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Developmentally regulated activity of CRM1/XPO1 during early *Xenopus* embryogenesis

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SUMMARY

In this work, we have investigated the role of CRM1/XPO1, a protein involved in specific export of proteins and RNA from the nucleus, in early *Xenopus* embryogenesis. The cloning of the *Xenopus laevis* CRM1, XCRM1, revealed remarkable conservation of the protein during evolution (96.7% amino acid identity between *Xenopus* and human). The protein and mRNA are maternally expressed and are present during early embryogenesis. However, our data show that the activity of the protein is developmentally regulated. Embryonic development is insensitive to leptomycin B, a specific inhibitor of CRM1, until the neurula stage. Moreover, the nuclear localization of CRM1 changes concomitantly with the appearance of the leptomycin B sensitivity. These data suggest that CRM1, present initially in an inactive form, becomes functional before the initiation of the neurula stage during gastrula-neurula transition, a period known to correspond to a critical transition in the pattern of gene expression. Finally, we confirmed the gastrula-neurula transition-dependent activation of CRM1 by pull-down experiments as well as by the study of the intracellular localization of a green fluorescent protein tagged with a nuclear export signal motif during early development. This work showed that the regulated activity of CRM1 controls specific transitions during normal development and thus might be a key regulator of early embryogenesis.

Key words: Nuclear export signal, Nuclear import signal, Gastrula-neurula transition, Leptomycin

INTRODUCTION

The recent discovery that CRM1/XPO1 functions in the nuclear export of proteins tagged with a short leucine rich motif (nuclear export signal or NES; Fornerod et al., 1997a,b; Fukuda et al., 1997a; Stade et al., 1997; Ossareh-Nazari et al., 1997), has elucidated an essential role for this protein in coordinating nuclear events such as mitosis and transcriptional activation with nuclear transport. It is now clearly established that a wide variety of major cellular regulators are exported from the nucleus in a CRM1-dependent manner. A common characteristic of these regulators is their transient action in the nucleus during either specific phases of the cell cycle or in response to certain signals. For instance, the regulators of mitosis, cyclin B1 and Cdc25 enter the nucleus during the G_2 phase of the cell cycle. During the rest of the cell cycle these proteins are maintained in the cytoplasm through continuous CRM1-dependent nuclear export (Hagting et al., 1998; Toyoshima et al., 1998; Yang et al., 1998; Lopez-Girona et al., 1999). In contrast, HsCdc6, a human homologue of budding yeast Cdc6p that is essential for DNA replication, is nuclear in G_1 phase and translocates to the cytoplasm at the start of S phase in a CRM1 dependent manner (Jiang et al., 1999). Transcription factors such as NF-AT and yeast transcription factors, Yap1p and Pap1, enter the nucleus after T-cell activation (NF-AT) and after oxidative stress (Yap1p and Pap1), respectively. Otherwise, these factors remain cytoplasmic in a CRM1-dependent fashion (Yan et al., 1998; Kuge et al., 1998; Zhu and McKeon, 1999; Kehlenbach et al., 1998; Toone et al., 1998; Kudo et al., 1999). The intracellular localization of several kinases involved in mitogen activated protein kinase signaling is also found to be regulated through the activity of CRM1 (Tolwinski et al., 1999; Engel et al., 1998; Fukuda et al., 1997b,c; Ferrigno et al., 1998). Most interestingly, the discovery of a functional NES in p53 showed that the response of this protein to stress could also be largely controlled by CRM1 (Stommel et al., 1999). Additionally, the intracellular localization of the p53-interacting protein, MDM2, involved in p53 degradation, is also controlled by a functional NES and most probably by CRM1. The CRM1-dependent export of MDM2 and p53 seems therefore to control the stability of the latter (Freedman and Levine, 1998; Tao and

Levine, 1999). All of these examples clearly identify CRM1 as a key regulator of cellular responses to diverse signals.

The use of an antibiotic known as leptomycin B (Hamamoto et al., 1983a,b) was an essential element in the understanding of the control of the intracellular localization of all the proteins mentioned above. The first indication concerning the role of leptomycin B (LMB) was obtained when Nishi and colleagues found that a mutated form of CRM1 in *S. pombe* was responsible for resistance of cells to this drug. Moreover, the phenotype of a cold-sensitive crm1 mutant at restrictive temperature was almost the same as that of the wild-type cells treated with LMB (Nishi et al., 1994). These findings strongly suggested that LMB inhibited CRM1 function. Further evidence of involvement of CRM1 in the export of NES-containing proteins became obvious when Wolff and colleagues observed that LMB could inhibit the export of NES-containing proteins (Wolff et al., 1997). It is now clearly established that LMB is a specific inhibitor of the NES-dependent nuclear export of proteins (Fornerod et al., 1997b) and that it can directly prevent the binding of NES to CRM1 (Fukuda et al., 1997a; Ossareh-Nazari et al., 1997; Kudo et al., 1998). LMB therefore allows investigators to specifically inhibit the activity of CRM1 in cells and to evaluate the effect of CRM1 dysfunction.

In this work, we have investigated the role of CRM1 in the control of early *Xenopus* embryogenesis. Considering the essential role of CRM1 in the control of the activity of a variety of key regulators (essentially those involved in signal-dependent control of transcription and cell cycle), it was expected that CRM1 would play a crucial role in early embryogenesis. Our data show that though CRM1 is maternally expressed and is present throughout early *Xenopus* development, its activity is tightly regulated as a function of developmental stage. Indeed, results presented in this paper show that CRM1 which is initially present in an inactive form, becomes functional during the gastrula-neurula transition (GNT) period. These data therefore indicate that in addition to the major cell cycle and transcription modifications associated with GNT, activation of CRM1-signaling is also an essential feature of this critical stage of development.

MATERIALS AND METHODS

Cloning of *Xenopus* CRM1-encoding cDNA

Based on sequence homology between yeast and human CRM1, degenerate oligonucleotides were designed for RT-PCR amplification of the *Xenopus* CRM1. The 5′ primer was GTNGARAAYGAYSA-RGG and the 3′ primer was, DATYTTDATRAANGTRTCRC-ANGCCATRTC, where N = A+G+C+T; R = A+G; Y = C+T; S = G+C; D = G+A+T. Total RNA was purified from *Xenopus* oocytes and subjected to reverse transcription and PCR, as described (Brocard et al., 1997).

Briefly, cDNA synthesis conditions were as follows. The 3′ primer (see above) was used to prime first strand cDNA synthesis. RNA and the oligonucleotide were annealed in 10 μl of the annealing buffer (20 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, 250 mM KCl) for 5 minutes at 65°C, followed by 30 minutes at 55°C, and finally for 5 minutes at room temperature. The cDNA synthesis was initiated by addition of 20 μl of RT buffer (20 mM Tris-HCl, pH 7.0, 10 mM $MgCl_2$, 100 μg/ml actinomycin D, each 0.5 mM dNTP and 12.5 mM dithiothreitol), containing 10 units RNasin (BRL) and 4 units avian myeloblastosis virus reverse transcriptase (Promega). The reaction was carried out for 30 minutes at 37°C, and then diluted to 100 μl with H_2O. 1 μl of this RT reaction was amplified. The PCR conditions were 1 minute at 96°C, 2 minutes at 50°C, and 2 minutes at 72°C for 40 cycles using VENT polymerase and supplied buffer (New England Biolabs). PCR products were resolved on a 2% agarose gel and DNA fragments of expected size (between 450 and 500 base pairs) were cloned into Bluescript vector (Stratagene) and sequenced. Five inserts of different size were sequenced and one showed high sequence homology with the human CRM1 cDNA. This clone was used to screen a *Xenopus* oocyte cDNA library and after several rounds of screening, overlapping cDNAs containing the whole reading frame of XCRM1 were isolated and sequenced.

CRM1 mRNA and protein analysis during *Xenopus* embryogenesis

Total RNA was isolated from embryos at different stages during development as described (Seigneurin et al., 1996). Briefly, 50 embryos taken at different stages were lysed in 1 ml of a buffer containing 7.6 M guanidine-HCl in 0.1 M potassium acetate, pH 5. DNA was sheared by passing the suspension 10 times through a 21G needle, then RNA was selectively precipitated by the addition of 0.6 volumes of 100% ethanol. The pellet was resuspended with 200 μl TES (10 mM Tris-HCl, 1 mM EDTA, 5% Sarkosyl, pH 8) and reprecipitated. The purified RNA was then dissolved in RNase-free water. Proteins were extracted from 40 embryos taken at different stages as described previously and used to obtained a western blot (Grunwald et al., 1995). CRM1 was detected using a polyclonal anti-CRM1 antibody raised against the C-terminal peptide sequence ‘EFAGEDTSDLFLEEREALR’ (Kudo et al., 1997).

In situ localization of XCRM1

Cryosections were taken from embryos at different stages of development and immunolocalization performed exactly as described before (Grunwald et al., 1995). Briefly, embryos at different stages were fixed on ice in 4% PFA, 0.6× PBS and impregnated with 10% and then 20% sucrose in PBS (1 hour each on ice). After embedding in tissue-Tek, embryos were frozen and cut with a cryostat into 10 μm-thick sections. Sections were mounted onto gelatin-coated slides, treated with 4% PFA for 10 minutes at room temperature, and washed with three changes of PBS at 4°C. Before immunolabeling, slides were treated with 0.25% Triton X-100 in PBS for 10 minutes. For immunodetection anti-CRM1 antibody was first diluted in PBS containing 0.1% BSA, 0.1% glucose and then used for immunodetection. The preparations were then observed under an epifluorescence microscope (Zeiss Axiophot).

Microinjection of plasmids and RNAs

Yeast (*S. pombe*) and human cDNA encoding CRM1 in bluescript vector were transcribed in vitro and 0.1 and 1 ng RNA in water were injected into the stage 1 embryos before the first cleavage. Control embryos were microinjected with water. Embryos were then photographed at different times after the injection. Plasmids used for microinjection were: pXHC1 and pFHC1 (Kudo et al., 1997) as well as pXGLFE1, which is a pcDNA3.1 vector containing an insert encoding glutathione S-transferase (GST) fused to SV40 T-antigen nuclear localization signal (NLS) and green fluorescent protein (GFP) and the HIV-1 Rev nuclear export signal (NES) motif (Kudo et al., 1998). Plasmids, 5 ng, in 30 nl of the microinjection buffer (88 mM NaCl, 15 mM Hepes, pH 7.6), were microinjected as above.

Pull-down assays

100 *Xenopus* embryos taken at different stages were lysed in 500 μl of a buffer containing 20 mM Hepes, 0.25 M sucrose, 100 mM NaCl and 2.5 mM $MgCl_2$, pH 7.5, and centrifuged twice at 13000 ***g*** for 10 minutes at 4°C. The cleared supernatant was overlaid with 500 μl of melting-point bath oil (Sigma) and centrifuged for 10 minutes as

above. 250 μl of this extract were incubated with 15 μl of glutathione beads coupled to a GST-GFP-HIV-1 Rev NES fusion protein (Tachibana et al., 1996). The incubation was carried out at 4°C over night. The beads were washed 5 times with NETN buffer (100 mM NaCl, 1 mM EDTA, 0.5% NP40, 20 mM Tris-HCl, pH 8) and once with PBS. Proteins were eluted in loading buffer and used to obtain a western blot.

RESULTS

Molecular cloning of the *Xenopus* CRM1 encoding cDNA

Comparison of the amino-acid sequence of CRM1 from human and yeast shows the existence of several absolutely conserved motifs (Fornerod et al., 1997a; Kudo et al., 1997). Based on these sequences we designed degenerate oligonucleotides and performed an RT-PCR reaction using total RNA isolated from *Xenopus* oocytes. A fragment of 475 bp obtained from the RT-PCR was sub-cloned and sequenced and appeared to be a fragment of the *Xenopus* leavis CRM1-encoding cDNA. This fragment was then used as a probe to screen a *Xenopus* oocyte cDNA library and several positive clones were isolated and sequenced. Fig. 1 shows the comparison of the deduced *Xenopus* CRM1 protein sequence with that of human. The two proteins appear surprisingly identical (96.7% sequence identity over 1071 amino acids). CRM1 appears therefore to be one of the most conserved proteins within vertebrates and this observation strongly suggests a crucial role for this protein in the regulation of diverse functions in cells and during development.

The expression of CRM1-encoding mRNA and protein during early *Xenopus* embryogenesis

Total RNA isolated from embryos at different stages of development was isolated and analyzed for CRM1 mRNA expression. Fig. 2A shows that the CRM1-encoding mRNA is maternally expressed and is present throughout early development. Previously, we have found that CRM1 mRNA accumulation does not always coincide with CRM1 expression. In somatic cells, CRM1 mRNA is mostly expressed in proliferating cells whereas in arrested cells, although CRM1 can be detected, mRNA accumulation was found to be under a strong negative control (Kudo et al., 1997). Therefore, we thought it important to assess variation of CRM1 protein expression during development. Using specific polyclonal antibodies raised against a peptide located in a conserved domain at the C-terminal part of the human CRM1 (Kudo et al., 1997), we were able to show that the protein is also constitutively expressed throughout development (Fig. 2B). This suggested that unlike several other maternally-expressed transcripts (i.e. histone H1; Bouvet and Wolffe, 1994), CRM1 mRNA is not under any obvious translational control.

Modification of the intranuclear localization of CRM1 during early embryonic development

Previous studies using yeast and mammalian cells in culture showed that CRM1 is essentially localized at the level of the nuclear membrane region in the nucleoplasm (Fornerod et al., 1997a; Kudo et al., 1997; Adachi and Yanagida, 1989). It was therefore of particular interest to analyze the intracellular localization of XCRM1 during early embryogenesis. Embryos were taken at different stages of development and cryosections were prepared and used for the immunodetection of XCRM1. Before and during the gastrula stage, in addition to a diffuse nucleoplasmic localization, XCRM1 appeared to be localized as a network in the nucleus. Indeed, CRM1 seems to form some type of filamentous interconnected intranuclear structure (Fig. 5B, see stage 9 and 10, Fig. 3A, stage 9 and 10, presented at low magnification). The low level staining observed outside the nuclei is very probably due to the yolk-related background fluorescence also observed in mock immunolabeled cryosections (not shown). Specific nuclear membrane-associated localization (‘adult-type’) of CRM1 was observed only at the beginning of the neurula stage and thereafter (Fig. 3A, see stages 13 and 36). These data clearly show that the intranuclear localization of CRM1 is under a developmentally controlled process. Moreover, these observations identify gastrula-neurula transition as a potentially important period in the regulation of the activity of CRM1 during early development.

Our data show that XCRM1 is present throughout early development. However, the redistribution of the protein in the nucleus leading to a marked association of the protein with the nuclear membrane region after gastrula stage, suggests that these changes may be related to a functional modification of the protein. In order to investigate this possibility, we first expressed a GFP-tagged CRM1 in embryos to follow its nuclear localization during early development. *Xenopus* embryos at stage 1 were microinjected with an expression vector expressing either a GFP-hCRM1 fusion protein or a non-tagged hCRM1. Microinjected embryos expressing the GFP-CRM1 fusion protein or CRM1 were taken at stage 9 and 10 and cryosections were obtained. Until stage 9 (MBT), the promoter of the microinjected plasmid (CMV promoter) like those of cellular genes is not active (Almouzni and Wolffe, 1995; Newport and Kirschner, 1982). In agreement with this, we could not detect the presence of the fluorescent protein in cells in any part of the embryos (not shown). At stage 10 (gastrula), the expression of GFP-CRM1 is visible in many cells and interestingly, it is essentially located in the nuclear membrane region (Fig. 3B). Nuclei from embryos expressing a non-tagged form of CRM1 do not show any kind of autofluorescence (not shown). We also observed that the development of embryos expressing exogenous CRM1 (native or fused to GFP), was severely affected (not shown). This experiment indicates that at stage 10, the ectopically expressed CRM1 shows an ‘adult type’ nuclear localization (compare Fig. 3B with A, stage 36). At this stage, the endogenous protein shows a specific intranuclear localization (Fig. 5B, stage 10). The particular association of the endogenous CRM1 with the nuclear membrane region is observed only after the gastrula stage (Fig. 3A, stage 13). These observations suggest that before gastrula stage, the ectopically expressed CRM1 escapes a mechanism that hinders the endogenous protein to have the specific nuclear-membrane associated localization.

This prompted us to study the effect of CRM1 overexpression during early development in more detail. RNA encoding either human or yeast CRM1 (HuCRM1 and YCRM1, respectively) were prepared using in vitro transcription and 1 ng of RNA injected in two groups of stage

1 embryos. Embryos microinjected with either RNA showed an arrest in development before, during or just after the neurula stage. Fig. 4A shows that in certain of the microinjected embryos there is early formation of the cement gland which is a sign of accelerated differentiation. The development of these microinjected embryos arrested and then they died, while

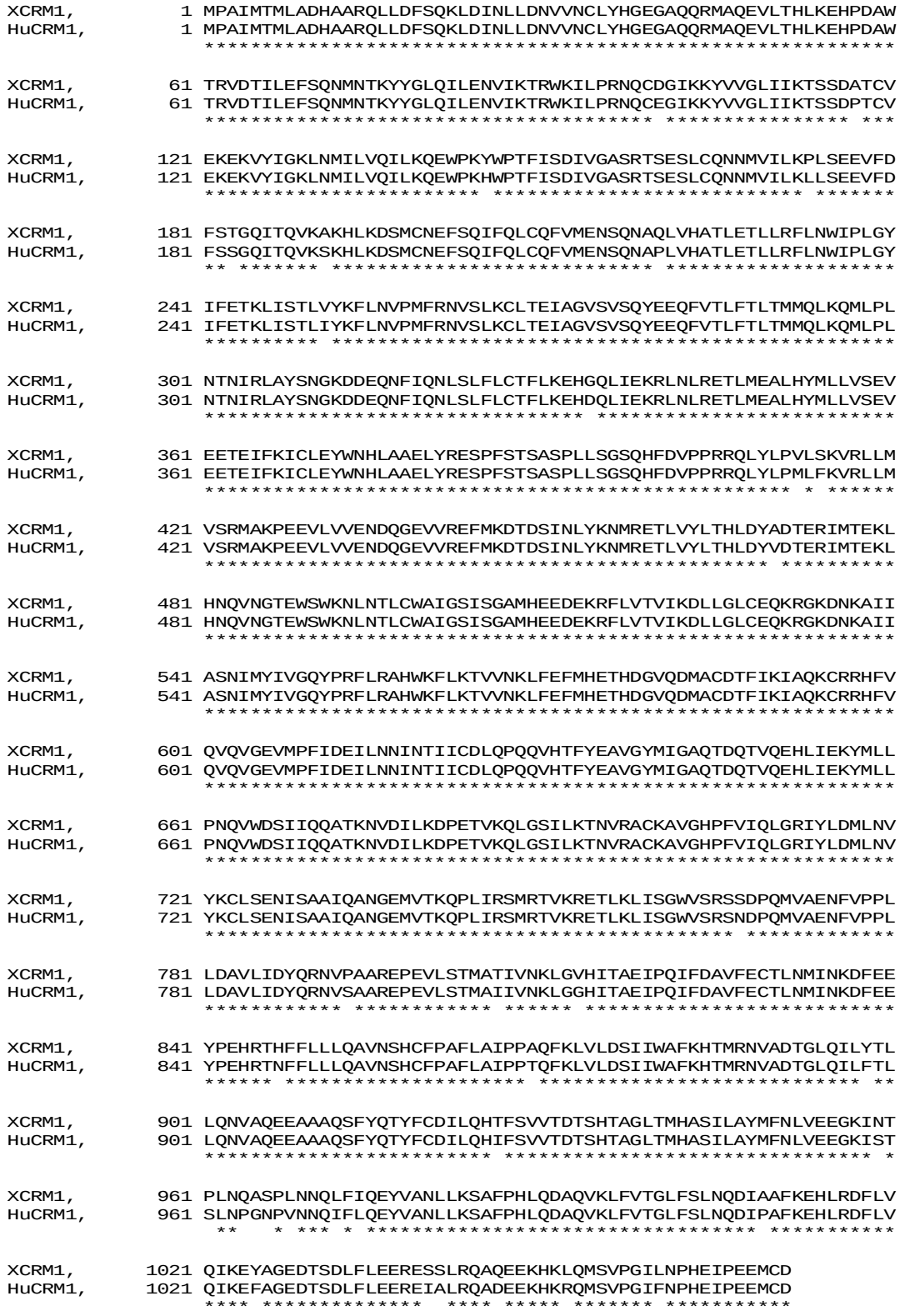

Fig. 1. CRM1 is a highly conserved protein in vertebrates. *Xenopus* leavis CRM1 encoding cDNA was cloned and the deduced amino acid sequence was compared with that of the human CRM1. Stars indicate amino acid identity.

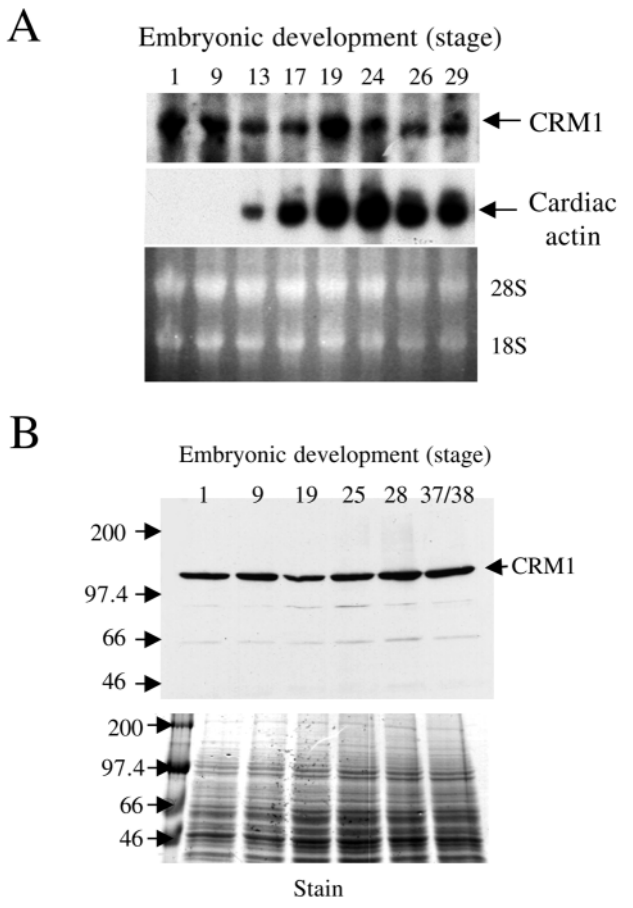

Fig. 2. CRM1 is constitutively expressed during *Xenopus* embryogenesis. (A) 20 μg of total RNA isolated from embryos taken at the indicated stages were used to obtain a northern blot. The blot was then probed with ^{32}P-labeled XCRM1 (upper panel) and Cardiac actin probe (lower panel). The ethidium bromide stained gel before the transfer of RNAs onto the membrane is also shown.
(B) Equivalent amount of proteins extracted from embryos at the indicated stages of development was analyzed on a 8% polyacrylamide gel (stained gel, lower panel) and then transferred to a membrane that was used to detect CRM1 using a polyclonal anti-CRM1 antibody (upper panel).

control embryos continued normal development (not shown). We then decided to microinject 0.1 ng of either human or yeast CRM1 RNA. Microinjected embryos showed the same phenotype as those microinjected with 1 ng RNA but in these conditions they did not die and could continue their development. However, compared to the control embryos, a significant delay in development was observed (Fig. 4B, compare control embryos with those microinjected with HuCRM1 and YCRM1 RNA). Development was arrested and YCRM1 and HuCRM1-expressing embryos died when control embryos reached stage 35/36 of development. From the external aspects of these embryos, it is obvious that problems in organogenesis were encountered during development. For instance, eye formation was affected (Fig. 4B, right panel). These experiments strongly suggest that an unprogrammed activity of CRM1 during the early development may have severe consequences on normal development.

Inhibition of CRM1 during early development

The results described thus far indicate that the GNT is a critical period for the activity of CRM1. Indeed, during this period the intranuclear localization of CRM1 changes: it becomes associated with the nuclear membrane region. This period also corresponds to the time during development when the CRM1 over-expression dramatically affects normal development. These results would predict that during GNT, embryos might be specifically sensitive to leptomycin-treatment. Leptomycin B is a specific inhibitor of CRM1 that interferes with the binding of NES-containing proteins to CRM1 (Fukuda et al., 1997a; Ossareh-Nazari et al., 1997; Kudo et al., 1998). The treatment of embryos with this drug, is therefore a very convenient way to inhibit CRM1 function and evaluate its consequence on embryonic development. LMB-treated and control embryos were taken at different stages and analyzed. As predicted, treatment of embryos with 1 μg/ml LMB blocked embryonic development at the neurula stage (Fig. 5A). This experiment suggests that the action of CRM1 is essential for embryos to go through the neurula stage and further that inhibition of CRM1 activity does not affect normal development before this stage. This observation suggests, firstly, that CRM1 becomes specifically activated during GNT and second, that an inhibitory mechanism might exist to maintain CRM1 inactive before this stage. In this respect, it is interesting to note that in LMB-treated embryos taken at stages 9 and 10, CRM1 is relatively homogeneously distributed in the nucleus and that the specific intranuclear localization of CRM1 observed in the control embryos, are not visible (Fig. 5B). Considering the fact that leptomycin has been shown to directly interact with CRM1 (Kudo et al., 1998) and to modify its structure (Fornerod et al., 1997b), one may suggest that LMB binding dissociates CRM1 molecules present in a stored form in the nucleus at those stages. We therefore propose that the network of CRM1 observed in the nuclei of embryos before GNT is consisted of stored CRM1 in inactive complexes. The interaction of CRM1 with LMB inhibits probably the participation of CRM1 to these structures.

Developmentally regulated activity of CRM1

Our data strongly suggest that CRM1, although present during early embryogenesis, becomes active during GNT. In order to directly test this hypothesis we set up a pull-down experiment using a fusion protein bearing GST, GFP and a functional NES from HIV-1 Rev. This fusion protein, attached to glutathione beads, was incubated with an extract prepared from embryos taken at different stages. After the incubation of these extracts with the fusion protein, the beads were washed and the retained *Xenopus* proteins were eluted and used to analyze the presence of CRM1 by western blotting. Fig. 6A shows that CRM1 present in the extract can bind to the fusion protein only after stage 11 and thereafter. This experiment shows that until the gastrula stage of development, CRM1 is unable to recognize NES motif. We therefore propose that after gastrula stage due to some unknown events that are probably related to the control of the localization of the protein, the stored inactive CRM1 becomes functional. It changes its nuclear localization and becomes able to recognize the NES motif present in target proteins.

In order to obtain more data in support of our conclusions, we cloned a DNA encoding a fusion protein containing GST-SV40 T NLS-GFP-Rev NES into the pcDNA 3.1 expression

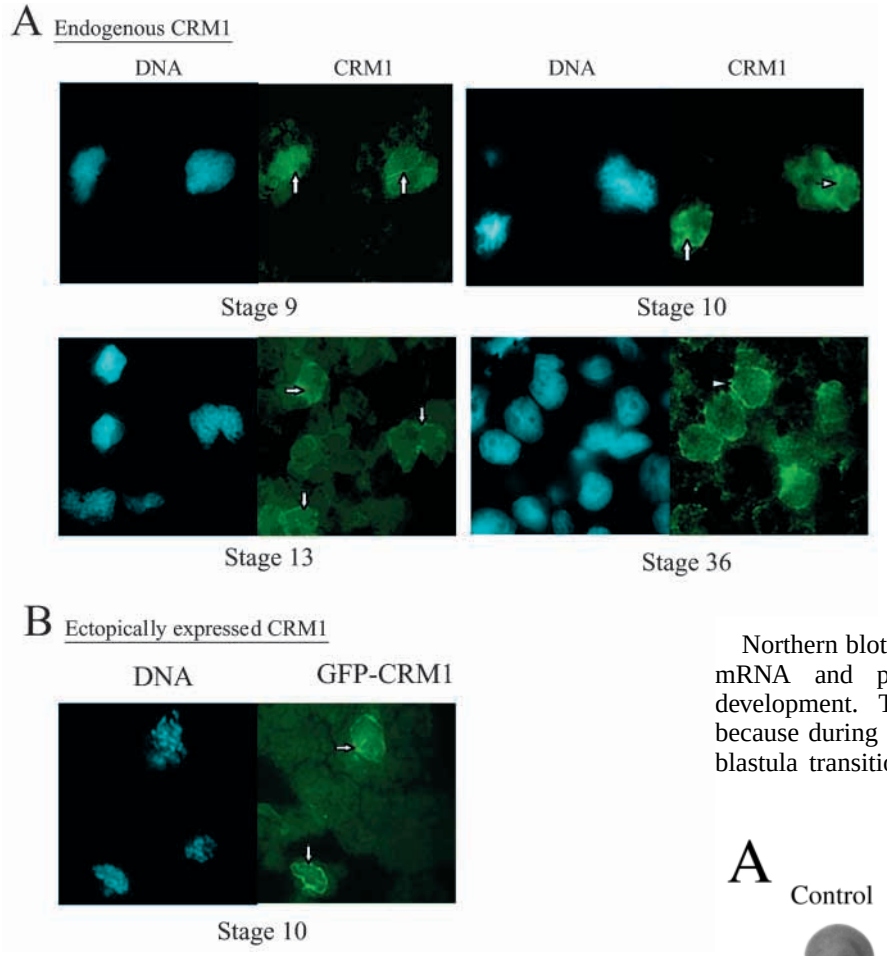

Fig. 3. Intranuclear localization of CRM1 changes during *Xenopus* embryonic development. (A) Cryosections obtained from embryos taken at the indicated stages were used for the immunodetection of CRM1. DNA column represents Hoechst labeled nuclei shown in the CRM1 column. Arrows indicate the specific intranuclear localization of CRM1 discussed in the text. (B) The ectopically expressed CRM1 becomes rapidly associated with nuclear membrane region. A plasmid containing the human CRM1 cDNA fused to GFP under CMV promoter was microinjected into stage 1 embryos. Embryos were then taken at the stage 10, fixed, cryosectioned and analyzed for GFP fluorescence.

vector. This fusion protein processes two well characterized signals; an active (nuclear localization signal) NLS from SV40-encoded T antigen and the HIV-1 Rev NES. The SV40 T NLS mediates the import of the protein to the nucleus which is then exported in a CRM1-dependent manner. This protein is therefore an excellent probe to monitor CRM1 activity during *Xenopus* development, since in the absence of CRM1 activity, it remains exclusively nuclear (Kudo et al., 1998). Stage 1 embryos were injected with this expression vector and embryos were taken at the stage 10 and 13. Cryosections were obtained and used to localize the GFP-related fluorescence. Fig. 6B shows that at stage 10, the GFP-related fluorescence can be detected essentially in the nucleus. Interestingly, at stage 13, besides the nuclei, significant GFP-related fluorescence could be observed in the cytoplasm which is indicative of the activity of CRM1 at this stage of development. This experiment provides additional arguments in favour of an activation of CRM1 function in stage 13 *Xenopus* embryos.

DISCUSSION

The cloning of a cDNA encoding XCRM1 revealed the extraordinary conservation of this protein during evolution. This finding is indicative of the crucial role played by this molecule in eukaryotic cells. Usually, within conserved proteins, only specific functional domains show high degree of sequence conservation during evolution. In the case of CRM1, sequence conservation, covers the entire protein length and this observation suggests the participation of the protein in a mechanism which is itself highly conserved in eukaryotes, i.e. various regions of the protein interact with conserved partners.

Northern blot and western blot analysis showed that CRM1 mRNA and protein are present throughout the early development. This observation is particularly interesting because during early *Xenopus* embryogenesis, up to the mid-blastula transition (MBT, 4096 cells), cells live only on the

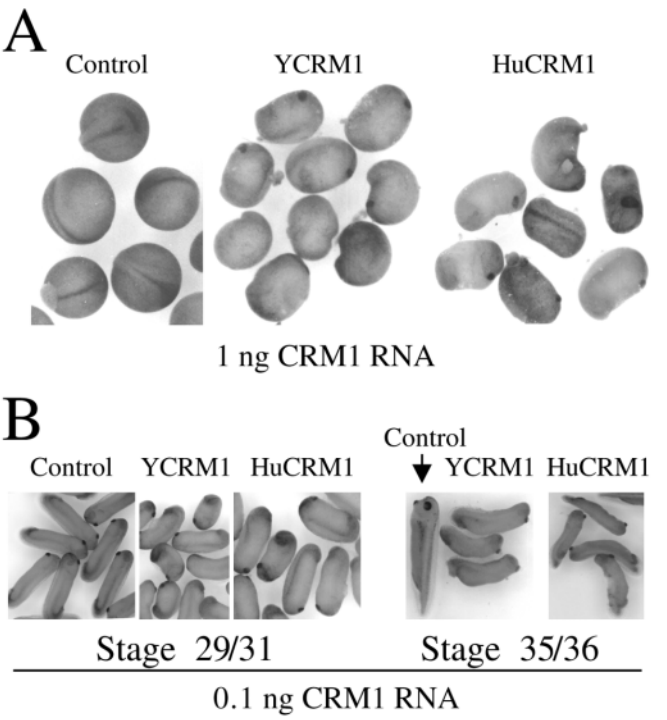

Fig. 4. Ectopic expression of CRM1 interferes with normal embryonic development. (A) 1 ng of RNA encoding either human or yeast (S. pombe) CRM1 (HuCRM1 and YCRM1, respectively), was microinjected into stage 1 embryos. Embryos were photographed when control embryos reached stage 18/19. (B) Stage 1 embryos were microinjected with 0.1 ng human or yeast CRM1-encoding RNA and embryos were photographed when control embryos reached stage 29/31 or stages 35/36 of development.

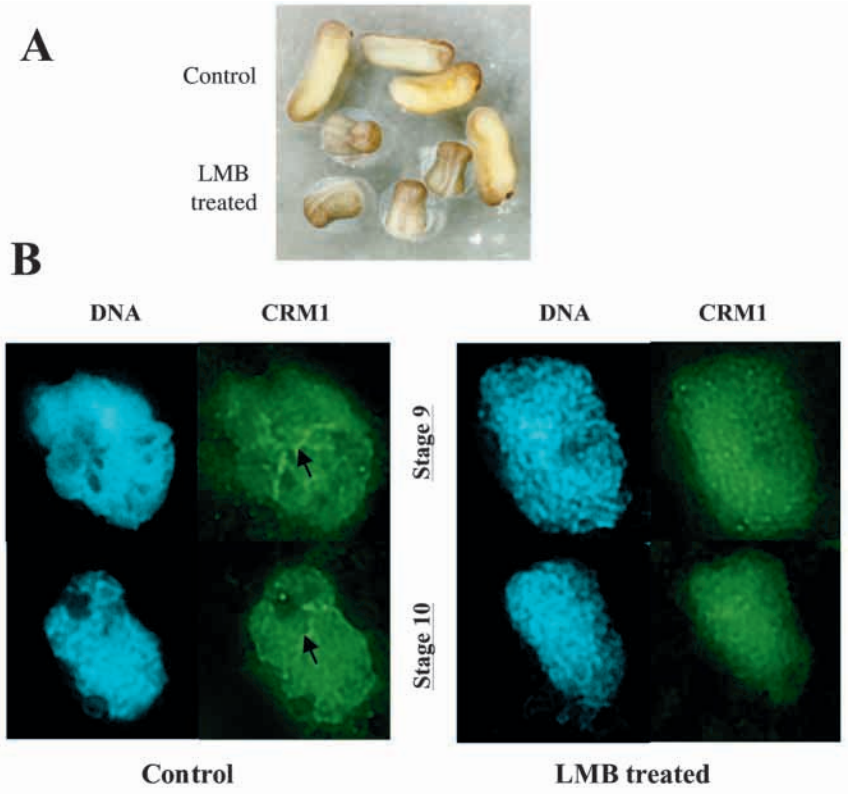

Fig. 5. Leptomycin B treatment blocks embryonic development at the neurula stage of development. (A) Stage 2 embryos were placed in the presence of 1 μg/ml of leptomycin B and were photographed when control embryos (untreated) reached stage 24/26 of development. (B) Leptomycin B treatment disturbs the specific nuclear localization of CRM1. Untreated (control) and LMB-treated embryos taken at the indicated stages were collected and fixed. Cryosections from these embryos were used for immunolocalization of CRM1 as in Fig. 3. The DNA column represents Hoechst-labeled nuclei shown also in the CRM1 column (CRM1-related immunofluorescence). Arrows indicate the specific nuclear localization of CRM1 discussed in the text.

maternal RNA stocks. Activation of zygotic gene expression is observed after MBT (Newport and Kirschner, 1982). One may therefore expect an important function of CRM1 during these periods. However, the treatment of embryos with LMB showed a stage-specific effect. In the presence of LMB, embryos reach MBT, accomplish the gastrula stage without any apparent phenotype but stop at neurula stage. We therefore concluded that CRM1 becomes active and plays an important role before the initiation of the neurula stage. Moreover, we observed a modification of the intranuclear distribution of CRM1 after gastrula stage, where an increasing number of cells show characteristic association of the protein with the nuclear membrane region. This observation suggests that CRM1 could be present in an inactive form before gastrula stage. The disappearance of the particular intranuclear localization of CRM1 after LMB treatment could be due to the dissociation of CRM1 from anchoring molecules under the action of the CRM1 binding activity of LMB. Indeed, the interaction of LMB with CRM1 seems to trigger an important modification of CRM1 conformation, as the binding of LMB causes a shift of the mobility of CRM1 on a native gel (Fornerod et al., 1997b).

The microinjection of 1 ng of either yeast or human CRM1 leads to an inhibition of development before, during or just after the neurula stage. It is interesting to note that in microinjected embryos an accelerated formation of cement gland was observed. This result suggests that CRM1 overexpression may accelerate the activity of one or several molecules involved in this specific epidermal differentiation. The microinjection of 0.1 ng of yeast or human CRM1 mRNA into embryos leads to a marked delay in normal development. The fact that microinjection of either yeast or human CRM1 gives the same phenotype is further evidence that yeast and human CRM1 are functionally similar (Kudo et al., 1997). The reason for the inhibition of development after the ectopic expression of CRM1 is not clear. However, we observed that

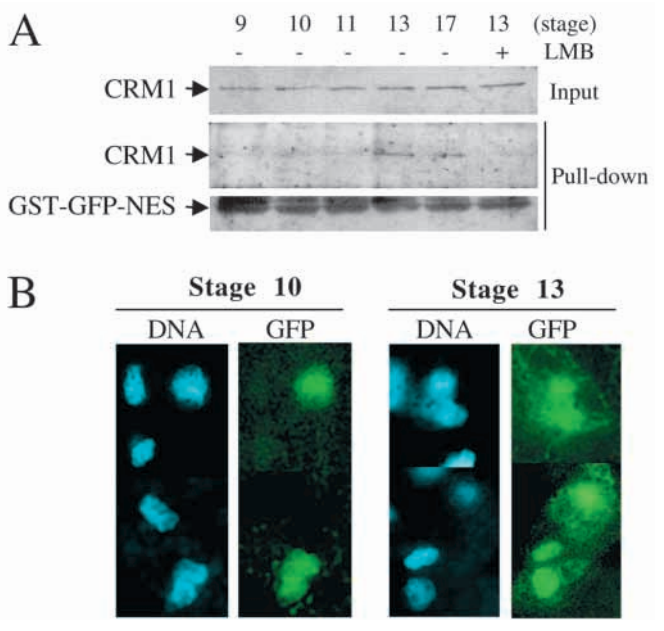

Fig. 6. Developmentally regulated activity of CRM1. (A) 100 *Xenopus* embryos were collected at indicated stages to prepare an extract. The extracts were then incubated with glutathione beads coupled to a GST-GFP-NES fusion protein. After incubation, beads were washed and the presence of CRM1 and the fusion GST proteins were determined by western blotting (pull-down panel). 10 μl fraction of the extract from each stage was analyzed for the presence of CRM1 before the pull-down procedure (input panel). In parallel, a pull-down was also performed from stage 13 embryos in presence of 100 nM leptomycin (+LMB line). (B) A plasmid encoding a GST-SV40 T NLS-GFP-Rev NES fusion protein was microinjected into stage 1 *Xenopus* embryos. Cryosections were prepared from embryos taken at stages 10 and 13, fixed and counterstained with Hoechst to visualize the nuclei (DNA panel). The corresponding GFP-related fluorescence is shown in the GFP panels.

CRM1 over-expression affects the normal development at the same stage as LMB treatment (during neurula stage). One may postulate that the activity of crucial regulators of neurula are under the control of CRM1 and that the ectopically expressed protein may escape the regulated activity of the endogenous CRM1, forcing an unprogrammed export of macromolecules and leading to an arrest of development. Indeed, the ectopically expressed CRM1 becomes rapidly associated with the nuclear periphery regions at a time when the endogenous protein is mostly associated with internal nuclear structures (see Figs 3B and 5B). All these data strongly suggest that CRM1 plays a major role during development at the gastrula-neurula transition, and more specifically during the neurula period. We then confirmed this conclusion using a direct approach to assess CRM1 activity during early *Xenopus* development.

The gastrula-neurula transition period, when embryonic development becomes dependent on CRM1 activity, is also a period of important modifications in gene expression and cell cycle parameters (Andrews et al., 1991; Grunwald et al., 1995). Previously we were able to show that this period corresponds to major changes in proliferative capacities of embryonic cells. The first significant accumulation of G_1 phase cells is observed after stage 10 during the gastrula-neurula transition period. Before stage 12, the majority of cells are in the S phase of the cell cycle and between stage 10 and 12, an important reduction of cells in S phase is observed (Grunwald et al., 1995). The frequent mitosis-dependent disruption of the nuclear membrane before gastrula stage may render the action of molecules involved in nucleo-cytoplasmic transport less critical. While, during gastrula-neurula transition the considerable elongation of the cell cycle most probably creates a need for the regulated transport of macromolecules as in adult type cells.

Our data show that CRM1 activity is essential during the GNT period, however, we can not define the way CRM1 controls this transition. The recent elucidation of the function of CRM1 revealed its involvement in many critical process including regulation of gene expression and cell cycle progression and cellular responses to environmental signals including stress. Factors controlling the recognition of NES motif by CRM1 play an important role in these CRM1-dependent regulated responses. For instance, the interaction of calcineurin with the transcription factor NF-AT masks its NES and allows nuclear localization of this factor after the T-cell activation (Zhu and McKeon, 1999). Furthermore, phosphorylation of cyclin B1-NES during the G_2 phase of the cell cycle leads to the non-recognition of this motif by CRM1 and the accumulation of MPF in the nucleus (Yang et al., 1998). Moreover, the yeast AP-1-like transcription factor, Yap1p, that activates genes required for the response to oxidative stress is translocated to the nucleus when cells are placed in an oxidative environment (Kuge et al., 1997, 1998). The nuclear localization of this protein is essentially due to the non-recognition of its NES by CRM1 after the oxidative shock (Yan et al., 1998).

The control of mitosis also seems to be highly dependent on the activity of CRM1. Specifically CRM1 is involved in the control of the intracellular localization of MPF (Yang et al., 1998; Hagting et al., 1998; Toyoshima et al., 1998) as well as the delay in mitosis observed after DNA damage (Toyoshima et al., 1998; Lopez-Girona et al., 1999). Consequently, CRM1 might also be involved in the maintenance of higher order chromosome structure. Indeed, in yeast the major phenotype observed in CRM1 mutants was the appearance of deformed and condensed chromosome domains (Adachi and Yanagida, 1989). This suggests that CRM1 might mediate the transport of crucial molecules involved in the maintenance of chromosome structure and in chromosome condensation processes. It is now clear that several molecules involved in the regulation of mitosis and chromosome condensation are specifically exported from the nucleus by CRM1. Indeed, besides cyclin B1, the activity of other kinases involved in formation of mitotic chromosomes is also under the control of CRM1. The cellular localization of protein kinase A inhibitor, PKI which is a NES-containing protein also seems to be controlled by CRM1 (Hauer et al., 1999; Wen et al., 1995; Ossareh-Nazari et al., 1997). Protein kinase A appears to function in the prevention of chromosome condensation as evidenced by the fact that microinjection of PKA peptide inhibitor into mammalian cells induces chromatin condensation regardless of the phase of the cell cycle (Lamb et al., 1991). Finally, MAPKK possesses also a functional NES (Fukuda et al., 1996, 1997b,c; Tolwinski et al., 1999) and it is now clearly established that CRM1 controls its cytoplasmic localization (Fukuda et al., 1997a). The inhibition of CRM1 activity by leptomycin B causes an accumulation of the MAKK in the nucleus which can have severe effects on MAPK activity and consequently on the regulation of mitosis and chromosome condensation.

Taken together these findings point out the crucial function of CRM1 in the regulation of critical events in cells. It is therefore expected that CRM1 would also play an essential role during embryonic development. Our study is the first attempt to elucidate a role for CRM1 during early development. We show that CRM1 is a key stage specific regulator of embryogenesis. These findings will help to identify molecules that control development through their intracellular localization. Increasing evidence points to this as a potentially developmental control mechanism in early embryogenesis. For instance, it has been shown that the *Drosophila* Hox cofactor, extradenticle (exd), is nuclear only in the presence of homothorax (hth), another homeodomain protein. Both hth and exd are required for the proximal leg development. In the absence of hth, exd is exported from nuclei due to the activity of CRM1 (Abu-Shaar et al., 1999). Moreover, a *Drosophila* Rel protein, Dorsal, involved in the determination of *Drosophila* dorsal-ventral polarity, is retained in the cytoplasm and becomes nuclear in response to activating signals (Drier et al., 1999). A third example concerns myogenesis during *Xenopus* development. It has been shown that at MBT, frog embryos, ubiquitously express myoD mRNA. Expression of XMyoD is at first cytoplasmic but muscle induction allows MyoD to enter the nucleus (Rupp et al., 1994). It is not clear whether CRM1 plays a role in the control of the intracellular localization of these proteins but our results show that it most certainly controls the activity of at least one important regulator of early *Xenopus* development.

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