TC4 protein (data not shown) (for assay details see Newport and Dunphy, 1992). Based on these results, we conclude that the T24N mutant of TC4 does not directly inhibit either chromatin decondensation or nuclear membrane growth.

Once an intact nuclear envelope has completely encapsulated chromatin, further growth of the nucleus is dependent on selective transport of proteins through the nuclear pores. However, by measuring uptake of a fluorescently labeled nuclear transport substrate into nuclei in the presence and absence of T24N we have found that nuclear transport was depressed at most twofold by the T24N mutant protein. In fact, because nuclei do not grow in the presence of the T24N mutation, transport substrate actually accumulates to higher concentrations within nuclei formed in the presence of T24N than within controlled nuclei (Fig. 4, *A–C*). Using WGA to limit nuclear transport we have investigated the relationship between nuclear growth and transport. These results demonstrated that WGA-treated nuclei which transported less efficiently and accumulated less total transport substrate than T24N-treated nuclei, were still able to grow. Together these observations indicate that the T24N mutation of TC4 does not restrict nuclear growth by inhibiting nuclear transport. Further they show that nuclear transport is necessary but not sufficient for nuclear growth.

Moore and Blobel (1993) have recently presented evidence that TC4 is an important factor for nuclear import of proteins in a permeabilized cell system. Similarly, Melchior et al. (1993) find that nuclear transport is GTPγS sensitive and that this sensitivity can be mediated by TC4. However, it is important to note that both these permeabilized cell assays were performed with dilute cytosol (2.5–10 mg/ml). Indeed, Melchior et al. (1993) found that when physiological concentrations of cytosol were used, GTPγS caused no TC4-mediated inhibition of nuclear transport. In this study, we used physiological concentrations (~40 mg/ml) of Xenopus cytosol in a system which closely approximates an in vivo situation in that the extract repeatedly cycles through the cell cycle. When we added equimolar (or less) amounts of mutant T24N TC4 to that present in the extract, very little effect on transport was observed, while major effects on nuclear growth were seen. While the high cytosol concentration in our assay system might account for the absence of any observed effect of TC4 on nuclear import, it should be noted that the T24N mutant TC4 protein had no marked effects on nuclear import even in the permeabilized tissue culture assay used by Moore and Blobel (1993) and Melchior et al. (1993).

Taken together, our results indicate that in the presence of the T24N mutant of TC4 an intact nuclear envelope containing functional nuclear pores can form. This suggests that the T24N mutation is inhibiting a process which is required for nuclear growth beyond this point. The observation that nuclei formed in extracts depleted of RCC1 protein arrest at a similar point in the assembly pathway (Dasso et al., 1992; Dasso, M., unpublished observations) strongly suggests that TC4 normally interacts with RCC1 to facilitate further nuclear growth beyond this initial stage. Thus, it is quite likely that the T24N mutant acts in a dominant negative fashion, either blocking interaction between wild-type TC4 and RCC1 or inhibiting interaction of endogenous TC4 protein with other cellular proteins, thereby inhibiting further nuclear growth. At present, relatively little is known about the structural dynamics which drive nuclear growth. Clearly, once a functional nuclear envelope has formed, new proteins will be selectively transported into nuclei where they will accumulate. It is likely that many of these proteins will be assembled into structural elements that both drive nuclear growth and maintain nuclear organization during interphase. For example, it has been shown that at the end of mitosis nuclear lamins, a major structural element of the inner nuclear envelope, enter the nucleus after initial envelope formation and then assemble at the nuclear periphery (Newport, 1987; Newport et al., 1990; Meier et al., 1991; Chaudhary et al., 1993; Foisner and Gerace, 1993). In lamin-depleted extracts, nuclei form a nuclear envelope but grow very slowly. These lamin-depleted nuclei are also unable to carry out DNA replication. The observation that nuclei formed in the presence of T24N are able to accumulate proteins via transport yet do not grow suggests that the T24N mutant may be blocking the assembly of nuclear proteins which, like the

lamin proteins, are required for envelope growth and establishing the structural environment needed to carry out basic nuclear functions such as DNA replication.

Effects of T24N on Regulating the Initiation of Mitosis

We have found separately that addition of the T24N mutant of TC4 protein to cycling extracts prevents these extracts from entering mitosis. Moreover, because this arrest can occur in the absence of nuclei it is independent of the effect of T24N on nuclear growth. Arrest of the cell cycle by T24N TC4 is consistent with a role for TC4 as a part of a checkpoint control system which regulates entry into mitosis. Consistent with this, we have shown that addition of the T24N protein to cycling extracts inhibits the mitotic regulator cdc2 kinase. This inhibition is not due to nonspecific effects such as the inhibition of cyclin synthesis or its association with cdc2 kinase. Rather we have shown that T24N causes cdc2 to become stably phosphorylated on the negative regulatory sites located at Thr 14 and Tyr 15. This strongly suggests that T24N is modulating the activities of the kinase/phosphatase system(s) which control the level of phosphorylation at these regulatory sites (Smythe and Newport, 1992). We have further found that the inhibitory effect of T24N on cell cycle progression is reversible. Specifically, addition of the phosphatase inhibitor okadaic acid to extracts arrested in interphase by the T24N mutant protein causes the cell cycle to progress into mitosis.

In all of the many instances studied to date, mutations in *ras*-related proteins equivalent to the T24N mutation in the TC4 protein generate a protein which acts in a dominant negative manner to suppress signaling by the wild-type protein. Our observations show that the addition of the T24N TC4 mutant protein to cycling extracts prevents initiation of mitosis. We would propose two possible models: a somewhat heretical explanation of this data is that the GDP-bound form of TC4 protein is an active intermediary in the signal transduction pathway inhibiting entry into mitosis prior to the completion of replication. This interpretation would imply that the TC4 protein was "active" in negatively regulating $p34^{cdc2}$ when in its GDP-bound conformation and that ongoing DNA replication might generate GDP-TC4 (Fig. 8, *Model 2*). According to this model, GDP-TC4 would serve (directly or indirectly) as an activator for the Tyr 15 and Thr 14 kinases negatively regulating cdc2.

Perhaps a more direct interpretation of the observation that the T24N mutant protein inhibits entry into mitosis is that wild-type TC4 is normally part of a positive signaling system which promotes entry into mitosis when certain premitotic processes are complete (Fig. 8, *Model 1*). According to this model, completion of some premitotic process(es) would promote exchange of GDP for GTP on TC4 by the RCC1 protein. The GTP-bound form of TC4 would then promote cdc2 activation. While it is possible that the functions of TC4 in maintaining proper nuclear structure and in regulating the cell cycle are unrelated, it is tempting to speculate that nuclear structural rearrangements which are prerequisites for entry into mitosis (such as adequate nuclear growth, completion of DNA replication or rearrangements of chromatin structure) are monitored by RCC1/TC4. Because RCC1 is a highly abundant DNA-binding protein, the protein may play a fundamental role in maintaining chromatin structure. A dual function for the RCC1/TC4 system in both maintaining chromatin structure and in monitoring certain functions associated with this structural arrangement would be consistent with our observations that addition of the T24N mutant of TC4 inhibits both nuclear growth and progression into mitosis. Regardless of the precise premitotic cellular function being monitored by TC4, our observation that the T24N dominant negative mutant appears to be blocking entry into mitosis by maintaining cdc2 in an inactive state strongly suggests that the wild-type TC4 signaling system normally promotes mitosis by modulating the activities of the kinase/phosphatase system(s) which regulates cdc2.

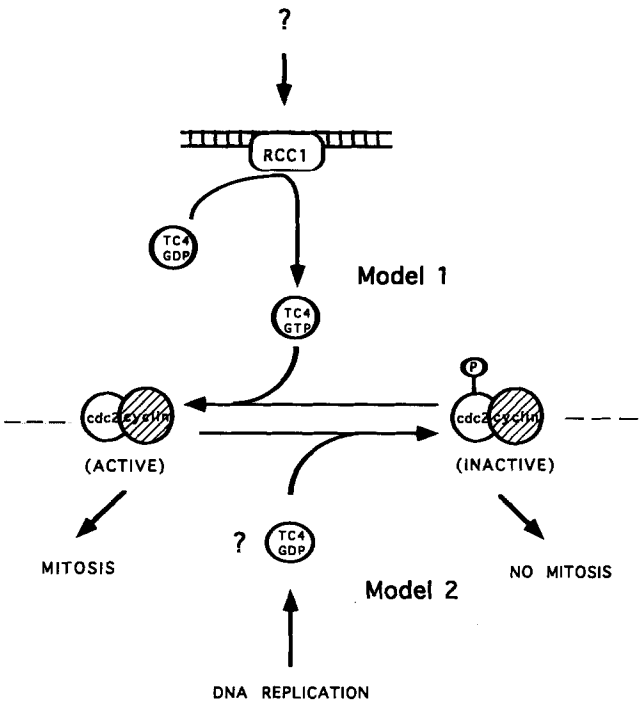

Figure 8. Regulation of cdc2 by TC4 protein. (Model 1) In this model, chromatin-bound RCC1, acting as a guanine nucleotide-releasing protein, catalyzes the exchange of bound GDP on TC4 for GTP. The GTP-bound form of TC4 could then act to stimulate entry into mitosis by positively regulating the activity of cdc2–cyclin complexes, perhaps by inactivating the tyrosine and threonine kinases which negatively regulate cdc2. This pathway involved in the positive regulation of cdc2 activity would act in opposition to the negative regulatory pathway inhibiting cdc2 activation prior to completion of DNA replication. The nuclear function(s) that are monitored such that RCC1 is stimulated to generate the GTP-bound form of TC4 and promote entry into mitosis are currently unknown. (Model 2) In this model, GDP-TC4 would be generated during replication and this GDP-TC4 could negatively regulate cdc2 activation, perhaps by stimulating the tyrosine and threonine kinases regulating cdc2.

A role for the RCC1/TC4 system as a positive regulator of mitosis, although consistent with our data, might at first appear to be in direct conflict with previously published observations describing the effects of RCC1 mutations on cell cycle progression. In particular, mutations which result in a quantitative degradation of RCC1 protein enter mitosis even though DNA replication has not been completed (Nishimoto et al., 1978). Importantly, this premature entry into mitosis is a slow process and requires ongoing protein synthesis. Based on the model presented above, we would expect that

in the absence of RCC1 protein the positive TC4 signal would not be generated. As such, the model predicts that cells would remain in S phase rather than enter mitosis. However, it should be remembered that in the absence of RCC1, DNA replication also stops. Therefore, in the absence of RCC1, we would expect both the postulated TC4-dependent positive regulatory system, as well as the replication-dependent negative regulatory system, to be inactivated. Under these conditions regulation of cdc2 kinase activity would depend on both the basal activities of the kinase/phosphatase system which regulate phosphorylation of cdc2 and the concentration of newly synthesized cyclin protein. At low levels of cdc2–cyclin complex the kinase/phosphatase system will keep cdc2 inactive. However, above a certain cdc2–cyclin concentration, the capacity of the unregulated kinase/phosphatase system to maintain all of the cdc2–cyclin substrate inactive will be exceeded and a small amount of active cdc2 will accumulate. Due to the ability of cdc2 to activate itself in an autocatalytic manner (Solomon et al., 1990), this small amount of active cdc2 would rapidly activate the remaining inactive cdc2 and the cells would enter mitosis spontaneously. The spontaneous oscillations between S phase and mitosis which occur even in the absence of nuclei during the early Xenopus embryonic cell cycle is an example of such an unregulated cell cycle. Therefore, when potential effects on both positive and negative checkpoint systems are taken into consideration, the observed entry into mitosis of cells lacking RCC1 could be reconciled with a model in which TC4 acts as part of a system positively regulating entry into mitosis.

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