

# A Glycine-rich RNA-binding Protein Mediating Cold-inducible Suppression of Mammalian Cell Growth

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**Abstract.** In response to low ambient temperature, mammalian cells as well as microorganisms change various physiological functions, but the molecular mechanisms underlying these adaptations are just beginning to be understood. We report here the isolation of a mouse cold-inducible RNA-binding protein (*cirp*) cDNA and investigation of its role in cold-stress response of mammalian cells. The *cirp* cDNA encoded an 18-kD protein consisting of an amino-terminal RNA-binding domain and a carboxyl-terminal glycine-rich domain and exhibited structural similarity to a class of stress-induced RNA-binding proteins found in plants. Immunofluorescence microscopy showed that CIRP

was localized in the nucleoplasm of BALB/3T3 mouse fibroblasts. When the culture temperature was lowered from 37 to 32°C, expression of CIRP was induced and growth of BALB/3T3 cells was impaired as compared with that at 37°C. By suppressing the induction of CIRP with antisense oligodeoxynucleotides, this impairment was alleviated, while overexpression of CIRP resulted in impaired growth at 37°C with prolongation of G1 phase of the cell cycle. These results indicate that CIRP plays an essential role in cold-induced growth suppression of mouse fibroblasts. Identification of CIRP may provide a clue to the regulatory mechanisms of cold responses in mammalian cells.

To adapt themselves to environmental temperature shifts, organisms have developed sophisticated strategies. To survive in the winter, some animals have developed a capacity for adaptive hypothermia (hibernation), in which they lower their body temperatures to a few degrees above the ambient (Willis, 1987; Aloia and Raison, 1989). Although nonhibernating mammals maintain high uniform body temperatures and have a poor tolerance for hypothermia, their cells may have a capacity for a far wider cold tolerance (Michl et al., 1966; Holecková et al., 1968). In mammals as well as other organisms, cold stress changes the lipid composition of cellular membranes and suppresses the rate of protein synthesis and cell growth (Rao and Engelberg, 1965; Siskin et al., 1965; Watanabe and Okada, 1967; Nelson et al., 1971; Nishimune and Komatsu, 1972; Burdon, 1987; Aloia and Raison, 1989). In clinics, hypothermia is now employed in heart and brain surgeries and in the preservation of donor organs (Fuller, 1987). However, the molecular mechanisms regulating the response to cold stress in mammalian cells are just beginning to be understood (Glofcheski et al., 1993; Matz et al., 1995).

The cold-shock responses of microorganisms have been extensively investigated (Jones et al., 1987; Goldstein et al.,

1990; Jones and Inouye, 1994). Cold stress induces the synthesis of several cold-shock proteins, which are involved in various cellular processes such as transcription, translation, and DNA recombination (Jones and Inouye, 1994). Recently, a family of proteins consisting of one amino-terminal consensus sequence RNA-binding domain (CS-RBD)<sup>1</sup> and one carboxyl-terminal glycine-rich domain has been isolated from cyanobacterium (Sato, 1994, 1995), plant cells (Gómez et al., 1988; Bergeron et al., 1993; Hirose et al., 1993; Carpenter et al., 1994; Heintzen et al., 1994), and human (Derry et al., 1995). This protein family is referred to as a glycine-rich RNA-binding protein (GRP) family and some of them have been demonstrated to be induced by cold stress (Bergeron et al., 1993; Carpenter et al., 1994; Heintzen et al., 1994).

CS-RBD, also referred to as ribonucleoprotein (RNP) motif, RNP consensus sequence, or RNA recognition motif, is one of the major RNA-binding motifs and is the most widely found and best characterized (Kenan et al., 1991; Burd and Dreyfuss, 1994). It is composed of ~90 amino acids, including two highly conserved sequences, an octamer designated RNP1 and a hexamer designated RNP2, and a number of other, mostly hydrophobic conserved amino

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1. *Abbreviations used in this paper:* CIRP, cold-inducible RNA-binding protein; CS-RBD, consensus sequence RNA-binding domain; GFP, green fluorescence protein; GRP, glycine-rich RNA-binding protein; GST, glutathione-S-transferase; ODN, oligodeoxynucleotide; RNP, ribonucleoprotein.

acids interspersed throughout the motif. A number of proteins with CS-RBD have been found in organisms ranging from *Escherichia coli* to human (Fukami-Kobayashi et al., 1993). They contain one to four CS-RBDs and auxiliary domains that are characterized by an abundance of specific residues in their amino acid sequences (Bandziulis et al., 1989). Proteins with CS-RBD are involved in the posttranscriptional regulation of gene expression (Burd and Dreyfuss, 1994). For example, poly(A)-binding proteins have one CS-RBD and a proline-rich domain and regulate mRNA stability and translation. SF2/ASF, B52, X16, and PR264/SC35, whose CS-RBDs make a cluster in the phylogenetic tree, have a serine-arginine-rich domain and are splicing factors. Some hnRNPs, such as hnRNP A1, have two CS-RBDs and a glycine-rich domain and play important roles in mRNA biogenesis, such as splicing and RNA export. Although GRPs, hnRNPs, and nucleolin have domains rich in glycine in common, they belong to phylogenetically distinct families, and no definite roles played by GRP have been clarified (Bergeron et al., 1993; Fukami-Kobayashi et al., 1993; Carpenter et al., 1994; Heintzen et al., 1994).

In the present study, we have screened for RNA-binding proteins expressed in mouse testis with a PCR-based cloning method and isolated a cDNA encoding a novel GRP. This protein was induced in response to cold stress and designated as cold-inducible RNA-binding protein (CIRP). Furthermore, we demonstrated that CIRP is involved in temperature-dependent regulation of cell growth.

## Materials and Methods

### Cells and Culture Conditions

BALB/3T3 cells were cultured in D-MEM (Gibco Laboratories, Grand Island, NY) supplemented with 10% calf serum (Dainippon Pharmaceutical Co., Osaka, Japan) and 100 IU/ml penicillin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. BMA1, TAMA26, and COS-7 cells were cultured in D-MEM (Gibco Laboratories) supplemented with 10% FCS (Dainippon Pharmaceutical Co.) and 100 IU/ml penicillin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air.

For temperature-shift experiments, BALB/3T3 cells ( $5 \times 10^3$  cells/cm<sup>2</sup>) were grown for 24 h at 37°C and then shifted to 15, 25, 32, 37, 39, or 42°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. After 6 h or other indicated times, the cells were scraped and frozen in liquid nitrogen. Extraction of total RNA, Northern blot analysis, and Western blot analysis were carried out as described (Furutani et al., 1995).

To assess the effects of antisense oligodeoxynucleotide (ODN), sense ODN (0.5 μM), antisense ODN (0.5 μM), or vehicle alone was added to the BALB/3T3 cells in exponential phase of growth, and the temperature was shifted from 37 to 32°C. After 12 h of incubation, the expression of p18<sup>crp</sup> was analyzed by Western blotting. To assess the effects on growth,  $5 \times 10^3$  BALB/3T3 cells were plated into each well of a 24-well plate. 16 h later, 0.1, 0.5, or 1.0 μM of antisense ODN, sense ODN, or vehicle alone was added to each well and the culture was continued. 2 d later, the numbers of cells were determined under a microscope. The experiments were repeated three times, and each assay was performed in triplicate. Statistical differences between sample means were calculated by analysis of variance, followed by unpaired Student's *t* test. The results are expressed as the mean ± SEM, and *P* < 0.02 was considered significant.

### ODNs

Antisense and sense ODNs containing phosphorothioates were purchased from Cruachem (Kyoto, Japan). The sequence of the sense ODN was (5'-AGCGGCTGCCATGGCATCAG-3') spanning the translation initiation site of *cirp* mRNA, and that of antisense ODN was complementary to this sequence.

## Cloning of *cirp* cDNA

The first-strand cDNA was prepared from the total cellular RNA of mouse testis (4-mo-old) by using a First-strand cDNA Synthesis Kit (Pharmacia LKB Biotechnology, Piscataway, NJ) with random primers, according to the manufacturer's instructions. For the PCR, we used a set of degenerate primers, (5'-TCGAATTCIAT[C]A[G]AAIC[G]CA[G]AA-ICC-3' [I; inosine]) encoding the amino acid sequence GFG/AFV/I and (5'TCAAGCTTTTC[T]A[G]TIGGIGGIC[T]T-3') encoding the amino acid sequence F/YV/IGGL. The PCR reaction was repeated for 40 cycles at 94, 37, and 72°C for periods of 1 min. The PCR products were cloned into the EcoRV site of TA vector and then sequenced. Using the cloned PCR fragment as a probe, clone RNP78 was isolated from a mouse testis cDNA library constructed in λ-Zap phage vectors (Stratagene, La Jolla, CA). The nucleotide sequence of RNP78 insert (*cirp*) was compared to known sequences in GenBank using the Genetics Computer Group software package.

## RNA Extraction and Northern Blot Hybridization

Cells were dissolved in TRIzol reagent (Life Technologies, Grand Island, NY), and RNA was extracted by following the manufacturer's instructions. 20 μg of total RNA of each sample was separated in 1.0% agarose/formaldehyde gels by electrophoresis and was blotted onto nylon filters (Hybond-N+; Amersham International, Buckinghamshire, UK). The filters were hybridized with [ $\alpha$ -<sup>32</sup>P]dCTP-labeled random-primed cDNA fragments, then washed under stringent conditions (65°C for 30 min in a washing buffer composed of 0.1 × SSC and 0.1% SDS), and detected by autoradiography. A 650-bp SacI-EcoRI fragment of *cirp* cDNA was used

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ctcgcgccttaggaagcttgggtgtgtgtggcgcgctgtcttcccgctcgcgtcagggac 60
ctgccgactcagcggctcggatccatcagatgaaggcaagctttctgtggaggactc 120
      M A S D E G K L F V G G L
agcttcgacaccaacgagcagcgtggtgagcaggtcttctccaogtatgggcagatctcc 180
S F D T N E Q A L E Q V F S K Y G Q I S
gaagtgggtggtaaaggacagggagaccagcagatcccgaggcttgggtttgtcacc 240
E V V V V K D R E T Q R S R G F G F V T
tttgaatgatcagcagcgttaaggacccatgatggctatgaatgggaagctctgtggac 300
E E N I D D A K D A M M A M N G K S V D
gggcggcagatcagagttgacagcagcgtggaagcttctgacaaccggctccgaggatac 360
G R Q I R V D Q A G K S S D N R S R G Y
cgggggtgctctgtggggcggggcttttctcgtgggggcaagccggggcgagggg 420
R G G S A G G R G F F R G G R C R G R G
ttctccagaggaggagaccgggctatggagtggtggcgcctttgagtcgccgagtggg 480
F S R G G G D R G Y G G G R F E S R S G
ggttatggaggctcagagactactatgacagcggagtcaggtggcagctatggttat 540
G Y G G S R D Y Y A S R S Q G G S Y G Y
cggagctcggagggtcctacagagacagctatgacagttatgtacacacaacagagtaa 600
R S S G G S Y R D S Y D S Y A T H N E *
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gcagcctgcttctgacccaagatggcctcgtgactagacctgttttaaggaaagcgtgt 780
ctgttttaagatattcttaaaacggtttgaaagcaactcagatattactgtttaccat
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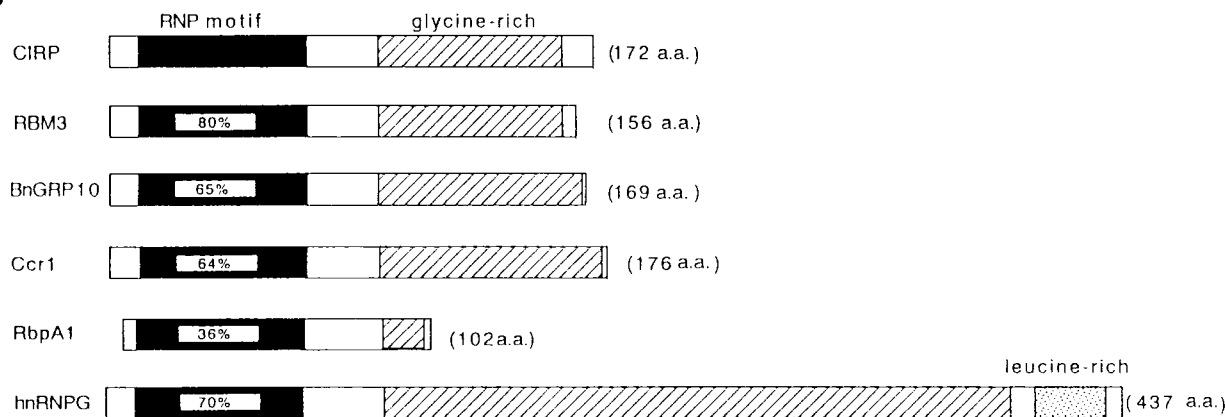
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Figure 1. Cloning of *cirp* cDNA. Nucleotide and deduced amino acid sequences of the clone RNP78 containing *cirp* cDNA. Amino acid sequence is shown in single-letter code below the nucleotide sequence. The putative RNP motifs are underlined. GYGGG and GYG G, well conserved among GRPs, are doubly underlined. Terminal codon is indicated by an asterisk. These sequence data are available from GenBank/EMBL/DDBJ under accession number D78135.

**a**

Protein	RNP-2	RNP-1
CIRP	MASDEGKLFVGGLSFDTNEQALEQVFSKYGQISEVVVVKDRETQRS	RGFGFVTFENIDDAK DAMMNGKSV DGRQIRVDQA
RBM3	.S.E.....N.N.D...DH..SF.P.....	.....I..T.PEH.SV..R...E.L.....H.
BnRG-10	.SEV.YRC.....AWA.GDAE..RT..QF.EVIDSKIIN.....G.....	.....KDEKSM...IDE...EL...T.T.NE.
Ccr1	.SEV.YRC.....AWA..DED.QRT..QF.DVIDSKIIN.....SG.....	.....KDEKAMR..IEE...EL...V.T.NE.
Ccr2	SGDV.YRC.....AWA.DDR...TA.AQ..DUIDSKIIN.....G.....	.....KDEKAM...IEG...QDL...S.T.NE.
hnRNPG	EADRP...I...NTE...K...A..G...R.V..LLM.....NK.....	.....A...SPA.....ARD...L...KA.K.EQ.
Consensus	L F V G N L E L F F G I I	R G F G F V X F A L G
	I Y I K G M D Y V V	K Y A Y I V

**b**



**Figure 2.** (a) Comparison of amino acid sequences in the RNA-binding domains of mouse CIRP, human RBM3 (Derry et al., 1995), *B. napus* BnGRP10 (Bergeron et al., 1993), *A. thaliana* Ccr1 and Ccr2 (Carpenter et al., 1994), and human hnRNP G (Soulard et al., 1993). The consensus sequence for the RNA-binding domain, as determined by Burd and Dreyfuss (1994), is also shown. Dots indicate amino acids identical to the CIRP sequence. The sequences, RNP1, RNP2, DRET, and MNGKXXDG, are boxed. (b) Structural comparison of CIRP with its related proteins. The length of each bar reflects the actual length of the sequences. The numbers in the black boxes indicate percent identity of amino acid sequence to CIRP in the CS-RBD (RNP motif). Note that the glycine-rich domain of cyanobacterium *Anabaena variabilis* RbpA1 (Sato, 1995) is smaller than that of CIRP.

as a probe. The filters were stripped and rehybridized with a cDNA probe for the S26 ribosomal protein as an internal control.

### Southern Blot Analysis

ZOO-BLOT was purchased from Clontech Laboratories, Inc. (Palo Alto, CA). The ZOO-BLOT contained EcoRI-digested genomic DNAs from nine eukaryotic species: human, monkey, rat, mouse (BALB/c), dog, cow, rabbit, chicken, and yeast (*Saccharomyces cerevisiae*). For Southern blot analysis of mouse genomic DNA, DNA was extracted from the mouse liver of 4-mo-old ddy/std and C57BL/6 mice as previously described (Kaneko et al., 1995). The extracted DNA was thoroughly digested with one of the restriction enzymes, EcoRI, PvuII, or KpnI. 20 µg of the digested DNA was electrophoresed in 0.8% agarose gel and transferred onto a nylon filter (Hybond-N+; Amersham International). Filters were hybridized with an [ $\alpha$ -<sup>32</sup>P]dCTP-labeled probe at 65°C and washed under high-stringent conditions (two times in 0.1 × SSC and 0.1% SDS at 65°C). The coding region of *cirp* cDNA, positions 82–600, was used as a probe.

### Protein Analysis

The anti-CIRP polyclonal antibody was produced by immunizing rabbits with a carboxyl-terminal oligopeptide (DSYDSYATHNE). For Western blot analysis, 10 µg of proteins were separated by 14% SDS-PAGE, blotted onto polyvinyl difluoride membranes (Millipore, Tokyo, Japan), and treated with the antiserum (1:20,000 diluted). Bound antibody was detected using a goat anti-rabbit IgG conjugated with horseradish peroxidase (BioRad Labs, Hercules, CA) and the enhanced chemiluminescence system (Amersham International). Protein concentrations were determined using the Bradford reagent (BioRad Labs).

### Fluorescence Microscopy

Immunofluorescence microscopy was performed on BALB/3T3 cells as previously described by Osborn (1994). The anti-CIRP polyclonal antibody was used at a dilution of 1:10,000. Detection was with FITC-conjugated goat anti-rabbit IgG (DAKO) diluted 1:1,000. As a control, preimmune serum was used at the same dilution.

Green fluorescence protein (GFP)/Thr<sup>65</sup> was purchased from Clontech Laboratories Inc., and was ligated into MluI and NotI sites of pMkit-neo vector (pGFP). *Cirp* cDNA encoding amino acids 1–162 was ligated in-frame into PstI site of GFP/Thr<sup>65</sup> (pGFP-CIRP). To assess the intracellular localization of GFP-CIRP fusion protein and GFP, we transfected pGFP-CIRP or pGFP DNA into COS-7 cells. After 3 d of culture at 37°C, the medium was removed and replaced with PBS. Then the cells were examined by using a fluorescence microscope (Olympus, Tokyo, Japan) equipped with the FITC filter set.

### Northwestern Analysis

A glutathione-S-transferase (GST)-fusion protein was prepared by cloning a 519-bp PCR fragment (positions 83–601) of *cirp* cDNA into the SmaI site of the expression vector pGEX-4T1 (Pharmacia LKB Biotechnology). Transformed DH5α *E. coli* cells were grown at 30°C and GST-CIRP fusion protein was purified with Bulk GST Purification Modules (Pharmacia LKB Biotechnology) following the manufacturer's instructions. 2 µg of GST or 1 µg of GST-fusion protein per lane was electrophoresed on 12% SDS-PAGE gels, one of which was stained with Coomassie blue. Others were blotted onto PVDF membrane (Millipore) and denatured and renatured with guanidine-HCl methods, and Northwestern analysis was carried out as described by Schumacher et al. (1995). Each

RNA homopolymer probe (Pharmacia LKB Biotechnology) was labeled with [ $\gamma$ - $^{32}$ P]ATP and incubated with the blot. To examine the strength of RNA binding, the blots were subsequently washed in the binding buffer containing increasing concentrations of NaCl up to 2.0 M.

### Flow Cytometry

To analyze the effect of CIRP on cell cycle, we cloned the *cirp* cDNA into the expression vector p $\beta$ Apr-1 (Gunning et al., 1987) in the forward orientation relative to the  $\beta$ -actin promoter (p $\beta$ Apr-1-CIRP). BALB/3T3 cells were transfected with the p $\beta$ Apr-1-CIRP DNA or the p $\beta$ Apr-1 DNA. Stable clones were selected in G418 and by limiting dilutions. Five p18<sup>cirp</sup>-overexpressors and seven vector-transfected controls were analyzed. Doubling times were determined from the growth curves. For cell cycle analysis, trypsinized cell suspensions were adjusted to a concentration of  $1 \times 10^6$  cells/ml in prechilled 70% ethanol. Samples stored at 4°C overnight were stained with propidium iodide and analyzed by Epics Elite flow cytometer (Coulter Corp., Hialeah, FL). A minimum of 10,000 events were collected, and cell cycle distribution was obtained using a computer modeling program (Multicycle AV; Phoenix Flow Systems, San Diego, CA). Experiments were repeated three times. Length of G1 phase was calculated from the percent of cells in G1 phase according to the equation  $Td \times [1 - \log [2 - F(G1)]/\log 2]$ , in which  $Td$  is the doubling time and  $F(G1)$  is the fraction of cells in G1 phase (Koyasu et al., 1989).

## Results

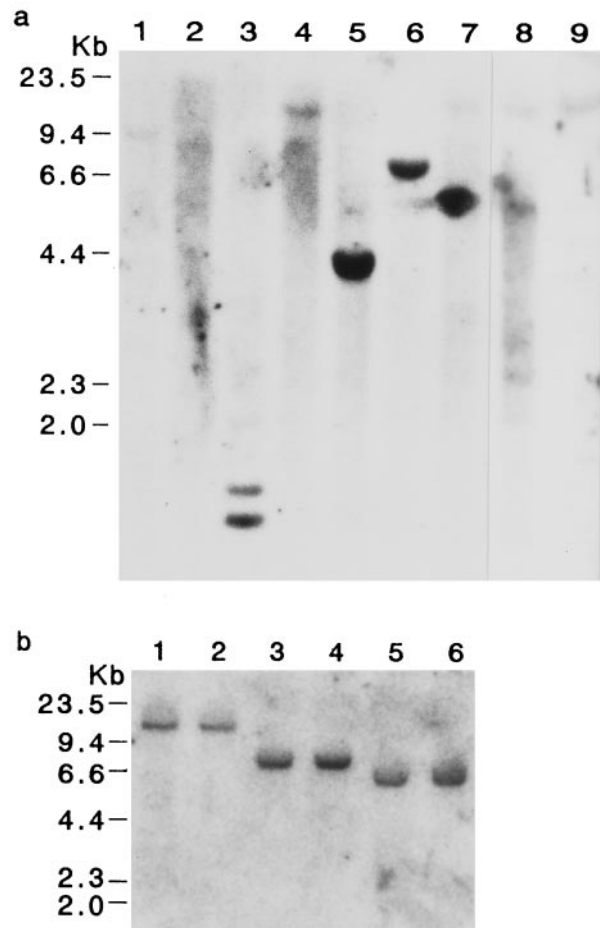
### Isolation of Mouse *cirp* cDNA

By screening adult mouse testis cDNAs for RNA-binding proteins with a PCR-based cloning method, we obtained one fragment that was similar to plant GRPs. After screening a mouse testis cDNA library with this fragment, a clone with a 1.3-kb insert (RNP78) was obtained. In Northern blot analysis, RNP78 hybridized to RNA bands of  $\sim$ 1.3 and 2.9 kb in size derived from mouse testis (data not shown).

RNP78 (*cirp*) contained a 1,264-bp insert with an open reading frame potentially encoding 172 amino acids (Fig. 1). The sequence around the first ATG codon at nucleotide position 81 provided a favorable context for translation initiation (Kozak, 1991), and the 5' untranslated region contained an in-frame stop codon at position 10. The predicted amino acid sequence displayed two main features: the presence of an amino-terminal CS-RBD and a carboxyl-terminal glycine-rich domain. The CS-RBD of *cirp* contained consensus sequences of RNP1, RNP2, and a number of other, mostly hydrophobic conserved amino acids interspersed throughout the motif (Fig. 2*a*). The carboxyl-terminal part was rich in glycine, serine, arginine, and tyrosine (38.8, 16.4, 19.4, and 10.4%, respectively).

### High Conservation of *cirp* Gene beyond Species

The predicted amino acid sequence of *cirp* was compared to known sequences in GenBank using the Genetics Computer Group software package. The overall amino acid sequence of CIRP was similar to human RBM3 (Derry et al., 1995) and plant GRPs such as *Brassica napus* BnGRP10 (Bergeron et al., 1993) and *Arabidopsis thaliana* Ccr1 (AtGRP7) and Ccr2 (AtGRP8) (van Nocker and Vierstra, 1993; Carpenter et al., 1994). In particular, CIRP was 60–80% identical in the CS-RBD to these proteins and also contained the sequences DRET and MNGKXXDG, highly conserved in plant GRPs and human hnRNP G (Soulard et al., 1993) (Fig. 2*a*). In addition, the putative



**Figure 3.** (a) Southern blot analysis of EcoRI-digested DNA from nine eukaryotic species (zoo blot analysis). DNAs were from human (lane 1), monkey (lane 2), rat (lane 3), mouse (lane 4), dog (lane 5), cow (lane 6), rabbit (lane 7), chicken (lane 8), and yeast (lane 9). The coding region of *cirp* cDNA was  $^{32}$ P-labeled and used as a probe. Autoradiography was done overnight (lanes 1–7) or for 3 d (lanes 8–9) at  $-80^{\circ}\text{C}$ . Mobilities of molecular size markers, HindIII-digested  $\lambda$  phage DNA, are indicated on the left. (b) Southern blot analysis of mouse genomic DNA. 20  $\mu\text{g}$  of liver DNAs extracted from either ddy/std mouse (lanes 1, 3, and 5) or C57BL/6 mouse (lanes 2, 4, and 6) were digested with one of the three restriction enzymes, EcoRI (lanes 1 and 2), PvuII (lanes 3 and 4), and KpnI (lanes 5 and 6), electrophoresed in 0.8% agarose gel, and transferred to a nylon membrane. The coding region of *cirp* cDNA was  $^{32}$ P-labeled and used as a probe. Autoradiography was done overnight.

CIRP protein is almost the same size as RBM3 (Derry et al., 1995) and plant GRPs (Bergeron et al., 1993; Carpenter et al., 1994) (Fig. 2*b*). These structural similarities suggest that CIRP and plant GRPs are members of the same family. *Cirp* is not a mouse counterpart of human RBM3 because we have recently isolated a human cDNA highly homologous to *cirp* (95.7% identical in predicted amino acids) and distinct from RBM3 (Nishiyama, H., and J. Fujita, unpublished data).

To assess the evolutionary conservation of the *cirp* gene, Southern blot analysis of genomic DNAs from nine eukaryotic species, human, monkey, rat, mouse, dog, cow, rabbit, chicken, and yeast, was carried out using the coding

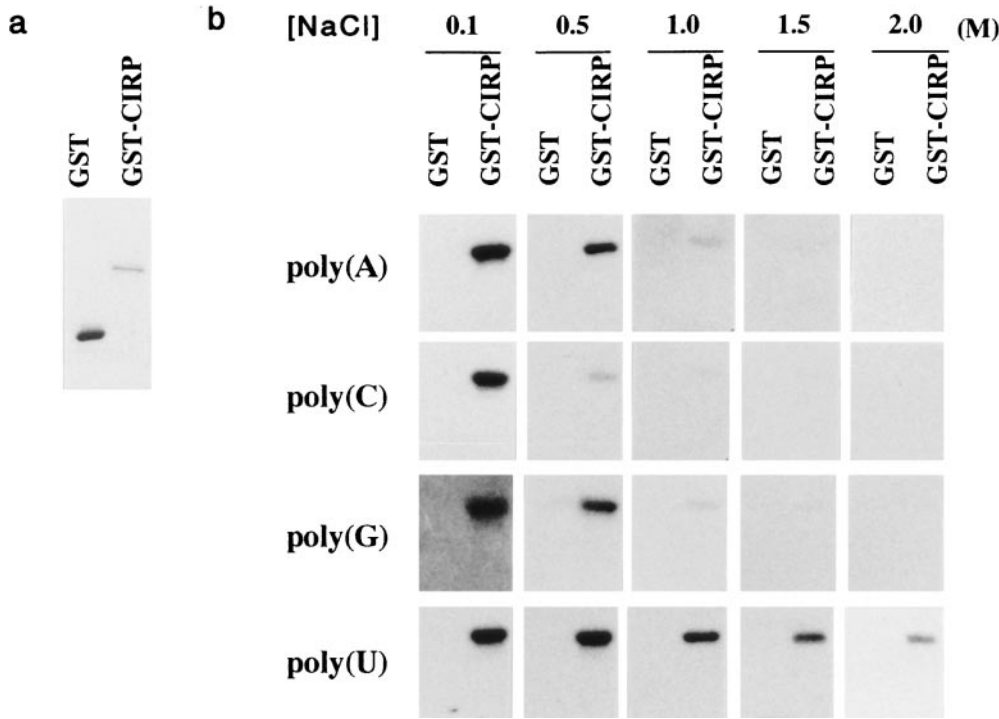


Figure 4. RNA-binding property of CIRP. (a) Bacterially expressed GST protein and GST-CIRP fusion protein demonstrated by Coomassie blue staining. (b) Northwestern blots showing binding of GST and GST-CIRP proteins to radiolabeled ribonucleotide homopolymers, poly(A), poly(C), poly(G), and poly(U), at different salt concentrations as indicated.

region of *cirp* cDNA as a probe. As shown in Fig. 3 a, signals were detected in all samples under high-stringent conditions, suggesting that the *cirp* gene family is well conserved beyond species. When the genomic DNA samples from two different strains of mice were digested with one of three restriction enzymes—EcoRI, PvuII, or KpnI—and analyzed, a single band was detected in each DNA sample (Fig. 3 b). Thus, the *cirp* gene is probably a single-copy gene in mice.

#### RNA-binding Activity of CIRP

We expressed and purified CIRP as a GST-fusion protein (Fig. 4 a) and analyzed its RNA-binding activity by Northwestern blot assay (Matunis et al., 1992; Schumacher et al., 1995). The GST-CIRP protein bound to all RNA homopolymers to a varying extent at low NaCl concentrations, while control GST did not (Fig. 4 b). At high NaCl concentrations, GST-CIRP bound only to poly(U), sug-

gesting that CIRP protein possessed a specific RNA-binding activity.

#### Accumulation of CIRP in Response to Cold Stress

To investigate the effect of temperature shifts on *cirp* expression, we exposed BALB/3T3 mouse fibroblasts to heat or cold treatment. As shown in Fig. 5 a, Northern blot analysis indicated that the levels of *cirp* mRNA increased markedly in response to mild cold treatment (32–25°C) but not to severe cold treatment (15°C). In contrast, heat treatment (39 and 42°C) decreased the levels of expression. Similar results were obtained in two other mouse cell lines, BMA1 bone marrow stromal cells (Fujita et al., 1983) and TAMA26 Sertoli cells (Kaneko et al., 1997) (Fig. 5 b and data not shown). To examine the expression at the protein level, we raised a polyclonal antibody against the predicted carboxyl-terminal peptide of CIRP. The antibody specifically recognized an 18-kD protein as expected

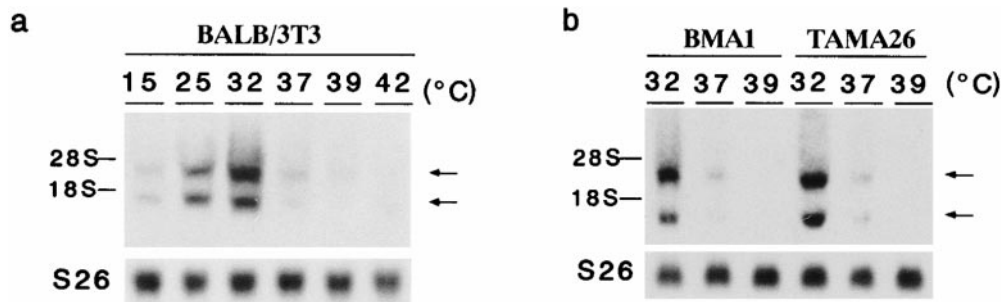
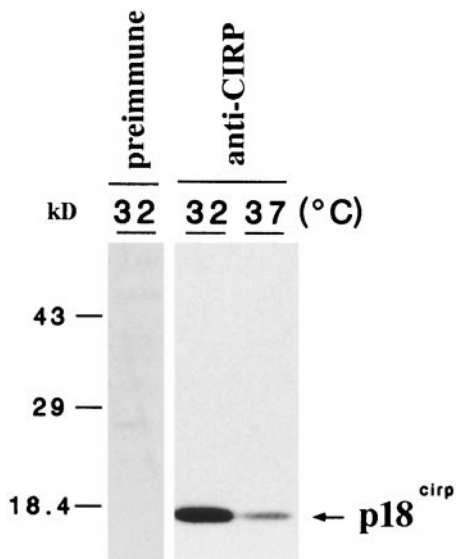


Figure 5. Temperature-dependent expression of *cirp* mRNA. Northern blot analysis of total RNAs from mouse cell lines, BALB/3T3 (a) and BMA1 and TAMA26 (b), harvested 24 h after the indicated temperature shift. The positions of 18S and 28S ribosomal RNAs are indicated on the left. As a control for the amount of RNA loaded, the filter was rehybridized with a mouse S26 ribosomal protein cDNA probe (lower).

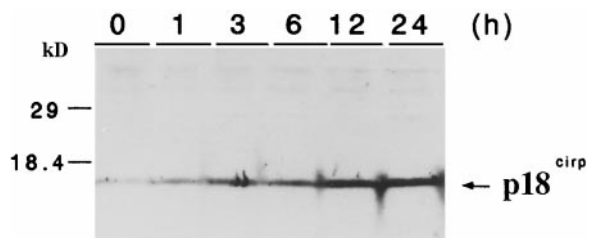


**Figure 6.** Western blot analysis of BALB/3T3 cell lysates using an anti-CIRP polyclonal antibody. BALB/3T3 cells were harvested 24 h after the indicated temperature shift. 10  $\mu$ g of samples was separated by 14% SDS-PAGE. Note specific recognition of an 18-kD protein by the anti-CIRP antibody. Mobilities of coelectrophoresed molecular size markers are indicated on the left.

from the amino acid sequence in the lysates of BALB/3T3 cells (Fig. 6). In agreement with the results of Northern blot analysis, the protein level was found to be increased 24 h after a temperature shift from 37 to 32°C. Subsequent Western blot analysis demonstrated that the level of p18<sup>cirp</sup> increased within 6 h after the temperature shift and peaked at 12 h, and the elevated level persisted for more than 24 h (Fig. 7).

#### Localization of CIRP in the Nucleoplasm

Immunofluorescence microscopy using the anti-CIRP polyclonal antibody showed that signals were detected in the nucleoplasm of almost all BALB/3T3 cells cultured at 32°C (Fig. 8, *a* and *b*), while no signals were detected using preimmune serum (Fig. 8, *c* and *d*). To confirm this result, we fused *cirp* and *Aequorea* GFP cDNAs in an expression



**Figure 7.** Kinetics of p18<sup>cirp</sup> induction in response to cold stress. BALB/3T3 cells were harvested at indicated times (hour) after a temperature shift from 37 to 32°C. Mobilities of coelectrophoresed molecular size markers are indicated on the left.

vector. GFP has been used as a molecular reporter to monitor patterns of protein localization, gene expression, and intracellular protein trafficking in living cells (Ogawa et al., 1995). When GFP-CIRP expression vector DNA was transfected into COS-7 cells, fluorescence was found only in the nucleoplasm (Fig. 8, *e* and *f*). On the other hand, when GFP expression vector DNA was transfected, fluorescence was detected in cytoplasm and nucleus (Fig. 8, *g* and *h*). These results suggest that CIRP is localized in the nucleoplasm.

#### Involvement of CIRP in Cold-induced Suppression of Cell Growth

After a temperature shift from 37 to 32°C, the growth of BALB/3T3 fibroblasts was impaired (Table I). To assess whether the induction of the *cirp* expression is related to the observed impairment of growth, we examined the effects of ODN antisense to *cirp* mRNA. The presence of antisense ODN partially inhibited the induction of p18<sup>cirp</sup> by temperature downshift (Fig. 9). Concomitantly, the growth impairment at 32°C was partially alleviated (Fig. 10), suggesting that the induction of p18<sup>cirp</sup> is necessary for growth suppression by cold stress.

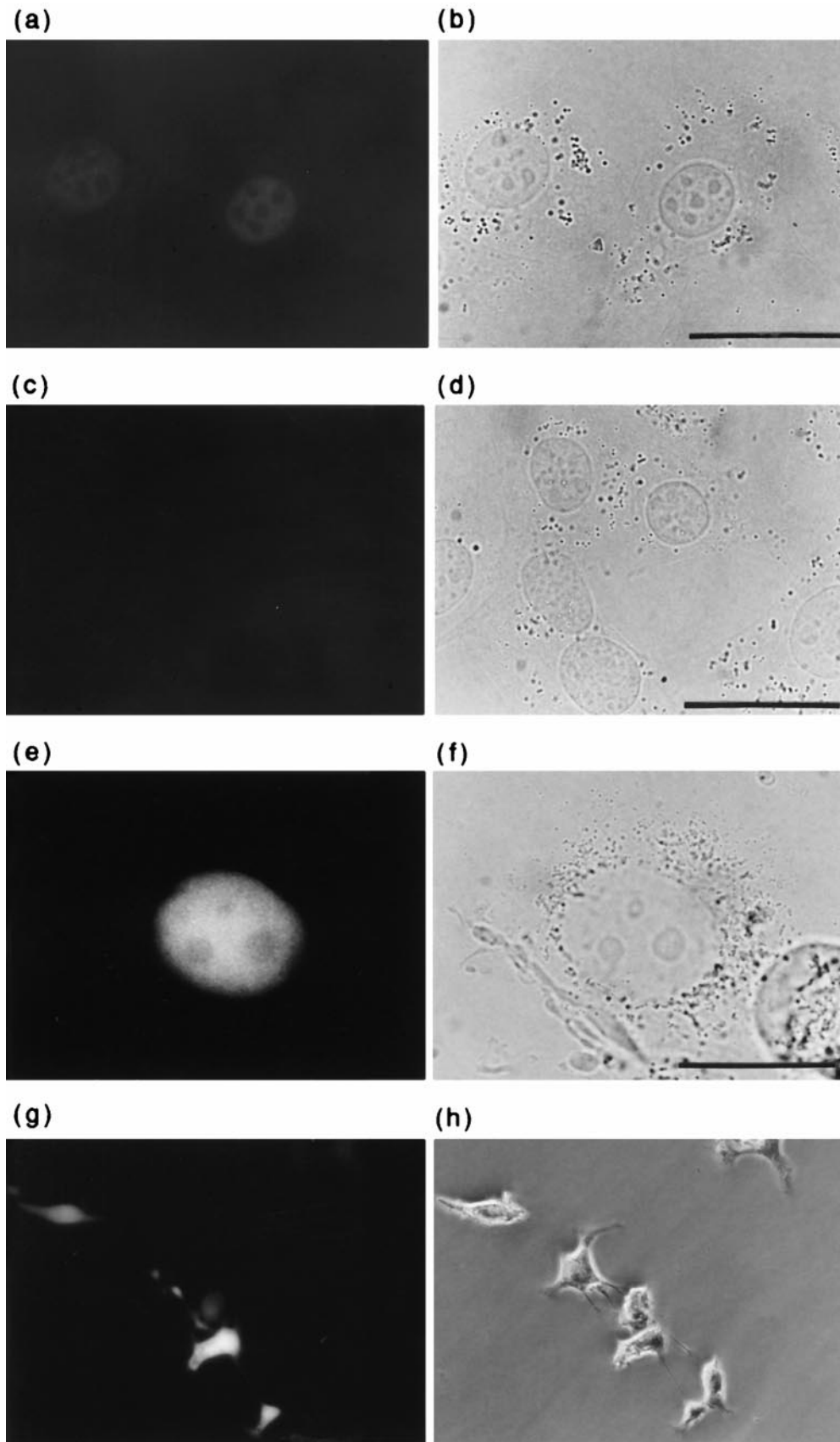
#### Prolongation of G1 Phase by Overexpression of CIRP

To further elucidate the role that p18<sup>cirp</sup> plays in the regulation of cell growth, a full-length *cirp* cDNA in an expression vector was stably transfected into BALB/3T3 cells. Five clones overexpressing p18<sup>cirp</sup> and seven control clones

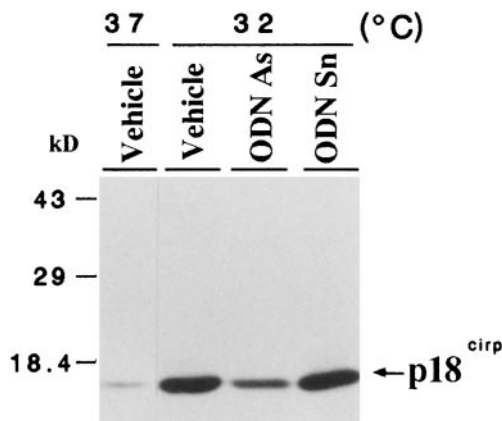
**Table I.** Effects of p18<sup>cirp</sup> Expression on the Cell Cycle of BALB/3T3 Cells

	BALB/3T3	BALB/3T3	Control transfectants (7 clones)	CIRP transfectants (5 clones)
Culture temperature (°C)	37	32	37	37
Doubling time (h)	19.3 ± 1.04	28.6 ± 1.53*	17.6 ± 1.07	23.5 ± 2.91*
Percent of cells in phase				
G1	39.4 ± 2.03	52.8 ± 4.75*	45.4 ± 4.16	53.6 ± 3.43*
S	33.5 ± 1.30	23.0 ± 1.94*	40.5 ± 2.59	32.4 ± 3.45*
G2	26.9 ± 2.42	24.2 ± 2.82	14.1 ± 3.16	13.7 ± 4.71
Length of phase (h)				
G1	6.11 ± 0.05	12.7 ± 1.99*	6.56 ± 0.78	10.5 ± 1.14*
S + G2 + M	13.2 ± 1.07	15.9 ± 0.53*	11.1 ± 0.99	12.9 ± 2.08

Doubling times were determined from the growth curves. Cell cycle analysis was performed with Epics Elite flow cytometer (Coulter Corp.), and cell cycle distribution was obtained using a computer modeling program (Multicycle AV, Phoenix Flow Systems). Experiments were repeated three times and the results are expressed as the mean ± SEM. \*Significantly different from controls by unpaired Student's *t* test ( $P < 0.02$ ).

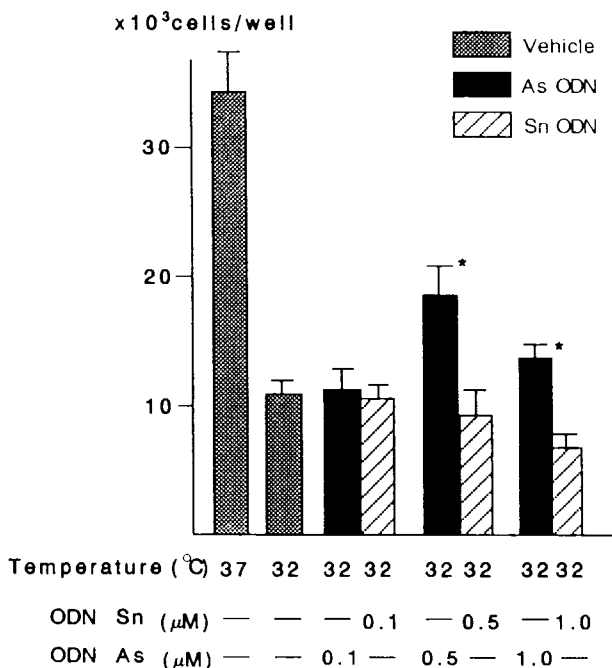


*Figure 8.* Localization of CIRP. Immunofluorescence microscopy of BALB/3T3 cells cultured at 32°C and stained with an anti-CIRP polyclonal antibody (a) or preimmune serum (c). The bound antibody was detected by an FITC-conjugated second antibody. Fluorescence microscopy of COS-7 cells expressing GFP-CIRP fusion protein (e) or GFP (g). Light (b, d, and f) or phase-contrast (h) microscopic images of the field of view identical to a, c, e, and g, respectively. Bars, 20  $\mu$ m.

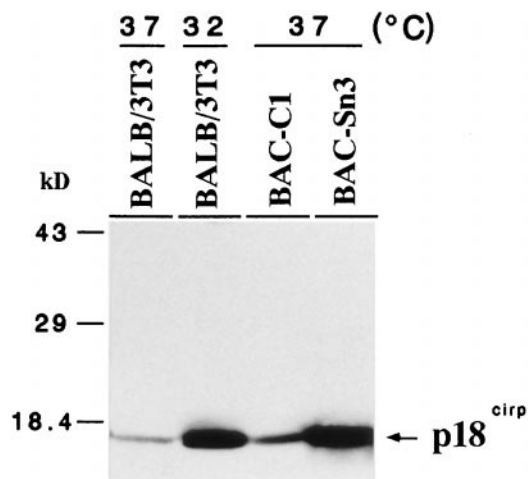


**Figure 9.** Effects of antisense (As) ODN on the cold-induction of p18<sup>cirp</sup>. BALB/3T3 cells were incubated at the indicated temperatures for 12 h in the presence of vehicle alone, As, or sense (Sn) ODNs (0.5  $\mu$ M). Note partial suppression of the cold-induced p18<sup>cirp</sup> expression in the presence of antisense ODN.

transfected with the vector DNA alone were analyzed. Expressions of p18<sup>cirp</sup> in a representative clone BAC-Sn3 and a control clone BAC-C1 are illustrated in Fig. 11. The p18<sup>cirp</sup> level in BAC-Sn3 cells maintained at 37°C was almost equal to that induced in BALB/3T3 cells upon cold stress. When these p18<sup>cirp</sup>-overexpressing clones and control clones were cultured at 37°C, the doubling time of the former was significantly longer than that of the latter (23.5 vs. 17.6 h, Table I). The cell cycle analysis revealed that



**Figure 10.** Effects of antisense (As) ODN on the cold-induced suppression of cell growth. Cell numbers were determined after 2 d of culture at the indicated temperature in the presence or absence of indicated concentrations of ODN. The results are expressed as the mean  $\pm$  SEM. \*Statistically different from controls by unpaired Student's *t* test ( $P < 0.02$ ).



**Figure 11.** Expression of p18<sup>cirp</sup> in representative transfectants. BALB/3T3 cells, cloned BALB/3T3 cells transfected with *cirp*-expression vector DNA (BAC-Sn3), or vector DNA (BAC-C1) were cultured for 12 h at the indicated temperature and analyzed by Western blotting.

the percentage of cells in G1 phase was 45.4% for control transfectants and 53.6% for CIRP transfectants. The percentage of cells in S phase was 40.5% for control transfectants and 32.4% for CIRP transfectants. Therefore, the duration of G1 phase was 6.6 h for control transfectants and 10.5 h for CIRP transfectants, suggesting that the lower growth rate of CIRP transfectants was due to the prolongation of G1 phase. Similar prolongation of G1 phase was observed when BALB/3T3 cells were transferred from 37 to 32°C (6.1 vs. 12.7 h). In addition to prolongation of G1 phase, the duration of S + G2 + M phase was prolonged in BALB/3T3 cells cultured at 32°C, but not in CIRP transfectants cultured at 37°C (Table I). These results indicate that CIRP plays a role in regulation of growth in response to temperature downshifts, but other factors also contribute to the cold-induced growth impairment.

## Discussion

In this paper, we identified a novel RNA-binding protein, CIRP, the expression of which was induced in mouse fibroblasts in response to the lowered culture temperature. Growth of mammalian cells is known to be regulated by various environmental factors, including temperature. Downshift of temperature from 37 to 31–33°C prolongs the total generation time of cultured mammalian cells (Rao and Engelberg, 1965; Watanabe and Okada, 1967). In mouse leukemic cells (L5178Y) and human amnion cells, the G1 phase was the most severely affected of the four phases of cell cycle, although other phases were also affected to varying degrees (Sisken et al., 1965; Watanabe and Okada, 1967). Our findings in mouse fibroblasts (BALB/3T3) were consistent with these previous observations. Furthermore, we demonstrated that overexpression of CIRP resulted in reduced growth rate and prolongation of the G1 phase. It remains to be determined how CIRP interacts with the known regulators of the G1 progression



(Sherr and Roberts, 1995) and how it affects duration of the G1 phase. Interestingly, CIRP and plant GRPs seem to belong to the same RNA-binding protein family, and some plant GRPs, such as *Sinapis alba* SaGRP and *Zea mays* ZMGRP, are expressed predominantly in meristematic and growing tissues (Gómez et al., 1988; Heintzen et al., 1994). These plant GRPs are probably involved in regulation of growth rate and/or response to external stimuli as demonstrated for CIRP in this study.

CIRP consists of CS-RBD and a glycine-rich domain. Binding experiments with ribohomopolymers and various RNAs have established that most of the CS-RBD-containing proteins have distinct RNA-binding characteristics (Burd and Dreyfuss, 1994). The Northwestern assay indicated that CIRP preferentially bound to poly(U). Similar nucleic acid-binding properties are known for hnRNP C, hnRNP A1, RGP-1b, and RGP-2 (Swanson and Dreyfuss, 1988a; Hirose et al., 1993, 1994). CIRP was localized to the nucleoplasm as known for hnRNP C and hnRNP A1 (Dreyfuss et al., 1984; Pinöl et al., 1989). The hnRNP C binds preferentially to U-rich polypyrimidine tracts found at the 3'-ends of introns and in 3' untranslated regions of mRNAs and is suggested to be important for 3'-end cleavage and polyadenylation (Swanson and Dreyfuss, 1988b; Wilusz et al., 1988). The hnRNP A1 is suggested to be involved in the 5'-splice site selection in an alternative splicing and mRNA transport (Mayeda and Krainer, 1992; Pinöl and Dreyfuss, 1992). In a similar fashion, CIRP may control the specific or general gene expression independently or by competing with hnRNP C and/or hnRNP A1 for the target binding sequences.

Recent studies on cold-shock response of *E. coli* have shown that CsdA, one of the major cold-shock proteins associated with ribosome, has a helix-destabilizing activity, and the *csdA* deletion impairs cell growth and the synthesis of a number of proteins at low temperature (Jones et al., 1996). The secondary structures in mRNAs that inhibit translation initiation are considered to become more stable at low temperature and are deleterious for growth of *E. coli* (Jones and Inouye, 1994). By unwinding stable secondary structures in mRNAs as an RNA chaperone and thus facilitating ribosomal functions, CsdA is supposed to play an essential role in mRNA translation at low temperature (Jones et al., 1996). The secondary structures and/or RNA-RNA annealing of pre-mRNA/mRNA are also proposed to play a critical role in processing of pre-mRNA into mRNA (Steitz, 1992, 1993; Lamm and Lamond, 1993). Therefore, the cold-inducible helix-destabilizing proteins are probably involved in processing of pre-mRNA at low temperature as well. Interestingly, hnRNP A1, hnRNP C, and its CS-RBD possess the ability to destabilize the helix and/or promote annealing of complementary nucleic acids (Görlach et al., 1992; Portman and Dreyfuss, 1994). It remains to be determined whether CIRP has such an RNA chaperone activity and how reduction of CIRP levels, contrary to the case with CsdA in *E. coli*, reverse growth inhibitory effects of low temperature in mammalian cells.

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