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Mutation of the hamster cell cycle gene *RCC1* is complemented by the homologous genes of *Drosophila* and *S.cerevisiae*

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The *RCC1* gene has been isolated from several vertebrates, including human, hamster and *Xenopus*. Genes similar to *RCC1*, namely *BJ1* and *SRM1/PRP20*, have been isolated from the insect *Drosophila* and from the budding yeast *Saccharomyces cerevisiae*. A mutation of the *RCC1* gene in the hamster BHK21 cell line, tsBN2, confers pleiotropic phenotypes, including G1 arrest and premature induction of mitosis in cells synchronized at the G1/S boundary. Similarly, mutations of the *SRM1/PRP20* gene are pleiotropic: the *srm1* mutant shows G1 arrest and suppression of the mating defect of mutants lacking pheromone receptors, and the *prp20* mutant shows an alteration in mRNA metabolism. Here we show that both *BJ1* and *SRM1/PRP20* complement the temperature sensitive phenotype of the tsBN2 cells. Like RCC1 proteins of vertebrates, the protein products of the *Drosophila* and yeast *RCC1* homologues were located in the nuclei of the mammalian cells. These results suggest that the *BJ1* and *SRM1/PRP20* genes are functionally equivalent to the vertebrate *RCC1* genes, and that the *RCC1* gene plays an important role in the regulation of gene expression in the eukaryotic cell cycle.

Key words: BJ1/cell cycle control/*RCC1/SRM1/PRP20*/tsBN2

Introduction

Mutants with a disrupted progression through the cell cycle have been extremely valuable in the cloning and characterization of the cell cycle genes of the fungi *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Aspergillus nidulans* (Weinert and Hartwell, 1989; Nurse, 1990; Yanagida, 1990; Morris *et al.*, 1989). In mammalian cells, temperature sensitive (ts) cell cycle mutants have also been isolated and characterized (reviewed in Siminovitch, 1976; Basilico, 1977; Baserga, 1985; Marcus *et al.*, 1985). However, unlike for fungi, only a few genes complementing the mutations of mammalian cells have been cloned (Kai *et al.*, 1986; Ohtsubo *et al.*, 1987; Greco *et al.*, 1987; Ittman *et al.*, 1987; Sekiguchi *et al.*, 1988; Zacksenhaus and Sheinin, 1990).

The tsBN2 cell line derived from the hamster BHK21/13 cell line was originally isolated as a ts DNA^- mutant. At non-permissive temperatures (39.5–40.5°C), this mutant shows either of two distinct phenotypes: asynchronous cultures exhibit G1 arrest, whereas cultures synchronized at the G1/S boundary exhibit premature chromosome condensation (PCC) (Nishimoto *et al.*, 1978). In addition to PCC, other mitotic specific events, such as depolymerization of the nuclear envelope, characteristic rounding of the cell, and formation of the mitotic spindle were simultaneously induced in tsBN2 cells that had been synchronized at the G1/S boundary prior to being shifted to the non-permissive temperature (Nishimoto *et al.*, 1978; H.Nishitani *et al.*, in preparation). PCC is the induction of chromosome condensation before the completion of DNA replication and can be induced in interphase cells by fusing them with mitotic cells (Rao and Johnson, 1970). Interphase cells also show PCC and nuclear envelope breakdown upon fusion with tsBN2 cells showing PCC (Hayashi *et al.*, 1982). Thus, at the non-permissive temperature, a *trans*-acting inducer of mitosis (presumably Mitosis Promoting Factor; MPF, reviewed in Dunphy and Newport, 1988; Lohka, 1989; Murray and Kirschner, 1989) seems to be newly produced in tsBN2 cells synchronized at the G1/S boundary. Since the tsBN2 mutation is recessive, the tsBN2 cell line was assumed to possess a ts defect in a negative regulator for the onset of mitosis, which inhibits the induction of mitosis until the completion of DNA replication (Nishimoto, 1988).

A human genomic DNA fragment and cDNA that complement the tsBN2 mutation have been isolated (Kai *et al.*, 1986; Ohtsubo *et al.*, 1987). These DNAs contain the *RCC1* gene (*Regulator of Chromosome Condensation*), which has been shown to be the mutated gene in tsBN2 cells (Uchida *et al.*, 1990). The *RCC1* cDNA encodes a protein with a molecular mass of 45 kd that contains seven homologous internal repeats of about 60 amino acids (Ohtsubo *et al.*, 1987). Ohtsubo *et al.* (1989) demonstrated that the RCC1 protein is a nuclear protein that is bound to the chromatin. Recently, by using antikinetochore autoimmune sera, the RCC1 protein was purified from HeLa cells (Bischoff *et al.*, 1990).

The *RCC1* gene has been isolated from several vertebrates, including human (Kai *et al.*, 1986), hamster (Uchida *et al.*, 1990) and frog (Nishitani *et al.*, 1990). Recently, genes similar to the *RCC1* gene were isolated from the insect *Drosophila* and from the budding yeast *S.cerevisiae* (Aebi *et al.*, 1990; Frasch, 1991; K.Clark, M.Ohtsubo, T.Nishimoto, M.Goebl, G.Sprague, in preparation). The *Drosophila BJ1* gene encodes a nuclear protein of 59 kd that is associated with nucleosomes. The deduced amino acid sequence of the first 421 amino acids of *BJ1* shows ~40% amino acid identity with the RCC1 protein (Frasch, 1991). The *SRM1/PRP20* gene of *S.cerevisiae* has been

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independently isolated by two groups, as a suppressor gene of a deletion of the **a**-factor receptor gene (Clark and Sprague, 1989) and as a gene involved in pre-RNA processing (Vijayraghavan *et al.*, 1989; Aebi *et al.*, 1990). The SRM1/PRP20 protein is also located in the nucleus (K.Clark, M.Ohtsubo, T.Nishimoto, M.Goebl, G.Sprague, in preparation). It has a predicted molecular mass of 52 kd and is 28% identical to the RCC1 protein throughout its length.

In eukaryotic cells, regulatory mechanisms of the cell cycle seem to be conserved through evolution. Genetic complementation has successfully demonstrated that regulatory elements of the cell cycle are likely to be functionally conserved between mammals and the single cell eukaryote, yeast (Lee and Nurse, 1987; Wittenberg and Reed, 1989; Sadhu *et al.*, 1990). To clarify the functional relationship of the RCC1 homologues, we expressed *BJ1* and *SRM1/PRP20* in tsBN2 cells. Both *RCC1* homologues efficiently complemented the tsBN2 mutation. This functional similarity suggests that the function of RCC1 gene is conserved in all eukaryotic cells.

Results

Comparison of the predicted amino acid sequences of RCC1 homologues

The predicted amino acid sequences of RCC1 proteins in vertebrates are well conserved; the amino acid identity is 95% between human and hamster RCC1 proteins (Uchida *et al.*, 1990), and 78% between human and *Xenopus* RCC1 proteins (Nishitani *et al.*, 1990). The RCC1 protein has a domain consisting of seven homologous repeats of about 60 amino acids (Ohtsubo *et al.*, 1987). Within this region of repeats, the amino acid identity increases for the human and *Xenopus* proteins (Nishitani *et al.*, 1990), thereby suggesting that the repeated domain is well conserved through evolution. This conservation is further confirmed by comparing the amino acid sequences of *Drosophila* BJ1, yeast SRM1/PRP20 and vertebrate RCC1 proteins (see below: Figure 1B). The amino acid identity is 42% between human RCC1 and the first 421 amino acids of BJ1, and 28% between human RCC1 and SRM1/PRP20. Using a computer program for homology searches developed by Toh *et al.* (1983), the amino acid sequences of human RCC1, BJ1 and SRM1/PRP20 were compared with each other (Figure 1A). As reported for human RCC1, seven homologous repeats were found within SRM1/PRP20 and BJ1, and these repeats were homologous to those of human RCC1.

On the basis of the homology matrices shown in Figure 1A, the amino acid sequences of the homologous repeated domain of the three proteins were aligned. As a comparison, the amino acid sequences of the repeated domains of hamster and *Xenopus* RCC1 are also shown (Figure 1B). The amino acid identity in this region is 45% between human RCC1 and BJ1 and 31% between human RCC1 and SRM1/PRP20, which is significantly higher than the total amino acid identity observed. Thus, the sequence of the repeated domain of RCC1 proteins seems to be well conserved through evolution.

In the repeated domain, the glycine residues shown by asterisks in Figure 1B, which are in identical positions in the seven repeats, are also conserved in all five organisms, suggesting that they are important for RCC1 function. The longest amino acid sequence conserved among the five organisms is the eight amino acid stretch (GALGRDTS) in the second repeat. The N-terminal domain outside of the repeated domain is rich in positively charged amino acids (31% of the 38 amino acids are positively charged in human RCC1). This region is also positively charged in the other organisms, although the amino acid identity is low.

Complementation of tsBN2 mutant by Drosophila BJ1 and yeast SRM1/PRP20

To investigate the functional conservation of the RCC1 proteins, the *BJ1* or *SRM1/PRP20* gene was cloned into expression vectors for mammalian cells under the control of the SV40 early promoter. These constructs were transfected into tsBN2 cells which were then shifted to the non-permissive temperature of 39.5°C as described in Materials and methods. As a control, we used the human *RCC1* cDNA clone, pcD51 (Ohtsubo *et al.*, 1987).

As shown in Figure 2, tsBN2 cells were transformed to the ts^+ phenotype not only with pcD51 but also with pBJ1 (the *BJ1* expression vector) and pSRM1 (the *SRM1/PRP20* expression vector). In our experimental system, the ability of *BJ1* or *SRM1/PRP20* to complement tsBN2 cells were reproducibly higher than that of the human *RCC1* cDNA (Figure 2). Plasmids in which the *BJ1* and *SRM1/PRP20* genes were inserted in the incorrect orientation did not efficiently complement the tsBN2 mutation indicating that the complementation is due to the presence of the genes in the correct orientation in the expression vectors. Furthermore, the complementation activity was specific for the *RCC1* gene and its homologues, as the *CCG1* gene which complements tsBN462/ts13 mutants (Sekiguchi *et al.*, 1988) did not complement the tsBN2 mutation (data not shown). These results suggest that the yeast and *Drosophila* genes share similarity in function as well as in structure with the *RCC1* gene.

Expression of transfected BJ1 and SRM1/PRP20 gene in tsBN2 cells

To confirm that expression of the *BJ1* and *SRM1/PRP20* genes was responsible for formation of the ts^+ transformants, we first verified that the transformants contained the appropriate DNAs. DNA was isolated from five independently isolated colonies of ts^+ cells transfected with *BJ1* or *SRM1/PRP20* and subjected to Southern blot analysis using ^{32}P-labelled *BJ1* or *SRM1/PRP20* DNA as a probe (Figure 3). As expected, a characteristic 2.3 kb *Eco*RI–*Bam*HI fragment of *BJ1* and a characteristic 2.4 kb *Pst*I–*Xho*I fragment of *SRM1/PRP20* were specifically detected in the ts^+ cells transfected with *BJ1* or *SRM1/PRP20* gene. Neither fragment was observed in the recipient tsBN2 cells, although some additional minor bands were detected in some transformants. It is notable that no hybridization signal was detected in tsBN2 cells, indicating that no cross-hybridization occurs between the hamster *RCC1* gene and the *Drosophila* and yeast homologues.

As a second step to confirm expression of the yeast and *Drosophila* genes, we prepared total RNAs from the same transformants used in the DNA analysis and analysed them by Northern blot hybridization using ^{32}P-radiolabelled *BJ1* or *SRM1/PRP20* DNA as a probe. As shown in Figure 4, an RNA transcript of ~3 kb, which is the predicted size of the mRNA, was specifically detected in the ts^+ cells transfected with *BJ1* or *SRM1/PRP20*. However, actin

mRNA was detected in all the cell lines, including untransformed tsBN2 cells (data not shown). These results indicate that the ts^+ transformation is correlated with, and presumably caused by, the expression of the *BJ1* or *SRM1/PRP20* gene.

Production and localization of BJ1 and SRM1/PRP20 proteins in mammalian cells

Nishitani *et al.* (1990) raised polyclonal antibodies to *Xenopus* RCC1 protein using bacterially expressed LacZ−*Xenopus* RCC1 fusion protein. The antibodies were

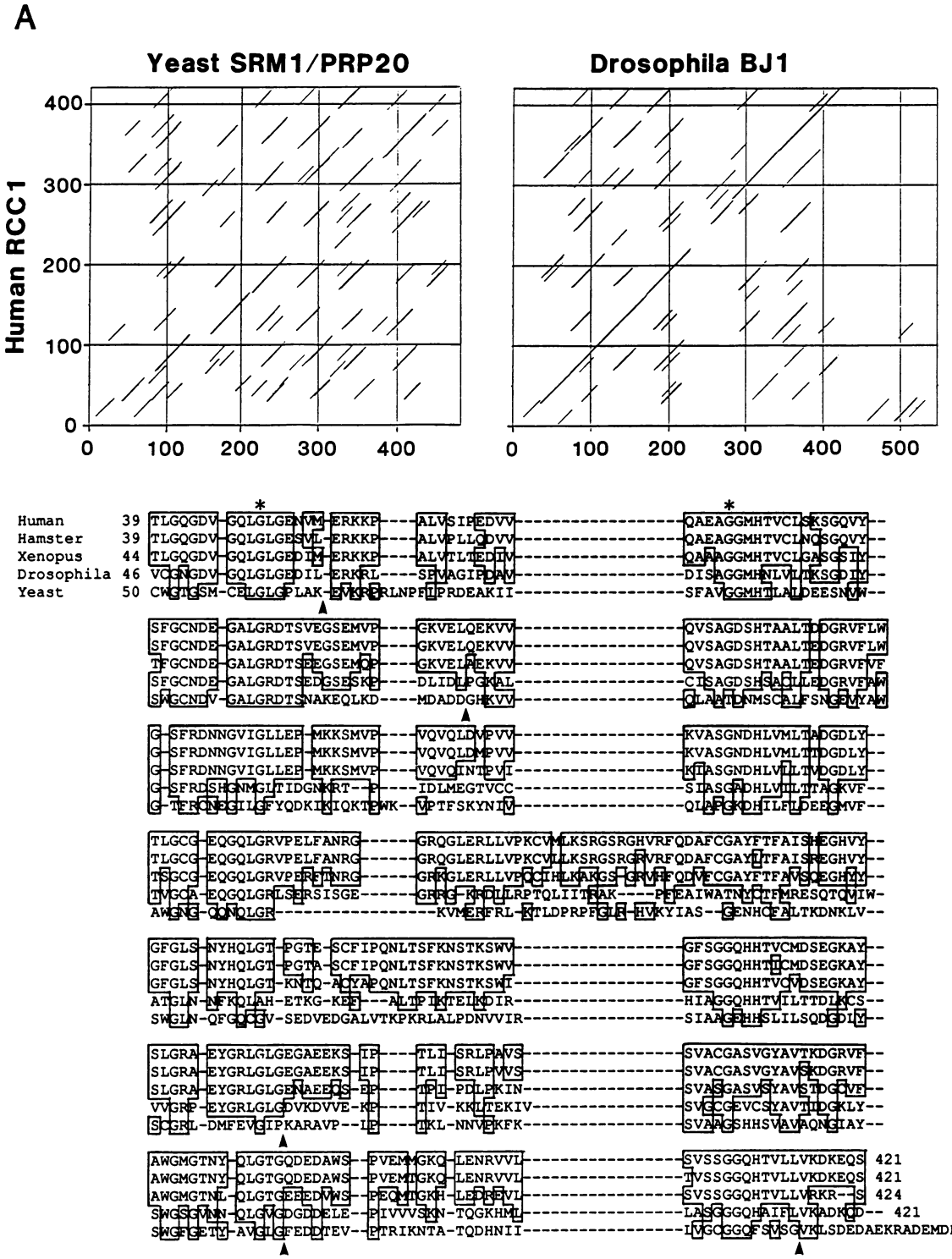

Fig. 1. Comparison of the predicted amino acid sequences of *RCC1* homologues from different species. (**A**) A computer program of Toh *et al.* (1983) was used to compare the predicted amino acid sequence of human RCC1 (vertical axis) with the sequences of *Drosophila* BJ1 and *S.cerevisiae* SRM1/PRP20, with a window size of 25 residues. (**B**) The predicted amino acid sequences for the conserved region of hamster RCC1 (Uchida *et al.*, 1990), *Xenopus* RCC1 (Nishitani *et al.*, 1990), *Drosophila* BJ1 (Frasch, 1991) and yeast SRM1/PRP20 (Clark and Sprague, 1989; Aebi *et al.*, 1990) are aligned with regions of the seven homologous repeats of human RCC1 (Ohtsubo *et al.*, 1987). Regions of identity with human RCC1 are boxed. Arrowheads indicate the positions of amino acid insertions in SRM1/PRP20: 67−68, NK; 135−163, SSDDEDGDLNELESTPAKIPRESFPPLAE; 366−379, KDQLPEYTYKDVHG; 430, P; 465, G.

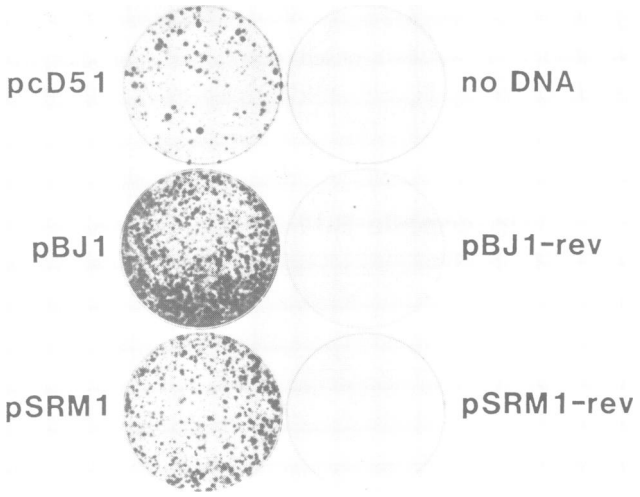

Fig. 2. Complementation of the tsBN2 mutation by *Drosophila BJ1* and yeast *SRM1/PRP20* genes. The tsBN2 cells (2×10^6 cells/100 mm dish) were transfected with the plasmid pcD51 (human, Ohtsubo *et al.*, 1987), pBJ1 (*Drosophila*), pSRM1 (yeast) or no plasmid DNA as described in Materials and methods. The pBJ1-rev and pSRM1-rev have the reverse orientation of the insert in each plasmid. Following transfection, cells were cultured at 33.5°C for two days and then shifted up to 39.5°C and the colonies were stained after 10 days as described in Materials and methods.

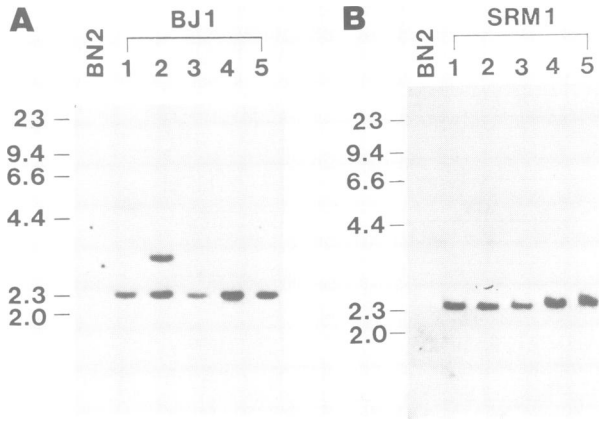

Fig. 3. Southern blot analysis of the ts^+ transformants. Genomic DNA (20 μg) isolated from tsBN2 cells (BN2) or independently isolated ts^+ transformants (lanes 1–5: **A**, *BJ1* transformants; **B**, *SRM1/PRP20* transformants) was completely digested with excess amounts of the restriction enzyme (A; *Eco*RI and *Bam*HI; B, *Hin*dIII) and electrophoresed in a 1% agarose gel. DNA was denatured, transferred to a nylon membrane filter and hybridized with a ^{32}P-labelled 2.1 kb *Xba*I fragment of the BJ1 cDNA (GP18-1 plasmid) (A) or with a ^{32}P-labelled 2.4 kb *Xho*I–*Pst*I fragment of the *SRM1/PRP20* gene (B) under high stringency conditions. Positions of fragments of *Hin*dIII-digested λ DNA are indicated in kb.

affinity-purified and observed to react not only with the *Xenopus* RCC1 protein but also with hamster and human RCC1 proteins (Nishitani *et al.*, 1990; Figure 7A). Using these antibodies, we examined the cross-reactivity to the BJ1 protein. Total proteins prepared from BHK21, tsBN2, and ts^+ cells transfected with the *BJ1* gene were subjected to immunoblot analysis (Figure 5A). The hamster RCC1 protein was detected in tsBN2 and BHK21 cells cultured at 33.5°C. The RCC1 protein specifically disappeared in tsBN2 cells and the *BJ1* transformants incubated at 39.5°C for 3 h

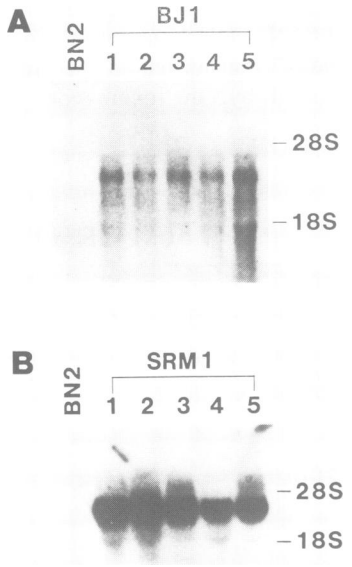

Fig. 4. Northern blot analysis of the ts^+ transformants. Total RNA (20 μg) from tsBN2 (BN2) or independently isolated ts^+ transformants (lanes 1–5: **A**, *BJ1* transformants; **B**, *SRM1/PRP20* transformants) was electrophoresed in a 1.2% formaldehyde agarose gel. RNAs were transferred to a nylon membrane filter and hybridized with the same probes used in Figure 3. Positions of 28S and 18S RNA are indicated.

(Figure 5A, see also Nishitani *et al.*, 1991). In the *BJ1* transformants, however, a specific protein with a molecular mass of 68 kd was observed in the five independently isolated ts^+ transformants. A protein of the same size was detected in *Drosophila* protein extract, using a monoclonal antibody to the BJ1 protein (Frasch, 1991). Although the molecular mass of the BJ1 protein estimated from the cDNA sequence is 59 kd (Frasch, 1991), we conclude that the 68 kd protein recognized by the anti-*Xenopus* RCC1 is the product of the transfected *BJ1* cDNA in the transformants of the tsBN2 cells. Indirect immunofluorescence experiments showed that this protein is located in the nuclei of the transformants (Figure 6A); this is consistent with the localization of mammalian RCC1 proteins (Ohtsubo *et al.*, 1989). However, as expected from the immunoblot analysis, no RCC1 was observed in the recipient tsBN2 cells kept at the nonpermissive temperature (data not shown; Nishitani, 1991).

We generated polyclonal antibodies to the carboxy-terminal half of the SRM1/PRP20 protein expressed in *Escherichia coli* (K.Clark, M.Ohtsubo, T.Nishimoto, M.Goebl, G.Sprague, in preparation). The antibodies were affinity-purified and found to detect a 54 kd protein from yeast, which is nearly identical to the predicted size of the SRM1/PRP20 protein. In addition, the antibodies revealed by indirect immunofluorescence that the SRM1/PRP20 protein is located in the yeast nucleus (K.Clark, M.Ohtsubo, T.Nishimoto, M.Goebl, G.Sprague, in preparation). We used these antibodies to examine the pSRM1 ts^+ transformants. Total proteins prepared from ts^+ cells were subjected to immunoblot analysis, and a major band of 54 kd was specifically observed in five independently isolated transformants (Figure 5B). Another slower migrating band

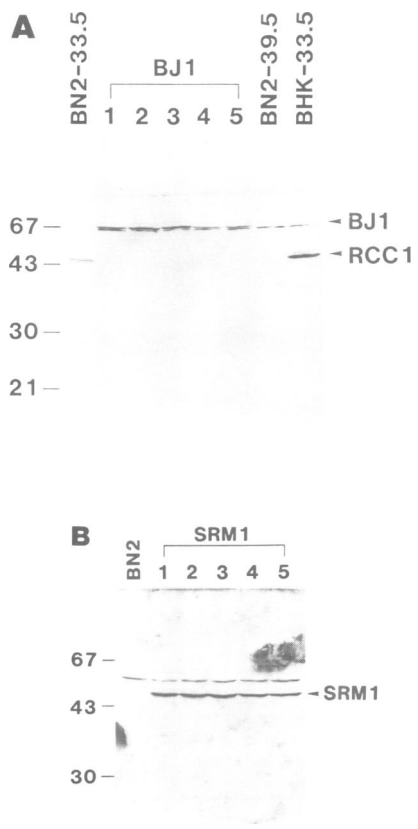

Fig. 5. (**A**) Immunoblot analysis of the *BJ1* transformants. Total cellular proteins (40 μg) prepared from tsBN2, BHK21 or each *BJ1* transformant were subjected to SDS−PAGE and analysed by immunoblot analysis, using affinity purified anti-*Xenopus* RCC1 antibodies. BN2-33.5, tsBN2 cells incubated at 33.5°C; BJ1, lanes 1−5, independently isolated clones of the *BJ1* transformants incubated at 39.5°C; BN2-39.5, tsBN2 cells incubated at 39.5°C for 3 h; BHK-33.5, BHK21 cells incubated at 33.5°C. The positions of RCC1 and the 68 kd protein (BJ1) are indicated by arrowheads, and the positions of the molecular mass markers are shown at the left in kilodaltons. Numbers of the clones correspond to numbers of those in Figure 3 and 4. (**B**) Immunoblot analysis of the *SRM1/PRP20* transformants. Total cellular proteins (40 μg) prepared from each cell line were subjected to SDS−PAGE and analysed by immunoblot analysis, using affinity-purified anti-SRM1/PRP20 antibodies. BN2, tsBN2 cells; SRM1, lanes 1−5, independently isolated clones of the *SRM1/PRP20* transformants. The position of the 54 kd protein is indicated by the arrowhead and the positions of the molecular mass markers are shown at the left. Numbers of the clones correspond to the numbers of those in Figure 3 and 4.

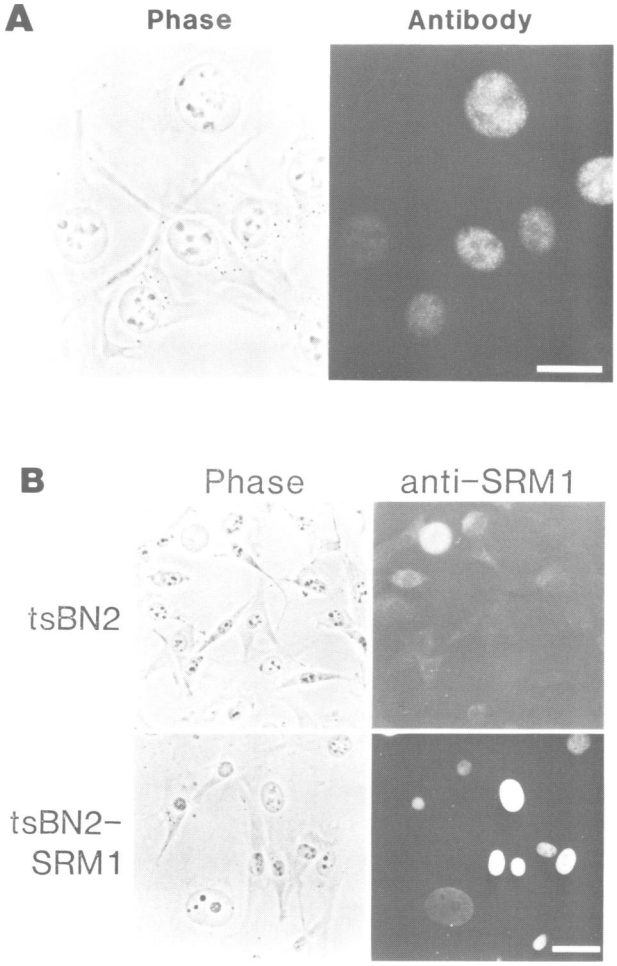

Fig. 6. (**A**) Immunolocalization of the BJ1 protein in a *BJ1* transformant. One of the clones of the *BJ1* transformants was grown on glass coverslips at 39.5°C, fixed, and then stained using affinity-purified anti-*Xenopus* RCC1 antibodies and rhodamine-conjugated goat anti-rabbit IgG. The phase contrast image (Phase) and immunofluorescence image (Antibody) of the same field are presented. Bar, 20 μm. (**B**) Immunolocalization of the SRM1/PRP20 protein in an *SRM1/PRP20* transformant. The tsBN2 cells (tsBN2) or one of the clones of the *SRM1* transformants (tsBN2-SRM1) were grown on glass coverslips, fixed, and then stained using affinity-purified anti-SRM1/PRP20 antibodies and rhodamine-conjugated goat anti-rabbit IgG. The phase contrast image (Phase) and immunofluorescence image (anti-SRM1) of the same field are presented. Both immunofluorescence photographs were taken at the same exposure time (11 s). Bar, 40 μm.

was also present, but it was observed at the same levels even in the tsBN2 cells. Thus, we concluded that the 54 kd protein was the *SRM1/PRP20* product. This protein was not detected by the anti-*Xenopus* RCC1 antibodies (data not shown). Indirect immunofluorescence experiments showed that the SRM1/PRP20 expressed in tsBN2 cells was also located in the nucleus, consistent with the finding in yeast (Figure 6B).

We reported previously that RCC1 is a DNA binding or chromatin-associated protein, which becomes dispersed throughout the cytoplasm during mitosis (Ohtsubo *et al.*, 1989). We therefore examined more closely the location of BJ1 and SRM1/PRP20 proteins during mitosis. We first reexamined the location of the human RCC1 protein in mitotic cells, using the affinity purified anti-*Xenopus* RCC1 antibodies, as they specifically recognize the human RCC1 protein in HeLa cells. The antibodies recognized both a 45 and a 46 kd RCC1 protein in HeLa cells as previously observed by Nishitani *et al.* (1990) (Figure 7A). These proteins were located in the nuclei of HeLa cells (Figure 7B). However, in mitotic cells the human RCC1 proteins had diffused into the cytoplasm (Figure 7C; a−d) and were relocalized to the nucleus after mitosis (Figure 7C; e). Essentially the same result was obtained using the anti-RCC1 peptide antibody, anti-peptide 1 serum (Ohtsubo *et al.*, 1989, data not shown). Similarly, hamster, *Xenopus* and *Drosophilia* RCC1 proteins had also diffused into the cytoplasm during mitosis in hamster cells or in the appropriate ts^+ transformants of tsBN2 (data not shown). In contrast, SRM1/PRP20 was predominantly located on condensed chromosomes throughout mitosis (Figure 7D). Thus, localization on the mitotic chromosomes seems to be a special feature of the SRM1/PRP20 protein.

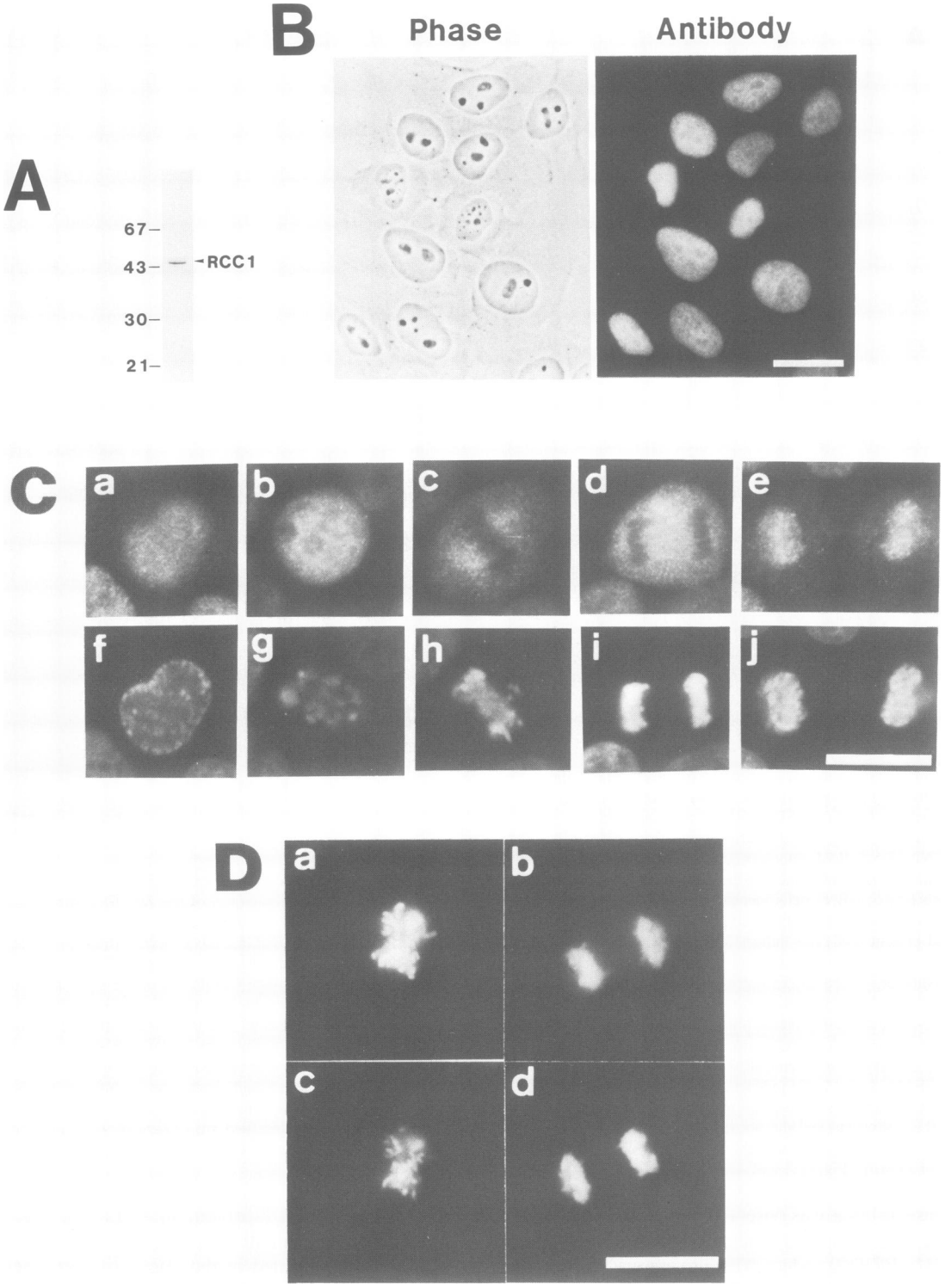

Fig. 7. (**A**) Immunoblot analysis of the HeLa S3 cells with anti-*Xenopus* RCC1 antibodies. Total cellular protein (10 μg) of HeLa S3 cells was prepared, subjected to SDS–PAGE, and analysed by immunoblot analysis using affinity-purified anti-*Xenopus* RCC1 antibodies. The position of the RCC1 protein is indicated by the arrowhead and the positions of molecular mass markers are shown at the left in kilodaltons. (**B**) Immunofluorescence analysis of HeLa S3 cells with anti-*Xenopus* RCC1 antibodies. The HeLa S3 cells were grown on glass coverslips, fixed, and then stained using affinity-purified anti-*Xenopus* RCC1 antibodies and rhodamine-conjugated goat anti-rabbit IgG. The phase contrast image (Phase) and immunofluorescence image (Antibody) of the same field are presented. Bar, 20 μm. (**C**) Immunolocalization of the human RCC1 protein in mitotic cells. The HeLa S3 cells were stained as described in (B). Typical figures of the cells in each stage of mitosis are indicated: a and f; prophase, b and g; prometaphase, c and h; metaphase, d and i; anaphase, e and j; late telophase. Images of Hoechst 33258 staining of DNA (f–j) and immunostaining (a–e) of the same field are presented. Bar, 20 μm. (**D**) Immunolocalization of the SRM1/PRP20 protein in mitotic cells. One of the clones of the *SRM1/PRP20* transformants was stained as described in Figure 6B. Typical figures of the cells in metaphase (a and c) and anaphase (b and d) are indicated. Images of Hoechst staining of DNA (c and d) and immunostaining (a and b) of the same field are presented. Bar, 20 μm.

Discussion

Complementation analysis has been used to assess the functional identity of the cell cycle genes between yeast and mammals (Lee and Nurse, 1987; Wittenberg and Reed, 1989; Sadhu *et al.*, 1990). Using this method, we have provided evidence that the *Drosophila BJ1* and yeast *SRM1/PRP20* genes are functionally homologous to the vertebrate *RCC1* genes. Both *BJ1* and *SRM1/PRP20* completely rescued the ts defect of the tsBN2 mutation, in contrast to the inefficient complementation by the *Xenopus RCC1* gene (Nishitani *et al.* 1990). Further evidence for homologous functions of the yeast and human homologues comes from the observation that two mutant alleles of the yeast gene, either *srm1* or *prp20*, can be complemented by the human *RCC1* gene, although the efficiency seems to be low (Fleischman *et al.*, 1991; K.Clark, M.Ohtsubo, T.Nishimoto, M.Goebl, G.Sprague, in preparation).

The human homologue of the fission yeast $cdc2^+$ gene, *CDC2*Hs which is 63% identical to cdc2, was isolated by complementation of a *cdc2* mutation in *S.pombe* (Lee and Nurse, 1987). This sequence identity is much higher than that between human RCC1 and SRM1/PRP20 (28%). However, a second example of cross-species complementation also involves proteins with only modest similarity. Specifically, the human CDC25 and *Drosophila* string proteins are < 40% identical to *S.pombe* cdc25, although they are functionally homologous to cdc25 by complementation tests (Edgar and O'Farrell, 1989; Jimenez *et al.*, 1990; Sadhu *et al.*, 1990). Thus, it appears that for some cell cycle functions relatively high amino acid identity in extensive regions is not necessary for functional complementation.

The RCC1 protein and its homologues in *Drosophila* and yeast showed similar subcellular localization in the transformants, suggesting that these proteins are functionally equivalent and that nuclear localization is significant for the function (Frasch, 1991; K.Clark, M.Ohtsubo, T.Nishimoto, M.Goebl, G.Sprague, in preparation). In mitotic cells, however, the SRM1/PRP20 protein consistently associated with the chromosomes during mitosis, in contrast to the human and *Drosophila* RCC1 homologues which were dispersed into the cytoplasm in mitosis. Thus, the diffusion of RCC1 protein into the cytoplasm during mitosis might not be necessary for RCC1 function.

Correlation between tsBN2 mutation and srm1/prp20 mutations

The tsBN2 cells showed pleiotropic phenotypes at the non-permissive temperature, such as G1 arrest and PCC induction (Nishimoto *et al.*, 1978, 1981). Recently, these phenotypes were proved to be induced by a point mutation at the 256th codon of the hamster *RCC1* gene, leading to a serine to phenylalanine substitution (Uchida *et al.*, 1990).

When cultures of tsBN2 cells that were first synchronized at the G1(0) phase by isoleucine deprivation were subsequently incubated at 39.5°C, both RNA and protein syntheses drastically decreased within 2 h after temperature shift (Nishimoto *et al.*, 1981). We previously thought that the reduction of RNA and protein syntheses was caused by partial condensation of the chromosomes, since a slight increase of phosphorylated histone H1 was observed in these G1-arrested cells upon a temperature shift (Ajiro *et al.*, 1983). However, no activation of $p34^{cdc2}$ kinase was observed in tsBN2 cells under these same conditions, suggesting that the chromosomes are not condensed (Nishitani *et al.*, 1991). Thus, the inhibition of RNA and protein syntheses may not be caused by chromosome condensation.

The RCC1 protein has DNA-binding activity, is located in the nucleus, and binds to the chromatin as a nonhistone protein (Ohtsubo *et al.*, 1989). This protein immediately decreases to undetectable levels in tsBN2 cells incubated at 39.5°C (Nishitani *et al.*, 1991). Therefore, it is quite possible that an alteration of chromatin structure, not necessarily chromosome condensation, resulting from the disappearance of RCC1 at 39.5°C may perturb mRNA metabolism in tsBN2 cells.

In keeping with the pleiotropic phenotypes of the tsBN2 cell line, two mutant alleles of *SRM1/PRP20* together show an array of pleiotropic phenotypes. One of the alleles, *srm1-1*, was isolated as a suppressor that restored mating to a strain that lacked pheromone receptors (Clark and Sprague, 1989). Even in the absence of the pheromone receptor genes, the *srm1-1* mutant was arrested in G1 and able to mate (Clark and Sprague, 1989). Another allele, *prp20-1*, was isolated as a ts mutant that was defective in mRNA metabolism (Vijayragharan *et al.*, 1989; Aebi *et al.*, 1990). At the non-permissive temperature, *prp20-1* mutants showed a change in their nuclear structure and accumulated abnormal RNAs, such as actin pre-mRNA and oversized mRNA species of intron-less genes. Thus, the *prp20* mutation leads to a pleiotropic effect on mRNA metabolism. Since cells must accumulate many RNA and proteins in G1 phase to initiate the cell cycle (Pardee, 1989), it is possible that the phenotype of *srm1-1* is caused by a defect in mRNA metabolism.

Mechanism of PCC induction in tsBN2 cells

Induction of PCC in the tsBN2 cells suggests that the RCC1 protein is involved in the regulation of the onset of mitosis and in the coupling mechanism between DNA replication and mitosis (Ohtsubo *et al.*, 1987, 1989; Nishitani *et al.*, 1991). By temperature shift to 39.5°C, the RCC1 protein of tsBN2 cells disappears due to the mutation and then $p34^{cdc2}$ histone H1 kinase is activated following to the dephosphorylation of $p34^{cdc2}$ (Nishitani *et al.*, 1991). Cycloheximide inhibits its PCC induction in tsBN2 cells and no activation of the $p34^{cdc2}$ kinase occurs in the presence of cycloheximide, though the RCC1 protein disappears by the temperature shift. Thus, the RCC1 protein seems to inhibit the synthesis of activator(s) for the $p34^{cdc2}$ kinase, either directly or indirectly. So far we have no evidence concerning the regulatory mechanism of RCC1 protein on the activation of $p34^{cdc2}$ kinase.

Based on the assumption that loss of RCC1 function perturbs mRNA metabolism and so protein synthesis, two views as to how loss of RCC1 can lead to PCC can be put forth. First, loss of RCC1 function may inhibit the synthesis of the mRNA for an unstable repressor(s), which normally prevents the activation of $p34^{cdc2}$ kinase until the completion of DNA replication. Alternatively, loss of RCC1 may induce the synthesis of an mRNA encoding an activator protein(s) for the $p34^{cdc2}$ kinase, an induction that is normally prevented until the completion of DNA replication. In either case, the regulatory protein(s) produced in the absence of the RCC1 function will induce mitosis in the presence of other proteins required for the induction of

mitosis. Thus, the RCC1 protein may be a regulatory for the onset of mitosis. However, we cannot exclude the possibility that the RCC1 is also involved in general RNA metabolism. We are now investigating the relationship between the loss of RCC1 function and the induction of mitosis.

Materials and methods

Construction of BJ1 and SRM1/PRP20 expression vectors

The *BJ1* expression plasmid, pBJ1, was created by cloning the 2.1 kb *Xba*I fragment of the *BJ1* cDNA from the GP18-1 plasmid (Frasch, 1991) into pcDL-SRα296 (Takebe *et al.*, 1988). The 2.1 kb *Xba*I fragment was blunt-ended with T4 DNA polymerase using the DNA blunting kit (Takara) according to the instructions of the manufacturer. *Sma*I linkers were added at the *Pst*I site of plasmid pcDL-SRα296, converting the *Pst*I site to a *Sma*I site. The blunt-ended 2.1 kb *Xba*I fragment was cloned into the *Sma*I site of the derivative of pcDL-SRα296. The resulting plasmid with the insert in the correct orientation is pBJ1 and the plasmid with the gene in the reverse orientation is pBJ1-rev. The plasmid pSRM1 was created by cloning the 2.4 kb *Xho*I−*Pst*I fragment of the *SRM1/PRP20* gene into pKCRH2-P4. pKCRH2-P4 was created by adding *Hin*dIII linkers at the *Eco*RI site of pKCR (O'Hare *et al.*, 1981) and subsequently adding a synthetic linker (5′-AGCTTCCGCGGGATATCCTCGAGCCCGGGACGCGTTCGAA-GAGCTCTGATTGATTGAAAGCT-3′) at the *Hin*dIII site. The 2.4 kb *Xho*I−*Sac*I fragment of *SRM1/PRP20* (*Sac*I site is derived from pBluescript-KS$^+$) was cloned into the *Xho*I−*Sac*I site of the pKCRH2-P4. Plasmid pSRM1 has the fragment inserted in the correct orientation. pSRM1-rev, which has the *SRM1/PRP20* insert in the reverse orientation, was created by digesting pSRM1 with *Hin*dIII and religating.

Cell culture and transformation

All cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum or 10% fetal bovine serum in a humidified atmosphere containing 10% CO_2. Cultures of tsBN2 cells were maintained at 33.5°C. Plasmid DNAs were transfected into tsBN2 cells by electric pulse-mediated gene transfer as described (Nishitani *et al.*, 1990) and the cultures were incubated at 33.5°C for 36 h and then at 39.5°C for 10 days. The ts$^+$ colonies were stained with crystal violet or independently isolated as single colonies.

Isolation of nucleic acid and filter hybridization

High molecular weight DNA was isolated from cultured cells by phenol extraction of the SDS-disrupted cells (Sekiguchi *et al.*, 1988). Plasmid DNA isolation and other recombinant DNA techniques were performed using the procedures of Maniatis *et al.* (1982). Cytoplasmic RNA was prepared according to the procedure of Chomczynski and Sacchi (1987). Nucleic acids were transferred to nylon membrane filters (PALL, Biodyne A). Blot hybridization was performed according to the procedures previously described (Kai *et al.* 1986; Ohtsubo *et al.*, 1987).

Immunoblotting

Immunoblot (Western blotting) analyses were performed by transferring the proteins electrophoretically to nitrocellulose after SDS−PAGE (Towbin *et al.*, 1979). The filters were reacted with 200-fold diluted affinity-purified antibodies to *E.coli*-expressed SRM1/PRP20 (K.Clark, M.Ohtsubo, T.Nishimoto, M.Goebl, G.Sprague, in preparation) or 1000-fold diluted affinity-purified antibodies to *E.coli*-expressed *Xenopus* RCC1 (Nishitani *et al.*, 1991).

Indirect immunofluorescence staining

Cells growing on glass coverslips were fixed in 3% (w/vol) paraformaldehyde in phosphate buffered saline (PBS), permeabilized with 1% NP-40 in PBS containing 10 mM glycine, and stained using 200-fold diluted affinity-purified anti-SRM1/PRP20 antibodies or 1000-fold diluted affinity-purified anti-*Xenopus* RCC1 antibodies and the rhodamine-conjugated goat anti-rabbit IgG (Tago Inc., Burlingame, CA), as described (Curran *et al.*, 1984). DNA was stained with Hoechst dye 33258 (1 μg/ml) for 5 min. Microscopy was done using a Zeiss Axiophot photomicroscope.

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References

Aebi,M., Clark,M.W., Vijayraghavan,U. and Abelson,J. (1990) *Mol. Gen. Genet.*, **224**, 72−80.
Ajiro,K., Nishimoto,T. and Takahashi,T. (1983) *J. Biol. Chem.*, **258**, 4534−4538.
Baserga,R. (1985) *The Biology of Cell Reproduction.* Harvard University Press, Cambridge, MA, pp. 69−80.
Basilico,C. (1977) *Adv. Cancer Res.*, **24**, 223−266.
Bischoff,F.R., Maier,G., Tilz,G. and Ponstingl,H. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 8617−8621.
Chomczynski,P. and Sacchi,N. (1987) *Anal. Biochem.*, **162**, 156−159.
Clark,K.L. and Sprague,Jr,G.F. (1989) *Mol. Cell Biol.*, **9**, 2682−2694.
Curran,T., Miller,A.D., Zokas,L. and Verma,I.M. (1984) *Cell*, **36**, 259−268.
Dunphy,W.G. and Newport,J.W. (1988) *Cell*, **55**, 925−928.
Edgar,B.A. and O'Farrell,P.H. (1989) *Cell*, **57**, 177−187.
Fleischmann,M., Clark,M., Forrester,W., Wickens,M., Nishimoto,T. and Aebi,M. (1991) *Mol. Gen. Genet.*, in press.
Frasch,M. (1991) *EMBO J.*, **10**, 1225−1236.
Greco,A., Ittmann,M. and Basilico,C. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 1565−1569.
Hayashi,A., Yamamoto,S., Nishimoto,T. and Takahashi,T. (1982) *Cell. Struct. Funct.*, **7**, 291−294.
Ittmann,M., Greco,A. and Basilico,C. (1987) *Mol. Cell. Biol.*, **7**, 3386−3393.
Jimenez,J., Alphey,L., Nurse,P. and Glover,D.M. (1990) *EMBO J.*, **9**, 3565−3571.
Kai,R., Ohtsubo,M., Sekiguchi,M. and Nishimoto,T. (1986) *Mol. Cell. Biol.*, **6**, 2027−2032.
Lee,M.G. and Nurse,P. (1987) *Nature*, **327**, 31−35.
Lohka,M.J. (1989) *J. Cell Sci.*, **92**, 131−135.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Marcus,M., Fainsod,A. and Diamond, G. (1985) *Annu. Rev. Genet.*, **19**, 389−421.
Morris,N.R., Osmani,S.A., Engle,D.B. and Doonan,J.H. (1989) *BioEssays*, **10**, 196−201.
Murray,A.W. and Kirschner,M.W. (1989) *Science*, **246**, 614−621.
Nishimoto,T., Eilen,E. and Basilico,C. (1978) *Cell*, **15**, 475−483.
Nishimoto,T., Ishida,R., Ajiro,K., Yamamoto,S. and Takahashi,T. (1981) *J. Cell. Physiol.*, **109**, 299−308.
Nishimoto,T. (1988) *BioEssays*, **9**, 121−124.
Nishitani,H., Kobayashi,H., Ohtsubo,M. and Nishimoto,T. (1990) *J. Biochem.*, **107**, 228−235.
Nishitani,H., Ohtsubo,M., Yamashita,K., Iida,H., Pines,J., Yasuda,H., Shibata,Y., Hunter,T. and Nishimoto,T. (1991) *EMBO J.*, in press.
Nurse,P. (1990) *Nature*, **344**, 503−508.
O'Hare,K., Benoist,C. and Breathnach,R. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 1527−1531.
Ohtsubo,M., Kai,R., Furuno,N., Sekiguchi,T., Sekiguchi,M., Hayashida,H., Kuma,K., Miyata,T., Fukushige,S., Murotsu,T., Matsubara,K. and Nishimoto,T. (1987) *Genes Dev.*, **1**, 585−593.
Ohtsubo,M., Okazaki,H. and Nishimoto,T. (1989) *J. Cell Biol.*, **109**, 1389−1397.
Pardee,A.B. (1989) *Science*, **246**, 603−608.
Rao,P.N. and Johnson,R.T. (1970) *Nature*, **225**, 159−164.
Sadhu,K., Reed,S.I., Richardson,H. and Russell,P. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 5139−5143.
Sekiguchi,T., Miyata,T. and Nishimoto,T. (1988) *EMBO J.*, **7**, 1683−1687.

Siminovitch,L. (1976) *Cell*, **7**, 1–11.
Takebe,Y., Seiki,M., Fujisawa,J., Hoy,P., Yokota,K., Arai,K. Yoshida,M. and Arai,N. (1988) *Mol. Cell. Biol.*, **8**, 466–472.
Toh,H., Hayashida,H. and Miyata,T. (1983) *Nature*, **305**, 5201–5205.
Towbin,H., Staehelin,T. and Gordon,J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4350–4354.
Uchida,S., Sekiguchi,T., Nishitani,H., Miyauchi,K., Ohtsubo,M. and Nishimoto,T. (1990) *Mol. Cell. Biol.*, **10**, 577–584.
Vijayraghavan,U., Company,M. and Abelson,J. (1989) *Genes Dev.*, **3**, 1206–1216.
Weinert,T.A. and Hartwell,L.H. (1989) *Science*, **246**, 629–634.
Wittenberg,C. and Reed,S.I. (1989) *Mol. Cell. Biol.*, **9**, 4064–4068.
Yanagida,M. (1990) *J. Cell Sci.*, **96**, 1–3.
Zacksenhaus,E. and Sheinin,R. (1990) *EMBO J.*, **9**, 2923–2929.

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