Premature Chromatin Condensation induced by loss of RCC1 is inhibited by GTP-and GTP γ S-Ran, but not GDP-Ran

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Premature Chromatin Condensation induced by loss of RCC1 is inhibited by GTP- and GTPγS-Ran, but not GDP-Ran

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Summary

RCC1 is a guanine nucleotide exchanging factor acting on nuclear G protein Ran. Premature chromatin condensation (PCC) occurs in the temperature sensitive (ts) *rcc1*$^{-}$ mutant of the BHK21 cell line, tsBN2 at the restrictive temperature. Since in the absence of RCC1, GDP-Ran predominates, this result indicates that the activation of MPF is inhibited by GTP-Ran. However, experiments with Ran mutants to determine whether GTP- or GDP-Ran prevents activation of MPF have yielded conflicting results. In order to clarify this point, we have microinjected nucleotide bound Ran, instead of mutated Ran, into the nuclei of tsBN2 cells treated to reduce RCC1-mediated guanine nucleotide exchange. GTP-Ran, GTPγS-Ran and GDP-Ran all inhibited chromatin condensation. However, the inhibition of chromatin condensation by GDP-Ran could be completely abolished by co-injection with GDP, but not GTP. Thus, we conclude that GTP-Ran blocks the activation of MPF and that hydrolysis of GTP is not required to prevent MPF activation.

Introduction

The events in the cell cycle of most organisms are ordered into dependent pathways in which the initiation of late events is dependent on the completion of early events. In eukaryote, for example, mitosis is initiated after DNA synthesis is completed. Some dependencies can be relieved by mutations (Hartwell and Weinert, 1989). tsBN2 cell line was isolated as a ts mutant from the BHK21/13 cell line that was derived from golden hamster (Nishimoto and Basilico, 1978). tsBN2 cells arrested at G1/S with hydroxyurea (HU) show PCC by shifting to the nonpermissive temperature of 39.5°C (Nishimoto and Basilico, 1978, Nishimoto *et al.*, 1981, Nishitani *et al.*, 1991). In this mutant, the onset of mitosis is uncoupled from the completion of DNA replication.

The human gene *RCC1* (regulator for chromosome condensation) complements tsBN2 mutation (Kai *et al.*, 1986; Ohtsubo *et al.*, 1987), and proved to be mutated in tsBN2 cells (Uchida *et al.*, 1990). RCC1 is abundant nuclear protein associated with chromosomes during the interphase of the cell cycle (Ohtsubo *et al.*, 1987). RCC1 is disappeared at the restrictive temperature in tsBN2 cells at any time in the cell cycle. From S phase onwards, loss of RCC1 induces a premature activation of MPF (the activation of $p34^{cdc2}$ histone H1 kinase), so that a normal mitotic cycle is induced, including the formation of a mitotic spindle, the nuclear membrane breakdown, and the appearance of the mitosis-specific MPM-2 antigen (Nishitani *et al.*, 1991). The microinjection of RCC1 inhibits PCC (Seino *et al.*, 1992, Azuma *et al.*, 1996). Thus, RCC1 has been considered to be involved in coupling S phase with mitosis. The RCC1 homologues, *pim1*$^{+}$, *SRM1/PRP20/MTR1*, and BJ1 have been isolated from *S. pombe*, *S. cerevisiae* and *Drosophila*, respectively (Dasso, 1993). A ts *rcc1*$^{-}$ mutant of *S. pombe*, *pim1-d1* is arrested at the end of mitosis with condensed chromosomes (Matsumoto and Beach, 1991, Sazar and Nurse, 1992), while *S. cerevisiae rcc1*$^{-}$ mutants have various phenotypes. The *srm1-1* is isolated as a suppressor restoring mating capacity to the receptorless mutants (Clark and Sprague, 1989). The *prp20* (pre-RNA processing) mutant is defective in mRNA splicing (Aebi *et al.*, 1990). In the *mtr1* (mRNA transport) mutant, mRNA accumulates in nucleus (Kadowaki *et al.*, 1993). It has also been shown that both nuclear

assembly and DNA replication were profoundly disrupted in RCC1 depleted *Xenopus* extracts (Dasso *et al.*, 1992).

Ran (originally named as TC4), a Ras-like small nuclear G protein, was discovered by virtue of its homology to Ras (Drivas *et al.*, 1990). Subsequently, Ran was co-purified with RCC1 protein from HeLa cells. Indeed, RCC1 functions as a guanine nucleotide exchanging factor (GEF) on Ran (Bischoff and Ponstingl, 1991a). In HeLa cells, the amount of Ran is approximately 25- fold molar excess over RCC1(Bischoff and Ponstingl, 1991a, 1991b).

Since Ran is involved in a nuclear transport (Moore and Blobel, 1993, Melchior *et al.*,1993, Görlich and Mattaj, 1996), the phenotypes of *rcc1*$^{-}$ mutants are considered to be a consequence of a defect in nucleocytoplasmic transport. In fact, tsBN2 cells are defective in both mRNA export and protein import (Kadowaki *et al.*, 1993, Tachibana *et al.*, 1994). Similarly to another member of the Ras super-family, GTP-Ran, but not GDP-Ran is active in nuclear transport (Moore and Blobel, 1993, Melchior *et al.*, 1993), and in recovering of DNA replication which is otherwise blocked in RCC1-depleted *Xenopus* extracts (Dasso *et al.*, 1994). However, there are conflicting evidences as to which form of Ran prevents the activation of MPF during S phase. The expression of G19V/Q69L-Ran which is blocked in GTP-form, causes a G2 block in cultured cells (Ren *et al.*, 1994), indicating that GTP-Ran prevents an activation of MPF in S phase. In contrast, Kornbluth *et al.*, (Kornbluth *et al.*, 1994) and Clarke *et al.*, (Clarke *et al.*, 1995) found that T24N Ran which was blocked in GDP-form, inhibited an activation of MPF in *Xenopus* extracts, arguing that GTP-Ran was required for activation of MPF. In order to clarify this disagreement, we have investigated the effect of Ran on PCC induced by the RCC1 mutation in the tsBN2 cell line. We used Ran prebound to guanine nucleotides *in vitro*, instead of Ran mutants, since there is no evidence that mutated Ran proteins behave as a normal GTP or GDP bound Ran, *in vivo*. Incubation of tsBN2 cells at the restrictive temperature prior to microinjection greatly reduces the endogenous guanine nucleotide exchanging activity acting on Ran (Bischoff *et al.*, 1995). Under such conditions, we directly examined the effect of injected Ran-guanine nucleotide complexes.

Materials and methods

Cell lines and culture conditions

The tsBN2 cell line (Nishimoto *et al.*, 1978) was cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% calf serum in a humidified atmosphere containing 10% CO_2, at 33.5°C (the permissive temperature). The restrictive temperature used was 40.5°C. In order to arrest a culture in early S phase, cells were incubated in DMEM medium without isoleucine with 3% dialyzed calf serum for 30 hours, and then fed with DMEM medium containing 10% fetal calf serum and 2.5 mM hydroxyurea for 16 hours at 33.5°C as described (Nishitani *et al.*, 1991).

Protein expression in *E. coli*, and purification

The pET8c expression constructs containing wild type Ran and T24N-Ran were introduced into *E. coli* [strain BL21 (DE3)]. Cultures of transformed bacteria were grown at 37°C (wild type) or 23°C (T24N-Ran). 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 (T24N), after which the bacteria were harvested by centrifugation and frozen at -20°C. Cell-pellet was suspended in the buffer containing 50 mM Tris-HCl (pH 8.0), 0.5 mM DTT, 0.1 mM *p*-amidinophenyl methansulfonyl fluoride and 1 mM EDTA, homogenized in the presence of lysozime (2 mg/g of cell-pellet) for 30 min, incubated for another 30 min in the presence of sodium deoxycholate (0.02%), DNase I (0.2 mg/g of cells) and 10 mM $MgCl_2$, and then centrifuged at 10, 000 x g for 30 min. The supernatant was applied onto DEAE Sephacel in the presence of 100 mM NaCl and the flaw through fraction was precipitated with 45-60% ammonium sulfate. The precipitate containing Ran was resolved in the buffer containing 20 mM HEPES-NaOH (pH7.6), 1 mM DTT, 1 mM $MgCl_2$, 50 mM NaCl, and was fractionated on Sephacryl S-100 column.

The fractions containing Ran were incubated for 45 min on ice, with 2.5 mM of one of GDP, GTP, and GTPγS in the buffer containing 20 mM HEPES-NaOH (pH 7.6), 1

mM DTT, 1 mM $MgCl_2$, 10 mM EDTA, 50 mM NaCl. After incubation, $MgCl_2$ was added to a final concentration of 20 mM to stop the binding reaction, and Ran was further purified by Mono-S HR 5/5 column with the NaCl linear gradient (0.0 - 0.6 M) in the buffer containing 20 mM HEPES-NaOH (pH 7.6), 1 mM DTT, 5 mM $MgCl_2$ and 1 mM CHAPS, and again incubated for 45 min on ice with 1 mM of a guanine nucleotide in the buffer containing 20 mM HEPES-NaOH (pH 7.6), 1 mM DTT, 5 mM $MgCl_2$, 25 mM EDTA. After dialysis in 50 mM HEPES-NaOH (pH 7.5), 1 mM DTT, 25 mM NaCl, 0.5 mM $MgCl_2$ for overnight at 4°C, Ran was concentrated by Ultra free CL.

RCC1 were expressed in *E. coli*, BL21 (DE3), and purified as described (Azuma *et al.*, 1996).

Guanine nucleotide binding

Bound nucleotides of wild type Ran protein were determined by incubating 20 pmol of protein with 2000 pmol of [^{3}H]-GDP or [^{3}H]-GTP (approximative 500 cpm/pmol) at 30°C in 25 µl of binding buffer (20 mM Tris-HCl pH 7.5, 1 mM DTT, 100 mM NaCl, 0.1 % Lubrol, 1 or 20 mM MgCl2, 6 or 1 mM EDTA). The reaction was stopped by the addition of the ice-cold stop buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 25 mM MgCl2). Nucleotides bound to protein were trapped by filtering the reaction mixture through nitrocellulose filters. Filters were washed three times with the same ice-cold buffer. Nucleotides bound to protein were quantitated by scintillation counting.

Preparation of substrates containing NLS

The substrates [fluorescein isothiocyanate labeled bovine serum albumin coupled to peptides containing the SV40 T antigen nuclear localization sequence (NLS)] were prepared as described (Yoneda *et al.*, 1987). 1 mg of synthetic peptide (CGGGPKKKRKVED) was mixed with 8 mg of fluorescein isothiocyanate labeled BSA activated with sulfosuccinimidyl 4-(p-maleimidophenyl) butyrate (Sulfo-SMPB; Pierce).

Microinjection of Ran

Cells were plated at 7 X 10^4 cells on 18 X 18 mm glass coverslips in 35-mm dishes and synchronized at early S phase as described above. Ran-guanine nucleotide complexes suspended in buffer containing 50 mM HEPES-NaOH (pH 7.5), 1 mM DTT, 25 mM NaCl, 0.5 mM $MgCl_2$ were injected into either the cytoplasm or nucleus of individual cells. In every injection, 0.3 mg /ml of Rabbit IgG was co-injected as an indicator. To maintain the pH of the medium during injection, 20 mM (final concentration) of HEPES-NaOH (pH 7.2) was added. In injection of T24N-Ran proteins, both tsBN2 cells and BHK cells were kept at the permissive temperature. 5 hr after injection, cells were fixed with cold (-20°C) methanol, stained with rhodamine-conjugated goat anti-rabbit IgG antibodies (TAGO, USA) and then with 1 μg/ml of DNA-specific dye, Hoechst 33342 (Calbiochem, USA), as described (Seki *et al.*, 1992). Photomicroscopy was performed using a Zeiss Axiophot. Frequency of cells showing PCC was normalized to that of cells injected with Rabbit IgG alone as described (Seki *et al.*, 1992).

The substrates containing NLS were injected with Ran proteins into tsBN2 which were plated and synchronized as described above, cells and 30 min after injection, cells were fixed as described above.

Results

Purification and characterization of Ran proteins expressed in *E. coli*

Lounsbury *et al.* (1994) have suggested that GST-Ran fusion proteins were able to bind guanine nucleotides and to interact with RCC1 in mammalian extracts, but that they might not interact normally with other proteins that associated with the endogenous Ran protein. We therefore performed the experiments using the non-fusion Ran protein. Bacteria containing wild type Ran or T24N-Ran plasmid which were kindly provided from Dr. M. Dasso (LME/NICHID, NIH.) were grown at 37°C or 23°C respectively and each expressed protein was induced efficiently as the soluble proteins. Each bacterially-expressed Ran was purified from the crude extract by a four-steps purification procedure as described in Materials and methods (Figure 1). Purified Ran protein was confirmed to be human Ran by a reactivity to the anti-human Ran antibody and to be a G-protein by an ability to bind to GTPγS. The preparation of GTP-Ran contained ~67% of GTP-form and 33% of GDP-form, and that of GTPγS-Ran contained ~80% of GTPγS-form and 20% of GDP-form, which were analyzed by high-performance liquid chromatography as described (Dasso *et al.*, 1994). Purified wild type Ran bound to either GTP or GDP with an almost equal efficiency (Figure 2), indicating that *E. coli* produced Ran behaved like a normal Ran protein as described (Klebe *et al.*, 1993).

T24N-Ran induced PCC at the permissive temperature

At the restrictive temperature, cultures of tsBN2 cells arrested at G1/S with HU show PCC, concomitantly with the disappearance of RCC1 (2). RCC1 acts as a GEF for Ran (6). Therefore the accumulation of Ran bound GDP which is presumably caused in tsBN2 cells results in PCC. In order to examine the relationship between loss of RCC1 function and PCC, the microinjection assay of T24N-Ran into either tsBN2 or BHK21 cells was carried out. T24N-Ran was predicted to be primarily in the GDP bound state according to the equivalent substitution of Ras (Feig and Cooper, 1988). M. Dasso *et al.* (1994) have shown that T24N-Ran proteins associated tightly with RCC1 proteins and blocked the

activity of RCC1 as a GEF for Ran *in vitro*. In both tsBN2 and BHK21 cells, PCC were induced by the injection of 5 mg/ml purified T24N-Ran proteins at 33.5°C, the permissive temperature (Figure 3A, B). The frequency of induced PCC by the injection of T24N-Ran in tsBN2 and BHK21 cells was almost consistent with that of induced PCC in tsBN2 cells at the restrictive temperature. As expected, GDP -form of Ran caused a MPF activation. Ren *et al.* (1993, 1994) have reported the expression of another Ran mutant (G19V, Q69L double mutant), analog of a Ras activated form blocks the cell cycle progression through G2/M. This result suggests that GTP -form of Ran inhibits an activation of MPF in agreement with our result.

In contrast to these results, it has been reported that T24N-Ran protein inhibited an activation of MPF and prevented entry into M phase in *Xenopuss* extracts (Kornbluth *et al.*, 1994, Clarke *et al.*, 1995). In *Xenopuss* egg extracts supplemented with nuclei, T24N-Ran proteins inhibit an activation of Cdc2/cyclin B (MPF) in the presence of replicating nuclear DNA and significantly delay or entirely arrest the cell cycle prior to mitosis. Thus, T24N-Ran, mutant form of Ran, caused the disagreement of effect on a MPF activation. Since it has been unclear how mutant form of Ran behaved in tissue culture cells or *Xenopuss* extracts, we set out to use Ran prebound to guanine nucleotide instead of Ran mutant.

GTP-Ran but not GDP-Ran inhibits PCC

The prebound guanine nucleotide of injected Ran might be exchanged by RCC1 so that the injection of Ran was carried out after tsBN2 cells were pre-incubated at the restrictive temperature for loss of endogenous RCC1 function. In order to determine an appropriate pre-incubation time at the restrictive temperature, series of cultures of tsBN2 cells were incubated at 40.5°C, and injected with RCC1 as described (Seino *et al.*, 1992), to determine the time at which PCC became irreversible. After 90 minutes, PCC was efficiently inhibited by injection of RCC1 (2 mg/ml), but not after 120 minutes (data not shown). Thus, we chose 90 minutes as a maximum length of pre-incubation at 40.5°C.

Under such conditions, the frequency of cells showing PCC was reduced to 1% by nuclear injection of RCC1(2 mg/ml).

Ran bound guanine nucleotide was directly injected into the nuclei, and as a control, into the cytoplasm. As shown in Figure 4, GTP-Ran inhibited PCC in both cases in a dose dependent manner. The inhibition of PCC is more pronounced with nuclear injection, compared to cytoplasmic injection. GDP-Ran also inhibited PCC weakly but significantly, in a dose dependent manner. If residual RCC1 exchanges GDP of injected GDP-Ran with GTP, GDP-Ran may inhibit PCC as GTP-Ran. Since the cellular concentration of GTP is much higher than GDP (Stocchi *et al.*, 1987), such an exchange could happen. In order to prevent an exchange of GDP-Ran for GTP-Ran, GDP-Ran was co-injected with more than an equal concentration of GDP and as a control, GTP. When 1 mM of GDP was included with the GDP-Ran, the ability of GDP-Ran to inhibit PCC was completely abolished (Figure 5). In contrast, co-injection with GTP did not affect the inhibitory activity of GDP-Ran (Figure 5). Injection of GDP or GTP alone had no effect on PCC induction (Figure 5). These findings indicate that GTP-Ran, but not GDP-Ran, inhibits PCC induced by loss of RCC1 function.

Hydrolysis of GTP bound to Ran is not required for inhibition of PCC

In order to further investigate whether the hydrolysis of GTP bound to Ran is required for inhibition of PCC, GTPγS-Ran was injected into the nuclei or the cytoplasm of tsBN2 cells. As shown in Figure 6, PCC was strongly inhibited by GTPγS-Ran, most markedly in the case of nuclear injection. The inhibitory activity of GTPγS-Ran was enhanced by co-injecting with 1 mM of GTPγS, while GTPγS alone could not inhibit PCC induction. Therefore, GTPγS inhibits PCC induction in the form of GTPγS bound Ran, indicating that the hydrolysis of GTP bound to Ran is not required to inhibit PCC.

PCC was not inhibited by block of the nuclear import.

The complex of transport factors, importin (karyopherin) α and β is essential for a nuclear localization signal (NLS) dependent nuclear import (Görlich and Mattaj, 1996).

Incubation of the importin heterodimer with GTP- but not GDP-form of Ran disassociate of importin α from β, resulting in the association of Ran with importin β (Rexach and Blobel 1995). This disassociation is taken place on the nucleoplasmic side of the nuclear pore complex (Görlich *et. al.* 1996). Görlich *et. al.*, (1996) suggested that the binding of GTP-Ran with the importin β causes the release of importin α. In our experiment, therefore the injected GTP -form of Ran (GTP-Ran, GTPγS-Ran) into cytoplasm might cause the dissociation of the importin α from β, resulting in the block of nuclear import. In order to address the question whether the inhibition of PCC is caused by the block of nuclear import, we examined the effect of injected Ran on a NLS dependent nuclear import in tsBN2 cells. Into tsBN2 cells which were incubated for 90 minutes at 40.5°C (the same experimental condition of the microinjection assay), GTP-Ran proteins and the fluorescein isothiocyanate- labeled substrates containing NLS were simultaneously injected, and as a control, the NLS-substrates alone were injected into tsBN2 cells as described in Materials and methods. In both cases, the nuclear accumulation was detected efficiently within 30 minutes after injection (Figure 7). It has been reported that when the substrates containing NLS was injected into G1-arrested tsBN2 cells at 39.5°C for 6 hours, the nuclear protein import in tsBN2 cells was inhibited (Tachibana *et al.* 1994). In our experiment, tsBN2 cells were pre-incubated for 90 minutes at 39.5°C. Under such condition, the nuclear protein import was not inhibited. These results show that the inhibition of PCC was not caused by the block of the NLS dependent nuclear import.

Discussion

Previously we found that the induction of PCC in tsBN2 cells was correlated with loss of RCC1 which might cause the accumulation of GDP-Ran (Nishitani *et al.*, 1991). To directly confirm which form of Ran inhibits an activation of MPF during S phase, we injected various forms of Ran into tsBN2 cells. At permissive temperature, PCC was induced by the injection of T24N-Ran that is assumed to be GDP form. On the contrary, T24N-Ran has been reported to inhibit an activation of MPF in *Xenopus* extracts. Since Ran mutant has yielded conflict results, we directly used Ran which was bound to guanine nucleotide instead of Ran mutant. As could be expected by analogy with other members of the Ras family, GTP-Ran, but not GDP-Ran, strongly inhibited PCC induction. Thus, our present data indicate that the activation of MPF is inhibited by GTP-Ran in S phase.

Cdc25C which is essential for PCC induced by loss of RCC1, is localized in the cytoplasm in BHK21 cells (Seki *et al.*, 1992). Therefore, it must enter the nuclei prior to PCC. A hydrolysis of GTP-Ran is essential for a NLS dependent nuclear import (Moore and Blobel, 1993, Melchior *et al.*, 1993), and a nuclear import of a protein having no NLS like cdc25C also seems to depend on a hydrolysis of GTP-Ran (Palacios *et al.*, 1996) so that the PCC inhibitory activity of GTPγS-Ran could be caused by an inhibition of the nuclear import of cdc25C. However, this is unlikely for the following reasons. First, PCC was inhibited by injection of GTP-Ran. Since GTP-Ran facilitates nuclear import, it should enhance the import of cdc25C which would increase PCC-frequency. Second, GTPγS-Ran inhibited PCC more strongly when injected into the nucleus compared with cytoplasmic injection. When a nonhydrolyzable GTP analogue is added to *in vitro* nuclear import system, Ran accumulates on the cytoplasmic side of the nuclear envelope where a large Ran binding protein, RanBP2, is located (Melchior *et al.*, 1995). If GTPγS-Ran inhibited PCC by blocking the nuclear import, therefore, the cytoplasmic injection should inhibit PCC more strongly than the nuclear injection.

The experimental evidences presented here indicates that there may be another Ran pathway which is involved in the regulation of MPF activity in which the hydrolysis of GTP bound to Ran is not be required. Our results are consistent with the previous report

that Ran blocked in GTP -form inhibits the cell cycle progression from G2 to M phase (Ren *et al.*, 1994). In S phase, the inhibition of MPF activation is critical for completion of DNA replication. This inhibition is due to the phosphorylation of cdc2 and possibly a putative inhibitor(s) analogous to those found associated with cyclin-cdks complexes (Nasmyth and Hunt, 1993). The completion of S phase may inactivate RCC1 and cause an accumulation of GDP-Ran, allowing the activation of MPF. These events probably occur in the nucleus and result in the entry of cdc25C. In support of this, the cycloheximide which inhibits the activation of MPF also inhibits entry of cdc25C into the nucleus (Seki *et al.*, 1992). It is not yet clear how the GTP-Ran which might be essential for the nuclear import of cdc25C is produced at the end of S phase when we suppose that RCC1 is inactivated.

GTP-Ran or GTPγS-Ran is not as effective as RCC1 in inhibiting PCC. This may be due to GDP-Ran in our preparations of GTP- or GTPγS-Ran (33% and 20% of GDP-Ran respectively), although we cannot exclude the possibility that RCC1 has action other than the guanine nucleotide exchange that results in the inhibition of MPF activation.

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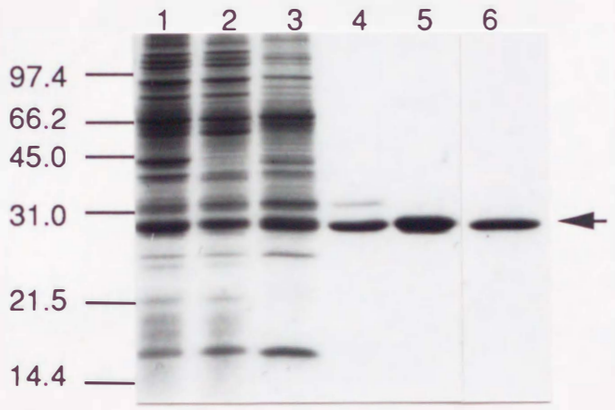


Figure 1

Figure 1. Expression and purification of Ran.

Wild type Ran was purified as described in experimental procedures. *E. coli* homogenate (lane 1), crude soluble extract (lane 2), DEAE fraction (lane 3), S-100 fraction (lane 4), mono-S fraction (lane 5), and mono-S fraction of T24N-Ran (lane 6) were analyzed by 15% SDS-polyacryamide gel electrophoresis, and stained with Coomassie Brilliant Blue. Arrow indicates a position of Ran.

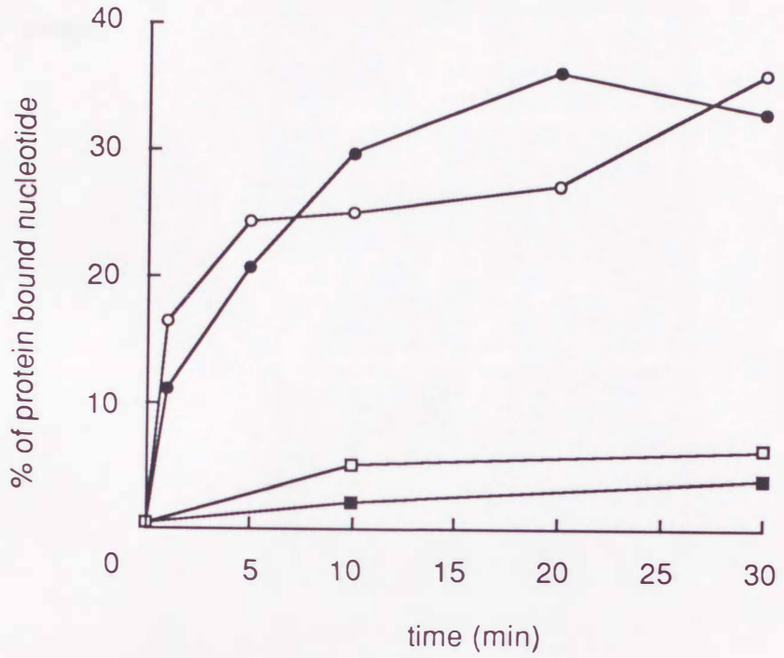

40
30
20
10
0
% of protein bound nucleotide
5
10
15
20
25
30
time (min)

Figure 2

Figure 2. Guanine nucleotide binding of wild type Ran.

A total 20 pmol of wild type Ran was incubated at 30°C with 6 nmol of [^{3}H]GDP (●, ■) or, 6 nmol of [^{3}H]GTP (○, □) in the presence of 1 mM $MgCl_2$ and 6 mM EDTA (●, ○) or, 20 mM $MgCl_2$ and 1 mM EDTA (■, □). The reaction was stopped by ice-cold stop buffer and the radioactive nucleotide bound to the protein was determined by filter assays.

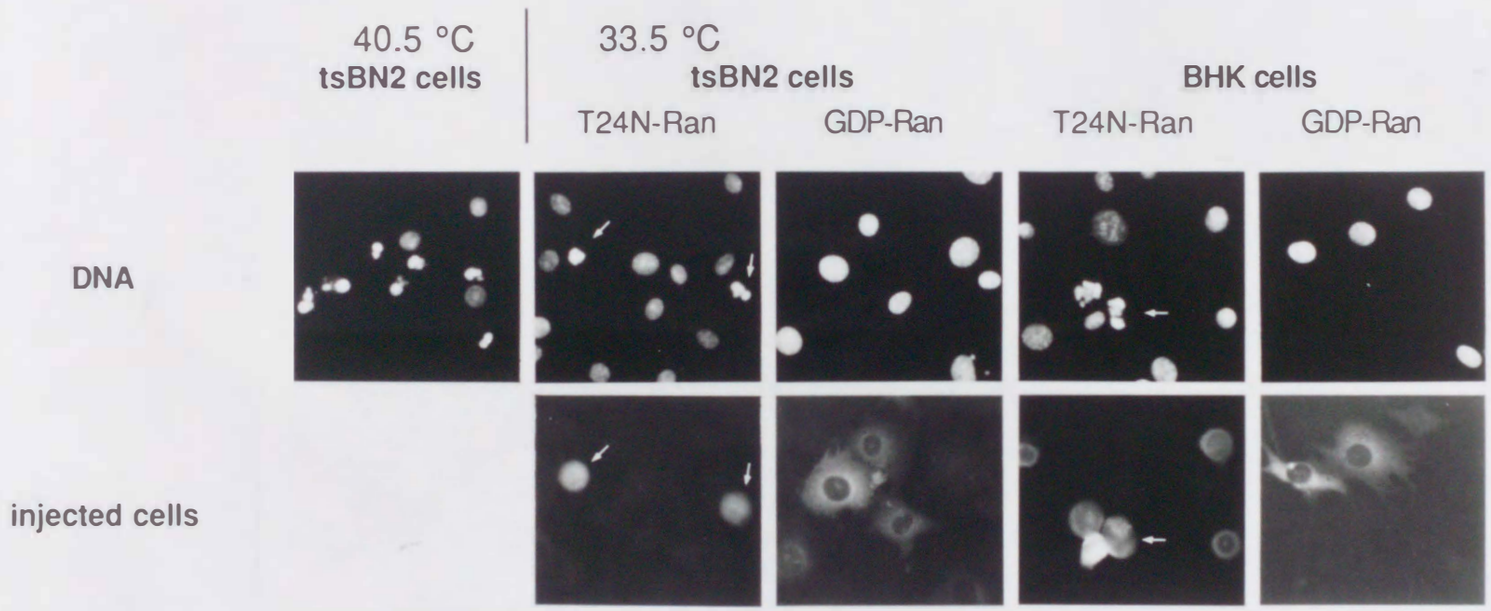

40.5 °C
tsBN2 cells
33.5 °C
tsBN2 cells
BHK cells
T24N-Ran
GDP-Ran
T24N-Ran
GDP-Ran
DNA
injected cells

Figure 3a

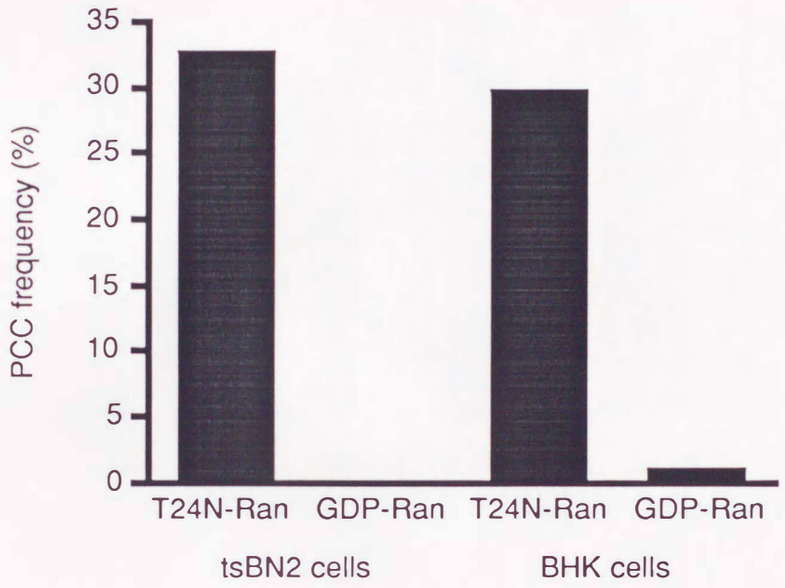

35
30
25
20
15
10
5
0
PCC frequency (%)
T24N-Ran
GDP-Ran
T24N-Ran
GDP-Ran
tsBN2 cells
BHK cells

Figure 3b

Figure 3. Induction of PCC by T24N-Ran mutant.

Either tsBN2 or BHK cells arrested with HU were incubated at 33.5°C, and given the injection of purified T24N- or GDP-Ran (5 mg/ml). Ran preparation was injected into the cytoplasm. In every injection, 0.3 mg /ml of Rabbit IgG was co-injected as an indicator of injected cells. The cells were stained with rhodamine-conjugated goat anti-rabbit IgG antibodies, and then incubated with Hoechst 33342 (a). Arrows indicate PCC.

PCC-frequency : induced PCC cells/ injected cells x 100 (b)

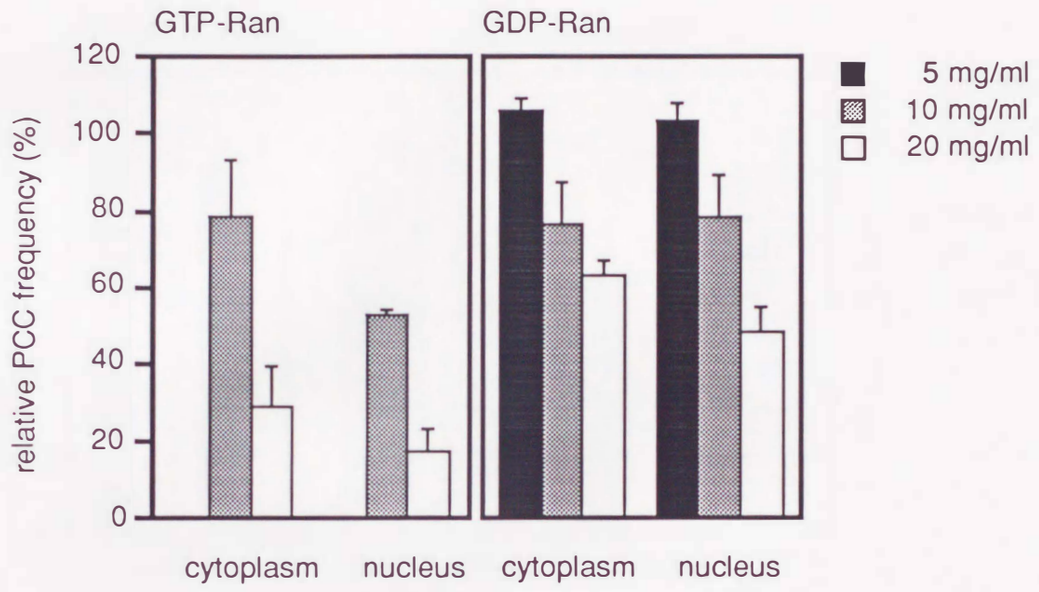

GTP-Ran
GDP-Ran
relative PCC frequency (%)
120
100
80
60
40
20
0
cytoplasm
nucleus
cytoplasm
nucleus
5 mg/ml
10 mg/ml
20 mg/ml

Figure 4

Figure 4. Inhibition of PCC by GTP- and GDP-Ran

Cultures of tsBN2 cells arrested with HU as described in Materials and methods, were incubated at 40.5°C for 90 min and then given the injection of indicated doses (5, 10 and 20 mg/ml) of GTP-, and GDP- Ran to either the nuclei or the cytoplasm. Five hour later, cells were fixed and stained to count the frequency of cells showing PCC. PCC frequency was normalized to that of cells injected immunoglobulin alone as described (16).

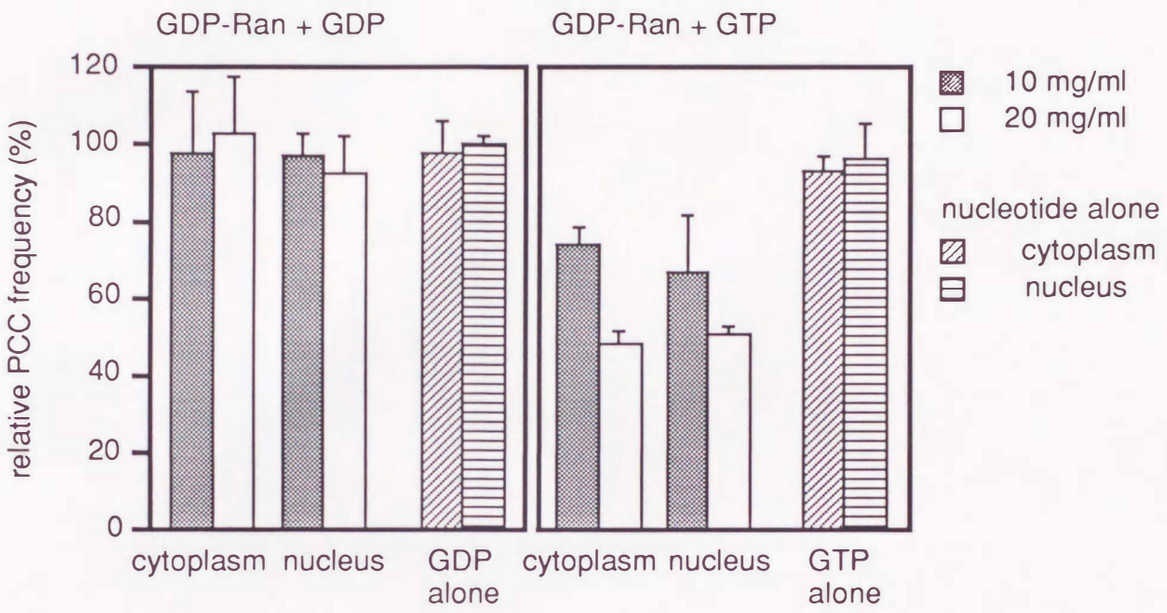

GDP-Ran + GDP
GDP-Ran + GTP
relative PCC frequency (%)
120
100
80
60
40
20
0
cytoplasm
nucleus
GDP alone
cytoplasm
nucleus
GTP alone
10 mg/ml
20 mg/ml
nucleotide alone
cytoplasm
nucleus

Figure 5

Figure 5. Co-injection of GTP and GDP with GDP-Ran

The injection was carried out as described in Figure 2. One mM of GDP or GTP was co-injected with GDP-Ran (10 and 20 mg/ml) as indicated. As a control, 1 mM GDP or GTP alone was also injected.

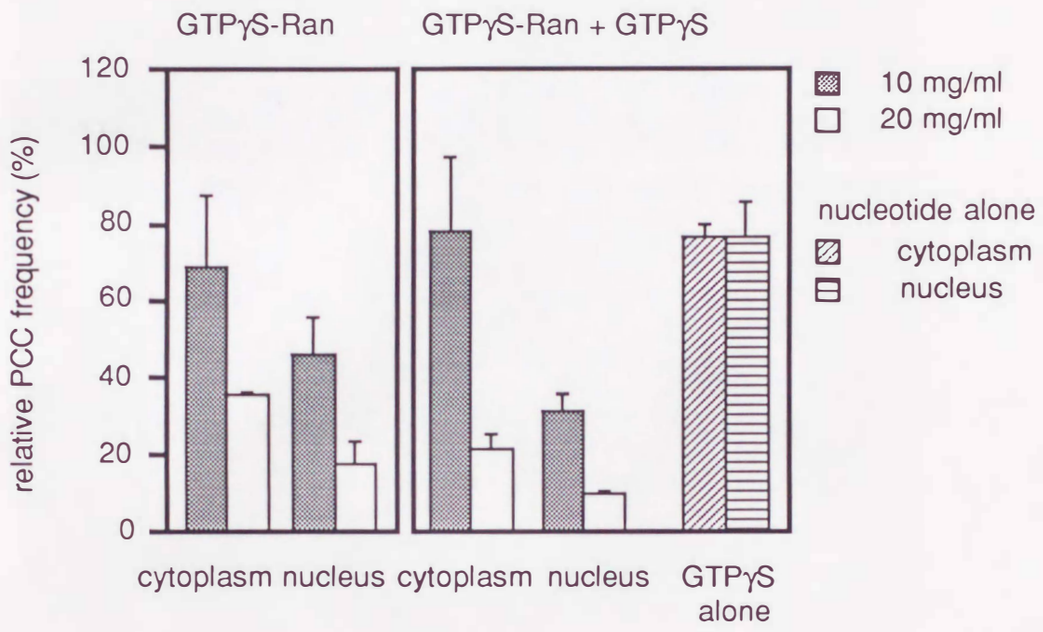
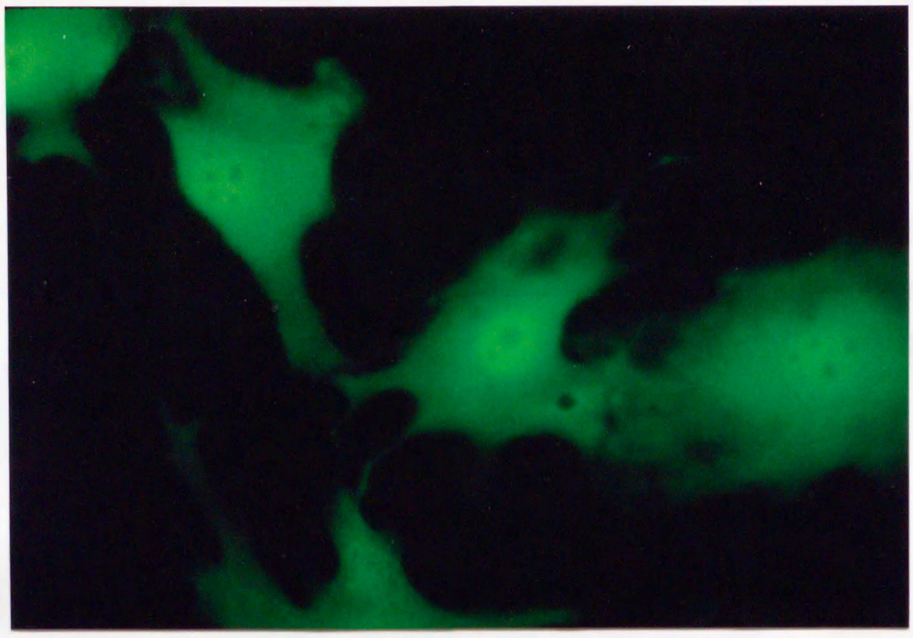

GTPγS-Ran
GTPγS-Ran + GTPγS
relative PCC frequency (%)
120
100
80
60
40
20
0
10 mg/ml
20 mg/ml
nucleotide alone
cytoplasm
nucleus
cytoplasm
nucleus
cytoplasm
nucleus
GTPγS
alone

Figure 6

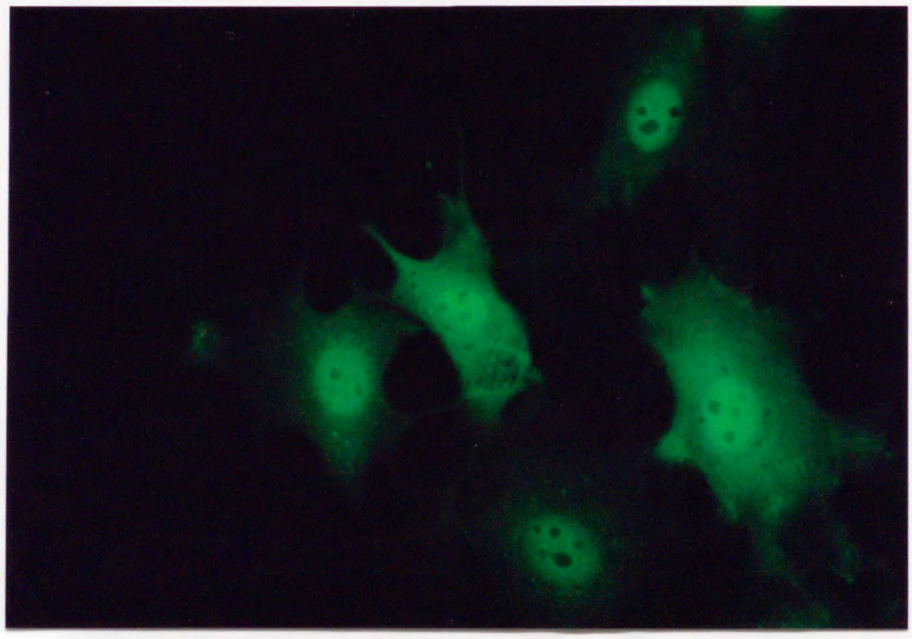
Figure 6. Inhibition of PCC with GTPγS-Ran

Injection of GTPγS-Ran (10 and 20 mg/ml) was carried out as described in Figure 4, either alone or with 1 mM of GTPγS.

a



b



c

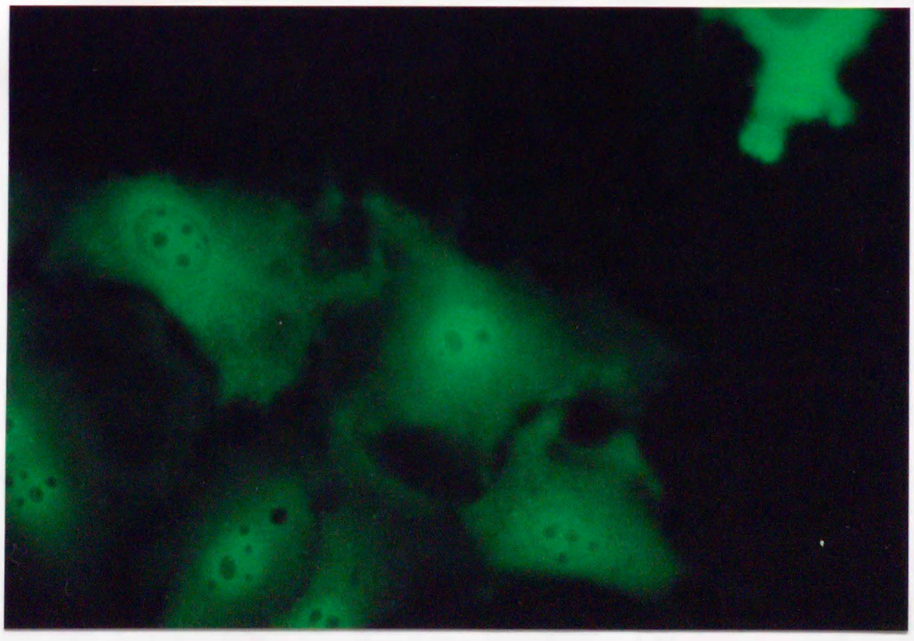
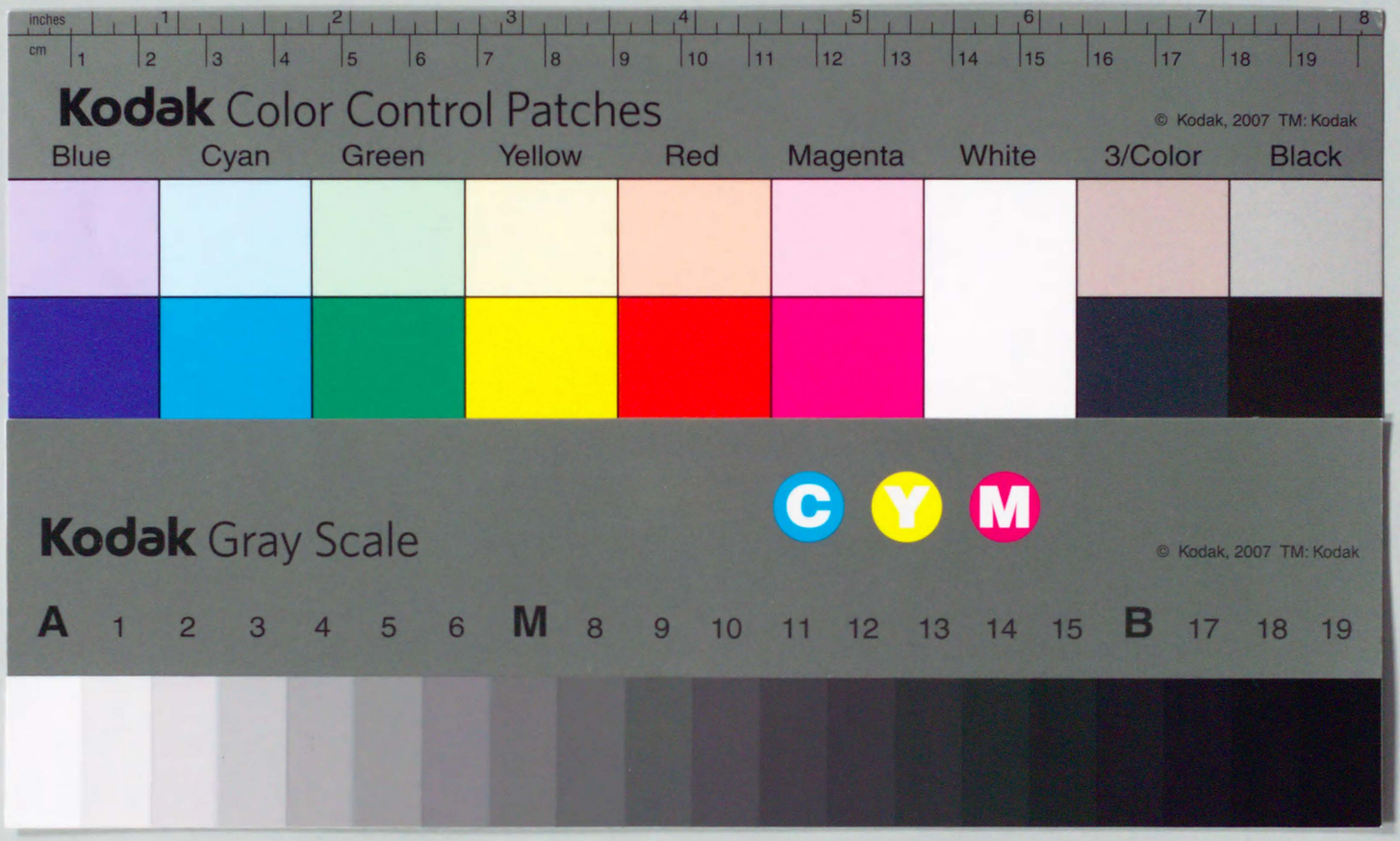


Figure 7

Figure 7. NLS dependent nuclear import assay in tsBN2 cells

Cultures of tsBN2 cells arrested with HU as described in Materials and methods, were incubated at 40.5°C for 90 min and then given the injection of the fluorescein isothiocyanate- labeled substrates containing NLS (a), NLS-substrates and 20 mg/ml GTP-Ran (b), and NLS-substrates and 20 mg/ml GTPγS-Ran (c) to the cytoplasm. Five hour later, cells were fixed and stained


inches
cm
Kodak Color Control Patches
© Kodak, 2007 TM: Kodak
Blue
Cyan
Green
Yellow
Red
Magenta
White
3/Color
Black
Kodak Gray Scale
C
Y
M
© Kodak, 2007 TM: Kodak
A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19