Evidence for overlapping, but not identical, protein machineries operating in vegetal RNA localization along early and late pathways in *Xenopus* oocytes

Maike Claußen, Katja Horvay and Tomas Pieler*

Institut für Biochemie und Molekulare Zellbiologie, Georg-August-Universität Göttingen, Justus-von-Liebig Weg 11, 37077 Göttingen, Germany
*Author for correspondence (e-mail: tpieler@gwdg.de)

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Summary

RNAs that localize to the vegetal cortex of *Xenopus* oocytes are involved in early embryonic patterning and cell fate specification. Two mechanistically distinct pathways lead to RNA enrichment at the vegetal cortex: the early and the late. While several candidate proteins that seem to operate in the late localization pathway have been identified, proteins involved in the early pathway remain to be identified. In this study, we report on the isolation of a novel vegetally localized RNA in *Xenopus* oocytes that makes use of the early pathway and encodes a protein with a conserved but functionally uncharacterized NIF-motif. The localization signal of XNIF was mapped to a 300-nucleotide region in the 5′-UTR, which is able to mediate both accumulation to the mitochondrial cloud in stage I oocytes, as well as vegetal transport in later stage oocytes. The XNIF-LE contains 16 copies of the previously defined CAC-containing signal motifs for RNA localization. A critical number of such repeats seems to be required for accumulation in the mitochondrial cloud along the early pathway, but additional repeats seem to be required for localization along the late pathway. Cross-linking experiments identify two novel proteins of 62 and 64 kDa that interact with the XNIF-LE but not with the Vg1-LE that operates in the late pathway. Conversely, at least two of the previously identified VgRBPs, Vg1RBP1 and Prrp, also bind to the XNIF-LE. Thus, overlapping, but not identical, protein machineries mediate vegetal RNA localization along early and late pathways in *Xenopus* oocytes.

Key words: RNA localization, RNA transport, *Xenopus laevis*, Oogenesis

Introduction

The subcellular localization of mRNAs is a common mechanism for generating cellular asymmetries in somatic cells, as well as for establishing polarities during early embryogenesis, when localized RNAs become inherited by a restricted population of cells and initiate regional differences in gene expression and cell fate. Localized RNAs have been found in a variety of different cell types and organisms ranging from yeasts to mammals.

In *Xenopus*, vegetally localized RNAs have been implicated in playing a role in early embryonic patterning and cell fate determination. There are two major pathways that mediate RNA localization to the vegetal cortex during oogenesis (reviewed by Kloc et al., 2001; Rand and Yisraeli, 2001). During the earliest stages of oogenesis (stage I/II), the first one of the localization pathways, the early one, utilizes a structure referred to as the mitochondrial cloud (Balbiani body), which is composed of mitochondria, lipids and diverse electron-dense materials. Most of the early pathway RNAs are later restricted to the germinal granules in the distal region of the vegetal cortex and seem to be involved in germ cell determination (for a review, see Kloc et al., 2001). The second pathway, which operates during stages III-IV, is referred to as the late pathway and localizes mRNAs encoding developmental determinants such as Vg1, VegT and XBic-c to the vegetal pole of the oocyte (Weeks and Melton, 1987; Wessely and De Robertis, 2000; Zhang and King, 1996). In stage I oocytes these RNAs are found to be dispersed throughout the cytoplasm but are excluded from the mitochondrial cloud, where at this time point the early pathway RNAs are already enriched. Starting with late stage II/stage III of oogenesis, the late pathway RNAs first localize to the wedge-shaped region beneath the germinal vesicle that has previously been occupied by the mitochondrial cloud, and are subsequently transported towards the vegetal hemisphere, where they remain until the end of oogenesis. Characteristically, late pathway RNAs occupy a broader region of the vegetal hemisphere in stage VI oocytes than the early pathway RNAs, which are restricted to a narrower region at the tip of the vegetal cortex. However, the early and late localization pathways seem to overlap in some respect, since several RNAs have been described as exhibiting features of both localization pathways and are therefore thought to make use of an intermediate pathway (Betley et al., 2002; Chan et al., 1999).

RNA transport to distinct subcellular foci has been found to be mediated by cis-acting sequences, which usually reside in the 3′-untranslated region of the RNA to be localized. The 340

nucleotide localization element of the Vg1 mRNA (Vg1-LE) has been mapped by microinjection experiments and possesses a number of different subelements with redundant function (Cote et al., 1999; Deshler et al., 1998; Mowry and Melton, 1992; Yaniv and Yisraeli, 2001). Based on the observation that the late localizing VegT-RNA contains reiterated VM1 and E2 elements, it has been proposed that these may serve as consensus signals for late pathway RNAs (Bubunenko et al., 2002; Kwon et al., 2002). A detailed analysis of the localization element of an early pathway RNA has been performed with Xcat2, which contains a composite localization element in its 3′-untranslated region, consisting of a mitochondrial cloud localization element (MCLE) (Zhou and King, 1996a) and an additional, independent element, which directs the mRNA to the germinal granules within the germ plasm-containing region of the mitochondrial cloud (Kloc et al., 2000). On the basis of statistical analysis, it has been proposed that clusters of CAC-containing motifs characterize the localization elements of the majority of RNAs localizing to the vegetal cortex in *Xenopus* oocytes (Betley et al., 2002). Interestingly, this seems to hold true for vegetal RNAs belonging to the early, late or intermediate localization pathway.

Candidate trans-acting factors that interact with localization elements and may mediate the directional transport of the RNAs have first been identified by UV cross-linking experiments; six proteins showed a specific cross-linking activity to the 340 nucleotide Vg1-LE (Mowry, 1996). Four proteins that bind to the Vg1-LE have been identified. One is Vg1RBP, or Vera, which contains two RRM domains and four KH domains (Deshler et al., 1998; Havin et al., 1998). The second protein is VgRBP60, a homolog of hnRNP I, which contains four RRM domains (Cote et al., 1999). Two additional Vg1-LE-interacting proteins, Prrp and the recently reported VgRBP71, have been identified by screening a cDNA expression library for Vg1 RNA LE interacting proteins (Kroll et al., 2002; Zhao et al., 2001). The proline-rich RNA-binding protein Prrp contains two RNP domains, as well as a C-terminally located proline-rich region. VgRBP71 is a KH-domain protein, which is highly homologous to the human FUSE-binding protein or KSRP. Very recently, it has been shown that VgRBP71 acts as a translational activator by promoting the cleavage of a translational control element of Vg1 mRNA, rather than directly participating in the vegetal transport steps themselves (Kolev and Huber, 2003). Vg1RBP and VgRBP71 have been reported to bind RNAs of both localization pathways (Havin et al., 1998; Kroll et al., 2002), whereas Prrp seems to have binding preferences for the late pathway RNAs Vg1 and VegT, but associates also, like VgRBP71, with RNAs localized to the animal hemisphere (Zhao et al., 2001).

We have screened a vegetal cortex cDNA library for novel localized maternal mRNAs. One of these is XNIF, encoding an evolutionary conserved protein of unknown biological function. Transport to the vegetal pole of *Xenopus* oocytes is mediated via a 300-nucleotide sequence element located in the 5′-UTR of XNIF. A subdomain of this element is sufficient to drive transport to the vegetal cortex but is not sufficient to mediate accumulation in the mitochondrial cloud. Co-immunoprecipitation assays, as well as UV cross-linking, reveal a protein-binding pattern that is overlapping, but not identical, with the one obtained for the localization element of the late pathway mRNA encoding Vg1.

Materials and methods

RNA isolations and cDNA library preparations

Oocytes were obtained from pigmented or albino *Xenopus laevis* as described previously (Claussen et al., 1999). To prepare a cDNA library enriched for cortically and vegetally localized RNAs, vegetal oocyte cortices and associated RNAs were prepared in principle as described in Zhang and King (1999). Of this preparation, 2 μg total RNA were used for the construction of a SMART cDNA phage library (Clontech) according to the manufacturer's protocol. To prepare a cDNA library representing all transcripts present in the *Xenopus laevis* oocyte, approximately 0.5 ml collagenase-treated stage V/VI oocytes were homogenized in a total volume of 5 ml extraction buffer (50 mM Tris/HCl, pH 7.5; 5 mM EDTA, pH 8.0; 40 mM NaCl; 0.5% SDS) containing 500 μl proteinase K and incubated for 1 hour at 45°C. After several extraction steps, the RNA was LiCl-precipitated, dissolved, re-precipitated with ammonium acetate and treated with DNAse. Poly $(A)^+$ RNA was isolated on oligo dT-cellulose according to Sambrook et al. (1998), and 5 μg were used for the construction of a λZAP cDNA library according to the manufacturer's protocols (Stratagene).

Preparation of antisense RNA probes and whole-mount in-situ hybridization

For the in-situ hybridization-based screening, the phage cDNA library was subjected to a mass excision in BM25.8 cells (according to the Clontech protocol) and single bacterial colonies were then picked and grown in 100 μl LB medium in microtiter plates. These bacterial suspensions served as templates for insert amplification by PCR using the following vector-specific primers: 5′λTriplEx2-LD, CTCGGGAAGCGCGCCATTGTGTTGGT and 3′λTriplEx2Seq, TAATACGACTCACTATAGGGC. These PCR products then served as templates for the subsequent in-vitro transcription of labeled whole-mount in-situ antisense probes (Hollemann et al., 1999). Alternatively, the antisense transcripts were generated from linearized plasmids containing either the full-length sequence or fragments of the corresponding cDNAs. Whole-mount in-situ hybridizations were in principle carried out as described (Harland, 1991; Hollemann et al., 1999). After the color reaction, the specimens were photographed using a digital video imaging system. For sections, whole-mount in-situ stained oocytes were embedded in gelatine/albumine, and vibratome sections of 10-30 μm were done.

Cloning procedures

For localization element mapping experiments the vector pBK-CMV (Stratagene) was used for subcloning different subfragments of the XNIF cDNA sequences. For the detection of injected RNAs all constructs contain a 320 nt fragment of the open reading frame of the bacterial lacZ gene, which has been amplified from the pAX4a plasmid (Markmeyer et al., 1990) with the following primers: forward primer 5′-TTGGCGCGCATGATTACGGATTCACTGGCCG; reverse primer 5′-CGGGATCCGACCGTAATGGGATAGGTTACG and inserted into *Bss*HII- and *Bam*HI-sites of the corresponding pBK-CMV plasmid (pBK-CMV-lacZ). Similarly, a fragment spanning the XNIF-ORF was cloned into a pGEM-T-plasmid, which also contained the 320 nt lacZ-fragment. Subfragments of the XNIF cDNA were generated by PCR and ligated into *Bam*HI/*Xho*I sites of the pBK-CMV-lacZ. The Vg1-LE was amplified with the following PCR primers from a cDNA clone of the vegetal cortex library that contains the 3′-UTR of Vg1-LE in addition to a fragment of the coding region: 5′-CGGGATCCTATTTCTACTTTATTTCTACACTG and 5′-CCGC-TCGAGCAAGTCATATGGACTATTATATAT and ligated into *Bam*HI/*Xho*I sites of the pBK-CMV-lacZ.

For co-immunoprecipitation experiments, the proteins had to be produced by in-vitro translation and required an epitope tag for the immobilization to the protein G sepharose. In order to obtain an in-vitro translatable myc-tagged version of the Vg1RBP protein, a *Nco*I/*Xho*I fragment of the corresponding Vg1RBP cDNA in pET21a+ vector (Havin et al., 1998) was subcloned into the pCS2+MT vector (Rupp et al., 1994). For the in-vitro translation of Prrp, we used the pMT-21 plasmid, which contains the Prrp coding sequence fused to a repeated myc epitope tag at its C-terminus (Zhao et al., 2001). For the in-vitro translation of VgRBP 71/KSRP, the corresponding coding sequence was cut out with *Nde*I and *Xho*I from the pET23a-KSRP vector (a kind gift from Paul W. Huber), overhanging ends were filled in by Klenow polymerase and ligated into the *Xho*I linearized and filled in pCS2+MT. The poly-myc-tagged ribosomal protein L5 served as a negative control in this study (Claussen et al., 1999).

Synthesis, injection and detection of lacZ-tagged RNA constructs

Capped RNAs for injection into *Xenopus* oocytes were prepared by in-vitro transcription using the T3 or T7 mESSAGE mMACHINE kit (Ambion) according to the manufacturer's instructions. Oocytes were obtained from albino female *Xenopus laevis* and staged either by visual inspection or by using nylon sieves with different mesh sizes. Injections were performed using an Eppendorf Transjector 5246 injection system. Depending on the oocyte size, 0.05-0.1 ng RNA in a 0.5-1 nl volume were injected into the nuclei. Injected oocytes were incubated at 18°C for 2-3 days in vitellogenin-enriched L-15 culture medium as described previously (Wallace et al., 1980; Yisraeli and Melton, 1988). Frog vitellogenin was prepared as described in Kloc and Etkin (1999). Injected RNAs were detected by whole-mount in-situ hybridization using a digoxygenin-labeled lacZ antisense probe.

UV cross-linking assays and co-immunoprecipitation experiments

For the preparation of *Xenopus* oocyte S100 extracts, collagenase-treated oocytes (stage I-IV) were homogenized on ice with syringe needles of different sizes in an equal volume of S100 buffer (50 mM Tris/HCl, pH 8.0; 50 mM KCl; 0.1 M EDTA; 25% w/v glycerol; protease inhibitors). After a 15 minute centrifugation in a tabletop cooling centrifuge (1900 ***g***), the supernatant was centrifuged for 2 hours at 10,0000 ***g*** in a Beckmann ultracentrifuge. The protein concentration was measured by the Bradford method and aliquots were frozen in liquid nitrogen and stored at –70°C until use. The S100 extracts were typically 10-15 mg/ml in total protein. Radioactively labeled RNA probes were synthesized by in-vitro transcription using the Stratagene T3 or T7 in-vitro transcription Kit, according to the standard reaction protocol. For each labeling reaction 5 μl of 20 μCi/μl [α ^{32}P]UTP (Amersham Biosciences) were used. Unincorporated nucleotides were removed either by MicroSpin G-50 columns (Amersham Biosciences) or by using RNeasy mini columns (Qiagen). In-vitro UV cross-linking reactions were performed based on the protocol described in Mowry (1996) and contained 5 mg/ml heparin, 1% glycerol, 50 mM KCl, 10 mM DTT, 5.2 mM HEPES [pH 7.9], 40 μg/ml yeast tRNA as competitor, different specific competitor RNAs (2.25-2.5 μg) and 25% S100 oocyte total protein extract in an end volume of 10 μl. The in-vitro binding reactions were pre-incubated for 10 minutes at room temperature prior to adding the radioactively labeled RNA transcripts (~0.2 μg). After 10 minutes incubation at room temperature, RNAs were UV-irradiated for 10 minutes at room temperature in a Stratalinker (Stratagene) with a 9 cm distance from the bottom of the reaction tube to the UV bulbs. Afterward 1 μl RNAse A (1 mg/ml) and 1 μl RNAse T1 (10,000 U/ml) were added and incubated for 15 minutes at 37°C. The cross-linked proteins were separated by SDS-PAGE (10% gels) and analyzed by phospho-imaging (Molecular Dynamics).

For the co-immunoprecipitation experiments, myc-tagged Vg1RBP, Prrp, VgRBP71 and L5 were in-vitro translated using the T_NT coupled transcription and translation system (Promega). Then 1 μl containing 200,000-250,000 cpm of the appropriate ^{32}P-labeled RNAs as well as 2 μl of 5×UV cross-linking mix as described above, were added to a 12.5 μl T_NT reaction and adjusted to a total volume of 20 μl and incubated for 1 hour at 20°C. Afterward the binding reactions were transferred onto an anti-myc-immunopellet in 400 μl NET 2 buffer (50 mM Tris/HCl, pH 7.4; 150 mM NaCl; 0.05% NP40; protease inhibitors). For preparation of anti-myc-immunopellets, 15 μl of protein G sepharose (Amersham Biosciences) and 1 μl of a monoclonal anti-c-myc antibody M5546 (Sigma) per pellet were used. Immunoprecipitations were incubated with rotation for 1 hour at 20°C. The supernatant (20 μl) containing unbound RNA was phenol/chloroform extracted and analyzed on a denaturing PA-gel. The pellets were washed three times with NET 2 and after adding 200 μl NET 2 containing 1% SDS the bound RNA was phenol/chloroform extracted, precipitated and analyzed by denaturing urea PAGE (6-8%) and phospho-imaging.

Results

XNIF is a member of a new protein family

In order to isolate novel vegetally localized maternal RNAs in *Xenopus*, we prepared a cDNA library enriched for RNAs associated with the vegetal cortex of the oocyte. Of this library, 400 randomly selected single clones were analyzed for their localization in *Xenopus* oocytes by whole-mount in-situ hybridization. In addition to the re-isolation of several known vegetally localizing transcripts, such as Vg1 and VegT, four novel localized RNAs were identified. One of these followed all characteristics of an early localization pathway RNA. The clone isolated from the vegetal cortex library represented only a partial cDNA sequence, and therefore the corresponding full-length cDNA was isolated from a *Xenopus laevis* oocyte cDNA library by PCR-mediated screening methods. This cDNA comprised 2600 base pairs in length and included a remarkably long 5′-UTR (nt 1-1308), an open reading frame of 828 base pairs (nt 1309-2136) and a 466 base pair 3′-UTR (nt 2137-2601) (Fig. 1A). Conceptual translation of the open reading frame revealed a protein sequence of 276 amino acids in length with a calculated molecular mass of 31.2 kDa (Fig. 1A). A database search for similar proteins and for conserved protein motifs using the BLAST program (Altschul et al., 1990) revealed several homologs from different species. The highest similarities were found with one of several isoforms of the so-called chicken NLI-interacting factor NIF (AF189773), and the clone was therefore referred to as *Xenopus* NIF (XNIF) (Fig. 1A). A protein alignment based on the clustal method revealed the following degrees of identity: chicken NIF isoform T1 (AF189773), 86.5%; mouse NIF-like protein (AJ344340), 78.3%; hypothetical human protein Hya22 (NM_005808), 76.2%; *Xenopus laevis* XOs4 (AF441288), 57.6%; and *Xenopus laevis* Dullard, which has only a 31.1% overall identity. Highest similarities have been found in the highly conserved, but functionally so far undefined, C-terminally located so-called NIF domain (XOs4 75% and Dullard 42.1% identity, respectively). The XNIF domain (highlighted in Fig. 1A) contained a conserved phosphatase DXDXT/V motif, which is boxed in Fig. 1A,B. An alignment of the NIF domains of NIF-like proteins from different species is shown in Fig. 1B. Several XNIF-related hypothetical proteins have also been identified in other eukaryotic species, such as *Drosophila melanogaster*, *Anopheles gambiae*, *Caenorhabditis elegans*,

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        TCCGACCTTAGACTCGACCGCCCAACCGACAGGCCCTCAGCCTTCCATCATCTC
  55 TATATAGCGGTGTTTGCTCTTCCAGGGAACCCCCCTCTTCCCTCACAGCGCCCCGCCTGG
 115 GCTTCATGCTGAGCAAGAGAAGGAAAGAGCAGGGAGCCGACCCGGCCTCGGACACACGGG
 175 GGATTCTGAGCCAACCGACACATTGGGACTTCTCCTCAGGCGGATAGAACTCTCCCTCCA
 235 TCTTCCATTTCACCCTCATTCCCCTCACACACACACACAGGACATAAAGCCATTTCTATA
 295 CACGGCCTGACTCCCAGTATTGATATACACACGTTATATTCACCTTTTATTCACCTACTG
 355 ACCCTGCCATCTCCCCCTCCTACACCAACATCACTATATATACTTCCCTCATTCTCCAGC
 415 AGCCTCTATACACCTATTACACACATATATATATATTTATATATTTATATACCTACACAC
 475 ACACATATATATATATATATATATATATCTACACACACATATATACCTACACAGACAGTC
 535 CCTTGCAGCTCAGTATATCCTCTTTATTTGCAGACATTTCCACAGCATAACCCCGAGTCC
 595 AGTCCCTACATCCAATGAGATCACCTGCCATCCGACATACAGGAATACACTGAGCCAGGC
 655 AGAGATCGTCCTCCCATAATACAGGCTTCTAGACCCCTCTGCTGCACAGTCCAGACAGCA
 715 CTACGTACCCTTCCTGCCCATAACATTGGATGCTTTCACCGTACAGCGTTCTTTACTGGC
 775 GGCACCAATTCCGCAAAGTATAGAGCAGAAAAAACGTAGATATTTCTCCACCAGCTCATT
 835 CTCTGCCAGCCTAGCGACATACATAATCGGTGTTTTATAAGCGTATGCGTAGAATTAGGT
 895 CATTTTGTTCGTTAACATTCACATAGAGTTCACTTGCAGTTTGTAGAACATGAACAAATA
 955 GCTCTCTCTCTTTCTCAAATACAACATTGTCTCATCTCCTTATATACATACTTTGCACAC
1015 TCTTCTTCTCCGACATTCAGAGCCAGAACTGCCTCCATTTTCCCCTAACATACACACCAG
1075 CAACTCTGCCACTCTCCCCGGCATATAAAACACTCCAGACAGATTAGACTTCACGGCAGC
1135 AAGCTCTTCTATAAGAAACACCTGCGTAACAGCTTTCACCCTCACAGTGATCCCCCTCAA
1195 GGCACAAAATACACTCTCACCATTTCATACACCCTATAACAGCACTGCATCCCCAAGCCA
                                                          M  D    2
1255 GTCTCGAACTGCGGATTAGATAAAGAGGACAAGAACAACAGCCAATTCGTTTCCATGGAC
       N  T  S  I  I  T  Q  V  S  N  P  K  E  E  G  I  L  S  C  A   22
1315 AACACGTCCATCATCACCCAGGTCTCTAACCCCAAGGAGGAGGGAATCCTGTCCTGTGCC
       Q  E  K  V  S  Q  C  N  I  S  L  K  K  Q  R  N  R  S  I  F   42
1375 CAGGAAAAGGTCTCCCAATGCAATATTAGCCTAAAAAAGCAAAGGAACAGAAGCATCTTC
       G  S  L  F  C  C  F  R  S  Y  S  V  E  P  P  N  S  N  N  N   62
1435 GGCTCCTTATTCTGCTGTTTCCGTAGTTACAGCGTAGAGCCACCAAACTCGAATAATAAC
       S  S  P  L  P  P  L  V  E  E  N  G  G  I  Q  K  G  D  Q  T   82
1495 AGTAGCCCTCTTCCTCCGCTGGTGGAAGAAAATGGGGGCATTCAGAAGGGTGATCAGACT
       Q  A  L  T  I  P  S  P  P  T  K  Y  L  L  P  E  L  K  V  S  102
1555 CAAGCCCTTACCATTCCCAGTCCACCTACTAAATACCTCCTTCCTGAACTGAAAGTATCC
       E  Y  G  K  K  C  V  V  I  D  L  D  E  T  L  V  H  S  S  F  122
1615 GAATATGGGAAGAAGTGCGTGGTCATTGACCTGGATGAAACCTTAGTGCACAGTTCATTT
       K  P  I  N  N  A  D  F  I  V  P  V  E  I  D  G  T  I  H  Q  142
1675 AAGCCTATAAACAACGCAGACTTCATTGTTCCAGTTGAAATAGATGGAACAATACATCAG
       V  Y  V  L  K  R  P  H  V  D  E  F  L  Q  K  M  G  E  M  F  162
1735 GTCTATGTGTTAAAACGACCCCACGTAGATGAATTTCTTCAAAAAATGGGCGAGATGTTT
       E  C  V  L  F  T  A  S  L  A  K  Y  A  D  P  V  A  D  L  L  182
1795 GAATGCGTTCTCTTCACAGCCAGCCTTGCGAAGTATGCCGATCCAGTGGCTGATCTGCTA
       D  R  W  G  V  F  N  A  R  L  F  R  E  S  C  V  F  H  R  G  222
1855 GACCGGTGGGGAGTGTTCAACGCACGACTCTTCCGAGAATCCTGTGTTTTTCACAGGGGA
       N  Y  V  K  D  L  S  R  L  G  R  E  L  S  K  V  I  I  I  D  242
1915 AACTACGTCAAAGATTTAAGCCGCTTAGGTAGAGAACTGAGCAAAGTGATTATAATCGAT
       N  S  P  A  S  Y  I  F  H  P  E  N  A  V  P  V  M  S  W  F  262
1975 AACTCTCCTGCGTCATACATCTTCCATCCAGAGAATGCCGTTCCTGTAATGTCTTGGTTC
       D  D  M  A  D  T  E  L  L  D  L  L  P  F  F  E  G  L  S  K  282
2035 GACGACATGGCGGATACAGAGCTGCTCGATCTCCTTCCTTCTTCGAGGGACTGAGCAAA
       E  E  N  V  Y  N  M  L  N  K  L  C  N  R  *                296
2095 GAAGAAAACGTTTACAACATGCTAAATAAGTTATGTAACAGGTAGCCCTGGCCAAGCTCT
2155 CCGCTGTGAAAAAGCTGCGAATTGCAGCAGCTTCAGACTGAGCCCCGTCTCTTGACTCTT
2215 CGCTTCTCCACCCTGACATGTTTTGCTCTGAACCAATAAGCTCTGCCAAACAGAAATCCA
2275 TCACAAGAAGATCAGCTCTTGTTCTCCCGAAGGGTTTGAATGATGCTGTGCAGCCTTTCA
2335 CAACTCCATTGAACTCAATTCTCTAACCGAGTAAAACATTTTGTTGCCGAATGGAAAAGC
2395 CGCTTGCCGATTCTTCGGCATCTCTGGGGGTCACTGAGCAATGGACGGTGGAAAGACAAT
2455 ATTTTTTAAAGATAAACTTTTTGTCTCTAATTTTTCTTAAAATTAATATGGACTTTCAGG
2515 CTTTTATAACCAGAAGAAAACAACACACCAAATGAAACTGAATCTACAAAGAAGAACATA
2575 GTAAATGGTTGATGCTAATGATGCTAAAAAAAAAAAAAAAAAAA
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Schizosaccharomyces pombe, *Neurospora crassa*, *Saccharomyces cerevisiae*, *Dictyostelium discoideum*, *Arabidopsis thaliana* and *Oryza sativa*.

RT-PCR analysis with transcript-specific primers revealed constant XNIF mRNA levels throughout embryogenesis (Nieuwkoop stages 0 to 48 tested), and the RNA could also be detected in all adult tissues that have been tested (data not shown). Interestingly, XNIF showed a characteristic spatial expression in the developing somites during tadpole stages of embryogenesis, as detected by whole-mount in-situ hybridization (Fig. 2F).

XNIF encodes a vegetally localized transcript

In order to determine the localization of XNIF transcripts during oogenesis, whole-mount in-situ hybridization with Digoxygenin-labeled antisense RNA probes was performed. As shown in Fig. 2A, the XNIF transcripts localized mainly to the mitochondrial cloud of stage I oocytes. Subsequently, the RNA was translocated to the vegetal pole of stage II oocytes and became localized to a small patch at the vegetal cortex (red arrow in Fig. 2B). XNIF mRNA remained localized to this discrete region of the vegetal pole until stage VI of oogenesis (Fig. 2B,C, respectively). For a more detailed analysis of the distribution of XNIF in early oocyte stages, sections of stained stage I and late stage II oocytes were prepared. In stage I oocytes, a characteristic enrichment in the mitochondrial cloud could be observed (Fig. 2D). In late stage II oocytes, the transcript localized to a wedge-shaped region between the oocytes' nucleus and the vegetal cortex, where it appeared to be enriched in granule-like structures (Fig. 2E). Based on these characteristic features, namely the enrichment in the mitochondrial cloud during stage I of oogenesis and the localization to a relatively small-sized, discrete region of the vegetal cortex, XNIF can be assigned to the early pathway RNAs, together with Xwnt11, Xcat2 and Xpat.

The 5′-UTR of the XNIF transcript contains an RNA localization sequence

To identify and delineate the signals responsible for the

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XNIF    X.l.  102   SEYGKKCVVIDLDETLVHS........SFKPINNADFIVPVEIDGTIHQVYVLKRPHVDEFLQKMGEMFECVLFTASLAKYADPVADLLDR.WGVFN   189
NIF     G.g.  101   -D-----------------.....-----S-----------------------------R---L-----------------------.----R   188
NIF-l   M.m.  174   LD----------------.....-----S-----------------------------R-QL-----------------------.----R   261
Hya22   H.s.  166   LD----------------.....-----S-----------------------------R-QL-----------------------.----R   253
OS4     X.l.   98   KDK--I-M----------.....-----S----------E--T---------Y-----ER--QLY----------------T----K.S---R   185
Dullard X.l.   57   -QVKR-VL-L------I--HHDGVLRPTVR-GTPP---LK-V--KHPVRFF-H------F--EVVSQWY-L-V----MEI-GSA---K--NNK--LR   153

XNIF    X.l.  190   ARLFRESCVFHRGNYVKDLSRLGRELSKVIIIDNSPASYIFHPENAVPVMSWFDDMADTELLDLLPFFEGLSKEENVYNMLNK      272
NIF     G.g.  189   -------------------------------V-----------------Q------T-------I----------E--S--H-      271
NIF-l   M.m.  262   -------------------------------V-----------------Q------T-------I-------R-DD--S--HR      344
Hya22   H.s.  254   -------------------------------V-----------------Q------T-------I-------R-DD--S--HR      336
OS4     X.l.  186   S-----A---HQ-C----------D-K-TV-L-----------------Q------S-----S-I-I--EF-YS-DI-TS-GQ      268
Dullard X.l.  154   R-FY-QH-TLEL-S-I----VVHSD--S-V-L----GA-RS--D--I-IK---S-PS--A--N---MLDA-RFTAD-RSV-SR      236
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Fig. 1. XNIF contains the conserved NIF motif. (A) Nucleotide and predicted amino acid sequence of XNIF (accession number: AY280863). The start codon is marked by bold letters. Upstream stop codons of the same reading frame are marked by boxes. The stop codon is marked by an asterisk. A fragment that directs vegetal localization in stage I and later stage oocytes is underlined (nt 252-551). The conserved NIF-motif of XNIF is highlighted in the amino acid sequence. The conserved phosphatase motif DXDXT/V is boxed. (B) Amino acid sequence comparison of NIF-domains of XNIF and related (hypothetical) proteins from chicken (NIF isoform T1; AF189773), mouse (NIF-like protein; AJ344340), human (Hya22; NM_005808) and two *Xenopus* proteins (XOs4, AF441288, and Dullard, AB084264). Hyphens indicate amino acid positions identical to XNIF sequence; dots reflect deletions. The conserved phosphatase motif DXDXT/V is boxed.

vegetal transport of the XNIF transcript, microinjection experiments were performed. For this purpose, different subfragments of the XNIF RNA were cloned behind a 320-nucleotide lacZ reporter sequence. In-vitro transcribed capped RNA generated from these constructs was then injected into the nuclei of stage II-III oocytes and the localization was detected by whole-mount in-situ hybridization using a lacZ-specific antisense probe. Localization was evident from preferential staining in the vegetal half of injected oocytes; failure to localize resulted in diffuse, ubiquitous staining. Full-length XNIF fused to the lacZ tag localized efficiently to the vegetal pole of the injected oocytes (data not shown). In a first attempt to define the position of the localization element within the XNIF transcript, fragments containing either the 5′-UTR, the open reading frame or the 3′-UTR were tested for localization. Whereas the open reading frame as well as the 3′-UTR become dispersed throughout the oocyte, the RNA that includes the 5′-UTR is capable of mediating efficient localization to the vegetal cortex (Fig. 3A,B,F).

For a more detailed delineation of the XNIF localization element, five overlapping subfragments of the 5′-UTR were fused to the short lacZ tag and tested for localization by injection experiments (a schematic drawing of injected fragments is shown in Fig. 3F). Only the transcript containing nucleotides (nt) 252-551 of the corresponding cDNA (XNIF-LE) was able to localize efficiently to the vegetal pole (Fig. 3C,E). Conversely, an internal deletion of nt 253-550 from the 5′-UTR led to a loss of localization activity, revealing that this portion is not only sufficient but also necessary for localization (Fig. 3F). The localization element nt 252-551 was then subdivided into two fragments for further analysis, and here only the 5′ portion RNA fragment (nt 252-381) showed localization activity in stage III oocytes (Fig. 3D,F). To further verify the vegetal localization of the injected transcripts, oocytes were sectioned after in-situ staining and the wedge and cortical localization of the injected XNIF nt 252-551 in stage II oocytes is shown as an example in Fig. 3E.

The vegetal localization element of the XNIF transcript is sufficient to mediate enrichment of the transcript to the mitochondrial cloud in stage I oocytes

It has previously been reported that early pathway RNAs, such as Xlsirts, Xcat2 and Xpat, are transported to the vegetal pole after injection into stage III and early stage IV oocytes, and that the localization achieved in such experiments resembles the characteristically broader distribution of late pathway RNAs (Hudson and Woodland, 1998; Kloc et al., 1993; Zhou and King, 1996b). Therefore, it seems possible that early pathway RNAs are also capable of being transported along the late localization pathway in later stages of oogenesis, when the corresponding endogenous transcripts have already reached their destination via the early pathway. Since it is difficult to distinguish if the RNAs shown in Fig. 3 followed the early or the late pathway, the RNA constructs that mediate an efficient vegetal transport in stage II-III oocytes (nt 252-551, nt 252-381) were also tested for their accumulation in the mitochondrial cloud after injection into stage I oocytes, which is a characteristic feature of early pathway RNAs. As can be seen in Fig. 4A, the transcript containing nt 252-551 of the

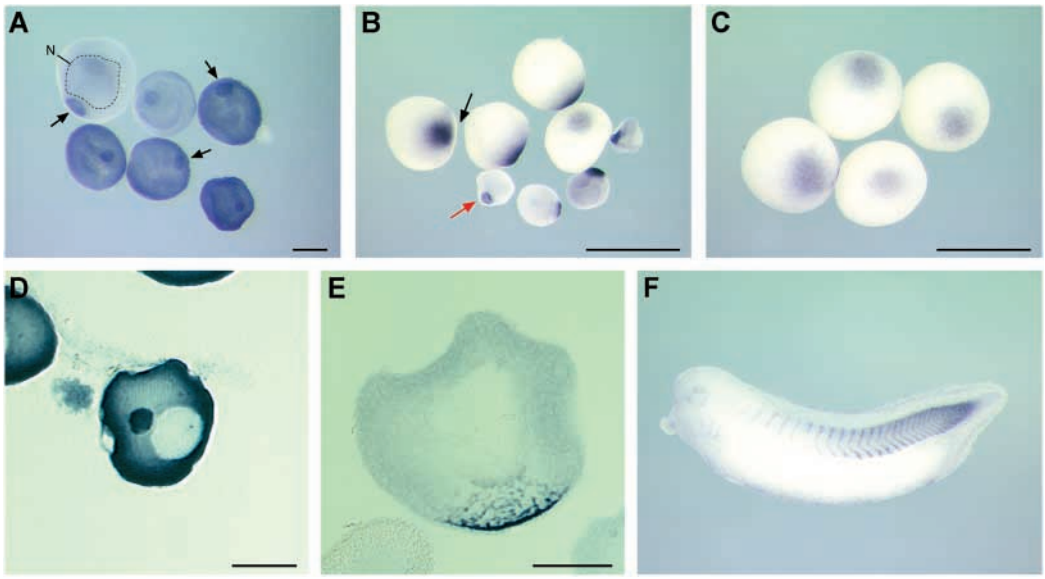

Fig. 2. XNIF encodes for a vegetally localized RNA in *Xenopus* oocytes. Whole-mount in-situ hybridization was carried out using albino oocytes of different stages. For a detailed analysis, sections of stained stage I and stage II oocytes were prepared. (A) In stage I oocytes, XNIF RNA is enriched in the mitochondrial cloud (examples marked by arrows). The cell nucleus is indicated by a dashed line in one oocyte. (B) In stage II oocytes, XNIF accumulates at the tip of the vegetal cortex (red arrow). In stage III-IV oocytes, XNIF RNA is associated with the vegetal cortex (black arrow). (C) Vegetal-cortical localization of XNIF in stage V/VI oocytes. (D) Section of whole-mount in-situ stained stage I oocyte. XNIF transcript is enriched in the mitochondrial cloud adjacent to the germinal vesicle. (E) Section of whole-mount in-situ stained stage II oocyte. XNIF is associated with reticular structures in a wedge-shaped region beneath the germinal vesicle. Scale bar: ~100 μm in (A,D,E) and 1 mm in (B,C). (F) XNIF expression in a stage 33 *Xenopus* albino embryo. Whole-mount in-situ hybridization staining with a XNIF specific antisense probe is shown.

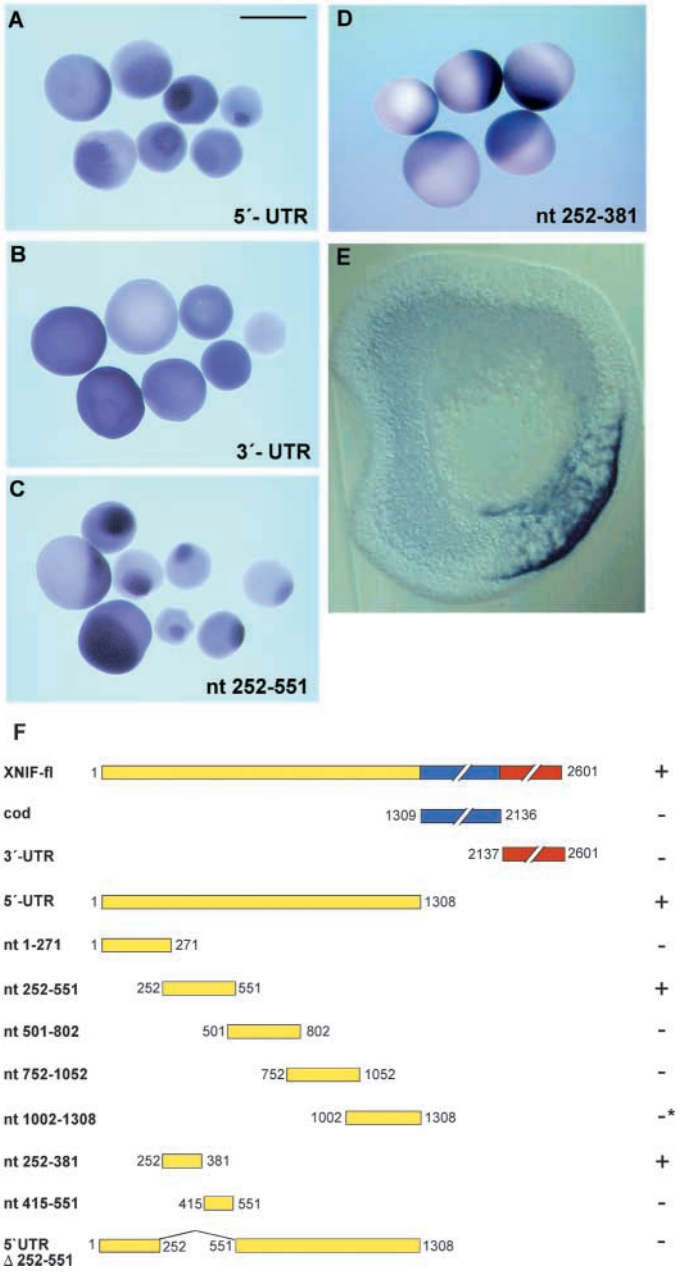

Fig. 3. The 5′-UTR of XNIF contains a vegetal localization element. *lacZ*-tagged RNAs were injected into stage II-III albino oocytes and visualized by whole-mount in-situ hybridization after culturing in vitellogenin-enriched L15 medium. Vegetal localization was observed in oocytes injected with transcripts containing the 5′-UTR (A) and with fragments containing nucleotides 252-551 (C) and 252-381 (D), but not with the coding region or 3′-UTR (B). Scale bar: ~500 μm. The section in (E) shows the wedge and cortical staining of a stage II oocyte that has been injected with a transcript containing the nucleotides 252-551 as shown in (C). (F) Schematic representation of the constructs used for XNIF localization element mapping experiments. 5′-UTR, ORF and 3′-UTR are represented by yellow, blue and red, respectively. Corresponding nucleotide positions of the XNIF cDNA are indicated. Capability of vegetal localization is marked with (+), absence of vegetal localization by (–). A weak and not always reproducible localizing activity was also observed for the fragment nt 1002-1308 and is marked by an asterisk.

XNIF cDNA showed an enrichment in the mitochondrial cloud after injection into stage I oocytes and also revealed vegetal localization after injection into later stage oocytes (Fig. 4F). However, the nt 252-381-containing element, even though it localized efficiently to the vegetal cortex in stage III oocytes, showed a homogeneous distribution after injection into stage I oocytes and was not found to be enriched in the mitochondrial cloud (Fig. 4B,G). An RNA construct that comprised the second half of the nt 252-551 XNIF-LE (nt 415-551) localized neither in early- nor in late-stage oocytes (Fig. 4C,H). In comparison, the 3′-UTR of XNIF, which bore no localizing activity, was not enriched in the mitochondrial cloud and was not localized in later stage oocytes (Fig. 4E,J). Visual inspection of the 300 nt region containing the XNIF localization element revealed a significant enrichment in CAC-containing repeats, which have been claimed to characterize the localization elements of the majority of RNAs localizing to the vegetal cortex in *Xenopus* oocytes, suggesting that these may also play a role in the localization of XNIF RNA (CAC-containing repeats are highlighted in Fig. 4K) (Betley et al., 2002). However, a critical number of CAC-containing motifs alone seems not to be sufficient for vegetal localization in stage III oocytes, since both elements exhibited comparable numbers of CAC-containing sequence elements (Fig. 4G,H,K). Interestingly, by contrast to an RNA construct containing one copy of nt 252-381, the same element duplicated in tandem repeat was found to be enriched in the mitochondrial cloud and also showed vegetal localization in later stage oocytes (Fig. 4D,I). Thus, our findings support the idea that, in contrast to vegetal localization per se, accumulation in the mitochondrial cloud may require a critical number of CAC-containing repeats (Fig. 4A,B,D).

Binding of the XNIF localization sequence to proteins from *Xenopus* oocyte extracts

To identify RNA-binding proteins with a function in the vegetal localization of XNIF RNA, in-vitro UV cross-linking assays have been performed. LE-containing fragments of XNIF and Vg1 mRNA fused to the lacZ-tag, as have been used for the localization studies, as well as the short lacZ RNA alone as negative control, were labeled with [^{32}P]-UTP by in-vitro transcription and incubated with *Xenopus* oocyte S100 extract in the presence of either tRNA, lacZ-tag RNA, Vg1LE RNA or XNIF-LE and subfragments of XNIF-LE, serving either as non-specific or specific competitors. As shown in Fig. 5, the non-localizing lacZ-tag RNA alone is mainly bound by a subset of proteins that is dominated by the 54/56 kDa protein doublet that may correspond to the very abundant FRGY proteins, as previously described (Bubunenko et al., 2002; Cote et al., 1999; Marello et al., 1992); binding of these proteins could be reduced by an excess of unlabeled lacZ-tag competitor RNA, but not by excess tRNA competitor (Fig. 5, lanes 1 and 2, and data not shown).

The cross-linking pattern observed for the XNIF-LE showed at least five additional protein bands, which could be competed in the presence of unlabeled XNIF-LE competitor RNA, but not with lacZ-tag RNA (Fig. 5, lanes 3, 4, 5). These proteins migrate with an estimated size of 62, 64, 69, 78 and 190 kDa. We also observed interaction of lower molecular weight proteins; these are, however, comparably weak, hampering the distinction between specific and non-specific binding, and

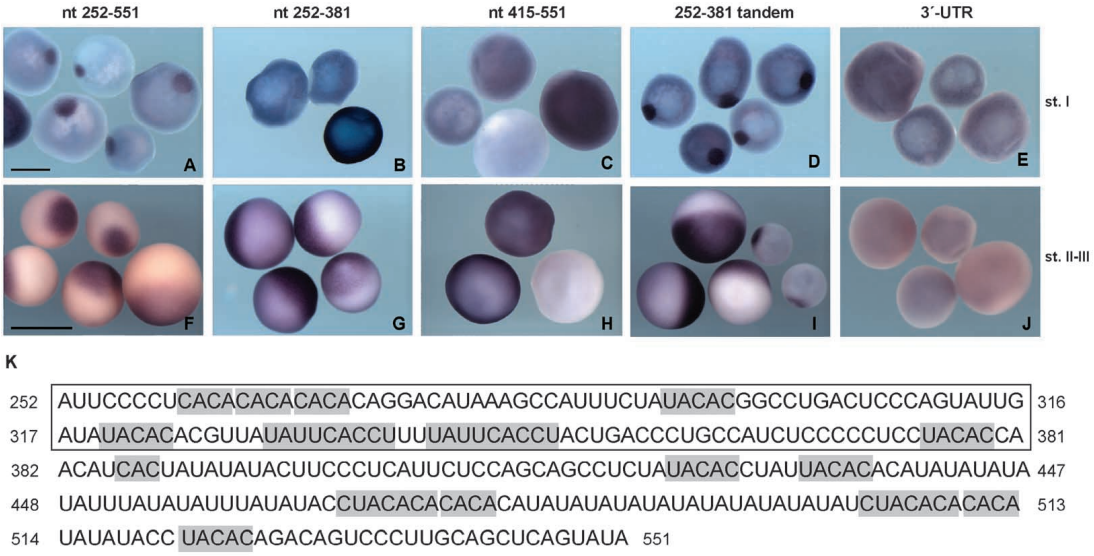

Fig. 4. XNIF-LE mediates accumulation in the mitochondrial cloud. *lacZ*-tagged RNAs were injected into stage I-III oocytes and localization was visualized by whole-mount in-situ hybridization. Localization to the vegetal cortex in stage III and enrichment in the mitochondrial cloud in stage I was observed with the transcript nt 252-551 (A,F). The transcript nt 252-381 does not accumulate in the mitochondrial cloud, but is transported in stage III oocytes (B,G). A transcript containing nucleotides 415-551 neither localizes in stage I nor in stage III oocytes (C,H). A transcript containing a duplication of nucleotides 252-381 in tandem repeat localizes to the mitochondrial cloud in stage I and to the vegetal cortex in stage II-III oocytes (D,I). No localization to the mitochondrial cloud or vegetal cortex was observed with a transcript containing the XNIF 3′-UTR (E,J). Scale bar: ~100 μm in A-D; ~500 μm in F-J. (K) The nucleotide sequence of the nt 252-551 localization element is shown. CAC-sequence element containing motifs and isolated CAC triplets are shaded. The nt 252-381 minimal sequence that mediates localization in stage III oocytes is boxed.

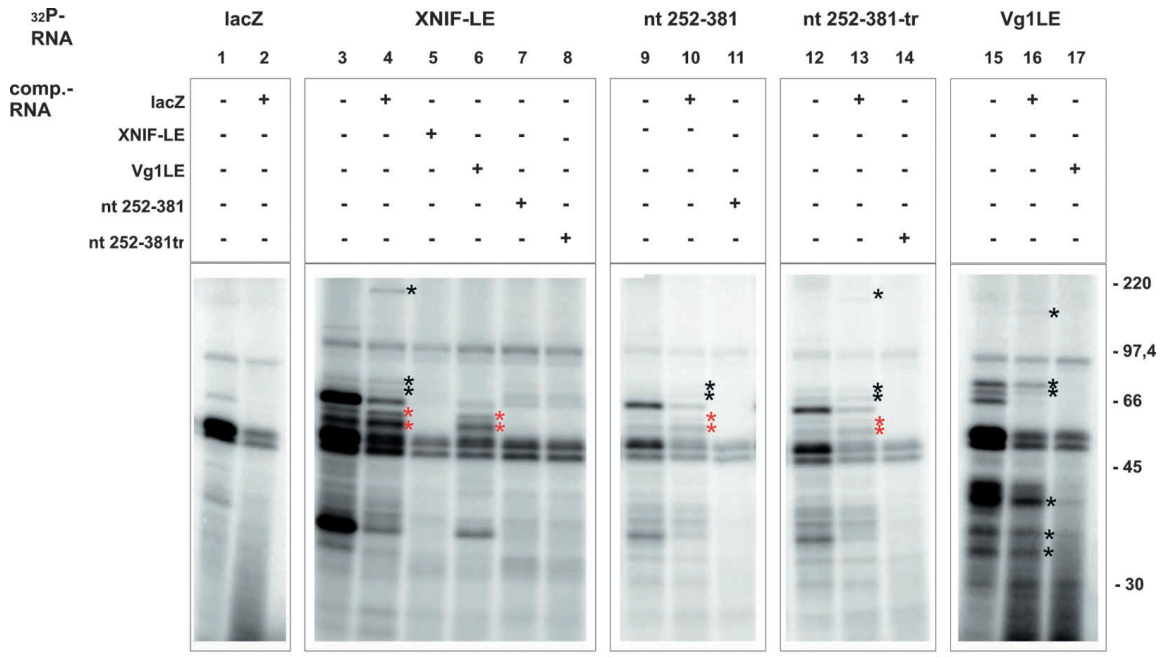

Fig. 5. XNIF-LE is recognized by specific proteins from *Xenopus* oocyte extract. RNA binding was assayed in vitro by UV cross-linking. RNA-binding reactions contained ^{32}P-labeled lacZ-tag RNA (lanes 1 and 2), lacZ-tag-XNIF-LE (252-551) (lanes 3-8), lacZ-tag-XNIF nt 252-381 (lanes 9, 10 and 11), lacZ-tag-XNIF nt 252-381 in a tandem repeat (nt 252-381-tr, lanes 12, 13 and 14) or lacZ-tag-Vg1-LE (lanes 15, 16 and 17) transcripts, S100 extract, and either solely tRNA competitor (1, 3, 9, 12 and 15), additional non-specific lacZ-tag competitor RNA (lanes 2, 4, 10, 13 and 16) or sequence specific competitor RNA, as indicated (lanes 5, 11, 14 and 17). For the XNIF-LE (252-551), additional unlabeled competitor and cross-competitor RNAs have been analyzed: Vg1LE (lane 6), XNIF nt 252-381 (lane 7) and XNIF nt 252-381 in a tandem repeat (lane 8). Cross-linked proteins were analyzed and detected by 10% SDS-PAGE and phospho-imaging. Specifically interacting proteins are marked by asterisks. Proteins that exclusively bound to the LE of the early pathway RNA XNIF are marked by red asterisks.

these are therefore not marked in Fig. 5. The cross-linking activity seemed to vary with different S100 protein preparations, and the 190-kDa protein was found to cross-link reproducibly solely in two batches of S100 preparations. In comparison, binding studies with Vg1-LE resulted in a protein pattern as described previously (Bubunenko et al., 2002; Mowry, 1996), revealing specifically interacting proteins of ~33, 36, 40, 69 and 78 kDa, as well the 190-kDa protein that has not been described before (Fig. 5, lanes 15, 16, 17). The previously described 60-kDa protein (VgRBP60) migrated close to the very abundant p54/p56 proteins and is not visible here (Cote et al., 1999). A comparatively weak interaction with the 62- and 64-kDa proteins was also observed for the nt 1002-1308 fragment and may reflect its very weak and not always reproducible localizing activity (see above, Fig. 3E).

To investigate whether the 69- and 78-kDa proteins, which interact with XNIF-LE as well as with Vg1LE, are identical, cross-competition experiments have been performed. An access of Vg1LE competitor RNA competed with XNIF-LE for the binding of the 69- and 78-, as well as the 190-, kDa proteins, whereas no cross-competition could be observed for the 62- and 64-kDa proteins, which specifically bind to the XNIF-LE (Fig. 5, lane 6). UV cross-linking experiments were performed with these RNAs to analyze whether the different transport capacities of the nt 252-381 XNIF subelement, which localized only in later stage oocytes, and the tandem repeat of this element, which regained the ability to localize to the mitochondrial cloud, were reflected by differential protein-binding activities. Both these RNAs bound to the 62- and 64-kDa proteins, as well as to the 69- and 78-kDa proteins, although with an overall weaker binding activity compared with the XNIF-LE (Fig. 5, lanes 9, 10 and 11 for nt 252-381 and lanes 12, 13 and 14 for the nt 252-381 tandem repeat). A very weak interaction with the 190-kDa protein could be observed only for the nt 252-381 tandem repeat (Fig. 5, lane 13). In cross-competition experiments, both RNAs nt 252-381 and nt 252-381tr competed with XNIF-LE for the binding of the 62- and 64-kDa proteins and the 69- and 78-kDa proteins (Fig. 5, lanes 7 and 8).

We conclude that XNIF-LE seems to bind to at least some of the previously described Vg1RBPs (p69/Vg1RBP and 78 kDa), but that it interacts also with additional proteins that do not bind to the Vg1-LE (62 and 64 kDa) and that may therefore correspond to proteins specifically involved in localization or anchoring of early pathway RNAs.

Binding of the XNIF localization element to specific proteins

Since the binding pattern of the early localizing XNIF-LE observed in the UV cross-linking studies differed from the one observed with the late localizing Vg1-LE, we wanted to test if this was also reflected by different binding preferences for known Vg1-LE-binding proteins in co-immunoprecipitation analyses, where Vg1RBP, Prrp and Vg1RBP71/KSRP were tested. The ribosomal protein L5 is a 5S rRNA-binding protein serving as a negative control here. RNA fragments that comprise nt 252-551 of the XNIF cDNA and that show localizing activity in both early and late stage oocytes (XNIF-LE), as well as the Vg1-LE, were radioactively labeled by in-vitro transcription. Due to their prior use in the injection experiments, these constructs also contained the lacZ tag sequence. The lacZ tag alone as a nonlocalizing RNA should not bind to LE-interacting factors and therefore served as a negative control. The proteins all contained an N- or C-terminally located poly-myc-epitope-tag, were produced by in-vitro transcription and translation in the coupled reticulocyte lysate system T_NT and were incubated with labeled RNAs to allow complex formation. RNA/protein complexes were immunoprecipitated with an immobilized anti-myc antibody. As can be seen in Fig. 6, the nonlocalizing lacZ RNA was mainly found in the supernatant of the binding reaction and showed only background binding in the pellet fractions. By contrast, the XNIF-LE exhibited a strong interaction with Vg1RBP, as was to be expected from the cross-linking data, and also a significant, albeit weaker binding to Prrp. No interaction above background could be observed with VgRBP71, as well as with the 5S rRNA-binding protein L5. Similar but reduced binding activities were observed for XNIF-nt 252-381, which exhibited transport activities only in stage III oocytes. The transcript that contained XNIF nt 252-381 in a tandem repeat, and which was capable of localizing also to the mitochondrial cloud in stage I oocytes, showed an increased binding to Vg1RBP comparable to the XNIF-LE.

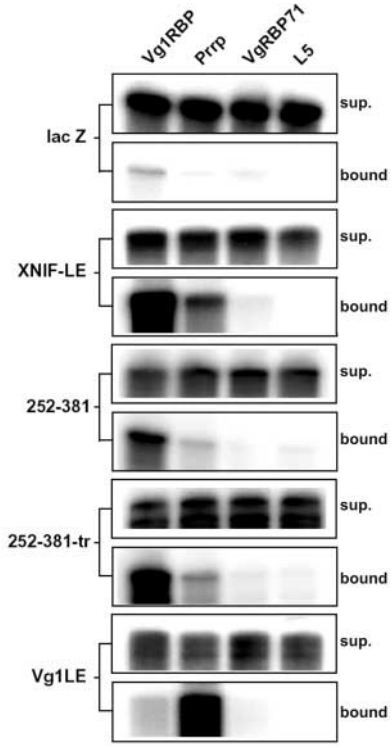

Fig. 6. The XNIF localization element interacts with Vg1RBP and Prrp in co-immunoprecipitation experiments. *lacZ*-RNA, XNIF-LE (nt 252-551) as well as XNIF nt 252-381, XNIF nt252-381 in a tandem repeat (nt 252-381-tr) and Vg1-LE are radioactively labeled by in-vitro transcription. Vg1RBP, Prrp, VgRBP71 and L5 were produced by coupled transcription and translation in the T_NT reticulocyte lysate system and incubated with radiolabeled RNAs. RNPs were immunoprecipitated by the myc-epitope and unbound RNA from the supernatant (sup.) and coprecipitated (bound) RNAs were analyzed by denaturing PAGE. Different labeled RNAs are marked on the left-hand side; in-vitro translated proteins are indicated on the top panel.

Vg1-LE also competed with XNIF-LE for Prrp binding (data not shown). By contrast, the Vg1-LE showed a strong interaction with Prrp, while it was not found to interact with Vg1RBP, VgRBP71 or L5 under the experimental conditions employed here.

Discussion

By random screening of a vegetal cortex cDNA library by whole-mount in-situ hybridization with *Xenopus* oocytes, we have isolated a gene referred to as XNIF, which localizes to the vegetal cortical region of *Xenopus* oocytes by making use of the early localization pathway. XNIF is a novel member of a large evolutionarily conserved family of proteins, which share a conserved C-terminal domain of ~200 amino acids. Most NIF family members have been identified through genome-sequencing projects. Chromosomal regions containing genes that encode human homologs of XNIF have been described as deleted or amplified in a variety of human tumors, but the exact biological function of these proteins remains elusive (Ishikawa et al., 1997; Protopopov et al., 2003; Su et al., 1997). Studies employing *Xenopus* explants and whole embryos show that XOs4, a *Xenopus* relative of XNIF, can induce mesoderm and dorsalize ventral mesoderm resulting in ectopic dorsal axis formation (Zohn and Brivanlou, 2001). Misexpression of XNIF by RNA injection into the ventral marginal region of one of four blastomeres of the 4-cell stage embryo did not cause the formation of a secondary axis (data not shown), even though XNIF exhibits a high degree of sequence identity with *Xenopus* Os4. Similarly, injection of XNIF RNA into the animal region of 2-cell stage embryos did not cause any significant effects (data not shown). Another *Xenopus* XNIF homolog, Dullard, has been identified recently (Satow et al., 2002). While overexpression of Dullard in early embryos caused apoptosis, results from antisense morpholino oligonucleotide experiments showed failure of neural tube closure and suggest a role for *dullard* in neural development (Satow et al., 2002). The expression pattern of XNIF during tailbud/tadpole stages suggests that its function during later stages of embryogenesis is fundamentally different from those of XOs4 and Dullard, and may be related to the process of somitogenesis.

Localized RNAs are classified as belonging to either the early or late localization pathway. Based on the observation that XNIF mRNA localizes to the mitochondrial cloud in stage I oocytes and is restricted to a discrete region of the vegetal cortex in late stage oocytes, it is assigned to the early localization pathway. Mapping analysis revealed that vegetal transport is mediated by a localization signal that resides in the 5′-UTR of XNIF mRNA. A 300 nt region of XNIF RNA is sufficient to mediate the accumulation of a heterologous RNA (lacZ tag) to the mitochondrial cloud in stage I oocytes. This is a remarkable feature, since in almost all localized RNAs analyzed to date, the transport signals have been found to reside within the 3′-UTR (reviewed by Palacios and Johnston, 2001). Proteins required for the enrichment in the mitochondrial cloud/vegetal transport may also participate in recruiting factors to 5′-UTR, which may be involved in translational repression during the transport process (Wilkie et al., 2003).

It has previously been reported that different early pathway RNAs are also capable to localize via the late localization pathway after injection into stage III oocytes, and that the localization achieved then resembles the one of a late pathway RNA (Hudson and Woodland, 1998; Kloc et al., 1993; Zhou and King, 1996b). Mapping analysis with Xcat2 mRNA showed that signals that mediate these localizing activities partially overlap (Zhou and King, 1996a, 1996b). In mapping experiments with XNIF, a 130 nt sequence element (nt 252-381) turned out to be sufficient to mediate vegetal localization in stage III oocytes, but failed to mediate the early pathway specific enrichment in the mitochondrial cloud; by contrast, a larger fragment, including this minimal element (nt 252-551), exhibited localizing activity in both early and late stage oocytes. We also observed that a duplication of this minimal localization element gained the ability to localize to the mitochondrial cloud. Interestingly, a comparable situation has been described for the late localizing Vg1-LE, where a duplication of the 5′ subelement (containing a VM1 motif) gains vegetal localization activity in late stage oocytes (Gautreau et al., 1997).

A recent publication (Betley et al., 2002) describes the existence of a conserved consensus signal for RNA localization in chordates. Based on the observation that short nt repeats are important for vegetal localization, a computer program called REPFIND was developed, which helps to identify repeat motifs in RNA. Using this program (http://zlab.bu.edu/repfind) it was shown that clusters of short CAC-containing motifs can be found in the localization elements of virtually all early and late localizing mRNAs investigated. Searches for CAC-containing sequence motifs in the 300 nt LE of XNIF revealed a total number of 16 such elements. Subfragments of this localization element (nt 252-381 and nt 415-551) contain a reduced number of CAC-containing repeats and loose their ability to localize to the mitochondrial cloud. However, as stated above, a duplication of nt 252-381, resulting in a total number of CAC-containing repeats comparable to the 300 nt XNIF mitochondrial cloud localization element, regains localization to the mitochondrial cloud, indicating that a critical number of CAC-containing repeats may indeed be required for mitochondrial cloud localization. Different types of CAC-containing repeats have also been discussed as being critical in localizing RNAs of the late localization pathway (Betley et al., 2002; Bubunenko et al., 2002; Deshler et al., 1997, 1998; Havin et al., 1998; Kwon et al., 2002). However, CAC-containing repeats alone seem not to be sufficient for localization of XNIF in later stages of oogenesis. Although both subfragments of the 300 nt XNIF-LE (nt 252-381 and nt 415-551) contain comparable numbers of CAC-motifs, only one (nt 252-381) mediates localization in late stage oocytes, indicating that there may be other structural features required for the late transport process.

UV cross-linking analysis reveals at least five proteins (62, 64, 69, 75 and 190 kDa) that interact with the localization element of XNIF. Two of these, namely the 62- and 64-kDa proteins, seem to be specific for the XNIF-LE and were not observed in UV cross-links with the LE of the late pathway Vg1 mRNA. Therefore, these define candidate proteins with a specific function in the early localization pathway. The UV cross-linking pattern of the XNIF-LE differs from the one of Vg1-LE in revealing only weak or no interaction with the low molecular weight VgRBPs (33, 36 and 40 kDa, respectively).

It is interesting to note that the protein-binding properties of the 252-381 element are virtually indistinguishable from those of its tandem repeat or of the entire XNIF-LE, even though the 252-381 element by itself is not capable of traveling via the early pathway in association with the mitochondrial cloud while still making use of the late pathway. These observations seem to have two implications. Firstly, they reemphasize the considerable overlap in respect to the protein machineries that appear to be involved in both of the vegetal transport routes. Secondly, they suggest that a higher order structure, with perhaps multiple copies of a basic RNP module, is required for association with the mitochondrial cloud in the context of the early localization pathway. Since UV cross-linking patterns give only information on the approximate size of interacting proteins, we also tested whether the known Vg1-LE-interacting proteins, Vg1RBP, Prrp and VgRBP71, bind to the XNIF-LE. Co-immunoprecipitation analysis revealed a very strong interaction of Vg1RBP with XNIF-LE, as was to be expected from the UV cross-linking experiments. The XNIF-LE also contains two copies of the previously defined E2-element (UUCAC), which has been shown to serve as binding site for Vg1RBP (Kwon et al., 2002). A specific but weaker interaction can also be observed with Prrp, which also strongly interacts with the Vg1-LE under these conditions.

As protein binding to early localization pathway elements has previously not been analyzed in detail, our results obtained from UV cross-linking studies and co-immunoprecipitation assays identify candidate protein factors for transport along the early pathway. Identification of the 62- and 64-kDa proteins, which specifically cross-link to the XNIF-LE, will provide further insight in the protein machinery involved in the localization of early pathway RNAs.

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