mRNP3 and mRNP4 are phosphorylatable by casein kinase II in *Xenopus* oocytes, but phosphorylation does not modify RNA-binding affinity

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Abstract mRNP3 and mRNP4 (also called FRGY2) are two mRNA-binding proteins which are major constituents of the maternal RNA storage particles of *Xenopus laevis* oocytes. The phosphorylation of mRNP3–4 has been implicated in the regulation of mRNA masking. In this study, we have investigated their phosphorylation by casein kinase II and its consequence on their affinity for RNA. Comparison of the phosphopeptide map of mRNP3–4 phosphorylated in vivo with that obtained after phosphorylation in vitro by purified *Xenopus laevis* casein kinase II strongly suggests that casein kinase II is responsible for the in vivo phosphorylation of mRNP3–4 in oocytes. The phosphorylation occurs on a serine residue in a central domain of the proteins. The affinity of mRNP3–4 for RNA substrates remained unchanged after the treatment with casein kinase II or calf intestine phosphatase in vitro. This suggests that phosphorylation of these proteins does not regulate their interaction with RNA but rather controls their interactions with other proteins.

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1. Introduction

Oocytes of *Xenopus laevis* accumulate large amounts of ribosomes, tRNA and mRNA which palliate the absence of transcription during the early embryogenesis until the mid-blastula transition [1]. Stored mRNA which accumulate during oogenesis are kept in a stable state and remain untranslated until they are recruited into polysomes at oocyte maturation and after fertilization [2]. In oocytes, most of the maternal mRNAs appear as complexes with a set of specific proteins [3,4]. These macromolecular structures, called mRNP, are apparently involved in both mRNA protection against degradation and masking mRNA from the translation apparatus (see e.g. [5,6]). mRNP3-4 are the major 'masking' proteins identified, and have been extensively characterized [3,7–11]. These proteins are phosphorylated during early oogenesis, and mRNA 'masking' in the oocyte depends on their phosphorylation state [7,12]. The nucleotide sequence of a mRNP4 cDNA is identical to that of FRGY2, a protein initially characterized as an oocyte transcription factor specific for Y-box containing promoters [9,13,14]. mRNP4/FRGY2 binds RNA with a high affinity and a limited sequence specificity [15,16].

While a number of studies have indicated that phosphorylation is involved in the regulation of maternal mRNA storage in *Xenopus* oocytes, no detailed analysis of this phosphorylation has been carried out yet. A casein kinase-II-'like' protein which is associated with mRNPs has been proposed to be responsible for the phosphorylation of mRNP3–4 [9,17]. Previous reports have discussed the possibility that phosphorylation could control mRNP assembly (e.g. by modulating the affinity of mRNP3–4 for RNA) or the fate of assembled mRNPs [9,15,18]. To address these issues, we have investigated several aspects of the phosphorylation process of mRNP3–4. We present experiments with highly purified CKII from *X. laevis*, which strongly suggest that this kinase is responsible for the phosphorylation of mRNP3–4 in oocytes. A biochemical approach allowed us to map the phosphorylated domain of mRNP3 and mRNP4. Finally, we have also analyzed the effects of phosphorylation on the affinity of mRNP3–4 for RNA.

2. Materials and methods

2.1. Purification of proteins and peptides

2.1.1. Proteins and peptides purification. Post-mitochondrial extract from immature ovaries (later called oocyte extract) was heated at 80°C for 10 min [10] and mRNP3/mRNP4 were purified from the extract using preparative electrophoresis [10,11]. The peptides were purified using the same method except that the gels were stained for 30 min with freshly prepared 0.1% Coomassie blue R-250 [19] and

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destained in 10% methanol [20]. The bands were cut out from the gel and peptide was electroeluted from the gel as described [21]. Protein concentrations were determined as in [11].

2.1.2. Refolding of mRNP3 and mRNP4. For in vitro phosphorylation and/or RNA-binding analysis of purified mRNP3/mRNP4, proteins were refolded as described by Jacquemin-Sablon et al. [16]. Previous studies indicated an effective renaturation of mRNP4 under these conditions [16].

2.1.3. Proteolysis of the proteins. Three kinds of hydrolysis treatments were used to produce mRNP3 or mRNP4 internal peptides. V8 protease digestion was carried out by mixing 3–5 µg of either the labelled or the unlabelled purified mRNP3/mRNP4 with V8 protease. Under our proteolysis conditions, staphylococcal V8 protease specifically cleaves Glu-X bounds except for Glu-Pro; [10,11]. For formic acid hydrolysis, proteins were incubated for 2–3 days in formic acid 80%. In the case of CNBr, proteolysis was performed in 70% formic acid and CNBr (molar ratio methionine:CNBr, 1:1000) for 12 h. After the reaction, 1 ml of water and 2–3 drops of glycerol were added per 100 µl and the sample was dried under vacuum to eliminate the formic acid. Dilution with water and drying was repeated once. Samples were resuspended in 25 µl of electrophoresis sample buffer [22].

2.2. Identification of the peptides produced by proteolysis

2.2.1. N-terminal sequencing. Identification of N-terminal sequence of peptides was performed according to the described procedure [10,11,20,21]. Note that the N-terminal sequences of several V8 peptides have already been determined [10].

2.2.2. Molecular mass determination by electrospray ionisation mass spectrometry. True molecular mass of the peptides was measured as described [20]. Peptides were purified by preparative gel electrophoresis as described above, treated to remove salt and SDS and resolu-

bilized in acetonitrile/H_2O/formic acid (50:49:1, v/v/v). Molecular mass determination of the peptides was performed on a Trio 2000 mass spectrometer (VG Biotech, Manchester, UK).

2.3. Phosphorylations and dephosphorylations

2.3.1. In vitro and in vivo phosphorylations of mRNP proteins. The mRNP proteins were phosphorylated in vitro as described [7,10]. In vivo phosphorylation of mRNP proteins was performed by incubating ovaries in $H_3^{32}PO_4$ (Amersham; 10 mCi/ml, carrier free) for 18–20 h at 20°C in Barth buffer. Phosphorylated mRNP3–4 were purified by preparative electrophoresis as described above.

2.3.2. In vitro phosphorylation of purified and refolded mRNP3/ mRNP4. Incorporation of phosphate into proteins (0.2–5 µg) using CKII was carried out in 25–50 µl reaction buffer [50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 5 mM DTT, 300 mM NaCl, 3 mM $MgCl_2$] containing 20 µM [γ-^{32}P]ATP and 0.1 to 1 µl of *Xenopus laevis* CKII (initial solution of 240 U/µg and 10–20 µg/ml), purified to homogeneity as previously described [23]. The reaction was carried out at 20°C and stopped after 40 min by addition of electrophoresis sample buffer [22].

2.3.3. Dephosphorylation of mRNP3 and mRNP4. Proteins dephosphorylation was performed by incubating either an oocyte extract or purified mRNP3–4 with calf intestine phosphatase (CIP; Boehringer). One hundred units of CIP were added per ml of extract and the sample was kept for 12 h at 37°C in CIP 10× buffer (Boehringer). Purified protein dephosphorylation occurred in the presence of 400 units of CIP per µg of protein for 2 h at 37°C. In all cases, in vitro ^{32}P-phosphorylated mRNP3–4 were used as dephosphorylation control.

2.3.4. Phosphoamino acids analysis. Partial acid or basic hydrolysis of in vitro labelled mRNP3/mRNP4 were performed according to

Table 1
Identification and phosphorylation of mRNP3 and mRNP4 peptides

Peptide	N-term amino acid[a]	Proposed aa sequence[b]	Phos[c]	M_r SDS-PAGE[d]	Expected M_r	M_r ESI-MS
mRNP4 peptides: V8, formic acid and CNBr proteolysis						
4a	94–	94–336	+++	44 600	26 845	
401	214–	214–336	+/−	24 500	13 351	13 358 ± 5
402	94–	94–213	+++	23 300	13 511	
403	94–	94–200	+++	20 600	12 137	12 143 ± 4
406	214–	214–293	−	17 300	9 156	9 158 ± 1
407	94–	94–155	+++	16 000	6 681	6 678 ± 5
408	6–	6–93	+/−	15 600	9 858	9 856 ± 7
410	21–	21–93	−	11 000	8 213	8 212 ± 6
4.CNBr.a	nd	154–336	+/−	37 000	20 412	
4.CNBr.b	nd	1–153	+++	25 000	16 852	
mRNP3 peptides: V8, formic acid and CNBr proteolysis						
3a	nd	94–324	+++	43 000	25 401	
301	94–	94–213	+++	23 100	13 289	13 500
302	99–	99–213	+++	21 300	12 700	
303–1	105–	105–213	+++	20 000	12 128	12 262 ± 6
303–2	214–	214–324 or −314		20 000	12 130 11 159	11 252 ± 1
304	214–	214–283	−	14 400	8 052	8 130 ± 1
305	11–	11–93	−	14 200	9 409	
3–MC	214–	214–257	−	12 000	4 937	4 939 ± 1
306	21–	21–93	−	11 000	8 403	8 403 ± 1
3.CNBr.a	nd	154–324	+/−	33 000	19 107	
3.CNBr.b	nd	1–153	+++	25 000	16 864	

[a]The N-terminal amino acid of the peptides were identified by sequencing. nd, not determined.
[b]Proposed peptide localisation. In the case of peptide 303-2, it was not possible to decide between two close C-terminal sites.
[c]Phosphorylation state of the peptide after [γ-^{32}P]ATP labelling. Occasionally very weak signals were observed for the peptides, we have indicated these as: +/−. For peptide 303, a positive signal was observed; it probably originated from peptide 105–213 (303-1) while the peptide starting at 214 (303-2) would be negative.
[d]Apparent molecular mass determination on SDS-PAGE.
[e]Molecular masses of the proposed peptide calculated with the amino acid sequence of FRGY2/mRNP4 [14] and of mRNP3 [13]. Note that the peptides starting at aa 1 are necessarily heavier than indicated since the first amino acid of mRNP3 and mRNP4 are blocked.
[f]Molecular masses determined by mass spectrometry after electroelution from SDS-PAGE. During this procedure [20] we noted a dephosphorylation of the labelled peptides (data not shown). Note that from our results, we can deduce that in our *Xenopus* oocytes, there is a T (and not an A) at the position 254 of mRNP4 [13,14].

[24]. Radiolabelled phosphoamino acids were separated using thin-layer chromatography and revealed by autoradiography [25].

2.4. RNA filter-binding assay

RNA binding was measured by a nitrocellulose filter assay [26] as described in [16]. The RNA substrate contains 456 nucleotides of the 5' end *unr* mRNA and a 60 residues poly A tail. Two ng of RNA were incubated with 2.5–50 ng of protein in 100 μl of binding buffer (20 mM sodium phosphate buffer pH 7.4, 2 mM MgCl$_2$, 1 mM DTT, 20 μg/ml BSA). Controls performed in the absence of mRNP3/mRNP4 but in the presence of CKII showed no RNA binding to CKII [27] under these conditions.

3. Results

3.1. mRNP3 and mRNP4 are phosphorylated by CKII on a serine residue

Sequence analysis of mRNP3 and mRNP4 shows 10 and 13 putative phosphorylation sites for CKII respectively (Fig. 1, peptides underlined once). CKII purified from *X. laevis* oocytes efficiently phosphorylated mRNP3 and mRNP4 in vitro (Fig. 2A and B, lanes 4, 5). Analysis of the peptides generated by proteolysis with the protease V8 indicated that only some of them were phosphorylated (Fig. 2A, lanes 1, 2) suggesting that only a few of the potential CKII phosphorylation sites were used. As the in vitro phosphorylation sites could differ from the in vivo ones, we performed the same peptide analysis on in vivo phosphorylated mRNP4. The V8 peptide pattern of in vivo labelled mRNP4 was identical to the one previously observed (compare Fig. 2B, lane 1 and Fig. 2C, lanes 3, 4). Note that when an oocyte extract is incubated with [γ-^{32}P]ATP, the purified mRNP4 digested by V8 also presented the same autoradiography pattern (Fig. 2C, lanes 5, 6). These results indicate that purification and renaturation did not modify the phosphorylation sites and strongly suggest that CKII is responsible for the in vivo phosphorylation of mRNP4 in oocytes. The same conclusion based on comparison of the in vivo and in vitro labelling were obtained for mRNP3 (data not shown).

To identify the amino acid(s) residue(s) which bind phosphate, radiolabelled mRNP3 and mRNP4 were submitted to

Table 2
Apparent-binding affinity of mRNP3 or mRNP4 for RNA after treatment with casein kinase II (CKII) or calf intestine phosphatase (CIP)

Pretreatment	mRNP3	mRNP4
	$K_d \pm$ S.D. (nM)^a	
None	1.8 ± 1.0	3.5 ± 2.0
CKII incubation buffer		
−CKII	2.2 ± 0.7	nd
+CKII	2.7 ± 0.5	nd
CIP incubation buffer		
−CIP	2.6	5.7 ± 2.3
+CIP	3.3	5.8 ± 2.6

^{a}Affinity constant was determined as in [16] (average of three experiments).
nd, not determined.

an acidic hydrolysis. Chromatographic analysis indicated that only serine residues and neither threonine nor tyrosine residues were phosphorylated (Fig. 3).

3.2. Identification of CKII phosphorylation domain in mRNP3–4

In order to identify the phosphorylation domain of mRNP3 and mRNP4, we used both radiolabelled proteins and analyzed their V8-peptides (Fig. 2) and two other sets of peptides resulting of either formic acid or CNBr treatment (see Table 1). Formic acid induces specific hydrolysis of Asp-Pro bounds and CNBr specifically hydrolyses Met-X bounds (see potential sites in Fig. 1). The digestion of mRNP3–4 with V8 resulted in the production of several peptides, but only a few of them were phosphorylated (see results in Table 1 for CNBr and V8 peptides and [28] for formic acid peptides). The N-terminal sequence of some peptides was determined by Edman degradation (Table 1 and [28]). Note that only the peptides resulting from an internal cut could be sequenced since the N-terminals of mRNP3 and mRNP4 are blocked, [10,11]. Knowing the N-terminal amino acids, the usual procedure to obtain a complete identification of the peptides is to measure their

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mRNP3   MSEAEPRETE  AVTQPEPGPE  IHKPDIVPPR  NQINKKLLAT  QVQGTVKWFN  VRNGYGFINR  NDSKEDVFVH   70
mRNP4   .SEAEAQ.P.  P.P...SEPE  .Q..G.AAA.  ..A...V...  ..........  ..........  ..TKED....

mRNP3   QTAIKKNNPR  KFLRSVGDGE  TVEFDVVEGE  KGAEAANVTG  PGGVPVKGSR  FAPNSTRFRR  QFYRPRADTA  140
mRNP4   ..........  ....SVGD..  ..........  ..........  ..........  ....RR....  R.......TA

mRNP3   GESGGEGVSP  EQMSEGEKGE  ETSPQQRPQR  RRPPPFFYRR  RFRRGPRPNN  QQNQGAEVTD  QSENKDPAAP  210
mRNP4   GESGGE....  ..MSEGE...  ..........  ..........  ..........  .........E  .....DPV..
                  ↑

mRNP3   TSEALASGDG  QQRPPPRRFQ  QRFRRPFRPR  PPPPQTPEGG  DGEAKAE-GE  -----PQRQR  NRPYVQRRRA  274
mRNP4   ......SGDD  P.........  ..........  .A.Q......  ...TKAESGE  DPRPE.....  .........R  280

mRNP3   QQPPTV----  QGESKAESE-  HPASEEGTPS  DAPTDDGAPV  ETSEAGVE--  DTTTAPE  324
mRNP4   .GATQ.AATA  ...G...PTQ  .......TPS  DSPTD.....  QSSAPDPGIA  ..P...    336
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Fig. 1. Position of various sites in mRNP3 [13] and mRNP4 [14] amino acids sequences. The phosphorylation minimum consensus sites by CKII (S/T-X-X-Acidic) are underlined. Hydrolysable sites by formic acid (D-P) are in italic and underlined twice and the only internal methionine residue in each sequence is indicated by an arrow. Except for the residues implicated in these sites, we have only indicated in mRNP4 the amino acids which are different from mRNP3.

IN VITRO PHOSPHORYLATION OF mRNP3 AND mRNP4 BY PURIFIED CKII
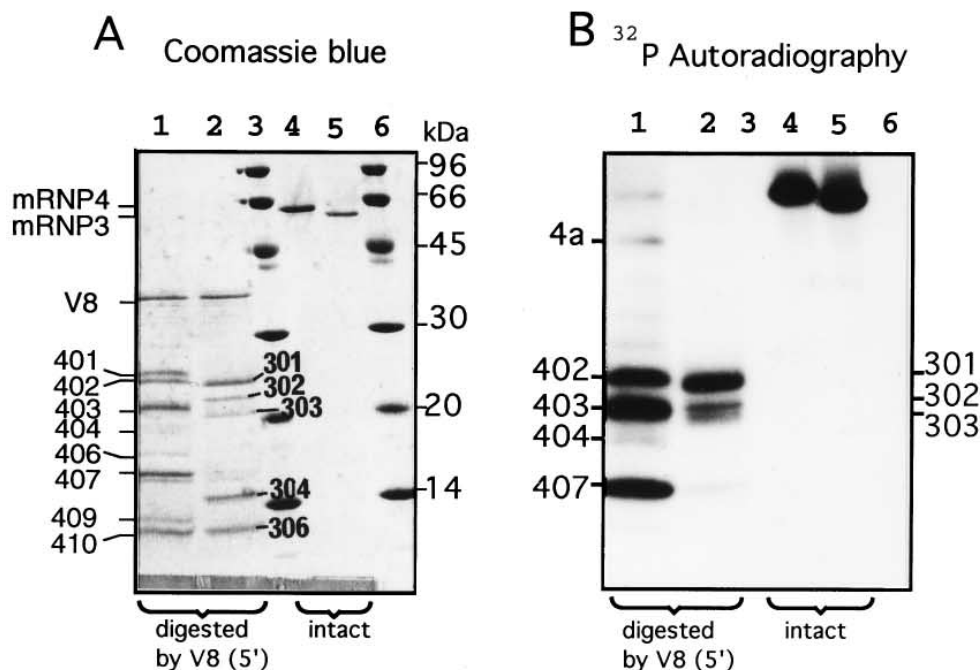
IN VIVO PHOSPHORYLATION AND IN VITRO PHOSPHORYLATION OF mRNP4
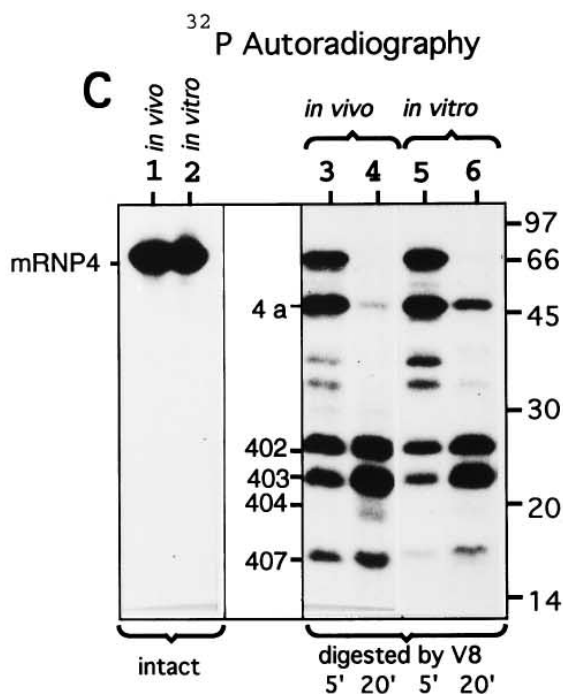
apparent molecular mass on SDS-PAGE and to deduce their C-terminal from the protein sequence and the specificity of the sites of proteolysis (V8, Glu-C; formic acid, Asp-Pro; CNBr, Met-C). This procedure was very difficult, if not impossible, to use in this case because both mRNP3 and mRNP4 present a

very unusual migration rate on SDS-PAGE [11]. We therefore used mass spectrometry to determine the true molecular mass of some peptides (Table 1) after SDS-PAGE and electroelution [20]. A calibration curve was then established relating true M_r and apparent M_r on SDS-gel (data not shown). In

Fig. 2. Phosphorylation of mRNP3 and mRNP4. (A and B) In vitro phosphorylation by purified casein kinase II. Five µg of purified and rena-
tured mRNP3 or mRNP4 were phosphorylated by purified CKII in presence of [γ-^{32}P]ATP. Radiolabelled proteins were digested by V8 pro-
tease for 5 min and the peptides produced separated by SDS-PAGE 12.5% and revealed by Coomassie blue staining (A) and autoradiography
(B). Lanes: 1, mRNP4 V8-digested; 2, mRNP3 V8-digested; 3 and 6, Pharmacia kit of low molecular mass markers; 4, intact mRNP4; 5, in-
tact mRNP3. (C) mRNP4 phosphorylation obtained in vivo by incubation of the ovaries in H$_3^{32}$PO$_4$or in vitro by incubation of an oocyte ex-
tract in [γ-^{32}P]ATP. Purified radiolabelled mRNP4 was digested by V8 protease, proteolysis products were separated on SDS-PAGE and radio-
labelled peptides revealed by autoradiography. Lanes: 1, intact mRNP4 in vivo labelled; 2, intact mRNP4 in vitro labelled; 3 and 4, in vivo
labelled mRNP4 digested by V8 for 5 min (lane 3) or 20 min (lane 4); 5 and 6, in vitro labelled mRNP4 digested by V8 for 5 min (5) or
20 min (6).
←

most cases, this procedure allowed us an unambiguous iden-
tification of the peptides (Table 1). For the formic acid and
CNBr peptides, we could even avoid N-terminal sequencing
(Table 1).

Results of peptide localization and phosphorylation are giv-
en in Table 1. It clearly shows that the phosphorylated V8
peptides derive from digestion of a central protein domain
from aa 94 to aa 213, see peptides 4a, 402, 403 and 407 for
mRNP4. No or very little phosphorylation can be observed in
the N- or C-terminal parts of the protein (see peptides 401 or
410 for mRNP4). Identical results are obtained with mRNP3.
Phosphorylation analysis of CNBr and formic acid peptides
confirm and refine this observation. For example, in the case
of CNBr proteolysis, only one peptide (from aa 1 to aa 153) is
phosphorylated (Table 1). In view of these results, we con-
clude that the phospho-acceptor site(s) is(are) present in the
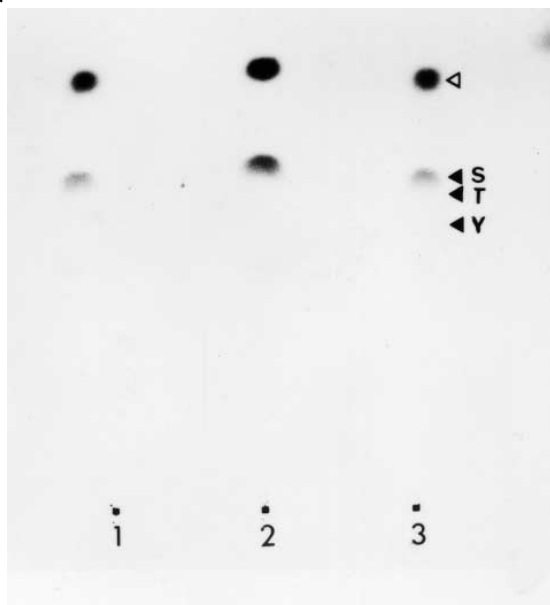central part of mRNP3 and mRNP4 between the aa 94 and
153.

Fig. 3. Phosphoamino acid analysis of mRNP3 and mRNP4.
mRNP3 and mRNP4 proteins were phosphorylated in vitro with
[γ-^{32}P]ATP. ^{32}P-labelled proteins were separated on SDS-PAGE,
electro-transferred to Immobilon membranes and subjected to parti-
al acid hydrolysis as described [24]. The products were mixed with
unlabelled phosphoamino acid standards and resolved on thin-layer
cellulose plate by electrophoresis at pH 3.5. Unlabelled phospho-
amino acids were visualized by ninhydrin staining and ^{32}P-labelled
phosphoamino acids were revealed by autoradiography. The posi-
tions of phosphoserine (S), phosphothreonine (T) and phosphotyro-
sine are indicated by dark arrows. White arrow indicates free ^{32}P in
the migration front. Lanes: 1, mRNP3; 2, mRNP3+mRNP4; 3,
mRNP4.

3.3. Influence of mRNP3–4 phosphorylation state on their affinity for RNA

The phosphorylation state of mRNP3 and mRNP4 might
directly affect their interaction with RNA. Previously, effect of
putative casein kinase II on RNA binding has been inferred
from an indirect test using heparin as a CKII inhibitor [9,15].
In this study, we compared the affinity of mRNP3 and
mRNP4 for RNA following a treatment in vitro with either
purified CKII or calf intestinal phosphatase (Table 2). We
previously used a filter binding assay to investigate the affinity
of mRNP3–4 for RNA [16]. We have shown that mRNP3–4
purified from oocytes has a high affinity ($K_d = 3$ nM) and only
limited specificity for long substrates of complex sequence
such as mRNAs. Using the same assay and a probe derived
from the unr mRNA, we show that the affinity constants are
identical after CIP or CKII treatment of mRNP3–4 (Table 2).
Incubation of mRNP3–4 in the appropriate buffers had no
significant effect on RNA binding (Table 2).

Therefore, phosphorylation by CKII or dephosphorylation
by CIP did not significantly alter the affinity of mRNP3–4 for
RNA.

4. Discussion

In this study we could show that the phosphopeptide map
of mRNP3–4 phosphorylated in vitro by purified Xenopus
CKII is indistinguishable from that of mRNP3–4 phospho-
rylated in vivo. This result strongly argues in favour of the
CKII involvement in the phosphorylation of mRNP3–4 in
vivo. It validates the suggestion from previous studies that a
CKII-'like' protein could be implicated in the phosphoryl-
ation of mRNP3 and mRNP4 in Xenopus oocytes [8,9,17].
The phosphorylated peptides were derived from a central do-
main (aa 94–153) which contains 3 conserved serine residues
(119, 143 and 149), a fourth one (125) is present only in
mRNP3 (Fig. 1). As during Edman degradation of the rele-
vant peptides (see [10] for the sequences of peptides 405 and
303) normal amounts of serine 119 were detected, we can
exclude that phosphorylation occurs on that residue (see for
example [29]). Finally, while serine 149 is followed by a pro-
line which is detrimental for phosphorylation by CKII [30,31],
serine 143 is present in the (S/T-X-X-Acidic) consensus se-
quence (Fig. 1) and is therefore, the most likely substrate of
CKII.

mRNP3 and mRNP4 are the principal constituents of the
mRNA storage particles and have been implicated in mRNA
masking from translation [7,12,32–34]. An appropriate phos-
phorylation of mRNP3–4 has been shown to be required for
mRNA masking [12,33] but its precise role is still poorly
understood. CKII could regulate mRNA masking in oocytes
by acting either on the interaction of mRNP3–4 with RNA or

by controlling the fate of assembled mRNPs. Previous reports have described conflicting results on the role of phosphorylation on RNA-binding affinity. Kick et al. [12] observed a lower affinity of mRNP4 for β-globin and poly A RNA following dephosphorylation, while Tafuri and Wolffe [35] found no difference in the RNA-binding properties of native mRNP4 from *Xenopus* oocyte or bacterially expressed mRNP4 which is not phosphorylated, suggesting that proper phosphorylation is not essential. In the present study we have used a quantitative assay to show that the affinity of native mRNP3–4 remains unchanged whether phosphorylated by CKII purified from *Xenopus* oocyte or dephosphorylated by calf intestine phosphatase. Our results conclusively indicate that CKII does not directly regulate the interaction of mRNP3 and mRNP4 with RNA. Therefore, regulation of mRNA masking by CKII has to occur at another level, one possibility being that the phosphorylation of mRNP3–4 controls their interaction with other proteins, either other constituents of mRNP, or, possibly, factors directly involved in translation.

The simplicity of the phosphorylation pattern of mRNP3–4 in oocytes should facilitate the investigation of its functional role. For instance, mutagenesis of the phosphorylation site will provide a tool to determine its role in mRNA masking. Moreover, the analysis of mRNP3–4 phosphorylation during early development and the search for the corresponding kinases should now be feasible.

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