Molecular Cloning and Cell Cycle-dependent Expression of Mammalian CRM1, a Protein Involved in Nuclear Export of Proteins*

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Crm1 of Schizosaccharomyces pombe, a nuclear protein essential for proliferation and chromosome region maintenance, is a possible target of leptomycin B, an antifungal and antitumor antibiotic with cell cycle-arresting activity. cDNA encoding a human homolog of Crm1 was cloned. Human CRM1 (hCRM1) consisted of 1071 amino acids, of which the sequence showed 52% homology with S. pombe Crm1. hCRM1 weakly complemented the cold-sensitive mutation of S. pombe crm1-809, as did S. pombe crm1*. Overproduction of hCRM1 under the control of a series of nmt1 promoters suppressed cell proliferation in wild-type S. pombe in an expression level-dependent manner. A similar inhibitory effect was also observed for crm1*. Cells overproducing either hCRM1 or S. pombe Crm1 were distinctly larger than uninduced cells and contained compacted and fragmented nuclei. Furthermore, calcofluor staining demonstrated that most of these cells formed two septa per cell and accumulated a large amount of chitin or its related polysaccharides around the septa. Closely similar phenotypes between hCRM1- and S. pombe Crm1-induced cells indicate that the cloned cDNA encodes a functional homolog of S. pombe crm1*. Northern blot analyses with RNAs isolated from synchronized mammalian cells showed that the expression of mammalian CRM1 was initiated in late G1 and reached a peak at G2/M, although its protein level unchanged during the cell cycle. Transient expression of hCRM1 fused to the green fluorescent protein (GFP) in NIH3T3 cells showed that hCRM1 was localized preferentially in the nuclear envelope and was also detectable in the nucleoplasm and the cytoplasm. A crm1 mutation of S. pombe caused nuclear import of a GFP fusion protein containing a nuclear export signal but no change in the distribution of a GFP fusion protein containing a nuclear localization signal. All of these data suggest that CRM1 is a novel cell-cycle regulated gene that is essential for the nuclear export signal-dependent nuclear export of proteins.

The structure of eukaryotic chromatin, which is folded and packed into the nucleus, dynamically changes during the cell cycle. In the M phase, the chromatin is highly condensed to become mitotic chromosomes with higher order structures. The condensed higher order structure of chromatin is also present in the interphase, which is postulated to be involved in transcriptional silencing related to position effect variegation and dosage compensation, etc. (1). These structures contain the solenoid, also called the 30-nm fiber consisting of about six nucleosomes per turn of the 10-nm fiber, and an ordered network consisting of chromatin loops (2). Although various classes of nuclear proteins are proposed to be involved in forming and maintaining the higher order chromosome structures (3–5), little is known about the functions of these proteins.

Crm1 of Schizosaccharomyces pombe is one of the proteins required for maintaining the chromosome structures, which was identified as the protein encoded by crm1* that could complement the crm1 mutation of S. pombe (6). Cold-sensitive crm1 mutants were isolated during visual screening by means of DAPI staining, which showed deformed filamentous or fragmented nuclear structures at the restrictive temperature. The crm1* gene encodes a 115-kDa protein that is essential for proliferation of S. pombe and is localized in the nucleus or its periphery. Crm1 is involved in the regulation of not only nuclear structure but also specific gene activity; the crm1 mutants produced an increased amount of p25 whose expression is regulated by an AP-1-like transcription factor. Genetic analysis demonstrated that Crm1 was a negative regulator for Pap1, the S. pombe AP-1 homolog (7, 8). We have previously cloned a mutant gene of crm1 (crm1-N1) as a gene that confers resistance to leptomycin B on wild-type S. pombe from a gene library of a leptomycin B-resistant mutant of S. pombe (9). Leptomycin B is a Streptomyces metabolite causing growth arrest of S. pombe and mammalian cells at G1 and G2 phases and is considered to be a clinically important antitumor drug (10–14). The wild-type crm1* gene could also give resistance to leptomycin B when introduced into the cells of the wild-type background with a multicopy vector. In contrast, the cold-sensitive mutant crm1–809 showed hypersensitivity to leptomycin B at the permissive temperature. Furthermore, treatment of wild-type S. pombe with leptomycin B caused abnormal nuclear morphology and p25 overproduction, which were almost identical to the terminal phenotypes of crm1 mutants (9). Taken together, we have proposed that Crm1 or its regulatory cascade is the cellular target of leptomycin B. Recently, Wolff et al. (15) reported that leptomycin B inhibited nuclear export of the human immunodeficiency virus type 1 Rev protein and Rev-dependent mRNA. Since Crm1 is suggested to be highly con-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) D89729.

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served in all eukaryotes by detecting a protein reactive with an anti-Crm1 antibody in HeLa cells (6), it seems possible that mammalian CRM1 is involved in nuclear export of proteins and that the cell cycle arrest of mammalian cells by leptomycin B is ascribable to inhibition of the mammalian CRM1 function.

In this paper, we describe cDNA cloning of a functional mammalian homolog of Crm1. The cloned cDNA, named human CRM1 (hCRM1), was able to complement the S. pombe crn1 mutation and cause growth inhibition and morphological abnormalities in S. pombe when overproduced. The mammalian CRM1 gene was ubiquitously transcribed in all the tissues tested and its transcription level in cultured cells was regulated during the cell cycle. hCRM1 expressed in mammalian cells localized preferentially in the nuclear envelope and the S. pombe crn1 mutant was defective in the NES-dependent nuclear export of proteins. These results suggest that CRM1 is a novel cell cycle-regulated gene that may be involved in nuclear export of proteins in eukaryotes.

MATERIALS AND METHODS

Yeast Strains, Plasmids, and Media—The S. pombe strains used in this study were: JY266 (h->), JY266 (h-> leu1-32), AC1 (h-> leu1-32 crm1-809 crn1-429), DHC1 (h-> leu1-32 crm1-809 crn1-429 orf517), pREP1, pREP41, and pREP81 were used for transformation of S. pombe cells. pREP1 plasmid contains the wild-type nmt1 promoter, which can strongly direct transcription when thiamine is absent in the medium (17). pREP41 and pREP81 possess mutations in their promoter sequences, which result in the moderate and weak promoter activities, respectively (18). pDH1 containing hCRM1 cDNA was constructed as follows. Multiple restriction sites were introduced into a 5'-region adjacent to the initiation codon of hCRM1 using polymerase chain reaction amplification of a 5'-portion of the ORF. The amplified fragment was sequenced and replaced by the corresponding 5'-portion of the ORF on pBluescript SK+ (Stratagene), resulting in pBHC1. pDH1 was constructed by inserting the BamHI fragment of pBHC1 into the BamHI site of pBluescript SK+ in such an orientation that the ORF was under the control of the vector-originated cryptic promoter (19). To construct pRIHC1, pRI41HC1, and hCRM1 ORF excised from pBHC1 by BamHI and NdeI that includes the initiation codon was introduced into pREP1, pREP41, and pREP81, resulting in constructions of pRI1HC1, pRI41HC1, and pRI81HC1, respectively. The crm1+ ORF with multiple restriction sites at the 5'-end of the ORF was similarly introduced into pBHC1 by pBluescript SK+ (pBHC1) and pREP-hCRM1 was determined on both strands as probe. The nucleotide sequence of the 4088-base pair cDNA clone was sequenced to be part of a putative Crm1 homolog by sequencing. A cDNA clone containing hCRM1 cDNA was constructed with the ΔZAP vector (Stratagene) and was sequenced by dideoxy method using a DNA sequencer model 4000L (LI-COR, Inc.).

Assay of Colony-forming Ability—The viability during induction of hCRM1 or S. pombe Crn1 by the nmt1 promoter was determined as colony forming units. S. pombe JY266 cells transformed with pREP1, plp1HC1 containing hCRM1 cDNA, and prp1PC1 containing crm1+ ORF were grown in minimal medium with 10 μg/ml thiamine at 30 °C and transferred to minimal medium lacking thiamine at time 0. Cells in cultures at the initial cell density of 1.6 × 10⁶ cells/ml were taken at intervals, counted, and plated on minimal medium containing thiamine. Colonies formed were counted and the colony forming units were determined. Data are the mean of duplicated assays.

Microscopic Procedures—S. pombe JY266 transformants grown in minimal medium containing thiamine were transferred into medium lacking thiamine at time 0. The cells were further cultivated for 24 h, harvested, and fixed with 70% ethanol. Cells were observed under a light microscope (Olympus BHS-323N Nomarski differential interference microscopy) or a fluorescent microscope (Zeiss Axiovert 135). The fixed cells were washed several times with PBS and stained with DAPI or calcofluor (fluorescent blightener 28, Sigma) as described (24). For propidium iodide/calcofluor double staining, fixed cells were washed three times with 50 mM sodium citrate (pH 7.0), incubated with 1 mg/ml RNase A for 2 h at 37 °C, and then washed with PEM buffer (100 mM PIPES, pH 6.9, 1 mM EGTA, 1 mM MgSO₄). The cells were resuspended in PEM containing 0.5 mg/ml calcofluor for 30 min at room temperature. After the cells had been washed three times with PEM, they were mounted with Vectashield mounting medium (Vector, Burlingame CA) containing 0.05 mg/ml propidium iodide and then observed under a Zeiss Axiovert 135 microscope.

Mammalian Cells and Culture Conditions—A human myeloma cell line K562 was cultured at 37 °C under a humidified atmosphere of 5% CO₂ in RPMI 1640 supplemented with 10% fetal calf serum. A murine fibroblast cell line NIH3T3, a murine erythroblastemia (MEL) cell line F4N00, and a rat embryonic fibroblast cell line transformed with ras and p53Wildtype, clone 6, were maintained in culture at 37 °C in Dulbecco’s modified minimal essential medium supplemented with 10% fetal calf serum.

Cell Synchronization—MEL cells were synchronized in G1 phase by cell fractionation. Briefly, cells (1 × 10⁶) were resuspended in 10 mL of PBS. Cells at various stages of the cell cycle were prepared by centrifugal elutriation in PBS, with a Beckman JE-6 elutriator rotor. From each 50-ml fraction, a portion was fixed (0.1% glutaraldehyde, 15 min at room temperature) and then analyzed by flow cytometry. The cells in the two first fractions (100 ml) were counted, centrifuged, seeded at 6 × 10⁶ cells/ml, and then cultured for 0–8 h. At different times, cells were taken for cell cycle analysis and RNA preparation.

Clone 6 cells were synchronized in G1 phase by a temperature shift to 32 °C. Briefly, cells were seeded at 5 × 10⁶ cells/ml and grown for 24 h, and then placed at 32 °C for arresting the cell cycle in G1. In the cell cycle resumption experiments, cells arrested at 32 °C were placed at 37 °C again. Cells were taken at various times after the temperature shift for RNA preparation and cell cycle analysis.

Northern Blot Analysis—Tissue distribution of hCRM1 mRNA was analyzed with multiple tissue Northern blots purchased from CLONE-TECH (Palo Alto, CA) and 1.7-kb SacI-HindIII fragment containing a C-terminal half of hCRM1 ORF. The blots were washed twice with 0.2× SSC, 0.1% SDS at 65 °C and hybridized with [32P]labeled probe. The blots were washed twice with 0.2× SSC and 0.1% SDS at 65 °C and hybridized with [32P]labeled probe. The blots were washed twice with 0.2× SSC and 0.1% SDS at 65 °C and hybridized with [32P]labeled probe. The blots were washed twice with 0.2× SSC and 0.1% SDS at 65 °C and hybridized with [32P]labeled probe.
EcoRI-NcoI fragment of clone ID 313609 of IMAGE consortium (GenBank accession number W10968) encoding part of a mouse CRM1 homolog (mCRM1). The EST clone includes an N-terminal region of mCRM1, corresponding to 1–110 amino acids of hCRM1, that has 95 and 100% identity in nucleotide and amino acid sequences, respectively.

**Western Blot Analysis**—Clone 6 cells were collected, washed with PBS and resuspended in 200 μl of SDS-polyacrylamide gel electrophoresis sample buffer and sonicated. Proteins were resolved on 8% SDS-polyacrylamide gels and transferred into a membrane. The membrane was washed with PBS and incubated for 1 h in PBS containing 10% skim milk and 0.3% Tween 20. The membrane was then rinsed and incubated for 1 h in PBS containing 10% fetal calf serum, 0.2% Tween 20, and a 1/500 dilution of the anti-CRM1 antiserum raised against a peptide corresponding to the C-terminal 20 amino acids (Glu1024-Arg1043). After the membrane had been washed with PBS containing 250 mM NaCl and 0.3% Triton X-100, the CRM1 protein was detected using ECL system (Amersham).

**Flow Cytometry**—Flow cytometry was used for determination of cellular DNA and CRM1 contents. Cells were collected and fixed in 70% ethanol in PBS for 30 min at room temperature. The fixed cells (MEL and clone 6) were stained with the DNA-specific fluorochrome Hoechst 33258 in the presence of 1% Triton X-100 and analyzed with a FACStar+ (Becton-Dickinson). For double staining, the fixed cells were permeabilized with 0.25% (v/v) Triton X-100 (Sigma), then incubated with anti-CRM1 antiserum, diluted to 1/200 in PBS containing 10% fetal calf serum. The detailed protocol was described by Khochbin et al. (25). The second fluorescein isothiocyanate-conjugated antibody was obtained from Jackson Immunoresearch Lab. After immunolabeling, the cells were stained with Hoechst 33258 (Sigma) and analyzed by flow cytometry. The background fluorescence (ranging from channel 1 to 100, not shown) was measured by analyzing the fluorescein isothiocyanate fluorescence of cells incubated with non-immune serum, instead of the anti-CRM1 antiserum, during the immunostaining procedure.

**Expression of GFP-fused hCRM1 in Mammalian Cells**—The hCRM1 expression plasmids for mammalian cells were constructed using pEGFP-N1 and pEGFP-C1 (CLONTECH), which allow transient expression of the enhanced green fluorescent protein (GFP) fused at either the C or N terminus of hCRM1. Transfection into NIH3T3 cells was performed with LipofectAMINE (Life Technologies, Inc.) according to the furnished instructions. The cellular distribution of GFP-fused proteins was observed under a Zeiss Axiosvert 135 fluorescent microscope.

**Expression of GFP-fused Proteins Containing NLS or NES in S. pombe**—
RESULTS

Cloning of Mammalian CRM1 cDNA—We used sequence information of the EST data bases and polymerase chain reaction-based strategy to identify and clone mammalian homologs of *S. pombe* crm1*. A 0.6-kb fragment corresponding to the 5'-region of a putative Crm1 homolog was amplified by reverse transcriptase-polymerase chain reaction with an oligo(dT)-primed cDNA mixture made from total RNA of the human chronic myelogenous leukemia cell line, K562, using synthetic primers based on clones 10h09 (GenBank accession number H26490) and H2CBU81 (Human Genome Sciences, Rockville, MD). The amplified fragment was used as a probe to isolate a cDNA encoding the entire ORF of putative Crm1 homolog from a cDNA library of K562. Because of its characteristics described below, we refer to the gene product as the human homolog of Crm1 (hCRM1). The deduced amino acid sequence revealed that hCRM1 consists of 1071 amino acids with a calculated relative molecular mass of 123,384 Da (Fig. 1). The entire amino acid sequence showed significant similarities to those of *S. pombe crm1* (51.6% identity) and *S. cerevisiae* CRM1 (45.8% identity). No other protein with significant homology with hCRM1 was found through a data base search. hCRM1 contained no known motif or region that allows us to predict its function.

Northern blot analysis probed with the C-terminal region of hCRM1 cDNA revealed a major 5.6-kb transcript that was expressed in all human tissues tested except for kidney (Fig. 2). Overexposure of the blot showed the presence of the 5.6-kb transcript as well as a smear signal around 3-kb in kidney mRNA (data not shown). The biological importance of the low level of the 5.6-kb hCRM1 mRNA in kidney is unknown.

Ectopic Expression of hCRM1 in *S. pombe*—The high similarity between the amino acid sequences of hCRM1 and *S. pombe* Crm1 suggests that the cloned cDNA encodes a functional homolog corresponding to yeast Crm1. To examine this possibility, we introduced hCRM1 cDNA into *S. pombe* crm1*–809* mutant cells with a multicopy vector pDB248 and expressed it using the promoter activity present in the vector (19). As shown in Fig. 3, hCRM1 cDNA weakly suppressed the cold sensitivity of the crm1*–809* mutation. *S. pombe* wild-type crm1* also partly complemented the mutation when expressed with the promoter of the pDB248 vector. These results suggest that the cloned human cDNA is capable of functioning in *S. pombe*. Since expression of crm1* under the authentic crm1* promoter (pYA284) fully complemented the mutation, the weak ability of both hCRM1 cDNA and crm1* on pDB248 to support the growth at the nonpermissive temperature may be due to the insufficient expression level by the promoter of pDB248.

We next controlled their expression levels by a series of nmt1 (no message in thiamine) promoters, whose transcription is induced only in the absence of thiamine (17). pREP1 contains the wild-type nmt1 promoter with very potent transcriptional activity, thereby causing overproduction. pREP41 and pREP81 possess mutations in their promoter sequences, which result in the moderate and weak promoter activities, respectively (18). However, we could not evaluate the ability of hCRM1 or crm1* expressed under the nmt1 promoters in the crm1*–809* mutant, since neither wild-type crm1* of pYA284 nor hCRM1 cDNA on pREP1 could rescue the mutant cells at the nonpermissive temperature when the minimal medium was used for complementation assay (Fig. 4, 18 °C MM + thiamine). When hCRM1 was overexpressed with pREP1 in the crm1*–809* mutant at the permissive temperature, cell growth was strongly inhibited (Fig. 4, 30 °C MM – thiamine). The deleterious effect of the hCRM1 overproduction was also observed in the wild-type background, which was dependent on its expression level (Fig. 5A); the inhibitory effect of hCRM1 was alleviated by the moderate expression from the pREP41 promoter, and was almost undetectable when expressed with pREP81. Surprisingly, overproduction of wild-type *S. pombe* Crm1 caused similar growth inhibition in a promoter activity-dependent manner. Cell viability upon induction of the hCRM1 or *S. pombe* Crm1 expression was analyzed by determining their colony-forming abili-
ties. The colony-forming abilities of cells overproducing either hCRM1 or \textit{S. pombe} 
Crm1 with pREPI were dramatically reduced 15 h after the induction, indicating that overproduc-
tion of these proteins was lethal for cells (Fig. 5B).

We compared the terminal phenotypes between the cells producing hCRM1 and \textit{S. pombe} 
Crm1 with pREPI. Light microscopic observation showed that both the arrested cells were distinctly 
resolved into three parts: cells with irregularly shaped nuclei, cells with normal nuclei and 
uninduced cells or the parental cells (Fig. 6). Fluorescent microscopy of cells stained with DAPI showed that the nuclei of the cells overexpressing hCRM1 were highly compacted and 
fragmented in contrast to the diffused and decondensed nuclei of \textit{crm1–809} mutant cells arrested at the nonpermissive temperature. The nucleus was often abnormally located in the cell body; localization near the pole, cell wall, and the septum was 
observed. The nuclear morphology of the cells overproducing \textit{S. pombe} Crm1 was essentially the same as that of the hCRM1-producing cells but was more severely fragmented. These results suggest that Crm1 is involved in chromosome compaction and nuclear positioning.

Visualization of the septum by calcofluor (fluorescent blightener 28) staining that allows detection of chitin or its related materials revealed that most of the hCRM1-producing cells contained two septa (or the septum-like structures) per cell. In some cases, a thin third septum was formed between the two septa or a single thick septum was produced around the growing apex. The amount of chitin in the cell was extremely larger than that in the cell with normal cytokinesis. Double staining with calcofluor and propidium iodide showed that the nucleus was absent between the two septa in the cell. In some cells, the chromosomal DNA was not equally distributed into both cyto-
plasm divided by the septa. Similar aberrant septum formation and then the cells were cultivated for 8 h, whose cell cycle 
progression was monitored by flow cytometry (Fig. 7A). The expression level of the \textit{GAPDH} gene, a housekeeping gene, was 
constant throughout the cell cycle, while that of histone H4 was 
increased in S phase and then decreased in G2/M (Fig. 7B).

Cell Cycle-dependent Expression of Mammalian CRM1—

Expression of the endogenous mammalian CRM1 gene was analyzed using cultured murine erythroleukemia cells by Northern blotting. For synchronization, the G1 population (containing the early S phase cells) was collected using centrifugal elutria-
tion and then the cells were cultured for 5 h, whose cell cycle progression was monitored by flow cytometry (Fig. 7A). The expression level of the \textit{GAPDH} gene, a housekeeping gene, was 
constant throughout the cell cycle, while that of histone H4 was 
increased in S phase and then decreased in G2/M (Fig. 7C).

On the other hand, the mouse CRM1 gene was transcribed at a 
very low level in G1, but the amount of the message progress-
ingly increased at G2/S and peaked at G2/M (Fig. 7B). These
results suggest that the transcription of mammalian CRM1 is regulated during the cell cycle.

To confirm the cell cycle-dependent expression of CRM1, we next used rat embryonic fibroblast cells transformed with ras and p53Val135 (clone 6). Since p53Val135 is a temperature-sensitive tumor suppressor, clone 6 cells can grow well at 37 °C, while the cell cycle is arrested in G1 at 32 °C due to a large amount of the functional p53 in the nucleus (26, 27). In the exponentially growing cells at 37 °C, no change in the levels of the CRM1, GAPDH, and histone H4 transcripts as well as the flow cytometric pattern was observed during the 24-h cultivation (Fig. 8A). When the temperature was shifted down to 32 °C, the exponentially growing cells were arrested in G1 phase, which was shown by accumulation of G1 phase cells in the flow cytometric histogram (Fig. 8B). During the G1 arrest, the transcripts of CRM1 and histone H4 decreased to an almost undetectable level in a time-dependent manner, while that of glyceraldehyde-3-phosphate dehydrogenase was still unchanged. Furthermore, the release from the temperature arrest by shifting up caused resumption of transcription of the CRM1 and histone H4 genes 6 and 9 h, respectively, after the temperature shift. The initiation of CRM1 transcription occurs at late G1.

To examine whether the CRM1 protein level correlates with its cell cycle-dependent oscillation of transcription, we determined the relative amount of CRM1 in cells at each cell cycle phase by flow cytometry and the total amount of CRM1 by Western blotting. Flow cytometric analysis demonstrated that the growing cells in G1 still contained a significant amount of CRM1 which was almost half the amount in G2/M phase cells (Fig. 8D). Furthermore, despite the undetectable amount of CRM1 transcripts in the arrested clone 6 cells (Fig. 8B), the total CRM1 protein level determined by Western blotting was unchanged even when the cell cycle was arrested (Fig. 8E). The pattern of gene and protein expression of CRM1 is similar to that of histones, suggesting that CRM1 is a component of nuclear architecture that is duplicated in the late cell cycle.

**Subcellular Localization of hCRM1 in Mammalian Cells**—Previous studies have shown that Crm1 localizes to the nucleus or its periphery in S. pombe (6). To examine the subcellular distribution of CRM1 in mammalian cells, we introduced hCRM1 cDNA conjugated with the gene for enhanced GFP (28) into murine NIH3T3 cells. The hCRM1 constructs tagged with GFP at either the N or the C terminus of hCRM1 were transiently expressed under the control of the cytomegalovirus promoter. Fluorescent microscopy demonstrated that cells highly expressing the GFP-fused protein were rounded up and sometimes detached from the dish substratum, while modestly expressing cells did not undergo such a morphological change (Fig. 9, A and B). These results suggest that hCRM1 overpro-
duction was also cytotoxic to mammalian cells. Both the GFP-fused proteins tagged at the N and C termini of hCRM1 showed essentially the same subcellular localization; the GFP signal was most prominently observed on the nuclear envelope but the fused protein was also detectable in the nucleoplasm and the cytoplasm (Fig. 9, C and E). The preferential localization on the nuclear envelope was also observed in the round cells overproducing the fused protein (Fig. 9D). The expression of GFP alone did not show the nuclear envelope staining (Fig. 9F).

The expression level found in the cytoplasm was lower than that of the nucleoplasm in modestly expressing cells but was increased to almost the same level as that of the nucleoplasm when hCRM1-GFP was overexpressed. These results suggest that hCRM1 is located mainly in nuclear envelope but a significant amount was also present in the nucleus. A small fraction of hCRM1 may be distributed into cytoplasm, although it is possible that the cytoplasmic localization is due to unphysiological overproduction by the strong cytomegalovirus promoter.

*S. pombe* crm1 Is Essential for NES-dependent Nuclear Export of Proteins—The subcellular localization of hCRM1 suggests that CRM1 plays a role in nuclear transport across the nuclear envelope. The import of proteins into the nucleus occurs through the nuclear pore complexes which allow diffusion of small molecules and can accommodate the active transport of large molecules (29–31). The active import is energy-dependent and is mediated by saturable import receptors, which recognize distinct import signals. The best characterized one is the NLS of SV40 large T antigen and nucleoplasmin. The export of proteins from nucleus into cytoplasm also occurs through nuclear pore complexes and requires the presence of specific targeting sequences within the proteins, called NES (29, 31, 32). The leucine-rich NES found in protein kinase A inhibitor and human immunodeficiency virus type 1 Rev protein have been well studied to date. To examine whether CRM1 is necessary for protein nuclear import and export, we introduced genes encoding GST-GFP fusion proteins containing the SV40 NLS or the Rev NES into the wild-type and the crm1 mutant *S. pombe*, and observed the subcellular distribution of the GFP-fused proteins in the presence or absence of functional Crm1. In the wild-type background, the SV40 NLS-containing protein localized only in the nucleus, whereas the Rev NES-containing protein was detected only in the cytoplasm (Fig. 10). These results demonstrate that both the SV40 NLS and the Rev NES can work in *S. pombe*. In the *crm1–809* mutant, the GFP-fused Rev NES-containing protein remained accumulated in the nucleus of the wild-type background, the SV40 NLS-containing protein localizes only in the nucleus, whereas the Rev NES-containing protein was detected only in the cytoplasm (Fig. 10). These results clearly show that the *S. pombe* Crm1
protein is essential for NES-dependent protein nuclear export but not for NLS-dependent protein nuclear import.

DISCUSSION

We cloned cDNA of hCRM1, encoding a 1071-amino acid protein highly homologous to yeast Crm1. Recently, Fornerod et al. (33) also identified a human homolog of yeast Crm1 as a protein interacting with CAN/Nup214, an oncogenic nucleoprotein (34, 35). The nucleotide sequencing indicated that these proteins are the identical gene products, although they contain an amino acid difference probably due to the somatic mutation. In the present study, we demonstrated that hCRM1 was a functional homolog of yeast Crm1 by analyzing the ability to complement the S. pombe crm1 mutation and comparing the phenotypes of the yeast that overproduce hCRM1 and S. pombe Crm1. We previously reported that leptomycin B, a potent mammalian cell cycle inhibitor, inhibited the S. pombe Crm1 function, which was evidenced by the identical phenotypes between crm1 mutant cells and leptomycin B-treated wild-type cells (9). Leptomycin B at nanomolar concentrations completely blocked the G1/S transition and partially inhibited the G2/M transition in the mammalian cell cycle (14). hCRM1 is a possible target protein of leptomycin B in human cells.

Although expression of either hCRM1 or S. pombe Crm1 by the cryptic promoter of pDB248 partially complemented the crm1 mutation in S. pombe, the use of the nmt1 promoters failed to rescue the crm1–809 mutant (AC1). Introduction of crm1 with the intrinsic crm1′ promoter by the multicopy vector (pYA284) was able to complement the cold sensitivity of AC1 in the complete medium (YPD) but was also incapable of complementing the defect in the minimal medium with or without thiamine (Fig. 4). Although the reason for the inability of crm1′ to complement AC1 under these conditions is yet unknown, it is possible that AC1 has some other cryptic defect in proliferation in the synthetic medium at 18 °C.

During these experiments, we found that overproduction of hCRM1 or S. pombe Crm1 caused growth arrest in wild-type S. pombe. Both proteins induced almost the same phenotypes: an enlarged and elongated cell shape, chromosome DNA compaction and fragmentation, aberrant nuclear positioning, and aberrant septum formation. These results strongly suggest that CRM1 is a multifunctional protein that is involved in both chromosome structure control and septum formation control. Previous studies have shown that Crm1 and Pad1/Sks1 (36, 37) regulate Pap1-dependent transcription; Crm1 and Pad1/Sks1 are negative and positive regulators, respectively, for Pap1, an S. pombe AP-1 like transcription factor (36). It was therefore possible to assume that the effects of Crm1 overexpression were ascribed to the inactivation of Pap1. However, the growth inhibition by crm1′ on pREP1 was also observed in the pap1-deleted cells, suggesting that Pap1 is independent of the aberrant chromosome structure and septum formation caused by

![Figure 9](https://example.com/figure9.png)

**FIG. 9.** Subcellular localization of hCRM1 transiently expressed in NIH3T3 cells. NIH3T3 cells were transfected with pFHC1 for the enhanced GFP-hCRM1 fusion (A-D), pHCF1 for the hCRM1-enhanced GFP fusion (E), and a vector pEGF-C1 (F). Cell morphology was observed by phase-contrast microscopy (B) and the fusion proteins expressed in the cells were visualized by fluorescence microscopy (A and C-F). Panels A and B are fluorescent and phase-contrast images, respectively, of the same microscopic field. Panels C and D show cells modestly producing and overproducing the GFP-hCRM1 fusion, respectively. Panel E shows two cells, one of which modestly produces and another overproduces the hCRM1-GFP fusion. Panel F shows a cell producing enhanced GFP.

![Figure 10](https://example.com/figure10.png)

**FIG. 10.** S. pombe Crm1 is essential for nuclear export of NES-containing proteins. S. pombe strains JY266 and AC1 were transformed with pR1GsvNLSF1 for the GST-SV40 NLS-GFP fusion and pR1GrevNESF1 for the GST-Rev NES-GFP fusion. Cells were cultured in the absence of thiamine for 12 h at 30 °C for induction of the proteins, and then further cultivated for 6 h in the same medium at 30 or 18 °C.
Crm1. This is not surprising because pap1 itself is a nonessential gene (38). On the other hand, pad1 has been shown to be essential and its deletion causes aberrant nuclear morphology as well as growth arrest with an elongated cell shape (36). The pad1 disruptants show chromosome condensation as observed for the Crm1 overproducers. These chromatin morphologies seemed not to be mitotic chromosome condensation. These observations suggest that Crm1 and Pad1 are the negative and positive regulators, respectively, also for interphase chromosome condensation. However, the phenotypes of the pad1 disruptants and the Crm1 overproducers are similar but not the same; the chromatin in the pad1 disruptants appears “streaked” in some instances but such the streaked chromatin was not observed in the Crm1 overproducers. Furthermore, the fragmented chromatin appearance and aberrant septum formation caused by Crm1 have not been observed for pad1 disruptants. It is unclear at present whether the crm1 and pad1 genes are genetically interrelated.

Northern blot analyses indicated that CRM1 is ubiquitously expressed in mammalian cells and tissues. However, the transcription level was much lower in the G1 phase cells than those observed in the cells of other cell cycle phases. In the cells arrested in G1 by active p53 overexpression, the CRM1 transcripts were almost undetectable. These results suggest that expression of mammalian CRM1 is strictly regulated in the cell cycle. Furthermore, release from the p53-induced arrest by the temperature shift-up allowed to reinitiate the CRM1 transcription prior to the expression of histone H4, an S phase-specific gene. An increase in the p53 level leads to induction of p21cip1/waf1, which strongly inhibits the activity of cyclin-dependent kinases (39). Since one of the major substrates of cyclin-dependent kinases is pRB and the phosphorylation of pRB dissociates its binding proteins, such as E2F from the pRB complexes (20), it seems likely that CRM1 expression is controlled by pRB-associated transcription factors.

In contrast to the oscillation of CRM1 transcripts during the cell cycle, flow cytometry, and Western blot analysis showed that the protein level of CRM1 in G1 phase did not decrease even after the arrest at 32 °C by p53. This behavior is one of the characteristics of architectural proteins such as histones. Histone genes are transcribed specifically in S phase but their protein levels are almost constant during the cell cycle. It seems likely that CRM1 is also a component of the nuclear architecture that is duplicated in the late cell cycle.

Transient expression of GFP-fused hCRM1 in mammalian cells revealed that hCRM1 is localized in the nucleus, particularly the nuclear envelope. This is consistent with the previous observation of subcellular localization of Crm1 in S. pombe (6). However, the present study showed that a small fraction of hCRM1 was also present in the cytoplasm, although it was not clear whether the cytoplasmic localization is physiological. Since the amount of cytoplasmic hCRM1 appears to increase as the total expression level increases, it is plausible that hCRM1 is accumulated in the cytoplasm when the rate of hCRM1 synthesis is faster than the rate of nuclear import or when the CRM1-binding capacity in the nucleus is saturated. An alternative possibility is that CRM1 dynamically changes its subcellular localization between the nucleus and the cytoplasm.

Since CRM1 has no typical nuclear localization signal, it seems probable that the Crm1-binding protein(s) that determines the CRM1 localization is present in the nucleus or the nuclear envelope. One of such Crm1-binding proteins is CAN/Nup214, which is associated with the nuclear pore complex (33). Recently, leukomycin B has been found to block translocation of human immunodeficiency virus type 1 Rev from the nucleus to the cytoplasm (15). Taken together with the preferential localization of hCRM1 to the nuclear envelope and nuclear pore complexes, we postulate that CRM1 functions to promote the protein nuclear export. The present study by use of S. pombe confirmed the hypothesis; the GFP fusion protein containing the Rev NES was distributed into the nucleoplasm when expressed in the crm1 mutant cells but not in the wild-type cells. Nuclear localization of the NES-containing protein in the crm1 mutant was observed even at the permissive temperature, probably due to insufficient activity of the mutated Crm1 protein at the temperature as described previously (6, 7). It is striking that the CRM1 function is unidirectional since the NLS-containing proteins could be fully translocated into the nucleus even in the absence of S. pombe Crm1. Thus, CRM1, a highly conserved protein in eukaryotes, may be a component of the nuclear export apparatus in the nuclear envelope. It is possible that a variety of phenotypes observed in yeast upon gain or loss of function of Crm1 are ascribed to the alteration of nuclear export of proteins. The molecular mechanisms by which CRM1 and leptocin B affect a variety of nuclear functions, such as higher order chromatin structure and gene expression, are currently under investigation.

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Molecular Cloning and Cell Cycle-dependent Expression of Mammalian CRM1, a Protein Involved in Nuclear Export of Proteins
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