Functional Dissection of the RCC1 Protein; the N-terminal Region of RCC1 Protein is a Nuclear Location Signal Rather Than a DNA-binding Domain

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Functional Dissection of the RCC1 Protein ; the N-terminal Region of RCC1 Protein is a Nuclear Location Signal Rather Than a DNA-binding Domain.

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ABSTRACT

The RCC1 protein is involved in coupling of mitosis with S phase. It has seven homologous repeated domain of about 60 amino acids A DNA binding domain is in the N-terminal region outside this repeat. We found that the RCC1 protein which has a deletion in this DNA-binding domain can complement the tsBN2 mutation. At 39.5°C, where the endogenous RCC1 protein disappeared in the ts^+ transformants of tsBN2 cells which were transfected with the deleted RCC1 cDNA. The majority of the deleted RCC1 protein was in the post-nuclear supernatant. However, a significant amount still remained in the nuclear fraction. The deleted RCC1 remaining at the nuclei was eluted at the same concentration of NaCl and DNase I as the wild-type RCC1 protein of BHK21 cells. Furthermore, the deleted RCC1 protein was co-migrated with the nucleosome-fraction by sucrose-density gradient analysis. Expression of the N-terminal region fused with lacZ in COS cell revealed that the fused protein was located in the nucleoplasm. These findings suggest that the N-terminal region of the RCC1 protein functions as a nuclear location signal rather than the DNA-binding domain and the RCC1 protein may associate with the chromatin with an aid of other unknown protein(s).

INTRODUCTION

Many temperature sensitive(ts) mutant cell lines have been isolated from cultured cells derived from hamster or mouse (Marcus et al., 1985). The tsBN2 cell line derived from the BHK21 cell line is one of these ts mutants for cell-growth (Nishimoto and Basilico, 1978). Even though in cell where DNA replication is not completed, tsBN2 cells enter mitotic phase at the nonpermissive temperature, so that the dependency of mitosis on the completion of DNA replication is abolished in this mutant at the nonpermissive temperature (Nishitani et al., 1991).

The RCC1 gene which encodes a nuclear protein of 45 kDa is mutated in the tsBN2 cells (Uchida et al., 1990). Because of this mutation, the RCC1 protein disappears in this cell line at the nonpermissive temperature. Upon loss of RCC1 function, cdc2/histone H1 kinase is activated and tsBN2 cells enter mitotic phase prematurely and chromosome is condensed (Nishitani et al., 1991). Induction of PCC (premature chromosome condensation) can be prevented by a microinjection of RCC1 protein into tsBN2 cells (Seino et al., 1992), indicating that the RCC1 protein itself is responsible for the phenotype of tsBN2 mutation. Based on these results, the RCC1 protein is thought to be involved in the control mechanism for transition from G2 to M, in which the RCC1 protein monitors DNA synthesis and prevents the initiation of mitosis until completion of DNA replication.

RCC1 protein has seven internal repeated domains carrying about 60 amino acids (Ohtsubo et al., 1987). Proteins homologous to the RCC1 have been found from Xenopus (Nishitani et al., 1987), Drosophila (Bj1) (Frasch, 1991), and S. cerevisiae (SRM1/PRP20/MTR1) and S. pombe ($pim1^+$) (Aebi et al., 1990; Clark and Sprague, 1989; Kadowaki et al., 1993, Matsumoto and Beach, 1991). In all these homologues, the repeated domains are well conserved. Except for $pim1^+$, the tsBN2 mutation can be complemented by the cDNAs encoding these homologues (Ohtsubo et al., 1991) (Seino, unpublished result). Vice versa, the srm1 and prp20, ts mutants that have the ts alleles of SRM1, can be complemented by human RCC1 cDNA (Fleischmann et al., 1991; Clark et al., 1991). Thus, they are conserved functionally.

RCC1 has been reported to form a complex with a small nuclear G protein: Ran, and to function as a guanine nucleotide exchange factor (GNRF) on the Ran protein (Bischoff and Ponstingle, 1991a, b). RCC1 protein has a DNA-binding activity in vitro (Ohtsubo et al, 1991). We assumed that the DNA-binding activity of RCC1 protein plays an important role in checking DNA synthesis to prevent the initiation of mitosis until the completion of DNA synthesis. The RCC1 protein without its DNA binding domain, however, inhibited tsBN2-induced PCC by microinjection (Seino et al., 1992). Thus, it seemed to be clear that a DNA-binding activity is not essential for RCC1 to couple DNA replication and mitosis. Since the RCC1 protein truncated in

the N-terminal region outside the repeat was unstable in mammalian cells, we could not confirm that the RCC1 protein lost its DNA-binding activity could complement tsBN2 mutation.

In this report, we made small deletions in the N-terminal region of the RCC1 protein. These deleted RCC1 cDNAs efficiently complemented the tsBN2 phenotype. Thus, the DNA binding activity of the RCC1 protein is not required for the function of RCC1 When the N-terminal region of the RCC1 cDNA fused with the lacZ gene was expressed in COS cell, this fused protein was located to the nuclei and was extracted from the nuclei under the condition where the chromosome protein remained in the nuclei This results indicate that the N-terminal region of RCC1 is a nuclear location signal rather than a DNA-binding domain. Furthermore, the RCC1 protein lacking the N-terminal region still associates to the chromatin, suggesting that RCC1 forms a complex with protein(s) to associate to the chromatin.

MATERIAL AND METHODS

Cell lines and culture condition: The tsBN2 cell line is a temperature sensitive mutant of the BHK21 cell line. Δ8-29 ts^+-1, 2 and 3 are ts^+ transformants of the tsBN2 cell line, which were transfected with the deleted RCC1 cDNA, Δ8-29. Cell lines were cultured in DMEM (Dulbecco's modified Eagle's medium) containing 10% calf serum under a humidified atmosphere containing 10% CO_2 at 33.5°C (tsBN2), at 37.5°C (BHK21) and at 39.5°C (ts^+ transformants).

Construction of deleted RCC1 cDNA: The 1.4 kbp Pst I fragment of pcD51 (Ohtsubo et al., 1987) was subcloned into the Pst I site of pUC119. Deletions were introduced into this fragment according to the site directed mutagenesis system (Amersham) by using the following synthetic oligonucleotide: Δ8-29, 5'-AAGCGCATAGCT/CACAGCACAGAA-3'; Δ12-29, 5'-AAAAGAAGGTCC/CACAGCACAGAA-3';`` Δ8-12, 5'-CCCAAGCGCATAGCT/CCAGCAGATGCCATC-3'; Δ21-25, 5')-GCCATCCCCAAAAGC/TCACACAGGTCCCAC-3' (The bars indicate the position of the deletions to be introduced). The sequence of the mutated cDNAs were verified by dideoxynucleotide sequencing (Sanger et al., 1977). The 1.3 kbp HgiA I-Pst I fragments of the mutated cDNAs were either subcloned into pcDL-SRα296 (Takebe et al., 1988) for expression in mammalian cells, or else trimmed with T4 DNA polymerase and then subcloned into the Sma I site of pUC8 for expression in E. coli.

Expression of the deleted RCC1 cDNAs in E. coli: E. coli, JA221 containing the deleted RCC1 cDNA subcloned into pUC8 were grown exponentially. At OD_{600} = 0.6, IPTG (Isopropyl ß-D-thiogalactopyranoside) was added to the culture at a final concentration of 1 mM. Bacterial cells were harvested 3 hr later.

Transformation of tsBN2 cells: tsBN2 cells growing exponentially were collected and suspended at a concentration of 2 x 10^7 cells/ml in Saline G buffer (One litter solution containing 8.0 g NaCl, 0.4 g KCl, 0.395 g $Na_2HPO_4 \cdot 12H_2O$, 0,15 g KH_2PO_4, 0.4 g $MgCl_2 \cdot 6H_2O$, 0.1 g $CaCl_2$, and 1.1 g glucose, adjusted pH to 7.1-7.2) (Nishitani et al., 1990). Two hundred microliter of this cell suspension received 2 µg of either full-length or the deleted RCC1 cDNAs subcloned in pcDL-SRα296, and 0.2 µg of pSV2 neo plasmid. The mixture was exposed to a single electric pulse with a field strength of 1 kV/cm and a length of 1 - 3 ms. (Nishitani et al., 1990). Following the electric pulse, cells were plated in a growing medium containing 10% fetal calf serum. After incubation at 33.5°C for 2 days, transfected cultures were either shifted up to 39.5°C, or else given G418 (final concentration of 800 µg/ml) and incubated at 33.5°C. Ten to 14 days later, cells were fixed with formaldehyde and then stained with crystal violet as described (Ohtsubo et al., 1987).

Transient expression in COS cells: The cDNA encoding the N-terminal region of RCC1 (amino acid residue 1-72) fused to

lacZ gene (Seino et al. 1991) was digested with EcoR I and subcloned into the EcoR I site of pcDL-SRα296. This plasmid was transfected into COS7 cell using Lipofectin™ Reagent (BRL). Twenty-four hours later, transfected cells were replated to glass coverslips for indirect immunofluorescence or to cell culture dishes for cell fractionation, and incubated with DMEM containing 10% fetal calf serum for another 48 hr. As for a control, plasmid pCH1110, which is a SV40 derived expression vector encoding the lacZ gene (Pharmacia) was used.

Indirect immunofluorescence: Cells on glass coverslips were washed with PBS, fixed with 3% paraformaldehyde in PBS, permealized with 1% NP40 in PBS, stained with an antibody against ß-galactosidase (kindly provided by Dr. Sakaguchi. Kyushu University) or an antibody against Xenopus RCC1 and detected with Texas-Red conjugated monoclonal antibody against rabbit IgG as previously described (Nishitani et al. 1990). The nuclei were stained with Hoechst 33342.

Microinjection: Needle microinjection of the RCC1 protein into the cytoplasm of cultured cells was performed as described (Graessmann and Graessmann, 1976). Cells were plated at 1 x 10^5 on 18 x18 mm glass coverslips in 35 mm dishes, and synchronized at G1/S boundary as previously described (Seino et al., 1992). After hydroxyurea treatment, 20 mM HEPES, pH 7.2 was added to the cultures from 200 mM stock solution and purified RCC1 protein was co-injected with BSA. Fields of about 50-100 cells were injected at each experiment. The cultures

were then shifted up to 40.5°C within 30 min after the completion of the injections. Cells which received proteins were identified by staining with the anti-BSA antibody, using indirect immunofluorescence. PCC induction of the injected cells was determined by staining DNA with Hoechst 33342. The loss of cells from glass coverslips was prevented by careful washing at each step (less than 10%).

DNA-binding assay: Proteins separated on 12.5% SDS-polyacrylamide gel, were transferred to immobilon PVDF membrane (Millipore), washed three times with 6 M urea containing 0.2% NP-40, and then four times with the DNA binding buffer (10 mM Tris-HCl pH 7.8, 1 mM EDTA, 50 mM NaCl). The filters were then incubated in the DNA-binding buffer containing 10% skim milk and then with the same buffer containing ^{32}P labeled pUC18 DNA (digested with EcoR I). After incubation, the filters were washed three times with the DNA binding buffer. Bound DNA was detected by an autoradiography. All procedures were performed at room temperature.

Immunoblotting analysis: Proteins separated on 12.5% SDS-polyacrylamide gel were transfered to nitrocellurose filter. RCC1 was detected using the antibody against Xenpous RCC1 and horse radish peroxidase conjugated mouse monoclonal antibody against rabbit IgG (Nishitani et al., 1991). Development was done using ECL™ antibody detection kit (Amersham).

Preparation and fractionation of nuclei: Cells were

collected from the dish by scraping, washed twice with ice cold PBS and suspended in hypotonic buffer (10 mM Hepes, pH 8.0, 5 mM KCl, and 2 mM $MgCl_2$) containing 0.5 % NP40 for 10 min on ice. Then the cells were disrupted using a tight-fitting pestle of a potter homogenizer (usually 25 strokes). Nuclei were separated by centrifugation at 1,000 g for 5 min. and washed with ice cold hypotonic buffer (Ohtsubo et al. 1989). All procedures of nuclei fractionation were performed using freshly prepared nuclei, at a concentration of 10^8 nuclei/ml. In the salt extraction, 5 M of stock NaCl solution were diluted to the desired concentration. In the case of DNase I extraction, the appropriate amount of a solution containing 10 mg of DNase I per milliliter were added to the nuclei preparation to obtain the desired concentration of nuclease. All experiments were carried out for 10 min at 0°C. Nuclease treatment was terminated by adding EDTA (final concentration, 5 mM), followed by an additional incubation for 10 min. on ice. Nuclear supernatant and residual nuclei were separated by centrifugation (1,000 g for 5 min). Each fraction was subjected to the immunoblotting analysis.

Velocity gradient sedimentation : The fraction containing nucleoprotein complex isolated from the nuclei with DNase I treatment (10 μg/ml) (see above) was sedimented through the linear 5 to 30 % sucrose gradients made in 10 mM HEPES (pH 8.0)-1 mM EDTA (low salt) or 10 mM HEPES (pH 8.0)-1 mM EDTA-500 mM NaCl (high salt) as described (Klempnauer and

Sippel., 1986). Centrifugation was performed for 3 hr at 4 °C and at 150,000 g. Samples from each fraction were subjected to the immunoblotting analysis. They were also treated with 0.1 % SDS and 100 μg of proteinase K per ml at 37 °C for 30 min, and then analyzed by electrophoresis on 1.2 % agarose gels and stained with EtBr to estimate the existence and size of DNA fragments in the fractions.

RESULTS

Complementation analysis of the N-terminal deleted RCC1 cDNA

The N-terminal region of RCC1 has a DNA binding activity in vitro (Seino et al., 1992). The RCC1 protein truncated in the N-terminal region outside the repeat has an ability to prevent the temperature-induced PCC of tsBN2 cells by microinjection (Seino et al., 1992). However, we could not obtain stable ts^+ transformants of tsBN2 cells by transfecting this deleted RCC1 cDNA. We, thus, investigated a role of the DNA-binding activity by introducing an internal small deletion in the N-terminal region outside the repeat as shown in Figure 1A. DNA binding activity of the deleted RCC1 protein was examined by so called south-western analysis using the labelled plasmid DNA as a probe (Figure 1B).

The deletion from the 8th to 12th amino acids did not affect the DNA binding activity, but RCC1 lost its DNA binding activity by the deletion from 21th to 25th amino acids. These internal deletions including the deletion from the 8th to the 29th amino acids did not affect the stability of the RCC1 protein in COS cells (data not shown) and complemented the ts phenotype of tsBN2 cells efficiently (Figure 1).

Ts^+ transformants of tsBN2 cells transfected with the deleted RCC1 cDNA had the same distribution of cellular DNA

content as the BHK21 cells by Facs analysis. In this transformant. Furthermore, PCC was not induced at nonpermissive temperature (data not shown). Thus, the loss of DNA binding activity in the RCC1 protein seems to have no effect in the progression of the cell cycle.

The ability of the N-terminal deleted RCC1 protein to inhibit PCC in tsBN2 cells caused by temperature-shift was confirmed by microinjection. Both intact and deleted RCC1 proteins expressed in bacteria inhibited PCC efficiently in a dose dependent manner (Figure 2). The kinetics of PCC-inhibition by the deleted RCC1 protein; Δ 8-29, was similar to that by the intact wild-type RCC1 protein as well. Thus, the deleted RCC1 protein directly inhibited PCC-induction, indicating that the DNA binding domain of RCC1 protein was not required for complementing tsBN2 phenotype.

Expression of N-terminal deleted RCC1 protein in ts$^+$ transformants

In order to clarify how the deleted RCC1 protein complements tsBN2 cells, we investigated whether the endogenous RCC1 protein of tsBN2 cells was stabilized at 39.5°C in the ts$^+$ transformants of tsBN2 cells which were transfected with the N-terminal deleted RCC1 cDNA. Cellular proteins were extracted from BHK21 cells or the ts$^+$ transformants; Δ 8-29 ts$^+$ clone 1, 2 and 3 which were maintained at 39.5°C or 33.5°C. As a control, cellular proteins of tsBN2 cells cultured at either 33.5°C

or at 39.5°C for 4 hr, were also prepared. The presence of RCC1 protein in these extracts was examined by immunoblotting analysis using the antibody against the Xenopus RCC1 protein (Figure 3). Due to the lower molecular weight, the deleted RCC1 protein can be easily distinguished from the endogenous one. The endogenous RCC1 protein was found in tsBN2 cells cultured at 33.5°C, but not in these cultured at 39.5°C for 4 hr, as reported (Nishitani et al., 1991). Consistently in ts^+ transformants cultured at 39.5°C, there was no band corresponding to the endogenous RCC1 protein of tsBN2 cells. After prolonged incubation at 33.5°C, however, the endogenous RCC1 protein of tsBN2 cells appeared in ts^+ transformants (Figure 3, Lanes 7 to 9). We thus concluded that the endogenous RCC1 protein of tsBN2 cells was not stabilized in ts^+ transformants of tsBN2 cells at nonpermissive temperature which were transfected with the deleted RCC1 protein.

Function of the N-terminal region of RCC1 protein

The above findings indicate that the N-terminal region of RCC1 protein is not required for complementing tsBN2 phenotype. Thus we considered that this region of the RCC1 protein may function as a nuclear location signal rather than a DNA-binding domain, since the amino acid sequence of this region is similar to the nuclear location signal of the Xenopus N1 protein (Fig. 1) (Robbins et al., 1991).

To examine this possibility, we investigated the location of

the RCC1-lacZ fusion gene's product. It was constructed by fusing the N-terminal region of RCC1 (amino acid residue 1-72) with the full length of lacZ gene (Seino et al. 1992). SV40 derived expression vector encoding this fused gene was transfected into COS cells using lipofection. Transfected COS cells were incubated for 48 hr and the location was examined with an antibody against ß-galactosidase. As a control, SV40 derived vector encoding lacZ gene alone was transfected into COS cells.

The fusion protein was located in the nuclei by examining with indirect immunofluorescence (Fig.4 A). In the contrary, ß-galactosidase alone was dispersed throughout the cytoplasm (Fig.4A) However, when transfected cells were fractionated into the nuclei and the post-nuclear supernatants, the fused protein was extracted to the supernatant with 0.5 % NP40 under the low salt condition (Fig. 4B). These results indicate that the fused protein locates in the nucleoplasm, but not associated to the chromatin. It is noticeable that the N-terminal deleted RCC1 protein, significant amount of which located in the nuclei, was not extracted at the same condition as the fused protein (see below). Thus, we conclude that the N-terminal region of RCC1 protein functions as a nuclear location signal and rather than as a DNA-binding domain <u>in vivo</u>.

Intracellular location of the N-terminal deleted RCC1 protein

In the ts^+ transformants transfected with Δ8-29 RCC1

cDNA, the deleted RCC1 protein was in the post-nuclear fraction, compared to the intact wild-type RCC1 protein, which was preferentially found in the nuclei fraction (Figure 5). However, a significant amount of the deleted RCC1 protein was also found in the nuclei (Figure 5, lane 4 of Δ8-29ts$^+$-1). To investigate the subnuclear location of the N-terminal deleted RCC1 protein, the nuclei were isolated from ts$^+$ transformants transfected with Δ 8-29 RCC1 cDNA, and then incubated in the buffer containing a increasing concentration of NaCl as described in Materials and Methods. Release of RCC1 protein from the nuclei was determined by immunoblotting using the antibody against Xenopus RCC1 protein. As shown in Figure 6 A, the deleted RCC1 protein was mainly eluted from the isolated nuclei at a concentration ranging from 0.2 to 0.4 M NaCl. With the same concentration of NaCl, the wild-type RCC1 protein was extracted from the isolated nuclei of BHK21 cells as reported previously (Ohtsubo et al., 1989) (Fig. 6A).

Furthermore, the N-terminal deleted RCC1 protein was extracted from the isolated nuclei with DNase I, at the concentration ranging from 10 to 100 μg/ml (Figure 6B). With the same concentration of DNase I, the wild-type RCC1 protein was also extracted from the nuclei of BHK 21 cells. These results suggested the possibility that some of the N-terminal deleted RCC1 protein was present on the chromatin in the same manner as the intact wild-type RCC1 protein.

The location of the N-terminal deleted RCC1 protein on the

chromatin was further investigated by velocity gradient sedimentation analysis. Nuclei isolated from exponentially growing BHK21 cells and the ts^+ transformants were digested with DNase I (10 mg/ml). After treatment with EDTA, the material released from the nuclei was subjected to velocity gradient sedimentation, either directly or after adjusting the concentration of NaCl to 0.5 M. Proteins of each fraction were analyzed with SDS-polyacrylamide gel electrophoresis and the distribution of RCC1 protein through the fraction was determined by immunoblotting as described in Materials and Methods (Figure 7). Under the low salt condition, RCC1 protein was sedimented with the fractions of nucleoprotein complex which contains histones H4, H2a, H2b, and H3 and fragmented DNA (Figure 7A, B and C). In the presence of 0.5 M NaCl, however, these deleted RCC1 proteins were separated from the nucleoprotein complex, floating to the top fractions (Figure 7D and E). The deleted RCC1 protein, thus, associates with the chromatin in a salt-labile fashion. A similar result was obtained from wild type RCC1 protein expressed in BHK21 cells (data not shown). These results suggest that RCC1 protein is associated to the chromatin with the aid of unknown other protein(s) with the salt labile fashion.

DISCUSSION

To date, the GTPase cycle was thought to be involved in the signal transduction from cell surface to the nucleus. The finding that RCC1 which locates on the chromatin catalyzes the guanine nucleotide exchange on the small G protein, Ran/TC4 (Bischoff and Ponstingle, 1991a, b) indicates the presence of signal transduction from the chromatin to the effector which might be in the nucleoplasm or in the cytoplasm. Based on the phenotype of the tsBN2 mutation, RCC1 protein was supposed to be involved in the coupling between DNA replication and mitosis. To perform this function, the RCC1/Ran complex may monitor the progression of DNA replication.

The Ran protein has no DNA binding activity (Bischoff and Ponstingle, 1991). On the other hand, the RCC1 protein is within the nuclei and has the DNA-binding activity in vitro. We therefore thought that a DNA binding activity of RCC1 is required to check the DNA replication. However, our present finding that the RCC1 protein without the DNA binding activity in vitro could efficiently complement tsBN2 phenotype indicates that a DNA-binding activity of RCC1 protein is not required for the function of this protein.

In the ts^+ transformants of tsBN2 cells transfected with the deleted RCC1 cDNA which has lost its DNA-binding activity in vitro, the majority of RCC1 was found to be in the post-nuclear supernatant fraction, where no wild type RCC1 protein was

detected. Furthermore, ß-galactosidase fused to the N-terminal region of RCC1 was located. in the nucleoplasm. These results therefore suggest that the N-terminal region is a nuclear location signal rather than a DNA-binding domain. Consistently this region of RCC1 protein was found to have a amino acid sequence similarity to the nuclear location signal of Xenopus N1 protein, that is, KR·····K··KK (Figure 1) (Robbins et al., 1991).

RCC1 protein in the nuclei was extracted with DNase I treatment. It was found to be co-sedimented with the nucleosomal fraction by the sucrose gradient sedimentation containing a low salt. In the presence of a high salt, however, RCC1 was released from the nucleosome fraction and remained in the top fraction, indicating that RCC1 associates to the chromatin in a salt labile fashion. This was also found in the case of the BJ1 that is the RCC1 homologue of Drosophila (Frasch, 1991). Similar to the wild-type RCC1 protein, the deleted RCC1 protein in the nuclei was extracted from the nuclei with salt treatment or DNase I treatment. The concentration of salt and DNase I required to extract the deleted RCC1 from the nuclei is the same as that of the wild-type RCC1 protein, and the velocity gradient analysis of the sample extracted with DNase I clearly indicated that the RCC1 protein without DNA binding activity was still able to associate to the nucleosome fraction in a salt-labile fashion. These results indicate that the RCC1 protein without its DNA binding activity can associate to the chromatin in the same manner as the wild-type RCC1 protein. RCC1,

therefore, may associate to the chromatin with the aid of other protein(s). What kind of protein(s) involved in the association of RCC1 with chromatin remains open to be examined. These proteins may be involved in the signal transduction from the DNA molecule to the RCC1/Ran complex.

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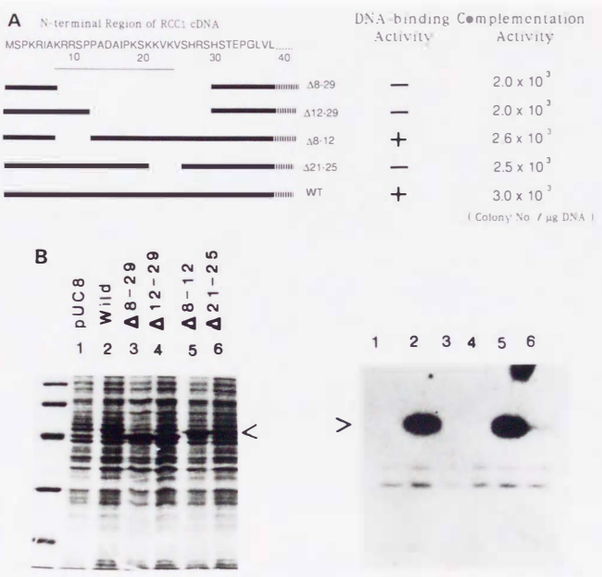

A
N-terminal Region of RCC1 cDNA
MSPKRIAKRRSPPADAIPKSKKVKVSHRSHSTEPGLVL.....
10
20
30
40
Δ8-29
Δ12-29
Δ8-12
Δ21-25
WT
DNA-binding Activity
Complementation Activity
—
—
+
—
+
2.0 x 10³
2.0 x 10³
2.6 x 10³
2.5 x 10³
3.0 x 10³
(Colony No / μg DNA)
B
pUC8
Wild
Δ8-29
Δ12-29
Δ8-12
Δ21-25
1 2 3 4 5 6
1 2 3 4 5 6

Figure 1. Deleted RCC1 cDNAs: their deletion sites, DNA-binding activity in vitro and complementation activity.

A) Relationship between DNA binding activity in vitro and its complementation activities.

Deletion of RCC1 was introduced as described in Materials and Methods. Exponentially growing tsBN2 cells were transfected with deleted RCC1 cDNA by electric pulse as described in Materials and Methods. The frequency of ts^+ transformation was normalized based on the frequency of neomycine resistant transformation which was same in all cases, and its ratio is shown. The frequency of ts^+ transformation with the intact RCC1 cDNA was 3×10^3 cells/μg DNA. The underline indicates the region homologous to the nuclear location signal of Xenopus N1 protein.

B) DNA binding activity of deleted RCC1 protein in vitro.

Total proteins of E. coli, JA221 expressing the wild type or the deleted RCC1 protein as indicated at the top of the panel was separated on 12.5% SDS-PAGE gel and stained with Coomassie Brilliant Blue (Left panel), or else transferred to immobilon PVDF membrane (Millipore). The DNA-binding activity of proteins blotted on filters was investigated using ^{32}P-labeled pUC18 DNA (digested with EcoR1) as a probe, as described in Materials and Methods (Right panel). The position of deleted and intact RCC1 protein was indicated by arrow heads.

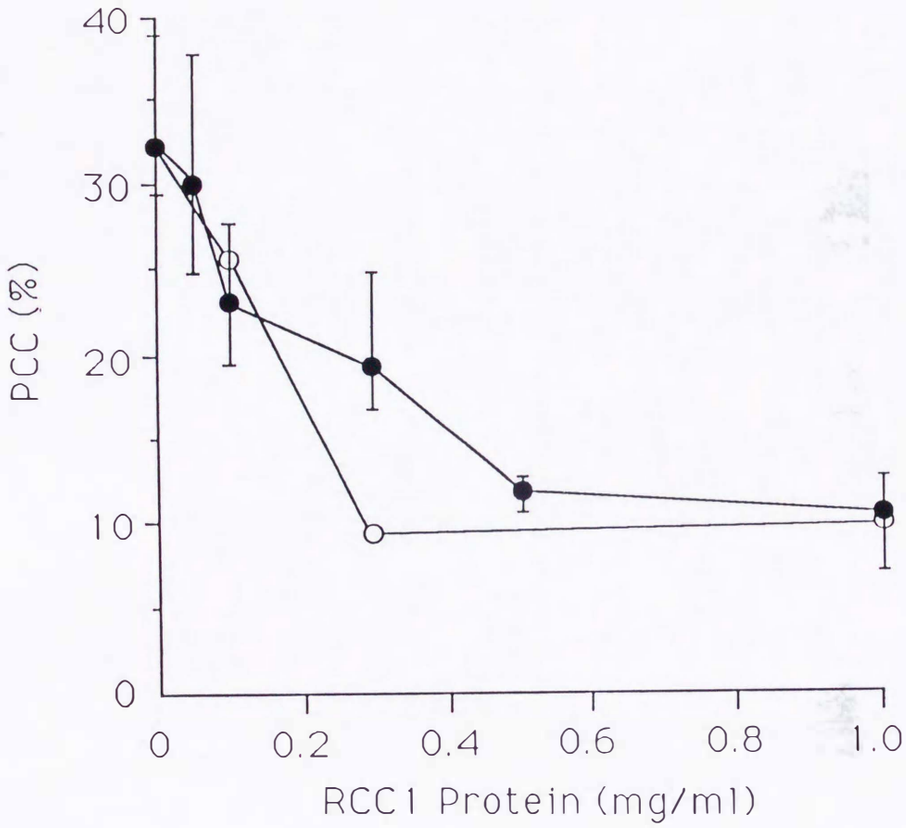


Figure 2. Inhibition of PCC caused in the tsBN2 cell with microinjection of the Δ 8-29 RCC1 protein

The RCC1 protein mixed with BSA was microinjected at the indicated concentration to tsBN2 cells synchronized at G1/S boundary as described in Materials and Methods. Total concentration of the protein injected solution was adjusted to 2 mg/ml. After microinjection, cells were incubated at 40.5°C for 4 hr, and the frequency of PCC was counted.

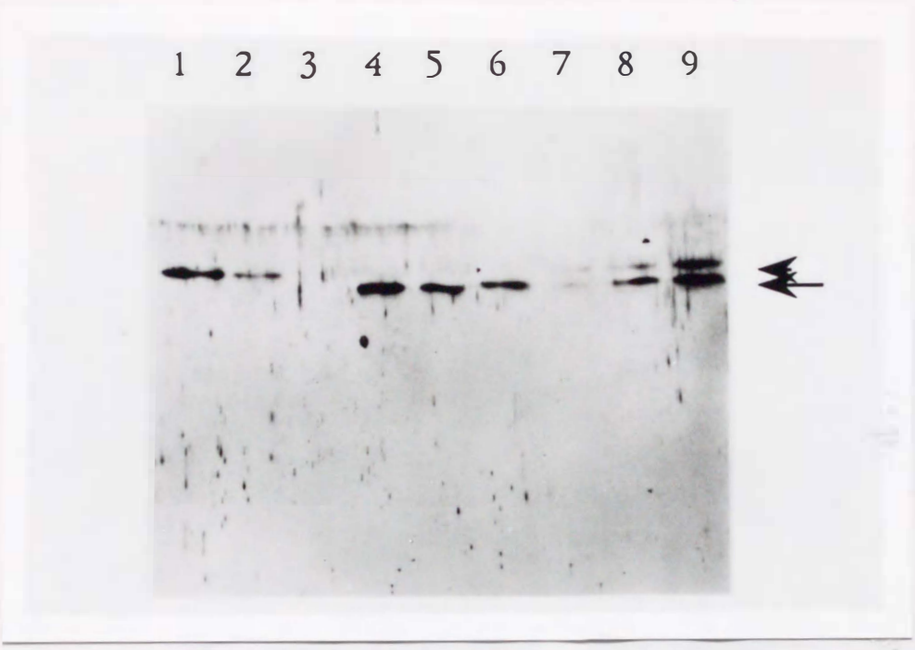


Figure 3. Immunoblotting analysis of RCC1 protein in ts^+ transformants of tsBN2 cells transfected with the Δ 8-29 RCC1 cDNA.

Thirty μg of total cellular protein were electrophoresed on 12.5% SDS-polyacrylamide gel and then subjected to immunoblotting as described in Materials and Methods using the antibody against Xenopus RCC1 protein (Nishitani et al., 1990). Loaded samples are extracted from BHK21 cells cultured at 37.5°C (Lane 1), from tsBN2 cells cultured at 33.5°C (Lane 2) or at 39.5°C for 4 hr (Lane 3), and from the ts^+ transformants cultured at 39.5°C (Lane 4 to 6) or at 33.5°C (Lane 7 to 9). Three independently isolated clones of ts^+ transformants were loaded. Clone 1 is Lane 4 and 7, clone 2 is Lane 5 and 8 and clone 3 is Lane 6 and 9.

The arrow head indicates the position of endogenous RCC1 and the arrow indicates the position of the deleted RCC1 protein.

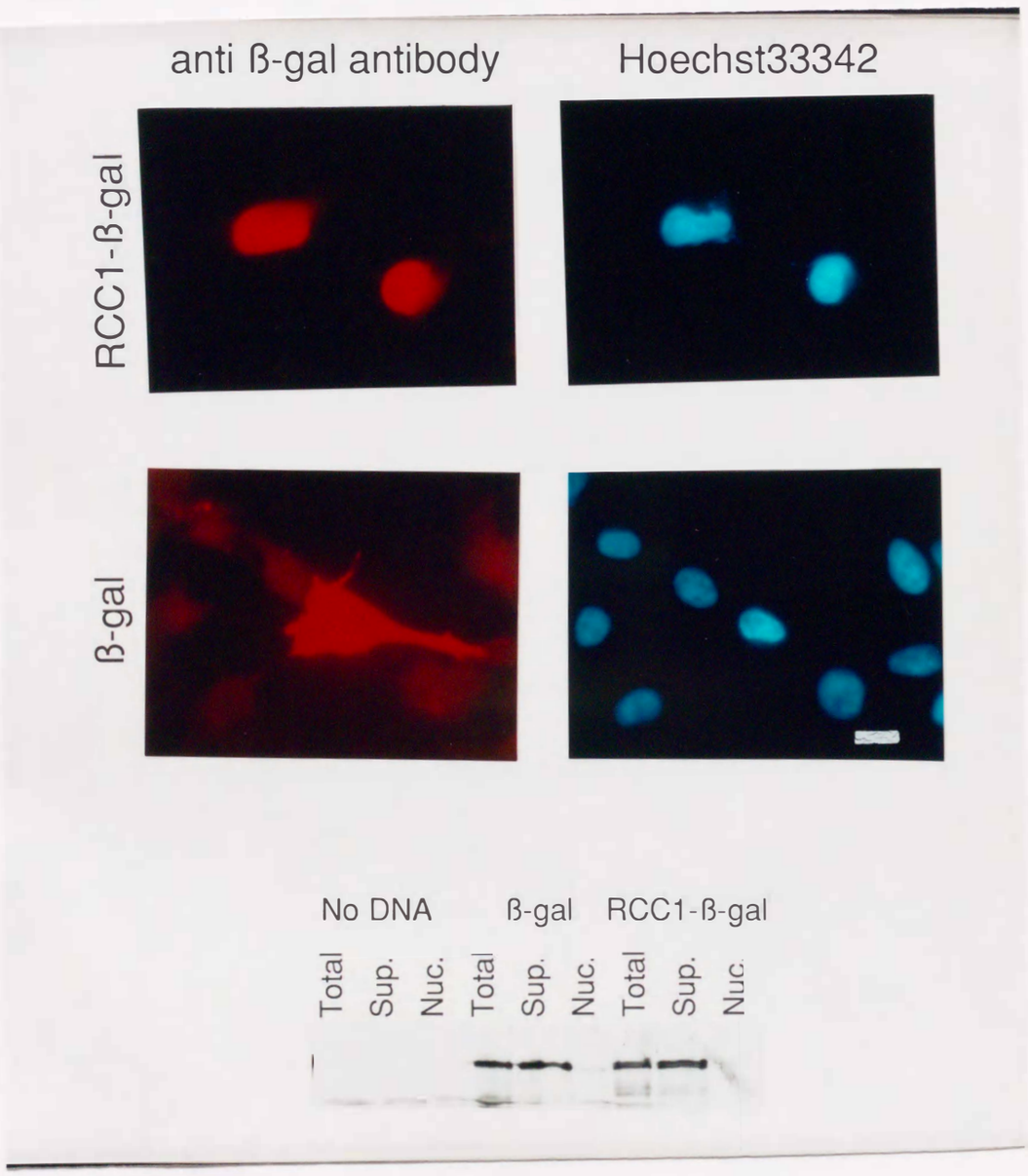

anti ß-gal antibody
Hoechst33342
RCC1-ß-gal
ß-gal
No DNA
ß-gal
RCC1-ß-gal
Total
Sup.
Nuc.
Total
Sup.
Nuc.
Total
Sup.
Nuc.

Figure 4. Location of the ß-galactosidase fused with the N-terminal region of RCC1 protein

Immunofluorescent staining (A); COS7 cells transiently expressing the fusion protein or ß-galactosidase alone were stained with an antibody against ß-galactosidase or Hoechst33342. The upper panels indicate the cells expressing the fusion protein and the lower panels indicate the cells expressing ß-galactosidase alone. Left panels are the staining pattern of the antibody against ß-galactosidase and right panels are the staining pattern of Hoechst33342.

Immunoblotting analysis (B); COS7 cells expressing the fusion protein or ß-galactosidase were fractionated into the nuclei and the post-nuclear supernatants, and then subjected with immunoblotting analysis using the antibody against ß-galactosidase. No DNA, ß-gal,and RCC1-ß-gal indicate the cells transfected with mock solution, plasmid encoding ß-galactosidase alone and the fusion protein, respectively. Total., Sup., and Nuc. indicate the total protein, post-nuclear supernatant and nuclei fraction, respectively.

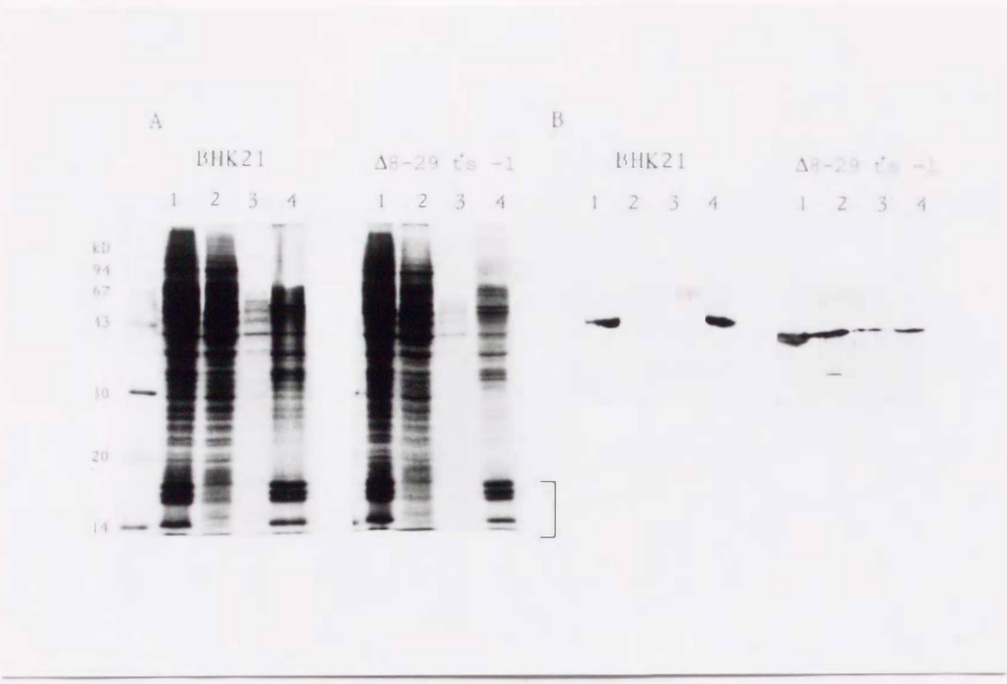


Figure 5. Location of the Δ 8-29 RCC1 protein in ts^+ transformants.

Cells of 1 x 10^6 of BHK21 cells cultured at 37.5°C, or of ts^+ transformants transfected with the Δ 8-29 RCC1 expression vector cultured at 39.5°C were fractionated into the nuclei fractions and the post-nuclear supernatant fractions as described in Materials and Methods. The nuclei were washed with the extraction buffer (Klempnauer and Sippel, 1986) (Washed fraction). The protein from the nuclear fraction and the post-nuclear supernatant fraction, and from the washed fraction were electrophoresed on either 15% or 12.5% SDS-polyacrylamide gel to analysis with Coomassie Brilliant Blue staining (A) or with Immunoblotting analysis using the antibody against the Xenopus RCC1 protein (B), respectively. Lanes indicate the total protein (Lane 1), and the proteins from the post-nuclear supernatant fraction (Lane 2), the washed fraction (Lane 3) and the nuclear fraction (Lane 4). The name of cell lines are indicated above the gel. The bracket indicates the position of histones.

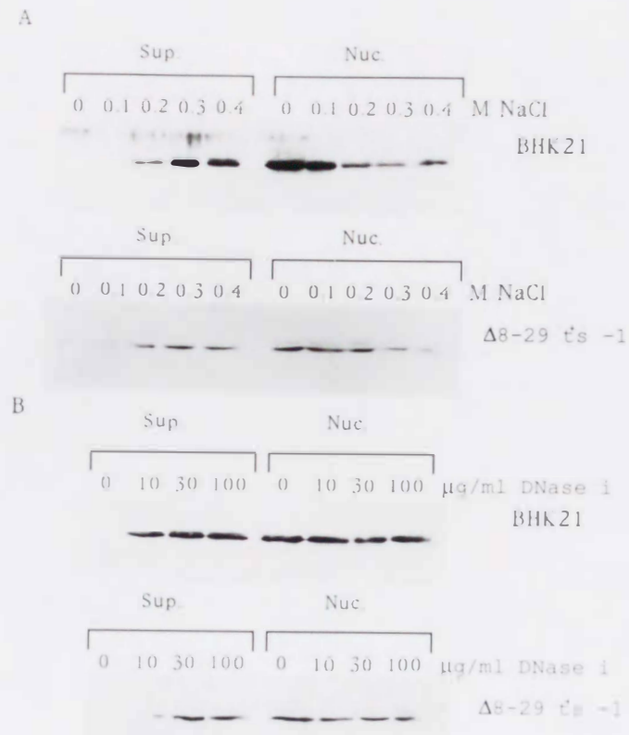
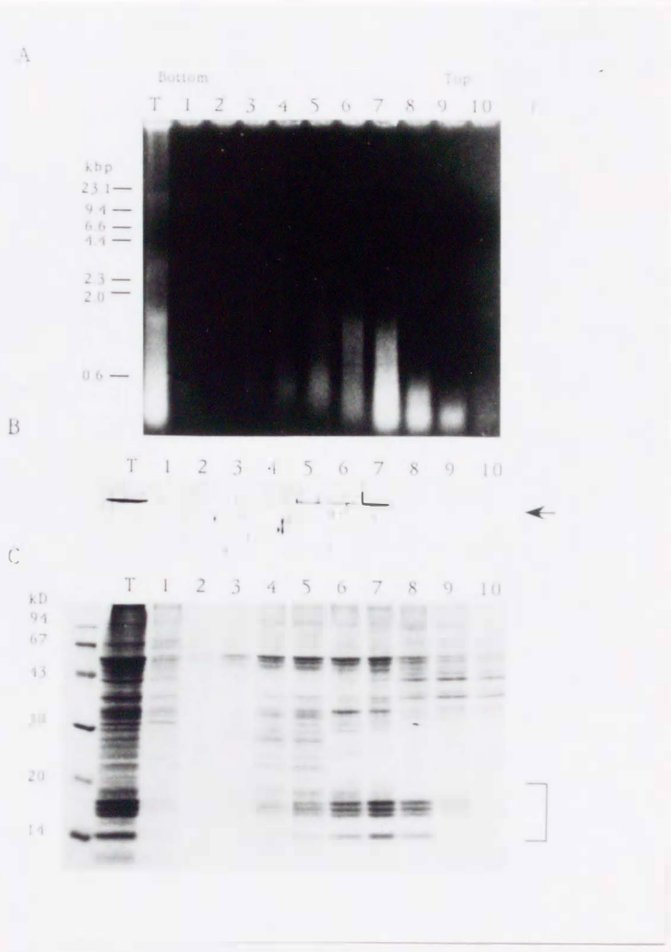


Figure 6. Releasing of the Δ 8-29 RCC1 protein from the isolated nuclei of the ts^+ transformants; Δ8-29ts^+ clone 1.

The nuclei isolated from BHK21 or the ts^+ transformants of tsBN2 cells transfected with the Δ 8-29 RCC1 cDNA were treated, either with NaCl at the concentrations ranging from 0 to 0.5 M (A) or with DNase I at the concentrations ranging from 0 to 100 μg/ml for 10 min at 0°C (B). The proteins released into the supernatant (Sup) or present in the nuclei (Nuc) were analyzed by immunoblotting, as described in Materials and Methods. The area of RCC1 protein is shown.


A
Bottom
Top
T 1 2 3 4 5 6 7 8 9 10
kbp
23.1
9.4
6.6
4.4
2.3
2.0
0.6
B
T 1 2 3 4 5 6 7 8 9 10
C
T 1 2 3 4 5 6 7 8 9 10
kD
94
67
43
30
20
14

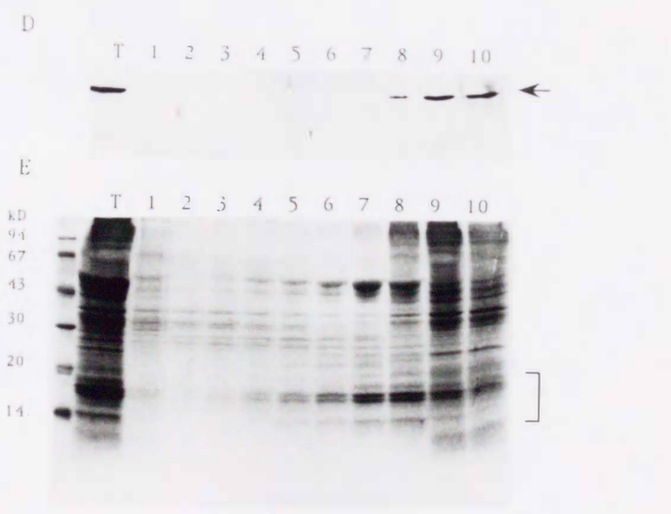
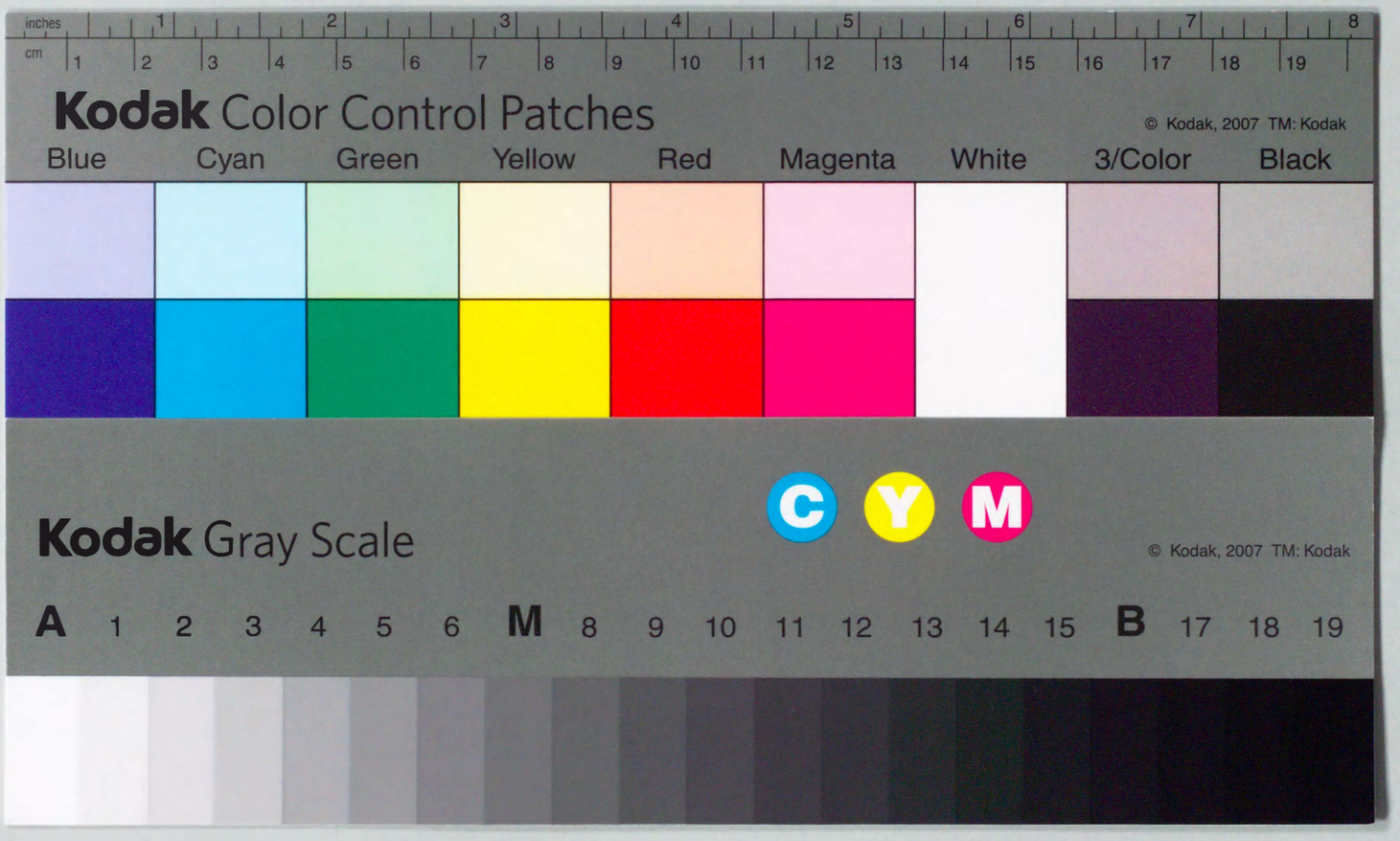

D
T 1 2 3 4 5 6 7 8 9 10
E
T 1 2 3 4 5 6 7 8 9 10
kD
94
67
43
30
20
14

Figure 7. Velocity gradient sedimentation analysis of nucleoprotein complexes released from the nuclei of the Δ8-29 ts^+ clone 1 with nuclease-treatment.

Nuclei from ts^+ transformants (1 x 10^8 cells) were digested with DNase I (10 μg/ml) as described in Materials and Methods. The materials released from the nuclei were divided into two samples. The salt concentration in one sample was adjusted to 0.5 M NaCl. One fiftieth of each sample (Lane T) was directly analyzed by electrophoresis in 15% SDA-polyacrylamide gel, and the remaining material was subjected to velocity gradient sedimentation as described in Materials and Methods. Numbered lanes refer to individual gradient fractions (total 10 fractions) : sedimentation was from right to left (top fraction is Lane 10). The size distribution of DNA fragments throughout the low-salt gradient as determined by agarose gel electrophoresis was shown in Panel A. Panel C and E show the distribution of proteins throughout the low-salt gradient (C) or the high-salt gradient (E) as determined by electrophoresis in 15% SDS-polyacrylamide gel. Panel B and D show an immunoblot analysis of the low-salt (B) or the high-salt containing 0.5 M NaCl (D) fraction. Only the relevant portions of the immunoblots are shown. The arrow-heads indicate the position of RCC1 protein. The positions of the core histones are marked by square.


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
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A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19