RNA helicase p54 (DDX6) is a shuttling protein involved in nuclear assembly of stored mRNP particles

David A. Smillie and John Sommerville*

Division of Cell and Molecular Biology, School of Biology, University of St Andrews, St Andrews, Fife KY16 9TS, Scotland
*Corresponding author (e-mail: js15@st-and.ac.uk)

Accepted 11 October 2001
Journal of Cell Science 115, 395-407 (2002) © The Company of Biologists Ltd

Summary

Previously, we showed that an integral component of stored mRNP particles in *Xenopus* oocytes, Xp54, is a DEAD-box RNA helicase with ATP-dependent RNA-unwinding activity. Xp54 belongs to small family of helicases (DDX6) that associate with mRNA molecules encoding proteins required for progress through meiosis. Here we describe the nucleocytoplasmic translocation of recombinant Xp54 in microinjected oocytes and in transfected culture cells. We demonstrate that Xp54 is present in oocyte nuclei, its occurrence in both soluble and particle-bound forms and its ability to shuttle between nucleus and cytoplasm. Translocation of Xp54 from the nucleus to the cytoplasm appears to be dependent on the presence of a leucine-rich nuclear export signal (NES) and is blocked by leptomycin B, a specific inhibitor of the CRM1 receptor pathway. However, the C-terminal region of Xp54 can act to retain the protein in the cytoplasm of full-grown oocytes and culture cells. Cytoplasmic retention of Xp54 is overcome by activation of transcription. That Xp54 interacts directly with nascent transcripts is shown by immunostaining of the RNP matrix of lampbrush chromosome loops and co-immunoprecipitation with de novo-synthesized RNA. However, we are unable to show that nuclear export of this RNA is affected by either treatment with leptomycin B or mutation of the NES. We propose that newly synthesized Xp54 is regulated in its nucleocytoplasmic distribution: in transcriptionally quiescent oocytes it is largely restricted to the cytoplasm and, if imported into the nucleus, it is rapidly exported again by the CRM1 pathway. In transcriptionally active oocytes, it binds to a major set of nascent transcripts, accompanies mRNA sequences to the cytoplasm by an alternative export pathway and remains associated with masked mRNA until the time of translation activation at meiotic maturation and early embryonic cell division.

Key words: *Xenopus*, Oogenesis, Maternal mRNA, Ribonucleoproteins, Nuclear export, Translation repression

Introduction

During various stages of early development, organisms are dependent on the translational control of stored mRNA to ensure regulated production of proteins (Standart, 1992). The ability of a particular mRNA to be translated depends on its dynamic association with different proteins: that is, its status as a ribonucleoprotein (mRNP) complex. The stored mRNP particles of *Xenopus* oocytes contain a set of abundant proteins whose role it is to maintain the mRNA sequences in a condition ready for translation at the appropriate stage of development. The most abundant of these is a core group of four proteins with apparent molecular masses on SDS-PAGE of 50/52, 54, 56 and 59/60 kDa. These proteins were initially designated RNP1-4 (Darnbrough and Ford, 1981). Other general components of oocyte mRNP particles regulate translation through interaction with sequences in the 3′UTR: among these are the poly(A)-binding protein (PABP) (Zelus et al., 1989), the protein that binds to a 3′ cytoplasmic polyadenylation element (CPEB) (Hake et al., 1994) and the CPEB-associated translation repressor, maskin (Stebbins-Boaz et al., 1999).

The first of the core proteins to be characterized was the pair of masking proteins pp60/mRNP4 and pp56/mRNP3, which were shown to block translation when bound to specific mRNAs (Richter and Smith, 1984; Kick et al., 1987). These are probably products of pseudoalleles, resulting from the tetraploid derivation of the genome of *X. laevis*. pp60 and pp56 are phosphoproteins (Dearsly et al., 1985; Cummings and Sommerville, 1988) that bind to single-stranded RNA with little sequence specificity (Marello et al., 1992). Cloning identified pp60/56 as members of the Y-box family of proteins (known as FRGY2a/b) (Deschamps et al., 1992; Murray et al., 1992; Tafuri and Wolffe, 1990), which show a preference for binding to single-stranded polynucleotides by means of two distinct structures formed by a cold-shock domain and a series of basic/aromatic islands (Wolffe et al., 1992; Ladomery and Sommerville, 1994; Murray, 1994).

The second core protein of *Xenopus* stored mRNP to be cloned and characterized was the 54 kDa component, which was shown to be an ATP-dependent RNA helicase (Ladomery et al., 1997). The possible interplay between Xp54, which is able to unwind mRNA, and pp60/56, which binds to single-stranded RNA sequences, has been discussed (Sommerville, 1999). Xp54 belongs to a small family (DDX6) of DEAD-box RNA helicases (de la Cruz et al., 1999). Orthologues of *Xenopus* p54 include RCK/p54 in mammals (Akao et al., 1995; Paynton, 1998); Me31B in *Drosophila* (de Valoir et al., 1991;

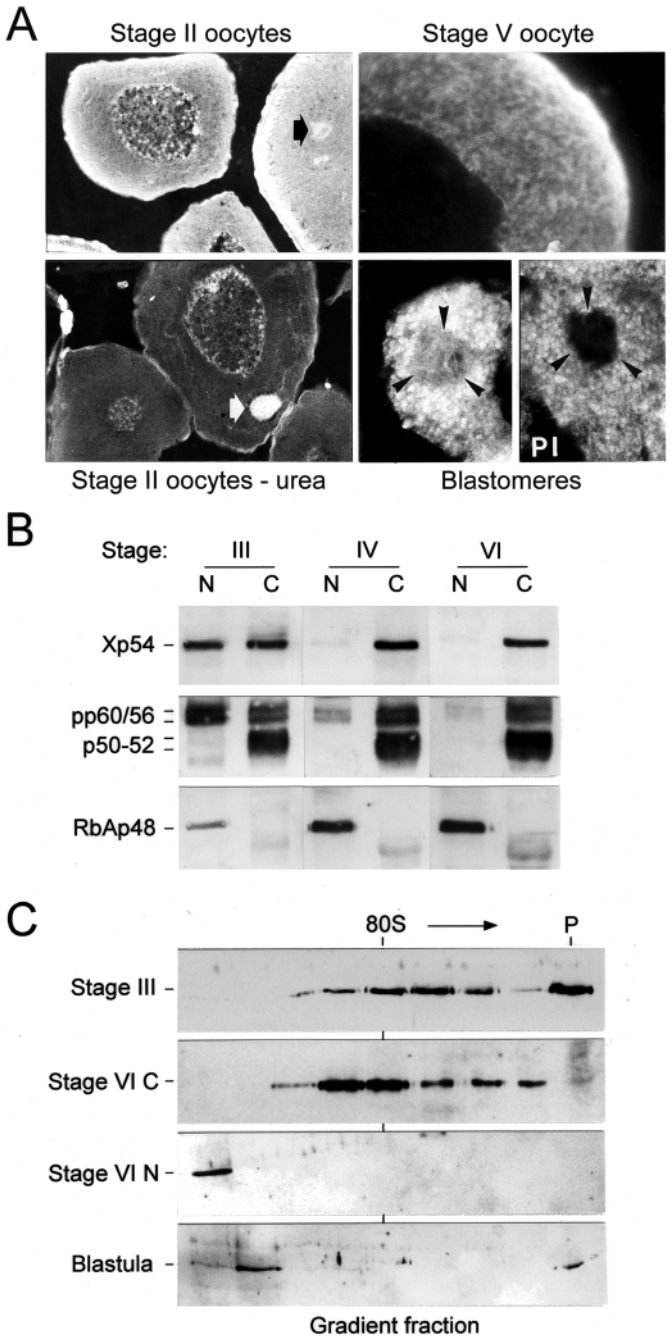

Fig. 1. Xp54 helicase is redistributed between the nucleus and the cytoplasm and between large and small complexes during the course of early development. (A) Sections through the ovary (with or without prewash in 4 M urea) and the blastula, both immunostained with anti-p54. Nuclear particles and fibres are obvious in stage I/II oocytes and in blastomere on left (arrowheads). The blastomere on the right is stained with preimmune serum (PI) and shows no nuclear reaction (arrowheads), only autofluorescent yolk. Arrows on oocytes indicate the Balbiani body. (B) The level of Xp54 in the nucleus relates to the level of transcriptional activity. An immunoblot of isolated nuclei (N) and cytoplasms (C) from mid- to late-oogenesis with anti-p54, anti-pp60/56, anti-p50-52 and anti-RbAp48. (C) Extracts (SN10) from stage III oocytes, stage VI cytoplasms, stage VI nuclei and blastula, separated on glycerol gradients and immunoblotted with anti-p54 are shown.

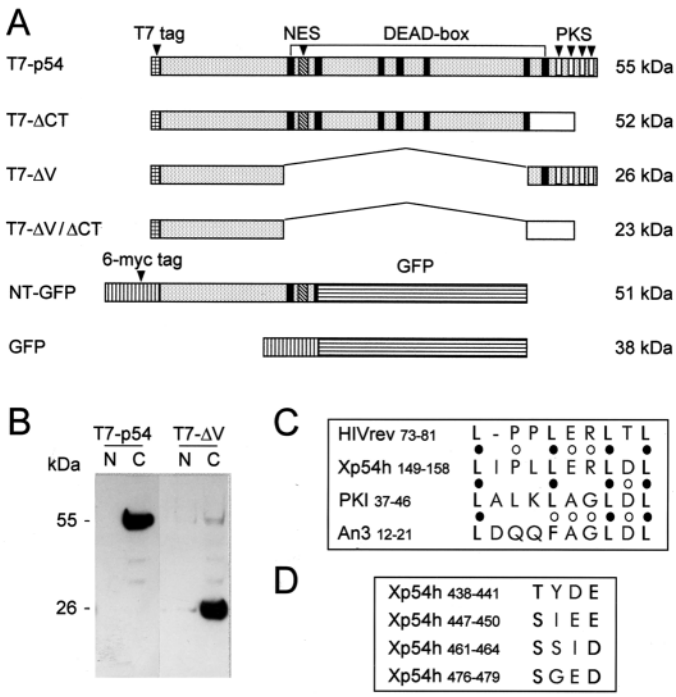

Fig. 2. Recombinant forms of Xp54 and identification of potential NES and phosphorylation sites. (A) A diagram of recombinant proteins expressed in this study. The black boxes represent the conserved motifs seen in all DEAD-box helicases. Locations of a potential NES (cross-hatched) and four potential CK2 phosphorylation sites (PKS, white boxes) are indicated. Various fragments of Xp54 (grey areas) were expressed either with a T7 epitope-tag or as GFP fusions containing a 6-myc tag. The white areas to the right in T7-ΔCT and T7-ΔV/ΔCT represent replacement of the C-terminus of Xp54 with an unrelated sequence encoded by the plasmid. (B) An immunoblot of T7-p54 and T7-ΔV, overexpressed in stage VI oocytes, showing cytoplasmic (C) but no nuclear (N) reaction. (C) Comparison of the leucine-rich sequence of Xp54 with the NES of HIV Rev (Fornerod et al., 1997), PKI (Hauer et al., 1999) and An3 (Askjaer et al., 2000). Positions of critical leucines (black circles) and other similarities (open circles) are indicated. The numbers refer to the residue positions starting from the initiating methionine. (D) The four potential CK2 phosphorylation sites (S/TxxD/E, where x is any non-basic residue) are located near the C-terminus of Xp54.

Nakamura et al., 2001); CGH-1 in *Caenorhabditis* (Navarro et al., 2001); p47 in *Spisula* (Minshall et al., 2001); Dhh1 in *S. cerevisiae* (Coller et al., 2001); and Ste13 in *S. pombe* (Maekawa et al., 1994). In metazoans, p54/Me31B/CGH-1/p47 is expressed mainly in germ cells: in yeast, Dhh1/Ste13 is essential for sexual reproduction. A common feature of all orthologues is that they are components of mRNP particles required for progression through meiosis.

Previous studies have shown that masking proteins bind to RNA transcripts in the oocyte nucleus (Braddock et al., 1994; Matsumoto et al., 1998). Because different sets of oocyte proteins may associate with different pre-mRNAs destined for immediate translation (hnRNP proteins) or for storage (masking proteins), there may be differences between the export pathways accessed by translatable and repressed mRNAs. Only recently have proteins involved generally in mRNA export been described (Conti and Izaurralde, 2001).

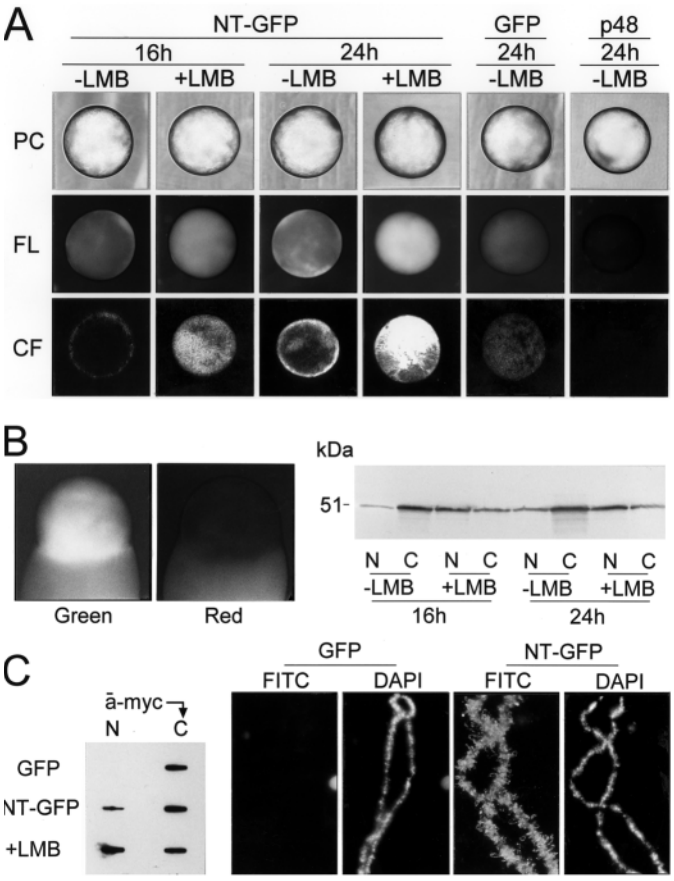

Fig. 3. Treatment of injected stage VI oocytes with 50 nM LMB results in nuclear accumulation of the N-terminal region of Xp54 fused to GFP. (A) Oocyte nuclei, isolated 16 and 24 hours after injection with plasmids expressing NT-GFP, GFP or T7-RbAp48 (p48), viewed by phase microscopy (PC), direct fluorescence (FL) and confocal fluorescence (CF) are shown. (B) (left) A nucleus and cytoplasm from an oocyte injected with NT-GFP, treated with LMB and viewed for green and red fluorescence. (right) An immunoblot, using anti-myc IgG, of expressed NT-GFP present in the nucleus and cytoplasm 16 and 24 hours after plasmid injection. Injected oocytes were incubated with or without LMB. Protein equivalents of five nuclei (N) and one cytoplasm (C) were separated by SDS-PAGE. (C) (left) Translocation into the nucleus of anti-myc IgG from 20 ng injected into the cytoplasm of oocytes expressing GFP and NT-GFP with or without LMB. The anti-myc IgG was detected on slot blots with an HRP-conjugated anti-mouse IgG. (right) Translocation of anti-myc IgG injected into the cytoplasm of stage IV oocytes onto the RNP matrix of lampbrush chromosomes is shown. Anti-myc IgG was detected, after incubation of chromosome spreads from oocytes expressing NT-GFP or GFP, with FITC-conjugated anti-mouse IgG. Location of the chromosomal axis is seen by post-staining with DAPI.

Associated with export of spliced mRNA are various proteins derived from the splicing reaction, which are retained as RNA-bound 'markers' near to the splice junctions (Stutz et al., 2000). Some of these RNA export factors have also been found on unspliced mRNA. A receptor identified in the recognition of RNA-bound factors and responsible for export of both spliced and unspliced mRNAs is the heterodimer TAP-p15 in metazoans and its equivalent, Mex67p, in yeast (Braun et al., 2001; Kang and Cullen, 1999; Hurt et al., 2000). An alternative pathway is described for HIV Rev, which is responsible for export of unspliced HIV-1 transcripts (Fischer et al., 1995). In this case, the arginine-rich NLS of Rev is recognized by the nuclear import receptor importin β, whereas the leucine-rich NES of Rev is recognized by the nuclear export receptor CRM1/exportin (Fornerod et al., 1997; Kudo et al., 1998).

In this report, we describe the ability of the mRNA-associated helicase Xp54 to shuttle between the nucleus and cytoplasm, to bind to nascent RNP transcripts and to accompany maternal mRNA sequences out of the nucleus and into the cytoplasm as mRNP storage particles.

Materials and Methods

Expression vectors

The pCS2*mt-SGP vector expresses a 6-myc epitope tag fused to GFP, driven from a CMV promoter, with an SV40 3′UTR that contains a poly(A) motif (Klymkowsky et al., 1999). The GFP sequence has been mutated from S_{65} to T in this version, and there is a stop codon after the GFP-coding sequence. A PCR fragment of cDNA clone 2B (Ladomery et al., 1997), encoding the first 164 residues of Xp54, was inserted between the unique *Eco*RI site immediately downstream of the 6-myc sequence and the *Xba*I site immediately upstream of the GFP sequence.site (Fig. 2A). The pCGT vector (kindly supplied by Javier Cáceras) contains a CMV promoter, an HSV tk 5′ leader sequence, a sequence encoding the T-epitope residues 1-11 of T7 gene 10 and the rabbit β-globin 3′ UTR, including intron B and the poly(A) motif. The complete coding sequence of clone 2B was inserted between the unique *Xba*I site immediately downstream of the T-epitope sequence and the unique *Bam*H1 site immediately upstream of the β-globin sequence. Deletions were generated by complete or partial digestion with *Eco*RV, removal of the small fragment and joining of the blunt ends with DNA ligase. ΔV deleted a fragment between the two *Eco*RV sites and removed 121 residues through the helicase core. In ΔCT, a fragment was deleted between the *Eco*RV and *Bam*HI sites, and the C-terminal 82 residues were removed and replaced by 49 residues encoded by the vector (Fig. 2A).

Antibodies

Antibodies to pp60 and pp56, Xp54 and p52 were raised in rabbits against native proteins (Ladomery et al., 1997; Sommerville and Ladomery, 1996). Antibodies to HDACm and RbAp48 were raised in rabbits against C-terminal peptides in the laboratory of Bryan Turner (Ryan et al., 1999). Mouse monoclonal antibodies to the bacteriophage T7 epitope (Novagen), the c-Myc epitope (clone 9E10, Sigma) and BrdU (Sigma) were used as recommended.

Oocyte isolation and extraction

Oocytes were isolated from *X. laevis* as described previously (Ryan et al., 1999) and were maintained in OR-2 medium (Evans and Kay, 1991). Oocytes were sorted into individual stages according to Dumont (Dumont, 1977). Pools of 50 oocytes (stages I-IV) or 25 oocytes (stages V and VI) were homogenized in 0.1 M NaCl; 2 mM $MgCl_2$; 2 mM dithiothreitol, 20 mM Tris-HCl, pH 7.5. Yolk and lipid were extracted with 1,1,2-trichlorotrifluoroethane (Evans and Kay, 1991). After centrifugation at 8000 ***g*** for 15 minutes at 0°C in a Sorvall SS-34 rotor, the clarified supernatant (SN10) was carefully removed.

Oocyte injection

Sets of 50-100 mid-vitellogenic (stage III/IV) or full-grown (stage VI) oocytes were isolated and 10 nl aliquots containing ~10 pg of purified plasmid DNA was injected, through the animal pole, into the nucleus

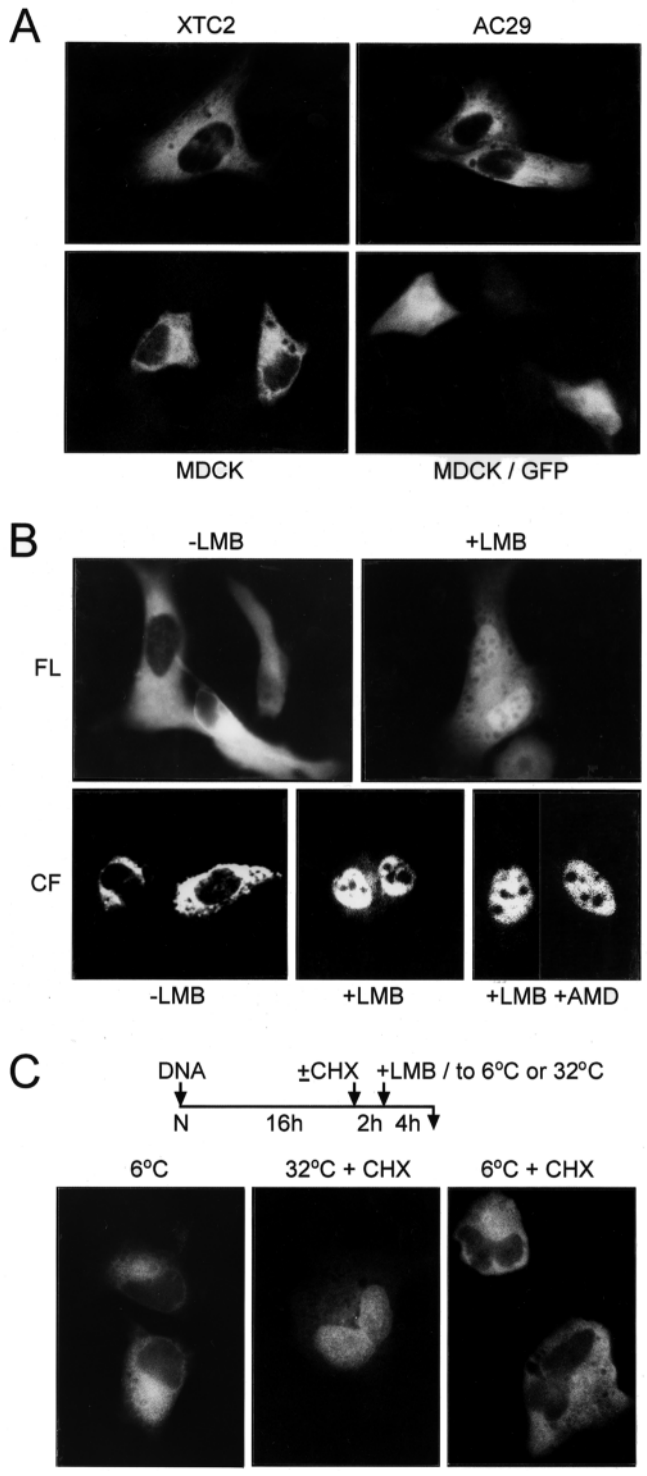

Fig. 4. The N-terminal region of Xp54 fused to GFP behaves like a shuttling protein in transfected culture cells. (A) Fluorescence images of *Xenopus* XTC2 cells, mouse AC29 cells and canine MDCK cells expressing NT-GFP and MDCK cells expressing GFP. (B) Treatment of transfected HeLa cells with 5 nM LMB for four hours prior to fixation results in nuclear accumulation of NT-GFP. Fluorescence (FL) and confocal (CF) images are shown. Cells were treated with 5 μg/ml actinomycin D (AMD) two hours before treatment with LMB. (C) Cold, but not treatment with cycloheximide (CHX), inhibits LMB-dependent nuclear accumulation of NT-GFP in transfected HeLa cells. The fluorescence images are of cells cooled to 6ºC after addition of LMB; cells treated with 20 μg/ml CHX for two hours prior to addition of LMB and maintained at 32ºC and cells treated with CHX and cooled to 6ºC.

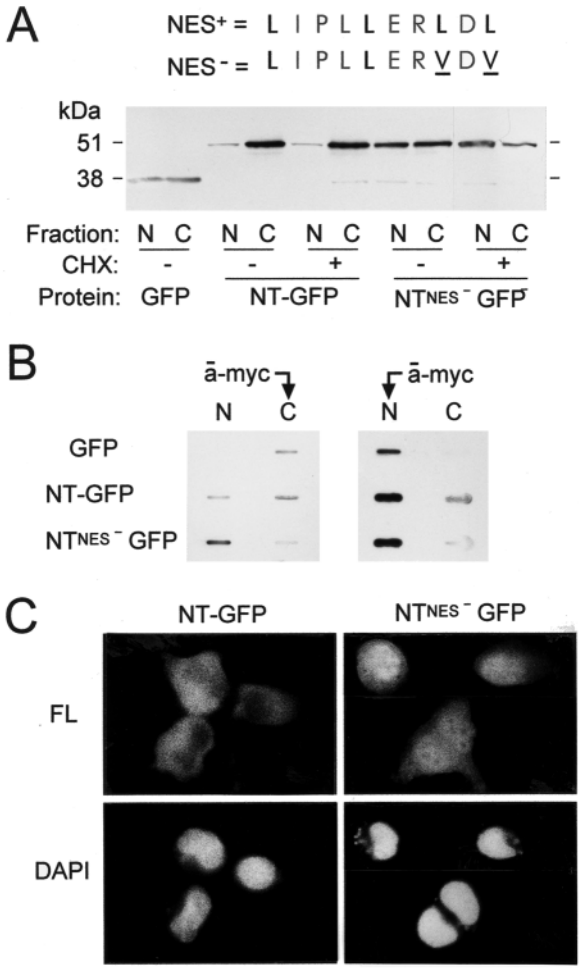

Fig. 5. Mutation of the leucine-rich NES impairs export of NT-GFP. (A) Leucine residues were mutated to valines at the two positions shown. An immunoblot of nuclei and cytoplasms isolated from oocytes expressing GFP, GFP-NT and GFP-NT$^{NES–}$ in the presence and absence of CHX (50 μg/ml) is shown. (B) Translocation of anti-myc IgG from the cytoplasm to the nucleus and from the nucleus to the cytoplasm in oocytes expressing GFP, GFP-NT and GFP-NT$^{NES–}$ is shown. (C) The green fluorescence (FL) and DAPI images are of HeLa cells expressing GFP-NT and GFP-NT$^{NES–}$.

of each oocyte. Oocytes were treated with 100 nM leptomycin B (LMB), 50 μg/ml cycloheximide (CHX), 5 μg/ml actinomycin D (AMD), 0.5 μg/ml α-amanitin (αAM), for four to six hours as in figure legends. Between 16 and 36 hours after injection, nuclei and cytoplasms were isolated under mineral oil (Sigma) from groups of 20-60 oocytes (Paine et al., 1992). Antibodies were introduced into the cytoplasm or nucleus of oocytes by injecting 10 ng of IgG. RNA was labelled in vivo by injecting oocytes with 0.1 μCi of [^{3}H]uridine (27 mCi/mmole, Amersham) or with 0.1 μg of BrUTP (Sigma). Poly(A$^+$) RNP was bound to and eluted from oligo(dT) cellulose as described previously (Ladomery et al., 1997). Radioactivity incorporated into RNA was measured after extraction at 60°C with phenol/chloroform buffered to pH 4.2 and precipitation with 2.5 vol

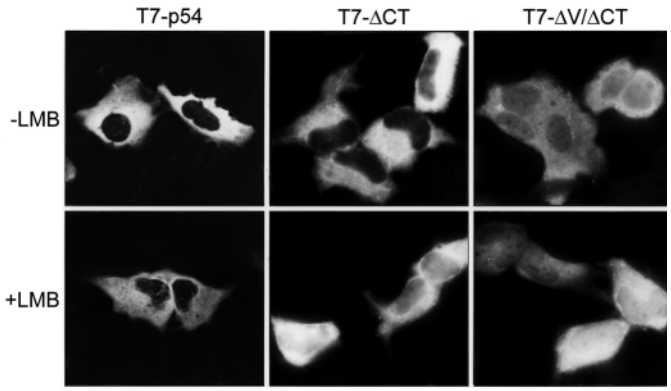

Fig. 6. The C-terminal region of Xp54 acts as a cytoplasmic retention signal in HeLa cells. Cells expressing T7-p54, T7-ΔCT and T7-ΔV/ΔCT were incubated for four hours in the presence or absence of 5 nM LMB. Fluorescence images were obtained by immunostaining with anti-T7 IgG and FITC-conjugated anti-mouse IgG.

ethanol. Lampbrush chromosomes from injected oocytes were prepared as described (Sommerville et al., 1993).

Cell transfection

Culture cells were grown in DMEM plus Glutamax (GibcoBRL) with 10% foetal calf serum (FCS; GibcoBRL) in eight-well chamber slides (Nalge Nunc). Mammalian cells expressing T7-tagged proteins were cultured at 37°C. Mammalian cells expressing GFP-fusion proteins were cultured at 32°C: *Xenopus* XTC-2 cells were cultured at 25°C. At mid-log phase, 20 ng of purified (Wizard Prep, Promega) plasmid DNA and 500 nl of 'Transfectene' (Qiagen) were added to 0.3 ml of fresh culture medium in each well. Transfection was allowed to occur over a 16-hour period. For further treatment, the medium was removed, the cells were washed in PBS and fresh medium with or without 5 nM LMB or 20 μg/ml cycloheximide or 5 μg/ml actinomycin D. After four to six hours of treatment, the cells expressing GFP fusion proteins were fixed with methanol at –20°C or with buffered 3% formaldehyde at 20°C. Cells expressing T7-tagged proteins were fixed with formaldehyde or methanol/acetone at –20°C.

Immunostaining

Proteins from the equivalent of one oocyte, embryo or cytoplasm or 5 nuclei were separated by SDS-PAGE, transferred to nitrocellulose (Protran, Schleicher & Schull) and immunoblotted as described previously (Ryan et al., 1999). Primary antisera or IgG, peroxidase-conjugated (HRP) anti-rabbit or anti-mouse IgG (Chemicon) were all used at a dilution of 1:10,000, and bands were developed using the ECL (Amersham) procedure. All immunoblots shown are representative of those obtained from at least two experiments. *X. laevis* ovary and early embryonic stages were fixed in 2% trichloroacetic acid, dehydrated, embedded in wax and sectioned on to glass slides. Dewaxed sections were rinsed with PBST, incubated for one hour at 20°C with anti-p54 diluted 1:200 in 10% FCS/PBST, rinsed five times in PBST for 10 minutes, then incubated with FITC-conjugated anti-rabbit IgG (Chemicon) diluted 1:200 in 10% FCS/PBST. After rinsing in PBST, a further five times, the sections were mounted in and viewed using a Leitz Ortholux fluorescence microscope. GFP-expressing and FITC-labelled cells were also examined by confocal laser microscopy (BioRad system). Lampbrush chromosomes were immunostained as described previously (Sommerville et al., 1993) using primary antisera diluted 1:5000 in 10% FCS/PBST. The secondary antibodies used were FITC-conjugated goat anti-mouse IgG and TRITC-conjugated goat anti-rabbit IgG (Chemicon) at a dilution of 1:5000 in FCS/PBST. In all immunostainings, the chromosomes or sections shown are representative of those seen in at least four different preparations.

Immunoprecipitation

IgG (2 μl) was linked to protein-A-glass beads (ProSep, Bioprocessing Ltd.) as described previously (Ryan et al., 1999). Poly(A^+) RNP extracted from 50 oocytes was mixed with the antibody-beads for 60 minutes at 20°C. The beads were washed with TBST to remove unbound material and then with 20 μl of 0.1 M glycine, pH 3.0, to remove bound RNP.

Gradient analysis

Nuclear lysates, clarified cytoplasmic extracts and poly(A) fractions were layered on 10-25% glycerol gradients made up in 0.1 M NaCl, 2 mM $MgCl_2$, 2 mM dithiothreitol, 20 mM Tris-HCl, pH 7.5. After centrifugation at 120,000 ***g*** in a 6×5 ml swing-out rotor (Beckman SW50Ti) at 0°C for two to three hours, the tube contents were fractionated. Fractions were analysed for proteins by immunoblotting. Sedimentation rates were calculated using 80S ribosomes and 60S and 40S ribosomal subunits run in parallel gradients and scanned through a flow cell measuring absorbance at 254 nm.

Results

Xp54 is detected in the nuclei of oocytes actively transcribing mRNA for storage

We showed previously that the DEAD-box helicase Xp54 accumulated in the cytoplasm of growing oocytes as a component of stored (maternal) mRNP particles (Ladomery et al., 1997). We also noted, in immunostained sections, some reactivity of anti-p54 with nuclear material. Upon examining sections from a range of oogenic and early embryonic stages, we see that the nuclear amount of Xp54 is highest in small, stage I/II oocytes (Fig. 1A), diminishing through stages III/IV, and being barely detectable at stages V/VI (Fig. 1A). As the level of RNA transcription from lampbrush chromosomes is highest in stage I/II oocytes, and the pool of stored cytoplasmic mRNP is established by the end of stage II, it is reasonable to assume that Xp54 is being incorporated into mRNP particles as they are formed in the nucleus. Excess Xp54 probably accumulates in the nucleus only before establishment of the cytoplasmic mRNP pool. An apparently nucleus-free situation then exists through late oogenesis and early embryogenesis until mid-blastula, when the cytoplasmic pool of mRNP is depleted and transcription is resumed. At this stage, Xp54 is seen in blastomere nuclei in the form of a fibrous network (Fig. 1A). A similar redistribution to the nucleus at onset of zygotic transcription is seen with p47 in early embryos of *Spisula* (Minshall et al., 2002).

Quantitation of nuclear to cytoplasmic ratios of Xp54 was checked by immunoblotting. By isolating nuclei and cytoplasms under oil (Paine et al., 1992; Ryan et al., 1999), we can ensure that there is no loss of protein nor leakage between cytoplasm and nucleus. Stage III nuclei are the earliest to be easily isolated: a clear transition is seen between stage III, with similar nuclear and cytoplasmic concentrations of Xp54, and stage IV, with little nuclear Xp54 (Fig. 1B). The immunoblotted samples are equivalent to ten nuclei and two cytoplasms, which give similar total amounts of protein in each track. Control immunoblots,

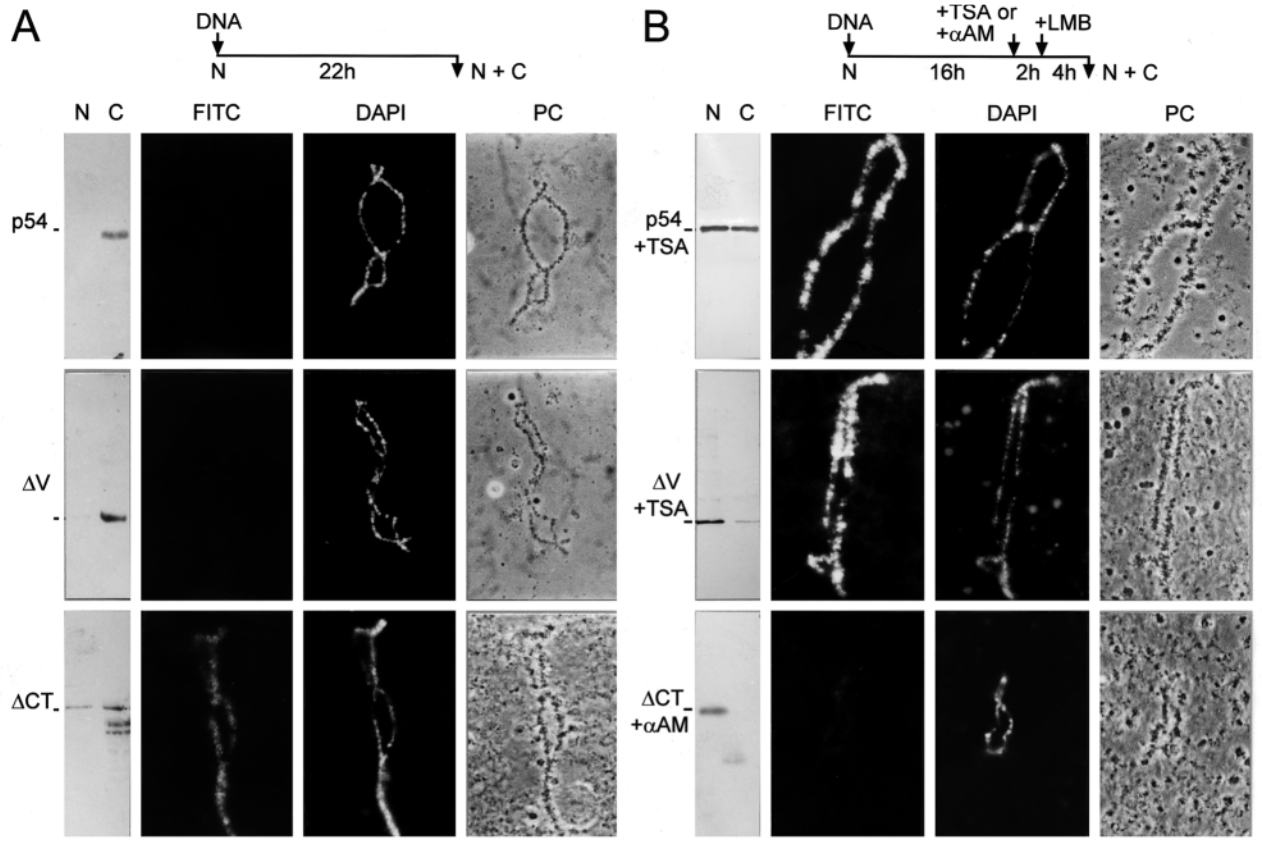

Fig. 7. Import of recombinant Xp54 into the oocyte nucleus and binding to nascent RNP is dependent on the transcriptional state of the chromatin. (A) The nucleocytoplasmic distribution of T7-p54, T7-ΔV and T7-ΔCT expressed in stage VI oocytes compared with binding of the recombinant proteins to the RNP matrix of lampbrush chromosomes is shown. The chromosome spreads were immunostained with anti-T7 IgG and FITC-conjugated anti-mouse IgG, post-stained with DAPI. Phase contrast (PC) microscopy images are also shown. (B) As described in (A) but with treatment of the oocytes with 2 ng/ml trichostatin A (TSA) or 0.5 μg/ml α-amanitin (α-AM) for two hours before addition of LMB. (C) Sedimentation of T7-p54 in complexes isolated from 50 stage VI oocytes and from nuclei and cytoplasms of 50 stage VI oocytes treated with TSA.

using the same transfer, show that the major components of nuclear mRNP assembly, the mRNA masking proteins pp60/56, have a similar distribution to that of Xp54, whereas the 50-52 kDa mRNA-associated proteins are restricted to the cytoplasm. A further control is provided by the nuclear factor, retinoblastoma-associated protein p48 (RbAp48), which is confirmed to be completely nuclear throughout (Fig. 1B). These results are consistent with Xp54 having a function in the assembly of mRNP particles in the nucleus.

One additional observation is that anti-p54 reacts strongly with material in the Balbiani body of stage I/II oocytes. Staining of this structure is seen to best effect when the more soluble material, including most of the cytoplasmic mRNP, is removed by washing the section in 4M urea (Fig. 1A). The Balbiani body contains not only an aggregation of mitochondria but also the germ-plasm (Wylie, 2000). This observation may relate to the findings that in *Caenorhabditis*, the p54 orthologue CGH-1 is expressed specifically in the germline and located in P granules in addition to other mRNA-containing particles (Navarro et al., 2001). It may also be related to the observation in *Drosophila* oocytes that the orthologue Me31B is highly concentrated in the sponge bodies, which may be functionally related to the Balbiani body of *Xenopus* oocytes (Nakamura et al., 2001).

Immunostaining of urea-washed sections of *Xenopus* ovary also reveals residual nuclear structures, both internally and at the nuclear periphery (Fig. 1A). It is likely, from the data presented later, that the residual material results from a tight interaction of Xp54 with pre-existing nuclear structures.

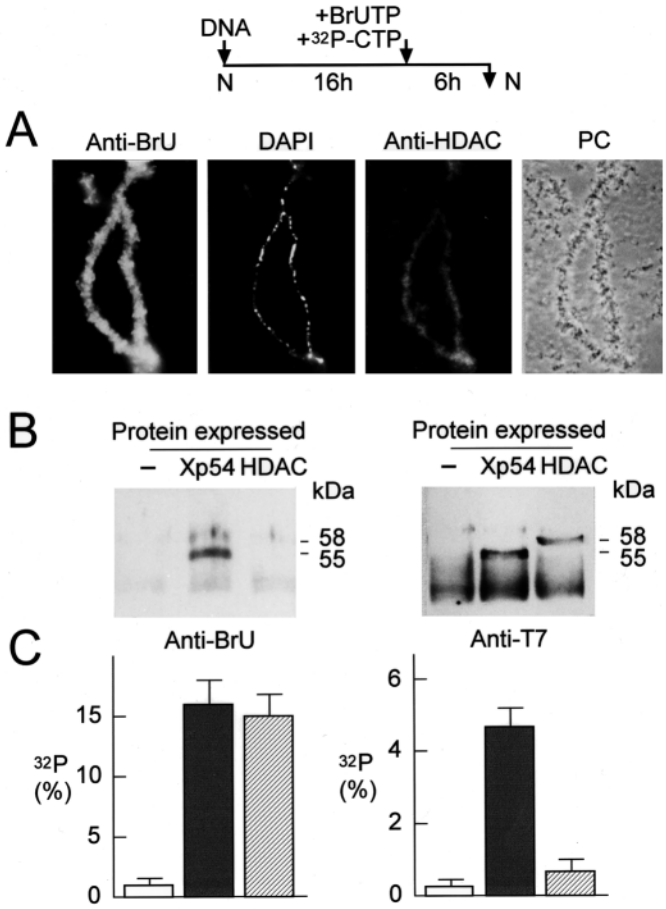

Fig. 8. Recombinant Xp54 associates with de novo synthesized RNA. Stage III/IV oocytes expressing T7-p54 and T7-tagged histone deacetylase (HDAC) were injected with 0.1 μg of BrUTP and 0.1 μCi of ^{32}P-CTP six hours before isolation of nuclei. (A) Double immunostaining of lampbrush chromosomes using anti-BrdU mouse monoclonal and anti-HDAC rabbit polyclonal. Immunofluorescent (FITC-conjugated anti-mouse IgG and TRITC-conjugated anti-rabbit IgG), DAPI and phase contrast (PC) images of a representative bivalent are shown. (B) Immunoprecipitation of T7-Xp54 and T7-HDAC from nuclear extracts using anti-BrdU and anti-T7 monoclonals. Nuclear extracts from non-injected oocytes (–) are shown as negative controls. (C) Immunoprecipitation of ^{32}P-labelled RNA from nuclear extracts similar to those in (B) using anti-BrdU IgG and anti-T7 IgG is shown.

Xp54 is detected in both mRNA-associated and soluble forms in oocytes and embryos

If Xp54 is shifted between different cell compartments, is there a corresponding shift in its association with other cellular components? To answer this question, extracts taken from nuclei and cytoplasms at different times of development were separated on glycerol gradients and the resulting fractions analysed for Xp54 by immunoblotting. Extracts from stage I oocytes show a broad range of sedimentation values, from soluble material at the top of the gradient to material sedimenting faster than polysomes in the pellet (not shown). This range includes poly(A)$^+$mRNP particles, which sediment at 30-120 S, and nuclear RNP particles and chromatin, which would pellet at this speed (Darnbrough and Ford, 1981; Cummings and Sommerville, 1988). By oogenic stage III, following centrifugation, particles sedimented around 80 S and other material was pelleted (Fig. 1C). The pelleted material can be recovered from isolated nuclei (not shown), and at least some of it represents the association of Xp54 with nascent transcripts as indicated by positive immunostaining of lampbrush chromosomes from stage III oocytes with anti-p54 (not shown). By stage VI, the only change is the detection of much less Xp54 in the pellet (owing to almost all of the protein being in particles in the size range of mRNP associated with a single ribosome and thus not pelleted) (Fig. 1C) (Sommerville, 1990). Isolation of nuclei from stage VI oocytes confirms that there is little Xp54 left in the largely inactive chromatin, instead, the small amount of protein detected is soluble (Fig. 1C). In embryos at mid-blastula, Xp54 is seen in the form of small particles and pelleted material (Fig. 1C), corresponding to a resumption of its association with active chromatin.

Expression of recombinant Xp54 in oocytes

The cDNA clone encoding Xp54 (Ladomery et al., 1997) was used to construct a series of T7 epitope-tagged and GFP-fusion proteins (Fig. 2A) for use in identifying sequences responsible for various cellular activities. All of the constructs could be expressed in oocytes from a CMV promoter. In general, recombinant protein expression was easily detected 18-24 hours after plasmid DNA injection into full-grown (stage VI) oocytes. For instance, T7-p54 could be expressed up to a level of about 50 ng/oocyte in 24 hours, which approaches the concentration of endogenous Xp54. Even at this high level of expression, T7-p54 was detected only in the cytoplasm (Fig. 2B). Surprisingly, a construct expressing a smaller protein, T7-ΔV, with a mass of 26 kDa, was also entirely cytoplasmic (Fig. 2B). A protein of this size would be expected to diffuse freely from cytoplasm to nucleus, as does the 38 kDa GFP protein used as a control in oocyte injections and cell transfections. To explain these observations, two possibilities are examined here: first, that Xp54 can gain entry to the nucleus, through diffusion or by active import, but is rapidly exported again; and second, that Xp54 contains a cytoplasmic retention signal that prevents its entry into the nucleus of full-grown oocytes.

Identification of a potential NES and phosphorylation sites in Xp54

On examining the amino-acid sequence of Xp54 for potential signals that might contribute to the cellular distribution of the protein, two motifs stand out. The first is a leucine-rich sequence similar to the nuclear export signal (NES) described for HIV Rev (Fischer et al., 1995) and possibly belonging to a family of NES motifs typified by critical positioning of leucine residues (Fig. 2C). Export of proteins containing a leucine-rich NES is sensitive to leptomycin B (LMB), a drug that specifically inhibits activity of the nuclear receptor CRM1 (Fornerod et al., 1997; Kudo et al., 1998). The potential NES is located in the N-terminal region of Xp54, which comprises the first 164 residues and is referred to henceforth as the N-terminal (NT) region. The second type of motif is the consensus site for phosphorylation by the protein kinase CK2. Only four CK2 sites are found in Xp54 (Fig. 2D), all of which are located within the C-terminal 44 residues. The C-terminal 83 residues is henceforth referred to as the CT region.

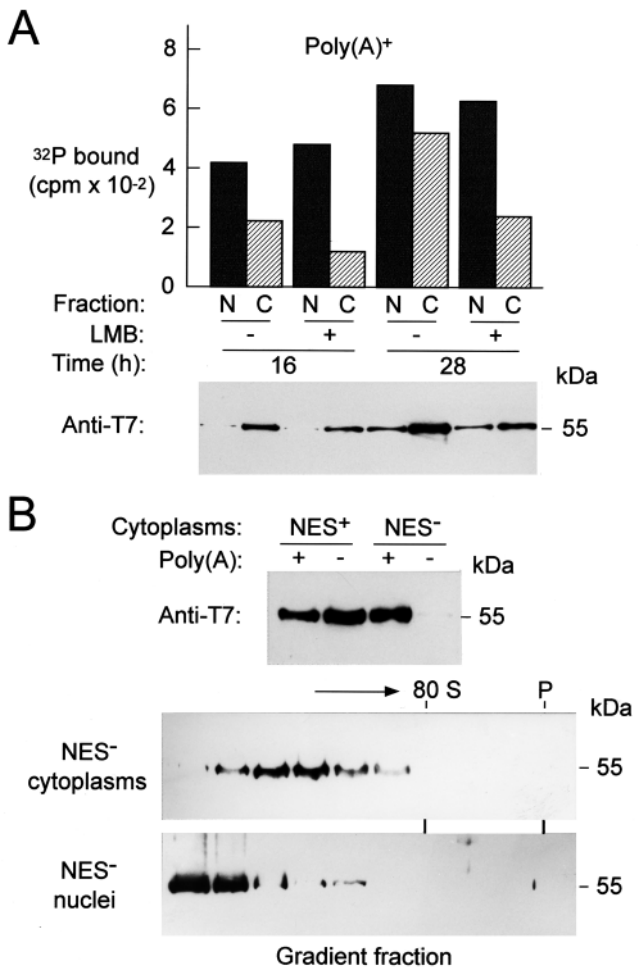

Fig. 9. Treatment of oocytes with LMB or mutation of the leucine-rich NES of Xp54 has little effect on nuclear export of mRNP particles. (A) Stage III/IV oocytes expressing T7-p54 were injected with 0.1 μCi of ^{32}P-CTP, and poly(A)$^+$ RNP was extracted to assay radioactivity incorporated (columns) and T7-p54 present (immunoblot) in nuclei and cytoplasms after 16 and 28 hours. (B) Immunoprecipitation, with anti-T7 IgG, of poly(A)$^+$ and poly(A)$^-$ RNP extracted from cytoplasms of stage III/IV oocytes expressing T7-p54 and mutant T7-p54^{NES-} for 60 hours. In addition, poly(A)$^+$ tails from cytoplasms of 50 oocytes expressing T7-p54^{NES-} were run on glycerol gradients, and sedimentation fractions were analysed by immunoblotting for the T7-tag. The sedimentation of T7-p54^{NES-} from 50 nuclei is also shown.

Interestingly, both the potential NES and the CK2 sites are conserved among vertebrate orthologues of Xp54 but are not evident in invertebrate orthologues.

Treatment of injected oocytes with LMB results in nuclear accumulation of Xp54

In contrast to the results obtained from expressing T7-p54 (Fig. 2B), expression of NT-GFP in oocytes resulted in fluorescence being detected directly in isolated nuclei. 16-24 hours after plasmid injection, NT-GFP was detected in increasing amounts around the nuclear periphery (Fig. 3A, first and third columns), a location that is consistent with efficient nuclear export. The nuclear concentration of NT-GFP was substantially increased by treating the injected oocytes with 50 nM LMB for four hours prior to nuclear isolation (Fig. 3A, second and fourth columns). Accumulation in the nucleoplasm indicated inhibition of NT-GFP export by the CRM1 nuclear pathway. From oocytes expressing GFP alone, a weak and evenly spread nuclear signal was seen, presumably due to diffusion from the cytoplasm (Fig. 3A, fifth column); from oocytes expressing T7-RbAp48, which served as a negative control, no nuclear fluorescence was seen (Fig. 3A, last column).

The nuclear concentration of NT-GFP increases (and the cytoplasmic concentration decreases) after treatment with LMB, which was demonstrated by comparing separated nuclei and cytoplasms, either directly by fluorescence or indirectly by immunoblotting (Fig. 3B, left). Viewed in blue light, which specifically activates GFP, the nucleus fluoresces more than attached cytoplasm: viewed in green light, no nuclear fluorescence is seen, whereas the cytoplasm still fluoresces, indicating that most of the cytoplasmic fluorescence is due to autofluorescence of yolk rather than presence of GFP (Fig. 3B, left). Upon immunoblotting NT-GFP with anti-myc (Fig. 3B, right), the protein from five nuclei (tracks 1 and 5) produces about a fifth (0.2) of the band intensity of protein from one cytoplasm (track 2 and 6) in the absence of LMB and three times the band intensity in the presence of LMB (tracks 3 and 4; 7 and 8). This equates to nuclear:cytoplasmic content ratios of 0.04 (0.2/5) and 0.6 (0.2×3), respectively. Assuming a nuclear:cytoplasmic available volume ratio of 0.05 for stage V/VI oocytes, the ratios of nuclear:cytoplasmic concentration of NT-GFP are estimated to be 0.8 (0.04/0.05) in the absence of LMB and 12 (0.6/0.05) in the presence of LMB. This 15-fold concentration in the nucleus after LMB treatment is consistent with inhibition of the CRM1 export pathway.

A stringent test of a protein being actively imported into, and exported out of, the oocyte nucleus is its ability to cotranslocate bound IgG molecules, the mass of IgG precluding its passive diffusion through the nuclear pores (Bellini and Gall, 1999). However, oocytes expressing NT-GFP, which also carries a 6-myc tag, translocate anti-myc antibodies from the cytoplasm to the nucleus (Fig. 3C, left). This effect is not seen with oocytes expressing myc-tagged GFP. Treatment of the NT-GFP-expressing oocytes with LMB results in enhanced accumulation of anti-myc IgG in the nucleus.

The NT region of Xp54 appears to be sufficient not only for nuclear import but also for binding to nascent transcripts on the extended loops of lampbrush chromosomes from stage IV oocytes. Furthermore, anti-myc IgG injected into the cytoplasm and translocated to the nucleus by NT-GFP also localizes to nascent transcripts, the anti-myc IgG being detected by immunostaining the chromosomes with FITC-labelled anti-mouse IgG (Fig. 3C). It is concluded that neither binding of IgG to the N-terminus nor fusion of GFP to the C-terminus impedes interaction of the NT fragment with nuclear import factors and nascent RNP transcripts. The same procedure using oocytes expressing myc-tagged GFP resulted in no chromosomal immunofluorescence and sufficed as a negative control (Fig. 3C).

Recombinant Xp54 can be expressed in a variety of culture cells

Owing to their large size and the accumulation of yolk, which obscures detail, oocytes are unsuitable for visualizing the

transport of GFP-fusion proteins. Transfected culture cells are more convenient. Various types of culture cells were transiently transfected with plasmid DNA expressing the NT-GFP fusion protein. These included *Xenopus* XTC-2 cells, which have embryonic features (Smith and Tata, 1991), mouse mesothelioma AC29 cells, canine kidney MDCK cells and human tumour HeLa cells. All cells tested showed essentially the same results, a cytoplasmic localization of NT-GFP (Fig. 4). Expression of the GFP protein itself resulted in approximately equal concentrations in the cytoplasm and nucleus, as would be expected if it were free to diffuse between compartments.

Treatment of HeLa cells with LMB results in nuclear accumulation of NT-p54

Initial transient transfection experiments indicated that the N-terminal region of recombinant Xp54 is required for nuclear export. In HeLa cells, NT-GFP appears to be exclusively cytoplasmic (Fig. 4B) even 48 hours after transfection (not shown). However, treatment of the cells with 5 nM LMB for four hours, 22 hours after transfection, resulted in most of the NT-GFP protein being relocated to the nucleus (Fig. 4B). Almost complete relocation was observed, as confirmed by optical sectioning (Fig. 4B, bottom row). Thus the initial absence of NT-GFP from the nucleus was considered to be due to efficient export rather than a failure of the protein to gain access through diffusion or active import. Treatment of the cells with 5 μg/ml actinomycin D (AMD) for two hours completely inhibits RNA synthesis (not shown) yet does not change the subsequent effect of LMB (Fig. 4B), indicating that neither nuclear uptake nor nuclear export of NT-GFP is dependent on continuing RNA synthesis. That nuclear import is dependent on an energy-requiring mechanism is indicated by the effect of reducing the culture temperature to 6°C, which inhibits nuclear accumulation in the presence of LMB (Fig. 4C, left). However, nuclear import of NT-GFP is not dependent on continuing protein synthesis: treatment of transfected cells with cycloheximide (CHX) at 20 μg/ml is sufficient to substantially inhibit protein synthesis (reduction of 95%) in two hours (not shown), but it does not prevent nuclear accumulation of NT-GFP in the presence of LMB (Fig. 4C, middle). CHX treatment has no additional short-term effect on the distribution of NT-GFP, irrespective of any subsequent treatment (Fig. 4C, right). These results show that in HeLa cells, at least, NT-GFP can shuttle between the nucleus and the cytoplasm and that signals sufficient for nuclear import and export are present in the NT-region of Xp54.

Mutation of the leucine-rich NES results in nuclear accumulation of NT-GFP

Pinpointing the nuclear-export function within leucine-rich sequence was achieved by mutating potentially critical leucine residues to valines. Described here are the effects of mutating both L_{156} and L_{158} to give an NES– form of NT-GFP (Fig. 5). These mutations were selected to have no obvious effect on the structure of the protein. The nucleocytoplasmic distribution of proteins expressed in oocytes shows a shift to nuclear accumulation of the NES– mutant compared with non-mutated NT-GFP and myc-tagged GFP, which represents a diffusible protein (Fig. 5A). The effect seen here with the NES– mutant is directly comparable with that of LMB treatment on non-mutated NT-GFP (Fig. 3B, right), indicating that the mutated sites are required, in non-mutated forms, for recognition by CRM1. Whereas CHX treatment has little effect on the distribution of non-mutated NT-GFP, it enhances the nuclear:cytoplasmic ratio of the NES– mutant, presumably owing to further depletion of the cytoplasmic pool on inhibition of protein synthesis. However, it is obvious that, as with LMB treatment of oocytes, mutation of the NES does not completely inhibit export of the protein.

On examining cotranslocation of antibody injected in the cytoplasm, it is seen that the NES- mutation has the effect of enhancing nuclear accumulation of anti-myc IgG (Fig. 5B, left). By injecting anti-myc IgG into the nucleus, a comparison can be made between the ability of NT-GFP and the NES– mutant to translocate the antibody to the cytoplasm. About ten times more IgG can be translocated to the cytoplasm in 16 hours in oocytes expressing NT-GFP than in oocytes expressing the NES– mutant (Fig. 5B, right). The antibody is translocated from neither cytoplasm nor nucleus in oocytes expressing myc-tagged GFP (Fig. 5B). Expression of the NES– mutant in HeLa cells results in nuclear accumulation of the GFP signal (Fig. 5C). These results are all consistent with the interpretation that the leucine-rich NES contributes to nuclear export of NT-GFP by the CRM1 pathway.

The C-terminal region of recombinant Xp54 can act as a cytoplasmic retention signal

The ability of NT-GFP to shuttle between the nucleus and the cytoplasm, with an equilibrium towards cytoplasmic accumulation, which is blocked by LMB or NES mutation, does not explain fully the influences determining the subcellular distribution of the complete Xp54 molecule. To address this point, constructs were made to express T7 epitope-tagged proteins with and without the carboxy-terminal (CT) region (Fig. 2A). As already noted, deletion of the enzyme core, including the putative NES, results in a small protein of 26 kDa, T7-ΔV, which is restricted to the cytoplasm of oocytes (Fig. 2B) and HeLa cells (not shown). The whole protein, T7-p54, behaves in a similar fashion (Fig. 2B), its cytoplasmic distribution in HeLa cells not being influenced by treatment by LMB (Fig. 6, left). Replacement of the C-terminal 82 residues of T7-p54 with an unrelated plasmid sequence results in a protein of 52 kDa, T7-ΔCT (Fig. 2A), which is predominantly cytoplasmic, but which now responds to LMB treatment by being accumulated in the nucleus (Fig. 6, middle). Finally, replacement of the CT region of T7-ΔV in the same way results in a protein of 23 kDa, T7-ΔV/ΔCT (Fig. 2A), which is now free to diffuse into the nucleus (Fig. 6, right). Furthermore, the distribution of T7-ΔV/ΔCT is not influenced by treatment with LMB, presumably because this protein lacks the 37 residues of the NT region containing the putative NES. Taken together, these results show that the CT region of Xp54 can be used to restrict proteins to the cytoplasm. It is possible, then, that this region is used in oocytes to regulate release of Xp54 for nuclear import.

Cytoplasmic retention of Xp54 in oocytes is relieved by activating transcription.

Xp54 is characterized as a protein associated with stored mRNA in the cytoplasm of oocytes. Results showing that Xp54 can shuttle between the cytoplasm and the nucleus raise

questions about its possible nuclear functions, particularly, does it interact with the nascent, pre-mRNA transcripts of lampbrush chromosomes?

As already noted, expression of T7-Xp54 in oocytes results in its cytoplasmic accumulation (Fig. 2B). Chromosomes isolated from full-grown oocytes have low levels of transcriptional activity and are often foreshortened owing to partial chromatin condensation. Such chromosomes, isolated from oocytes expressing T7-Xp54, show no evidence of the T7-epitope (Fig. 7A, top row). Likewise, the recombinant protein lacking the enzyme core, T7-ΔV, remains cytoplasmic and, as expected, is not detected on chromosomes (Fig. 7A, middle row). However, replacement of the C-terminal domain of Xp54 with an irrelevant sequence gives a protein, T7-ΔCT, which has access to the nucleus and is detected on chromosomal loops (Fig. 7A, bottom row). The immunoblot shows that T7-ΔCT is unstable in the cytoplasm of oocytes, giving rise to breakdown products, and that nuclear concentration of T7-ΔCT is low (as with transfected HeLa cells not treated with LMB) (Fig. 6, middle) and gives poor chromosome staining (Fig. 7A, bottom row). However, there is poor development of transcribing loops in all of the chromosomes shown in Fig. 7A. It has been noted previously that treatment of stage VI oocytes with inhibitors of histone deacetylase activity, butyric acid (Sommerville et al., 1993) and trichostatin A (TSA) (Ryan et al., 1999), can lead to reactivation of transcriptional activity. Treatment of injected oocytes with 2 ng/ml of TSA for six hours prior to isolating nuclei and making chromosome spreads resulted not only in more transcriptionally active chromosomes but also in nuclear uptake of T7-p54 and localization on the loop RNP matrix (Fig. 7B, top row). T7-ΔV was also imported into the nucleus and localized on chromosomal loops after TSA treatment (Fig. 7B, middle row). Thus the ability of Xp54, and recombinant fragments containing the CT region, to be released from the cytoplasm and enter the nucleus appears to be dependent on transcriptional activity of the endogenous chromatin. On inhibiting RNA polymerase II by treating injected oocytes with 0.5 μg/ml of α-amanitin (α-AM) for six hours, the opposite effect was achieved: the chromosomes condensed, and T7-ΔCT, which had been spontaneously taken up by the nuclei, is lost from the chromosomes (Fig. 7B, bottom row). Therefore, T7-ΔCT, and by implication Xp54, would appear to associate with RNA polymerase II transcripts in response to activation of transcription.

One prediction from the association of Xp54 with nascent transcripts is that the expressed recombinant protein should be found in large nuclear aggregates corresponding to the loop matrix. On expressing T7-p54 in stage VI oocytes, which have a low level of transcriptional activity, almost all of the protein is found in a soluble form or as small complexes sedimenting below 80 S on glycerol gradients: less than 2% of the T7-p54 sediments in large (>300 S) complexes (Fig. 7C, top). However, on expressing T7-p54 in stage VI oocytes that have been activated by treatment with TSA, more than 50% of the protein has moved into the nucleus where almost all is in the pelleted fraction (Fig. 7C, middle and bottom). These observations are consistent with the interpretation that Xp54 is incorporated into transcribing chromatin as already described (Fig. 7B).

T7-Xp54 binds newly synthesized RNA in oocyte nuclei

It has been shown previously that 5-bromo-UTP (BrUTP) injected into *Xenopus* oocytes is incorporated into lampbrush loop transcripts as a marker for RNA polymerase II activity (Gall et al., 1999). Incorporation of BrUTP can be detected using antibodies directed against bromodeoxyuridine, which give a specific crossreaction with nascent transcripts (Fig. 8A). In order to check that Xp54 binds, either directly or indirectly, to nascent pol II transcripts, immunoprecipitation studies were carried out using nuclei isolated from oocytes expressing T7-p54. RNA was double-labelled with BrUTP and ^{32}P-CTP for six hours prior to nuclear isolation to permit precipitation using anti-BrU and the assay of incorporated radioactivity. Co-precipitated protein was detected by immunoblotting. In addition to oocytes expressing T7-p54, oocytes expressing a chromatin-associated protein, maternal histone deacetylase (HDACm) (Ryan et al., 1999), which was also tagged with the T7-epitope, were used as controls. Anti-HDACm detects protein on lampbrush chromosomes with a distribution more coincident with the chromomeric axis than that of nascent transcripts detected with anti-BrU (Fig. 8A). Whereas anti-BrU precipitated roughly equal amounts of radioactive RNA from the nuclear extracts of oocytes expressing either T7-Xp54 or T7-HDACm, anti-BrdU co-precipitated much more Xp54 than HDACm from the corresponding extracts (Fig. 8C, left). Using the same extracts, anti-T7 co-precipitated more radioactive RNA from oocytes expressing T7-Xp54 than from oocytes expressing T7-HDACm, although the two proteins were precipitated in roughly equal amounts (Fig. 8B, right). Nuclear extracts from non-injected oocytes gave little precipitated RNA or protein. These results indicate that there is a specific association of Xp54 with nascent polymerase II transcripts.

Export of mRNA-bound T7-Xp54 is not affected by LMB or mutation of the NES

In order to assess the significance that the CRM1 pathway might have on the export of maternal mRNA, stage III/IV oocytes, expressing T7-p54, were labelled with ^{32}P-CTP in the presence and absence of LMB. Although cytoplasmic accumulation of labelled poly(A)$^+$ RNA appeared to be lower in oocytes treated with LMB, the nucleus:cytoplasm ratio of T7-p54 showed little difference between LMB treated and untreated oocytes (Fig. 9A). What was apparent was that the total amount of T7-p54 present was less, especially on extending the LMB treatment for a further 12 hours. It was concluded that extended treatment with LMB has a detrimental effect on the continuing synthesis or stability of T7-p54 and that this negative effect might extend to the production of mRNA. The contribution of the CRM1 pathway over the longer term is better studied using the NES– mutant. After expression for 60 hours in stage III/IV oocytes, Xp54 is found in both cytoplasmic poly(A)$^+$ and poly(A)$^-$ fractions, whereas T7-p54^{NES-} is found almost exclusively in the poly(A)$^+$ fraction (Fig. 9B). The partitioning of soluble T7-p54^{NES-} in the nucleus and mRNP-incorporated T7-p54^{NES-} in the cytoplasm is clearly shown by sedimentation analysis (Fig. 9B). Thus, mutation of the NES sequence, which traps soluble Xp54 in the nucleus, has little effect on export of poly(A)$^+$ that contains T7-p54^{NES-}. Although we cannot exclude the possibility of native Xp54 being included in these same mRNP particles, the failure of LMB to affect export of T7-p54 (Fig. 9A) makes this less likely. Also, the average sedimentation rate of RNP

particles containing T7-p54 (Fig. 9B; Fig. 7C) appears less than seen with native Xp54 (Fig. 1C), which may relate to an aggregation of particles or interaction with other components that may develop over a longer period in vivo.

The relevance of these results to the packaging and export of maternal mRNAs was checked by RT-PCR from RNA extracted from anti-T7-precipitated cytoplasmic poly(A)$^+$ RNP. DNA fragments of predicted length were specifically generated from primer pairs corresponding to cDNAs encoded by genes essential for meiotic maturation (*cyclin B1* and c-*mos*), cell proliferation during early embryogenesis (c-*fos*) and chromatin remodelling (*histone B4*; data not shown).

Discussion

Xenopus oocytes accumulate mRNA sequences to a pool size of about 2×10^{11} molecules per oocyte. This pool is established relatively early in oogenesis (prior to stage III) and is derived from intense transcriptional activity on the loops of lampbrush chromosomes (Sommerville, 1981). During the remainder of oogenesis, transcriptional activity gradually decreases, replacing some mRNA sequences lost through decay and producing new mRNA species in a stage-specific manner. At any one time, less than 2% of poly(A)$^+$ RNA is assembled in polysomes, the remainder existing in a translationally repressed state as RNPs, eventually to be used, in the absence of further transcription, during oocyte maturation, egg fertilization and early embryogenesis. The molecular composition of these stored, maternal mRNP particles has been a major focus of our studies.

The mRNP particle fraction contains a small set of abundant proteins that appear to be associated with most, if not all, translationally repressed mRNA species. A core group of proteins, originally designated RNP1-4 (Darnbrough and Ford, 1981), has been further characterized: the Y-box-masking proteins FRGY2a/pp60/mRNP4 and FRGY2b/pp56/mRNP3 (Ladomery and Sommerville, 1994; Murray, 1994; Wolffe et al., 1992); the DEAD-box RNA helicase Xp54/RNP2 (Ladomery et al., 1997); and an Xp54 partner protein p52/RNP1 (D.A.S. and J.S., unpublished). Previous studies have shown that FRGY2a/pp60/mRNP4 and FRGY2b/pp56/mRNP3 bind to nascent transcripts of lampbrush chromosomes (Sommerville and Ladomery, 1996) and are exported from the nucleus in association with the RNA products of reporter genes (Braddock et al., 1994; Matsumoto et al., 1998), indicating an early association with endogenous mRNA sequences. A close interaction of these two masking proteins with translationally repressed mRNA is thereafter maintained throughout the storage period. In this report, we describe the occurrence of endogenous Xp54 in the nucleus of stage I-III oocytes and the ability of recombinant Xp54 to be imported into the nucleus to bind to nascent transcripts and to be exported back to the cytoplasm. Because the masking proteins and Xp54 always occur in the same biochemical fractions and are detected at the same cytological locations, it is assumed that they may have co-operative activity in assembling a stable, translationally repressed mRNP, the ‘maskosome’ (Sommerville, 1999).

The permanence of association of Xp54 with the mRNP particle would argue that its helicase activity could be (re)activated at particular times in development, but specific signals for activation remain to be determined. In this report, we describe the effect of the C-terminal tail region on retaining Xp54 in the cytoplasm, thereby restricting its tendency to shuttle between the nucleus and the cytoplasm. The presence of four consensus CK2 protein kinase sites within this region suggests regulation by phosphorylation. In previous studies, it has been shown that the two mRNA masking proteins pp60 and pp56 are the most efficiently phospholabelled proteins through most of oogenesis (Dearsly et al., 1985). Phospholabelling occurs both in vivo and in vitro, by a CK2 catalytic subunit that is an integral component of the mRNP particle (Cummings and Sommerville, 1988). This early and continuing turnover of phosphates is not detected in Xp54. In the present study, we found no effect of activators or inhibitors of CK2 or protein phosphatases on nuclear import or export (results not shown). Since Xp54 is phosphorylated, both in vivo and in vitro, early in oocyte maturation (D.A.S. and J.S., unpublished), when translation of maternal mRNAs is first being activated, we conclude that phosphorylation by a maturation-specific protein kinase may lead to unmasking of mRNAs encoding cell cycle functions.

At the start of this investigation it seemed reasonable to ask if the maternal mRNAs, which are destined to be stored as translationally repressed RNA/protein complexes, have some unique form of nuclear export. Maternal RNA transcripts are distinct, in as much as a major fraction derives from intron-less genes that may employ a rapid alternative export pathway from that of spliced mRNA. For instance, pre-mRNA transcripts from reporter genes containing introns are properly spliced and fed into the pathway leading to translation, whereas transcripts from genes lacking introns or containing only late (3′) introns pass, by default, into the stored mRNP pool (Braddock et al., 1994; Matsumoto et al., 1998). A specific pathway for nuclear export of unspliced transcripts is described for HIV Rev in which a leucine-rich NES in Rev is recognized by the nuclear export receptor CRM1 (Fischer et al., 1995; Fornerod et al., 1997). Here, we have reported the occurrence of a similar leucine-rich sequence in Xp54, the ability of Xp54 to shuttle between cytoplasm and nucleus, the inhibition of nuclear export of Xp54 by LMB, which is a specific inhibitor of CRM1 (Kudo et al., 1998) and the inhibition of nuclear export of Xp54 by mutation of its leucine-rich NES. These various findings suggested that Xp54 might be directly responsible for the nuclear export of maternal mRNA. CRM1 is developmentally regulated during early development of *Xenopus* (Callanan et al., 2000), and the activity of the mRNA-associated helicase An3 is required for its nuclear export in oocytes by CRM1 (Askjaer et al., 2000), although no study has shown, unequivocally, a significant CRM1-dependent mRNA export pathway (Neville and Rosbash, 1999). In examining poly(A)$^+$ fractions from oocytes expressing Xp54, we have found no evidence that nuclear export of the in vivo assembled mRNP complexes is compromised by LMB treatment or mutation of the leucine-rich NES.

We conclude that the leucine-rich sequence may be used to remove excess Xp54 from the nucleus in situations in which nascent transcripts are already saturated with the helicase. The steady-state distribution of Xp54 in both oocytes and culture cells would suggest that nuclear export of overexpressed protein is more efficient than nuclear import. However, nuclear import of recombinant Xp54 can be enhanced by using transcriptionally active (stage III/IV) oocytes or by activating

transcription in stage VI oocytes by treatment with TSA, which is a specific inhibitor of maternal histone deacetylase activity and relaxant of chromatin structure (Ryan et al., 1999). At least some of the transcription-enhanced nuclear import of Xp54 may be due to release from cytoplasmic anchoring by a mechanism so far unknown. As already mentioned, the C-terminal tail region of Xp54 is implicated in cytoplasmic retention, but activation or inhibition of phosphorylation appears to have no effect on its nucleocytoplasmic distribution. Environmental stress has been described as a factor influencing nuclear export of mRNA-binding proteins. For instance, oxidative and osmotic shock, but not UV-irradiation or heat shock, leads to cytoplasmic accumulation of HIV-1 Rev, which can also be induced by treating the cells with inhibitors of CK1/2 (Soros and Cochrane, 2001). Also, the nucleocytoplasmic distribution of hnRNP A1 is influenced by osmotic shock and UV-irradiation, which acts through the MAP kinase kinase $MKK_{3/6}$-p38 signalling pathway to phosphorylate hnRNP A1 and divert it to the cytoplasm (van der Houven van Oordt et al., 2000). However, none of the various environmental stresses applied to oocytes expressing recombinant Xp54, including heat shock, cold shock, centrifugation and osmotic shock, had any effect on its nucleocytoplasmic distribution (data not shown).

Recent reports on orthologues of Xp54 not only support the proposal that these proteins ensure expression of mRNA species required for progress through meiosis but also add new insights into their involvement in mRNA metabolism. As in *Xenopus* oocytes, p54 in mouse oocytes is found associated with a Y-box-masking protein in maternal mRNP particles (Paynton, 1998). In addition, in *Drosophila* ovaries, the p54 orthologue Me31B associates with a Y-box protein yps and prevents premature translation of maternal mRNA as it is transported from nurse cells to the oocyte (Nakamura et al., 2001). In *C. elegans*, the p54 orthologue CGH-1 is present not only in the germline P granules but also in other particles (possibly mRNP) that appear in gonads coincidentally with entry into meiosis (Navarro et al., 2001). In addition, a novel function has been attributed to CGH-1, in that it is required to prevent a physiological germline apoptosis mechanism from killing all oocytes (Navarro et al., 2001). A mechanistic function for the p54 orthologue Dhh1p in *S. cerevisiae* has been demonstrated by its ability to interact with the decapping proteins Dcp1p, LSM1p and Pat1p, to stimulate decapping of mRNA and possibly to control translation (Coller et al., 2001). Furthermore, Dhh1p has been found to bind not only to the decapping enzyme in yeast but also to the major RNA deadenylase (Coller et al., 2001). A link between $poly(A)^+$ tail modification, p54 helicase activity and translation control is suggested by recent evidence showing that Xp54 and its orthologue p47 of *Spisula* both interact with the polyadenylation element binding protein CPEB (Minshall et al., 2002).

In *Xenopus* oocytes, Xp54 is a component of mRNP particles encoding proteins, such as Mos, cyclinB1, Fos and histone B4 (D.A.S. and J.S., unpublished), all of which have to be tightly regulated during meiotic maturation and early development. Whatever their specialized functions in different organisms, it must be recognized that the DDX6-type RNA helicases are key components of mRNP particles, ensuring appropriate translation of mRNA in meiotic cells.

We thank Javier Cáceras, for the T7-tag expression vector pCGT, Mike Klymkowski for the GFP expression vector pCS*mt-SGP, Minori Yoshida for generous gifts of leptomycin B and trichostatin A, Keith Blackwell, Jeff Coller and Nancy Standart for discussion and communicating unpublished results and Mike Ladomery for early contributions to the project. This work was supported by The Wellcome Trust.

References

Akao, Y., Marukawa, O., Morikawa, H., Nakao, K., Kamei, M., Hachiya, T. and Tsujimoto, Y. (1995). The rck/p54 candidate proto-oncogene product is a 54-kD DEAD box protein differentially expressed in human and mouse tissues. *Cancer Res.* **55**, 3444-3449.

Askjaer, P., Rosendahl, R. and Kjems, J. (2000). Nuclear export of the DEAD box An3 protein by CRM1 is coupled to An3 helicase activity. *J. Biol. Chem.* **275**, 11561-11568.

Bellini, M. and Gall, J. G. (1999). Coilin shuttles between the nucleus and cytoplasm in *Xenopus* oocytes. *Mol. Biol. Cell* **10**, 3425-3434.

Braddock, M., Muckenthaler, M., White, M. R. H., Thorburn, A. M., Sommerville, J. Kingsman, A. J. and Kingsman, S. M. (1994). Intronless RNA injected into the nucleus of *Xenopus* oocytes accesses a developmentally regulated translation control pathway. *Nucleic Acids Res.* **22**, 5225-5264.

Braun, I. C., Herold, A., Rode, M., Conti, E. and Izaurralde, E. (2001). Overexpression of TAP/p15 heterodimers bypasses nuclear retention and stimulates nuclear mRNA export. *J. Biol. Chem.* **23**, 20536-20543.

Callanan, M., Kudo, N., Gout, S., Brocard, M., Yoshida. M., Dimitrov, S., Khochbin, S. (2000). Developmentally regulated activity of CRM1/XPO1 during early *Xenopus* embryogenesis. *J. Cell Sci.* **113**, 451-459.

Coller, J. M., Tucker, M., Sheth, U., Valencia-Sanchez, M. A. and Parker, R. (2001). The DEAD box helicase, Dhh1p, functions in mRNA decapping and interacts with both the decapping and deadenylase complexes. *RNA* (in press).

Conti, E. and Izzaurralde, E. (2001). Nucleocytoplasmic transport enters the atomic age. *Curr. Opin. Cell Biol.* **13**, 310-319.

Cummings, A. and Sommerville, J. (1988). Protein kinase activity associated with stored messenger ribonucleoprotein particles of *Xenopus* oocytes. *J. Cell Biol.* **107**, 45-56

Darnbrough, C. H. and Ford, P. J. (1981). Identification in *Xenopus laevis* of a class of oocyte-specific proteins bound to messenger RNA. *Eur. J. Biochem.* **113**, 415-426.

Dearsly, A. L., Johnson, R. M., Barrett, P. and Sommerville, J. (1985). Identification of a 60 kDa phosphoprotein that binds stored messenger RNA of *Xenopus* oocytes. *Eur. J. Biochem.* **150**, 95-103.

de la Cruz, J., Kressler, D. and Linder, P. (1999). Unwinding RNA in *Saccharomyces cerevisiae*: DEAD-box proteins and related families. *Trends Biochem. Sci.* **24**, 192-198.

Deschamps, S., Viel, A., Garrigos, M., Denis, H. and Le Maire, M. (1992). mRNP4, a major mRNA-binding protein from *Xenopus* oocytes is identical to transcription factor FRG Y2. *J. Biol. Chem.* **267**, 13799-13802.

de Valoir, T., Tucker, M. A., Belikoff, E. J., Camp, L. A., Bolduc, C. and Beckingham, K. (1991). A second maternally-expressed *Drosophila* gene encodes a putative RNA helicase of the DEAD-box family. *Proc. Natl. Acad. Sci. USA* **88**, 2113-2117.

Dumont, J. N. (1977). Oogenesis in *Xenopus laevis*. *J. Morphol*. **136,** 153-180.

Evans, J. P. and Kay, B. K. (1991). Biochemical fractionation of oocytes. *Methods Cell Biol.* **36**, 133-148.

Fischer, U., Huber, J., Boelens, W. C., Mattaj, I. W. and Luhrmann, R. (1995). The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. *Cell* **82**, 475-483.

Fornerod, M., Ohno, M., Yoshida, M. and Mattaj, I. W. (1997). CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell* **90**, 1051-1060.

Gall, J. G., Bellini, M., Wu, Z. and Murphy, C. (1999). Assembly of the nuclear transcription and processing machinery: Cajal bodies (coiled bodies) and transcriptosomes. *Mol. Biol. Cell* **10**, 4385-4402.

Hake, L. E. and Richter, J. D. (1994). CPEB is a specificity factor that mediates cytoplasmic polyadenylation during *Xenopus* oocyte maturation. *Cell* **79,** 17-27.

Hauer, J. A., Barthe, P., Taylor, S. S., Parello, J. and Padilla, A. (1999).

Two well-defined motifs in the cAMP-dependent protein kinase inhibitor (PKIα) correlate with inhibitory and nuclear export functions. *Protein Sci.* **3**, 545-553.

Hurt, E., Strässer, K., Segref, A., Bailer, S., Schlaich, N., Presutti, C., Tollervey, D. and Jansen, R. (2000). Mex67p mediates nuclear export of a variety of RNA polymerase II transcripts. *J. Biol. Chem.* **275**, 8361-8368.

Kang, Y. and Cullen, B. R. (1999). The human Tap protein is a nuclear mRNA export factor that contains novel RNA-binding and nucleocytoplasmic transport sequences. *Genes Dev.* **13**, 1126-1139.

Kick, D., Barrett, P., Cummings, A. and Sommerville, J. (1987). Phosphorylation of a 60 kDa polypeptide from *Xenopus* oocytes blocks messenger RNA translation. *Nucleic Acids Res.* **15**, 4099-4109.

Klymkowsky, M. W., Williams, B. O., Barish, G. D., Varmus, H. E. and Vourgourakis, Y. E. (1999). Membrane-anchored plakoglobins have multiple mechanisms of action in Wnt signaling. *Mol. Biol. Cell* **10,** 3151-3169.

Kudo, N., Wolff, B., Sekimoto, T., Schreiner, E. P., Yoneda, Y., Yanagida, M., Horinouchi, S. and Yoshida, M. (1998). Leptomycin B inhibition of signal-mediated nuclear export by direct binding to CRM1. *Exp. Cell Res.* **242,** 549-547.

Ladomery, M. and Sommerville, J. (1994). Binding of Y-box proteins to RNA: involvement of different protein domains. *Nucleic Acids Res.* **22**, 5582-5589.

Ladomery, M., Wade, E. and Sommerville, J. (1997). Xp54, the *Xenopus* homologue of human RNA helicase p54, is an integral component of stored mRNP particles in oocytes. *Nucleic Acids Res.* **25**, 965-973.

Maekawa, H., Nakagawa, T., Uno, Y., Kitamura, K. and Shimoda, C. (1994). The *ste13*$^{+}$ gene encoding a putative RNA helicase is essential for nitrogen starvation-induced G1 arrest and initiation of sexual development in the fission yeast *Schizosaccharomyces pombe*. *Mol. Gen. Genet.* **244**, 456-464.

Marello, K., LaRovere, J. and Sommerville, J. (1992). Binding of *Xenopus* oocyte masking proteins to mRNA sequences. *Nucleic Acids Res.* **20**, 5593-5600.

Matsumoto K., Wassarman, K. M. and Wolffe, A. P. (1998). Nuclear history of a pre-mRNA determines the translational activity of cytoplasmic mRNA. *EMBO J.* **17**, 2107-2121.

Minshall, N., Thom, G. and Standart, N. (2002). A conserved role of a DEAD-box helicase in mRNA masking. *RNA* (in press).

Murray, M. T. (1994). Nucleic acid binding properties of the *Xenopus* oocyte Y-box protein mRNP3+4. *Biochemistry* **33**, 13910-13917.

Murray, M. T., Schiller, D. L. and Franke, W. W. (1992). Sequence analysis of cytoplasmic mRNA-binding proteins of *Xenopus* oocytes identifies a family of RNA-binding proteins. *Proc. Natl. Acad. Sci. USA* **89**, 11-15.

Nakamura, A., Amikura, R., Hanyu, K. and Kobayashi, S. (2001). Me31B silences translation of oocyte-localizing RNAs through the formation of cytoplasmic RNP complexes during *Drosophila* oogenesis. *Development* **128**, 3233-3242.

Navarro, R. E., Shim, E. Y. Kohara, Y., Singson, A. and Blackwell, T. K. (2001). *Cgh-1*, a conserved predicted RNA helicase required for gametogenesis and protection from physiological germline apoptosis in *C. elegans*. *Development* **128**, 3221-3232.

Neville M. and Rosbash M. (1999). The NES-Crm1p export pathway is not a major mRNA export route in *Saccharomyces cerevisiae*. *EMBO J*. **18**, 3746-56.

Paine, P. L., Johnson, M. E., Lau, Y.-T., Tluczek, L. J. M. and Miller D. S. (1992). The oocyte nucleus isolated in oil retains *in vivo* structure and functions. *BioTech.* **13**, 238-246.

Paynton, B. V. (1998). RNA-binding proteins in mouse oocytes and embryos: expression of genes encoding Y-box, DEAD-box RNA helicases and poly(A)-binding proteins. *Dev. Genet.* **23**, 285-298.

Richter, J. D. and Smith, L. D. (1984). Reversible inhibition of translation by *Xenopus* oocyte-specific proteins. *Nature* **309**, 378-380.

Ryan, J., Llinas, A. J., White, D. A., Turner, B. M. and Sommerville, J. (1999). Maternal histone deacetylase is accumulated in the nuclei of *Xenopus* oocytes as protein complexes with potential enzyme activity. *J. Cell Sci.* **112**, 2441-2462.

Smith J. C. and Tata J. R. (1991). *Xenopus* cell lines. *Methods Cell Biol.* **36**, 635-654.

Sommerville, J. (1981). Immunolocalization and structural organization of nascent RNP. In *The Cell Nucleus* **8,** (ed. H. Busch), pp. 1-57. Academic Press, New York.

Sommerville, J. (1990). RNA binding phosphoproteins and the regulation of maternal mRNA in *Xenopus*. *J. Reprod. Fert*. **42**, 225-233.

Sommerville, J. (1999). Activities of cold-shock domain proteins in translation control. *Bioessays* **21**, 281-297.

Sommerville, J., Baird, J. and Turner, B. M. (1993). Histone H4 acetylation and transcription in amphibian oocytes. *J. Cell Biol.* **120**, 277-290.

Sommerville, J. and Ladomery, M. (1996). Transcription and masking of mRNA in germ cells: involvement of Y-box proteins. *Chromosoma* **104**, 469-478.

Soros, V. and Cochrane, A. (2001). Alterations in HIV-1 Rev transport in response to cell stress. *Virology* **280**, 199-210.

Standart, N. (1992). Masking and unmasking of maternal mRNA. *Semin. Dev. Biol.* **3**, 367-379.

Stebbins-Boaz, B., Cao, Q., de Moor, C. H., Mendez, R. and Richter, J. D. (1999). Maskin is a CPEB-associated factor that transiently interacts with eIF4E. *Mol. Cell* **4**, 1017-1027.

Stutz, F., Bachi, A., Doerks, T., Braun, I. C., Séraphin, B., Wilm, M., Bork, P., Izaurralde, E. (2000). REF, an evolutionary conserved family of hnRNP-like proteins, interacts with TAP/Mex67p and participates in mRNA nuclear export. *RNA* **6**, 638-650.

Tafuri, S. R. and Wolffe, A. P. (1990). *Xenopus* Y-box transcription factors: molecular cloning, functional analysis and developmental regulation. *Proc. Natl. Acad. Sci. USA* **87**, 9028-9032.

van der Houven van Oordt, W., Diaz-Meco, M., Lozano, J., Krainer, A., Moscat, J. and Cáceras, J. F. (2000). The MKK3/6-p38-signaling cascade alters the subcellular distribution of hnRNP A1 and modulates alternative splicing. *J. Cell Biol.* **149**, 307-316.

Wolffe A. P., Tafuri, S., Rajan, M. and Familiari, M. (1992). The Y-box factors: a family of nucleic acid binding proteins conserved from *Escherichia coli* to man. *New Biol.* **4**, 290-298.

Wylie, C. (2000). Germ cells. *Curr. Opin. Genet. Dev.* **10**, 410-413.

Zelus B. D., Giebelhaus, D. H., Eib, D. W., Kenner, K. A. and Moon, R. T. (1989). Expression of the poly(A)-binding protein during development of *Xenopus laevis*. *Mol. Cell. Biol.* **6**, 2756-2760.