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# Cytoplasmic retention and nuclear import of 5S ribosomal RNA containing RNPs

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**Nuclear export of newly transcribed 5S ribosomal RNA in *Xenopus* oocytes occurs in the context of either a complex with the ribosomal protein L5 (5S RNP) or with the transcription factor IIIA (7S RNP). Here we examine nuclear import of 5S RNA, L5 and TFIIIA. The 5S RNP shuttles between nucleus and cytoplasm and only 5S RNA variants which can bind to L5 gain access to the nucleus. The 7S RNP is retained in the cytoplasm. Only TFIIIA which is not bound to 5S RNA is imported into the nucleus. As a novel mechanism for cytoplasmic retention, we propose that RNA binding masks a nuclear localization sequence in TFIIIA. In contrast to the nuclear import of L5, import of TFIIIA is insensitive towards the nuclear localization sequence (NLS) competitor p(lys)-BSA, suggesting that these two proteins make use of different import pathways.**

*Keywords*: cytoplasmic retention/nucleocytoplasmic transport/5S ribosomal RNA/TFIIIA/*Xenopus laevis*

## Introduction

Intracellular transport of RNA and protein is regulated via a repertoire of distinct signal structures which are capable of directing macromolecules to the different compartments of the eukaryotic cell. Regulated trafficking between nucleus and cytoplasm involves proteins and RNA. Considerable progress has recently been made in respect of our understanding of protein import into the nucleus. The signal structures that target proteins to the nucleus are referred to as nuclear localization sequences (NLSs) and two major classes of such sequences have been distinguished, the SV40 type and the bipartite type (Dingwall and Laskey, 1991). Zinc finger proteins may contain a third type of motif located within their zinc finger cluster (Van Wijk *et al.*, 1992; Gashler *et al.*, 1993; Matheny *et al.*, 1994). *In vitro* transport assays have led to the identification of at least two distinct soluble cytoplasmic activities involved in protein import (Adam *et al.*, 1990; Moore and Blobel, 1992). A complex of two polypeptides including the NLS receptor activity selects karyophilic proteins from the cytoplasm for interaction with the nuclear pore complex (Adam and Adam, 1994; Görlich *et al.*, 1994; Moroianu *et al.*, 1995; Radu *et al.*, 1995; Weis *et al.*, 1995). A second pair of proteins mediates the energy-dependent translocation step through the nuclear pore; one of these proteins is the GTPase Ran/TC4 (Melchior *et al.*, 1993; Moore and Blobel, 1993), the other one a small, 10 kDa Ran-interacting protein (Moore and Blobel, 1994).

Molecular mechanisms of protein export from nucleus to cytoplasm have only very recently been elucidated; it had been proposed that some proteins may not require positively acting export signals at all (Schmidt-Zachmann *et al.*, 1993), but other proteins, such as Rev, rely on the presence of a nuclear export signal (NES) to gain exit into the cytoplasm (Fischer *et al.*, 1995; Wen *et al.*, 1995). The protein import of GTPase Ran/TC4 has also been reported to be required for protein export (Moroianu and Blobel, 1995). Interaction of nuclear proteins with other intranuclear components, such as DNA or RNA, is generally believed to influence protein import/export rates.

Following transcription, most, but not all RNA molecules get exported from the nucleus to the cytoplasm; only a subset of specific small RNA molecules is able to migrate in the opposite direction (De Robertis *et al.*, 1982). Data accumulated on RNA export indicate that the substrate for this process is RNP rather than naked RNA (reviewed in Izaurralde and Mattaj, 1995). As with protein export, nuclear retention appears to be a mechanism that can regulate the RNA export rate (Jarmolowski *et al.*, 1994; Boelens *et al.*, 1995). The export of different classes of RNA that normally leave the nucleus is mediated by specific, saturable factors (Zasloff, 1983; Jarmolowski *et al.*, 1994). Candidate export proteins for 5S ribosomal RNA are TFIIIA and L5 (Guddat *et al.*, 1990). Other RNA binding proteins which have been implicated in RNA export are the HIV Rev protein (Fischer *et al.*, 1994) and the SRP proteins 9 and 14 (He *et al.*, 1994). The hnRNP protein A1 shuttles between nucleus and cytoplasm and since it associates with poly$(A)^+$ RNA in both compartments, it may be involved in mRNA export (Pinol-Roma and Dreyfuss, 1992).

It is of primary interest to identify export signals in nuclear RNPs (if they exist) and to identify protein components of the export RNPs. The $5'm^7G$ cap structure has been demonstrated to be important for U snRNA and mRNA export (Hamm and Mattaj, 1990; Jarmolowski *et al.*, 1994). A protein binding to the monomethyl-cap structure has been purified and demonstrated to be important for the export of U snRNA (Izaurralde *et al.*, 1995). As with protein import and protein export, the GTPase Ran/TC4 and the associated GTP exchange factor RCC1 are known to be involved in mRNA export in yeast (Amberg *et al.*, 1993; Kadowaki *et al.*, 1993; Schlenstedt *et al.*, 1995). More recently, the importance of RCC1 in RNA export was also demonstrated for mammalian cells; here, RCC1 was found to be required for export of snRNAs (Cheng *et al.*, 1995).

After U snRNP assembly and hypermethylation of the $5'm^7G$-cap to $m_3G$-cap in the cytoplasm (Mattaj, 1986), particles migrate into the nucleus where they exert their

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biological function. Such snRNPs utilize a bipartite NLS composed of the Sm-core domain and the $m_3G$-cap (Fischer and Lührmann, 1990; Hamm *et al.*, 1990). Differential sensitivity to various competitors of nuclear import indicates that most karyophilic proteins make use of a different import pathway than the majority of snRNPs (Fischer *et al.*, 1991, 1993; Michaud and Goldfarb, 1991, 1992).

In stage V/VI *Xenopus* oocytes, 5S ribosomal RNA synthesized in excess over other ribosomal components is found in the context of small, non-ribosomal RNPs. These can be either 7S RNPs, i.e. 5S RNA plus the zinc finger protein TFIIIA (Picard and Wegnez, 1979; Honda and Roeder, 1980; Pelham and Brown, 1980), or 5S RNPs, i.e. 5S RNA plus the ribosomal protein L5 (Guddat *et al.*, 1990; Allison *et al.*, 1991). Oocyte-type 5S RNA, which differs in only 6 of 120 nucleotides from somatic-type 5S RNA, appears to have a preference for TFIIIA binding, whereas somatic-type 5S RNA appears to prefer L5 for RNP formation (Allison *et al.*, 1995). Both particles form in the nucleus with newly transcribed 5S RNA and they are then exported for storage in the cytoplasm (Guddat *et al.*, 1990). 5S RNA re-import into the nucleus is a selective process (DeRobertis *et al.*, 1982) that can be inhibited by wheatgerm agglutinin and appears to be an energy-requiring process (Allison *et al.*, 1993). Early immunohistochemical data had provided first evidence for nuclear exclusion of 7S RNPs (Mattaj *et al.*, 1983). It has been suggested that 5S RNA can be exchanged from microinjected 7S RNP to 5S RNPs. Such 5S RNPs produced by RNA exchange in the cytoplasm can also be detected in the nucleus (Allison *et al.*, 1991). Taken together, these findings suggest that, in the oocyte, 5S RNA is exported from the nucleus either as 7S RNP or 5S RNP, and that, in association with ribosomal protein L5, this ribosomal RNA can return to the nucleus for ribosomal assembly in the nucleoli.

This study generates further support for the validity of this working hypothesis and provides insight into the molecular mechanisms that regulate the nucleocytoplasmic distribution of the three components in this system. 5S RNA binding is found to act as the key element in cytoplasmic retention of TFIIIA. In contrast, ribosomal protein L5 is imported into the nucleus in association with 5S RNA, and differential sensitivity towards the competitor p(lys)-BSA suggests that the two proteins make use of different import pathways. The ability of 5S RNA to be imported into the nucleus correlates with its ability to bind to ribosomal protein L5, indicating that 5S RNA import occurs in the context of an RNP rather than as naked RNA.

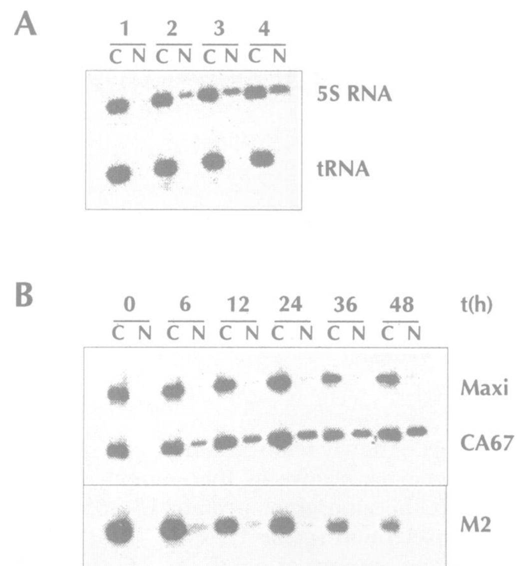


**Fig. 1.** Nuclear import of 5S RNA correlates with the ability to bind to ribosomal protein L5. (**A**) Intracellular distribution of $^{32}$P-labelled 5S RNA and tRNA after co-injection into the cytoplasm of *X.laevis* oocytes and after incubation at different temperatures. The oocytes were dissected either immediately after microinjection (1) or 16 h later after incubation at 6°C (2), at 12°C (3) or at 19°C (4). RNAs extracted from cytoplasmic (C) or nuclear (N) fractions were separated on denaturing polyacrylamide gels and visualized by use of a phospho-imager. (**B**) Nuclear import of $^{32}$P-labelled 5S RNA mutants Maxi, CA67 or M2 after cytoplasmic microinjection. Maxi and CA67 were co-injected (top) whereas M2 was injected in a separate experiment (bottom). At different times after injection (indicated at the top), oocytes were dissected and RNAs from cytoplasmic or nuclear fractions were analysed as described in (A).

## Results

### Nuclear import of 5S RNA variants

The ability of *Xenopus laevis* somatic 5S RNA to enter the nucleus after microinjection into the cytoplasm of *Xenopus* oocytes is a selective and temperature-dependent process (Figure 1A), as was to be expected from similar experiments performed with either human or *Xenopus* oocyte 5S RNA (De Robertis *et al.*, 1982; Allison *et al.*, 1993). With the intention to correlate the ability of 5S RNA to bind TFIIIA or ribosomal protein L5 with its ability to be imported into the nucleus, we have performed cytoplasmic injections of mutant variants of 5S RNA which were either impaired in their capacity to bind to TFIIIA, or to L5, or to both proteins (Figure 1B) (Guddat *et al.*, 1990). The Maxi 5S RNA variant, which will only interact with TFIIIA, is sequestered in the cytoplasm, whereas the CA67 variant, which will only interact with ribosomal protein L5, is imported into the nucleus at a rate comparable with WT 5S RNA. Mutant M2, which is reduced in its ability to interact with both proteins, remains in the cytoplasm. Note that, probably as a direct consequence of this lack of protein-binding activity, M2 RNA exhibits significantly reduced stability in comparison with the other RNAs tested. These findings indicate that 5S RNA enters the nucleus in a complex with L5 (5S RNP), whereas the complex with TFIIIA (7S RNP) is retained in the cytoplasm.

### Interaction of TFIIIA with 5S RNA results in cytoplasmic retention

In order to assay the nuclear import of L5 and TFIIIA proteins in the stage V/VI oocyte situation, myc-tagged protein versions were produced *in vitro* and microinjected into the cytoplasm of such cells (Figure 2A). L5 accumulates in the nucleus, whereas TFIIIA remains sequestered in the cytoplasmic compartment. Although this result matches exactly that which we would infer from the results obtained with mutant RNA on the nuclear import of the corresponding RNPs (as described above), this lack

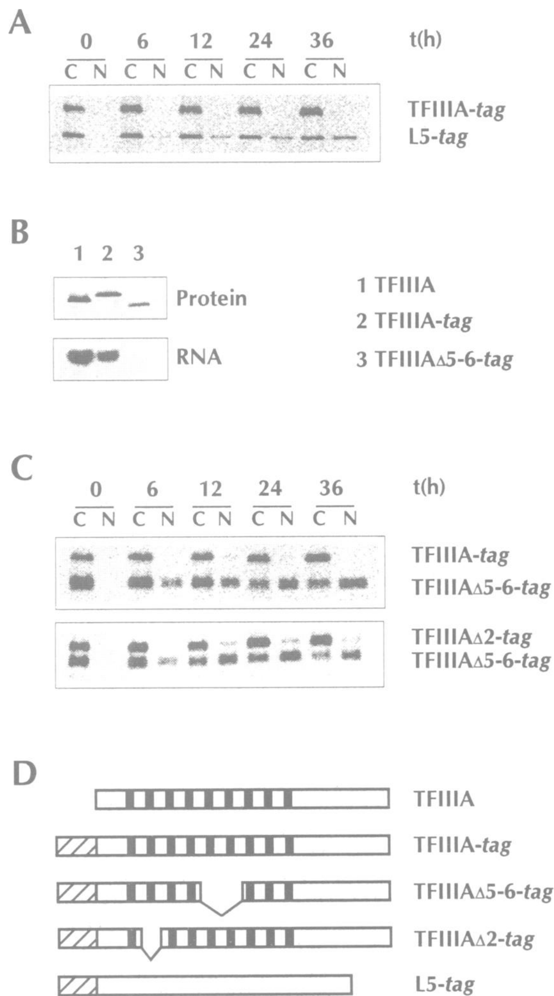


**Fig. 2.** Cytoplasmic retention of TFIIIA correlates with the ability to bind to 5S RNA. (**A**) Nuclear import of L5 and TFIIIA in *Xenopus* oocytes. *In vitro* translated $^{35}$S-labelled L5-*tag* and TFIIIA-*tag* proteins were co-injected into the cytoplasm of *Xenopus* oocytes. At different times after injection (indicated at the top) the oocytes were dissected and the proteins were immunoprecipitated from cytoplasmic or nuclear fractions using the myc-Ab. Extracted proteins were separated by SDS–PAGE and visualized by use of a phosphoimager. (**B**) Binding of 5S RNA to *in vitro* translated WT and mutant TFIIIA proteins. The proteins were *in vitro* translated in the presence of $^{32}$P-labelled 5S RNA. This reaction was followed by an immunoprecipitation with α-TFIIIA-Ab. The immunopellet was divided into two fractions, and either proteins (top) or the co-precipitated 5S RNA (bottom) were analysed by SDS or denaturing polyacrylamide gel electrophoresis, respectively. (**C**) Nuclear import of TFIIIA and TFIIIA mutant proteins in *Xenopus* oocytes. *In vitro* translated and $^{35}$S-labelled myc-tagged versions of either TFIIIA and TFIIIAΔ5-6 (top) or TFIIIAΔ2 and TFIIIAΔ5-6 (bottom) were co-injected into the cytoplasm. At different times after injection (indicated at the top) oocytes were dissected and the proteins immunoprecipitated from cytoplasmic and nuclear fractions using the α-TFIIIA-Ab. The extracted proteins were analysed as described in (A). (**D**) Schematic representation of proteins used in the experiments described above. The hatched boxes denote the myc-tag, closed boxes denote the zinc finger units in TFIIIA proteins. Internal deletions are indicated.

of TFIIIA import raises further questions. TFIIIA function as a transcription factor and the formation of export RNPs with newly transcribed 5S RNA requires prior uptake of the protein into the nucleus. This apparent dilemma prompted us to ask if TFIIIA import could be blocked by RNA binding. Two different lines of experimental evidence

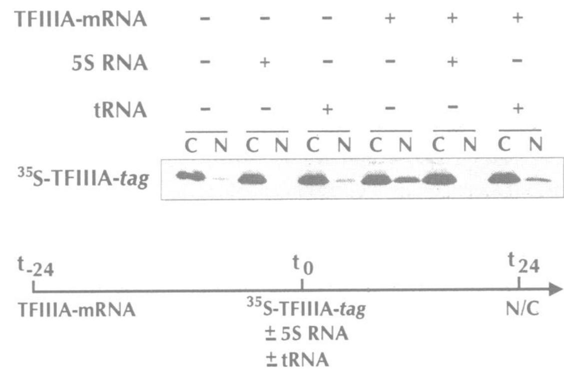


**Fig. 3.** 5S RNA inhibits nuclear import of TFIIIA in *X.laevis* oocytes. Microinjections, extraction of injected proteins and analysis of extracted proteins were performed as described in Figure 2A. All oocytes used in this experiment were injected with either TFIIIA mRNA (+) or with water (–) 24 h before the injection of *in vitro* translated $^{35}$S-labelled TFIIIA-*tag*. Where indicated, the protein was co-injected with 2.5 pmol *X.laevis* 5S RNA or with 2.5 pmol bovine liver tRNA. The injected oocytes were dissected (N/C) either immediately or 24 h after injection of $^{35}$S-labelled TFIIIA-*tag* protein (indicated at the top).

argue that this is indeed the case. First, a mutant version of TFIIIA that has lost the ability to bind to 5S RNA due to internal deletion of fingers 5 and 6 (Figure 2B) is readily imported into the nucleus after microinjection into the cytoplasm (Figure 2C). A different variant of TFIIIA, that has lost its ability to bind to DNA due to internal deletion of finger 2 but maintains RNA binding activity (Theunissen *et al.*, 1992) is retained in the cytoplasm, as is WT TFIIIA (Figure 2C). This result indicates that the cytoplasmic retention of TFIIIA depends on its 5S RNA binding activity. However, the finger 5/6 region of TFIIIA could equally interact with a different cytoplasmic anchor. Therefore, we designed an experiment that provides more direct evidence for the importance of 5S RNA in the cytoplasmic retention of TFIIIA.

The number of 5S RNA molecules available for TFIIIA binding should be saturable; saturation should be achieved by increase of the TFIIIA concentration in the oocyte above a critical threshold value. Such an increase in the intracellular TFIIIA concentration was obtained by microinjection of TFIIIA mRNA into the oocyte before injection of radiolabelled, full-length TFIIIA (Figure 3). A substantial portion of the radioabelled TFIIIA is now indeed able to enter the nucleus. Therefore, cytoplasmic retention of TFIIIA is a saturable process. The nuclear import of TFIIIA that is achieved by this increase in protein concentration can be blocked by a parallel increase of 5S RNA concentration via oocyte microinjection, but not with extra tRNA. Note that the residual level of TFIIIA import observed in this particular experiment also without prior overexpression of unlabelled TFIIIA is equally inhibited by 5S RNA but not by tRNA.

The strict correlation of RNA binding and cytoplasmic retention of TFIIIA observed could be the result of either one out of two different molecular mechanisms. Either 5S RNA binding makes TFIIIA unavailable for the import machinery by masking the NLS function, or 5S RNA serves as a linker with a cytoplasmic anchor. In order to distinguish between these two possibilities, an SV40-type NLS was fused to TFIIIA, which should bring the 5S

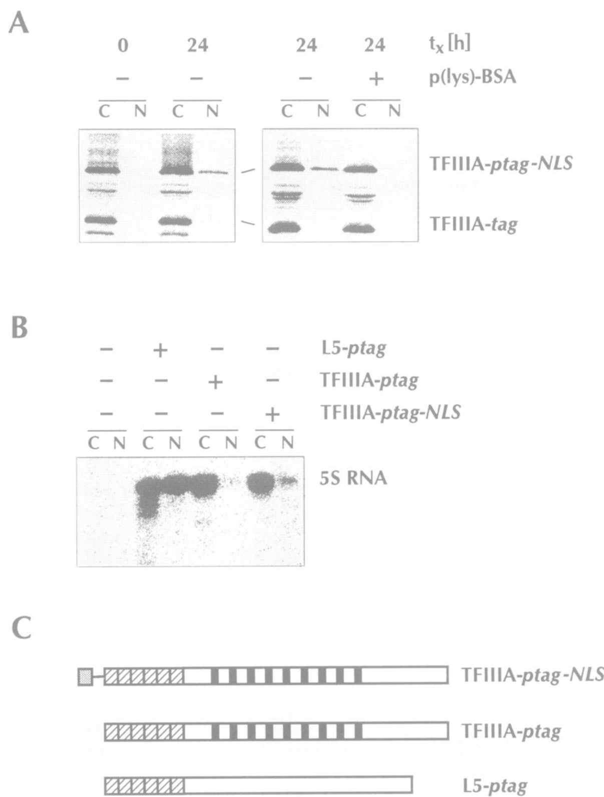


**Fig. 4.** SV40-NLS fusion relieves cytoplasmic retention of TFIIIA. (**A**) SV40-NLS-fusion mediates nuclear import of TFIIIA and can be inhibited by p(lys)-BSA. *In vitro* translated, $^{35}$S-labelled TFIIIA-*ptag-NLS* and TFIIIA-*tag* were co-injected into the cytoplasm and the nucleocytoplasmic distribution of the injected proteins was analysed either immediately or 24 h after injection. Where indicated, oocytes were injected with p(lys)-BSA (20 mg/ml) before the injection of the $^{35}$S-labelled proteins. The experiments shown in the left- and in the right-hand panels were performed separately with different preparations of *in vitro* translated $^{35}$S-labelled proteins. (**B**) Association of 5S RNA with L5-*ptag*, TFIIIA-*ptag* or TFIIIA-*ptag-NLS* in *Xenopus* oocytes. Oocytes were injected with either water, or L5-*ptag* mRNA, or TFIIIA-*ptag* mRNA or with TFIIIA-*ptag-NLS* mRNA and incubated for 4 h at 19°C, followed by a second, cytoplasmic injection with $^{32}$P-labelled 5S RNA. Twenty-four hours after the second injection the oocytes were manually dissected and cytoplasmic and nuclear fractions were subjected to immuno-precipitation with a myc-tag antibody. Co-precipitation of $^{32}$P-labelled 5S RNA was analysed by denaturing polyacrylamide–gel electro-phoresis and by phosphoimaging. (**C**) Schematic representation of TFIIIA-*tag-NLS*, TFIIIA-*ptag* and L5-*ptag*. The grey, hatched and closed boxes denote the SV40-NLS, the polymyc-tag (*ptag*) and the zinc finger units, respectively.

RNA/TFIIIA complex into the nucleus if it was not bound to a cytoplasmic anchor. A similar strategy had been employed in the analysis of the mechanism that regulates nuclear transport of NF-κB (Beg *et al.*, 1992). TFIIIA that contains an SV40 type NLS is no longer retained in the cytoplasm (Figure 4A). Note, however, that nuclear transfer of this TFIIIA–NLS fusion is very inefficient (see Figure 2C for comparison). This effect indicates that the SV40-type NLS-mediated pathway for the nuclear import of proteins is not very effective for RNPs. The idea that the SV40-type NLS fusion to TFIIIA is indeed responsible for the protein import observed is also supported by the finding that nuclear transport of TFIIIA–*ptag–NLS* is inhibited by the basic competitor protein p(lys)-BSA (Figure 4A). Finally, the fact that 5S RNA is co-immuno-precipitated with TFIIIA–*ptag–NLS* from both cytoplasmic and nuclear fractions (Figure 4B) is in line with the idea that it is the RNP and not the free protein that gets imported into the nucleus. Taken together, observations described in this section provide strong evidence for the idea that 5S RNA binding makes TFIIIA unavailable for the protein import machinery, perhaps by masking the NLS in TFIIIA.

### *Differential sensitivity of 5S RNA, TFIIIA and L5 towards inhibitors of nuclear import*

In contrast to L5, TFIIIA does not contain a primary sequence element that would match the NLS consensus sequences as defined by Dingwall and Laskey (1991). Several agents have been employed successfully in order to inhibit the nuclear import of proteins and RNA. The monoclonal antibody 414 (mAb 414) is directed against nucleoporins (Davis and Blobel, 1986) and it inhibits RNA import and export in *Xenopus* oocytes (Michaud and Goldfarb, 1992; Terns and Dahlberg, 1994). Similarly, wheatgerm agglutinin (WGA) is a lectin that binds to glycosylated components of the nuclear pore complex and thereby inhibits protein import as well as, in a dose-dependent manner, nuclear import of different U snRNAs (Michaud and Goldfarb, 1992). BSA cross-linked with synthetic peptides based on the SV40 large T-antigen nuclear localization sequence [p(lys)-BSA] inhibits protein and U6 snRNA import, but not import of other U snRNAs (Michaud and Goldfarb, 1991, 1992). We tested the effect of these different agents on nuclear import of 5S RNA, ribosomal protein L5 and the import-competent TFIIIA variantΔ5/6 (Figure 5). The inhibition of 5S RNA import observed with WGA confirms an earlier report by others (Allison *et al.*, 1993). The second inhibitor that is known to interact with nucleoporins, mAb 414, equally interferes with import of 5S RNA (Figure 5A).

The two agents which interact with components of the nuclear pore, WGA and mAb 414, both inhibit nuclear transport of the two proteins L5 and TFIIIAΔ5/6 (Figure 5B and C, respectively). However, differential effects were observed with p(lys)-BSA; this inhibitor reduces nuclear import of L5, which contains a canonical NLS (Figure 5B). In marked contrast, import of neither TFIIIA Δ5/6 nor 5S RNA is affected by the same dose of p(lys)-BSA (Figure 5A and C). In respect to TFIIIA, this finding supports the idea that this protein makes use of an import pathway that does not operate via an NLS receptor that interacts with the SV40-type nuclear localization sequence. The exact identity of the NLS in TFIIIA remains to be established, but preliminary experimental evidence suggests that it will be located within the zinc finger cluster (F.Rudt and T.Pieler, unpublished observations) as has been reported for other zinc finger proteins (Van Wijk *et al.*, 1992; Matheny *et al.*, 1994). The failure of 5S RNA mutants that cannot interact with ribosomal protein L5 to enter the nucleus (as described above) would suggest that inhibition of L5 import should equally result in cytoplasmic retention of 5S RNA. This, however, is not the case; at a concentration that is sufficient to inhibit nuclear transfer of L5 to a significant extent, p(lys)-BSA has no effect on the import of 5S RNA (Figure 5B and A, respectively). One way to explain this apparent

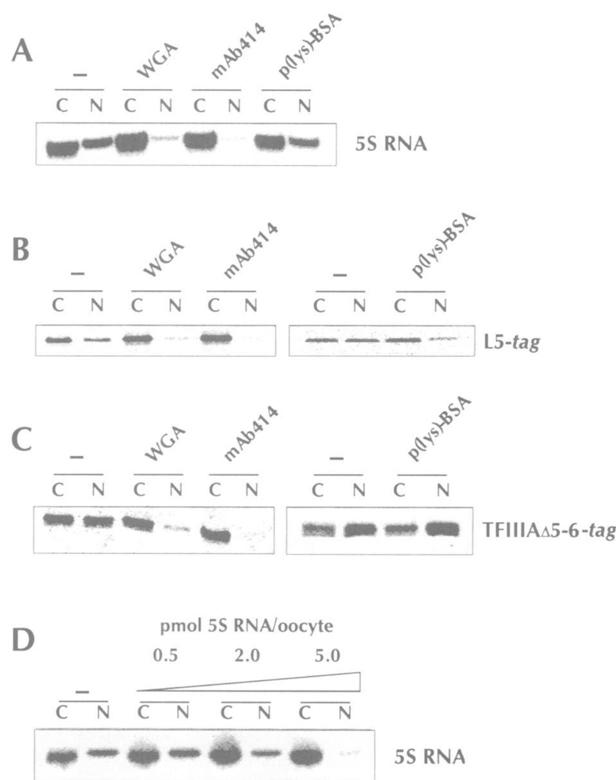


**Fig. 5.** Inhibitors of nuclear import of 5S RNA, L5 and TFIIIAΔ5-6. In the experiments shown in (**A**), (**B**) and (**C**) either 20 nl of water (–), WGA (10 mg/ml), mAb414 (25 mg/ml) or p(lys)-BSA (20 mg/ml) were injected into the cytoplasm of oocytes before a second injection of either $^{32}$P-labelled 5S RNA (A), or myc-tagged versions of L5 (B) or TFIIIAΔ5-6 proteins (C). The oocytes were incubated for 20 h and nuclear import of the RNA or of the proteins was analysed as described. (**D**) Saturability of the nuclear import of 5S RNA. The nuclear import of $^{32}$P-labelled 5S RNA was determined 24 h after its injection either in the absence of unlabelled 5S RNA or the presence of increasing amounts of unlabelled 5S RNA (indicated at the top).

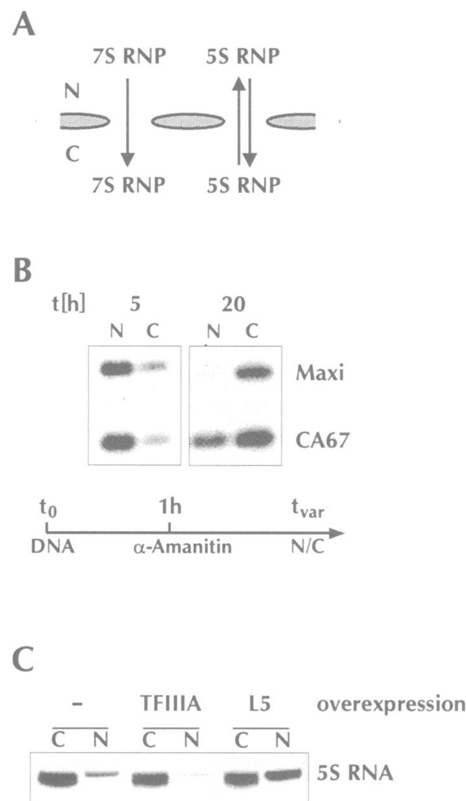


**Fig. 6.** Nucleocytoplasmic transport of the 5S RNP and the 7S RNP. (**A**) Schematic representation of nucleocytoplasmic transport of 5S RNP and 7S RNP in *Xenopus* oocytes. (**B**) Comparison of the nuclear export of 5S RNA mutants Maxi and CA67. Plasmids containing the Maxi or the CA67 genes were co-injected together with [α-$^{32}$P]GTP into the nucleus of *Xenopus* oocytes. At 1 h after injection the oocytes were injected a second time with 20 nl α-amanitin (300 μg/ml) in order to inhibit the transcription of the injected genes. The nucleocytoplasmic distribution of $^{32}$P-labelled RNAs was analysed after 5 and 20 h as described above. (**C**) Effect of increased levels of L5 or TFIIIA concentration on the nuclear import of 5S RNA. Oocytes were injected with either water as a control, or with L5 mRNA or TFIIIA mRNA. Two hours after this first injection, $^{32}$P-labelled 5S RNA was injected into the cytoplasm of these oocytes and its intracellular distribution was analysed 24 h later.

contradiction is to claim that free L5 and 5S RNA/L5 complex (5S RNP) make use of different import pathways. If this were true, it could have the important implication that the NLS in the protein is functionally distinct from the NLS in the RNP. Experiments which address this problem have been initiated in our laboratory.

If a saturable cytoplasmic protein factor like L5 would indeed be required for nuclear import of 5S RNA, this process should be competed by excess 5S RNA. In order to test this idea, oocytes were preinjected with unlabelled 5S RNA and the nuclear import of radiolabelled 5S RNA from a secondary injection analysed following the standard protocol (Figure 5D). The results obtained demonstrate that 5S RNA import in *Xenopus* oocytes is saturable, providing further evidence for an important function of a specific 5S RNA binding protein like L5 in the nuclear transport of this RNA molecule. Note, however, that import of 5S RNA can also be inhibited by a similar quantity of tRNA although there is only residual tRNA binding activity measured with L5 (data not shown). This unexpected finding raises the possibility that the import/export pathways for the different RNPs overlap and that even though tRNA is not imported into the nucleus, it may bind directly or indirectly to a factor that is required for 5S RNA transfer to the nucleus. As observed for 5S RNA export with nuclear injections (Jarmolowski *et al.*, 1994), cytoplasmic injection of poly(G) similarly interferes with nuclear transfer of 5S RNA (data not shown), perhaps by unspecific interaction with RNA binding protein(s) involved in (5S) RNA import into the nucleus.

### *Further evidence for the shuttling of 5S RNPs*

We have previously shown that nuclear export of 5S RNA correlates with its ability to interact with either TFIIIA or ribosomal protein L5 (Guddat *et al.*, 1990). Together with the data reported in this communication, our findings suggest a simple scheme for nucleocytoplasmic transport of 5S ribosomal RNA containing RNPs which is illustrated in Figure 6A. The 7S RNP forms in the nucleus and becomes exported to the cytoplasm where it remains. In contrast, the 5S RNP, which also forms in the nucleus, is able to shuttle back and forth between nucleus and cytoplasm. Such a simple model would suggest that a 5S RNA variant which will only bind to TFIIIA should be

quantitatively sequestered in the cytoplasm, whereas a mutant version that will only bind to ribosomal protein L5 should shuttle constantly between nucleus and cytoplasm and therefore reach an equilibrium distribution between these two major cellular compartments. Figure 6B illustrates that this is indeed the case. In contrast to our previous experiments, also using nuclear injection of cloned 5S RNA genes (Guddat *et al.*, 1990), RNA transcription was stopped 1 h after injection of the gene by a secondary injection of a critical dose of α-amanitin in order to stop the constant supply of newly transcribed 5S RNA in the nucleus; 5 h later, both RNA mutants are found to migrate to the cytoplasm at a similar rate. However, 20 h after the first injection, the TFIIIA binding variant (Maxi) is quantitatively transferred to the cytoplasmic compartment, whereas the L5 binding 5S RNA mutant (CA67) is distributed between nucleus and cytoplasm. Longer periods of incubation do not further affect the nuclear/cytoplasmic ratio of CA67 (data not shown) which we therefore assume to reflect the equilibrium concentration of 5S RNPs.

The dynamics of 7S RNP/5S RNP migration between nucleus and cytoplasm as depicted in Figure 6A would also suggest that an increase in the concentration of ribosomal protein L5 should stimulate nuclear import of 5S RNA, whereas an increase in TFIIIA concentration should enhance the rate of cytoplasmic retention. Such modulations of protein concentration were achieved by microinjection of mRNA corresponding to either TFIIIA or L5 into *Xenopus* oocytes. Oocytes manipulated in this way were then injected into the cytoplasm with radiolabelled 5S RNA and import rates analysed following the standard protocol. Increase in L5 concentration leads to enhanced import, increase in TFIIIA concentration to enhanced retention, as predicted from our model (Figure 6C).

## Discussion

We have analysed the correlation of protein-binding ability and nuclear import of 5S ribosomal RNA in *Xenopus* oocytes. Previous work had shown that newly transcribed 5S RNA associates with either ribosomal protein L5 or TFIIIA and that the ability of RNP formation correlates with 5S RNA export capacity (Guddat *et al.*, 1990). Experiments described in this report demonstrate that RNA import is also correlated with RNP formation. Only 5S RNA molecules with ability to interact with ribosomal protein L5 gain access to the nucleus. Conversely, RNA associated with TFIIIA remains sequestered in the cytoplasm. Cytoplasmic retention is also observed for exogenous TFIIIA microinjected into *Xenopus* oocytes. Sequestration in the cytoplasm correlates with the ability of the protein to bind to 5S RNA. Cytoplasmic retention of TFIIIA is saturable; above a critical concentration, TFIIIA is imported into the nucleus. This nuclear import of excess TFIIIA can be inhibited by increasing the concentration of 5S RNA.

One possible way in which 5S RNA could inhibit the nuclear transport of TFIIIA is by masking a nuclear localization sequence in the protein. NLS masking via protein–protein interaction has been demonstrated to regulate nuclear import of several proteins. The nuclear localization sequence of NF-κB is either masked by interaction with IκB (Beg *et al.*, 1992), or, as in the case of the p110 NF-κB precursor, by intramolecular folding (Henkel *et al.*, 1992). A different mechanism that leads to NLS inactivation is chemical modification; phosphorylation in close proximity to the NLS in the yeast zinc finger protein SWI/5 inhibits nuclear import (Moll *et al.*, 1991). A further example for cytoplasmic retention of a nuclear, DNA binding protein in *Xenopus* oocytes is provided by xnf7; a cytoplasmic retention domain (CRD) has been mapped, which is dominant over a second NLS added to xnf7, suggesting that CRD interacts with a cytoplasmic anchor which could be connected to the cytoskeleton (Li *et al.*, 1994). Interaction with RNA constitutes a novel molecular mechanism for cytoplasmic retention. Although the NLS in TFIIIA has not been mapped precisely, the fact that it is localized within the zinc finger cluster (F.Rudt and T.Pieler, unpublished observations), suggests that it may be masked by RNA binding, since all zinc fingers were found to be in tight contact with the RNA molecule (Theunissen *et al.*, 1992). The fact that the vast majority of the TFIIIA/5S RNA complex is found in the soluble, low molecular weight fraction of oocyte extracts provides a further argument against interaction with the cytoskeleton or sequestration in large complexes to be the reason for cytoplasmic retention of the 7S RNP (Mattaj *et al.*, 1983; Guddat *et al.*, 1990).

Reduced sensitivity of TFIIIA against the import inhibitor p(lys)-BSA suggests that TFIIIA makes use of a different import pathway than L5 and the majority of nuclear proteins (Michaud and Goldfarb, 1993). Since saturating amounts of p(lys)-BSA have been described not to reduce the rate of U2 snRNP import (Michaud and Goldfarb, 1991), import pathways for TFIIIA and U2 snRNP could overlap. The existence of a family of proteins structurally related to the NLS binding protein hSRPα (Görlich *et al.*, 1994; Weis *et al.*, 1995) indicates that a typical eukaryotic cell may indeed use different but perhaps overlapping import pathways for different nuclear proteins and/or RNPs. Further support for such a notion comes from a recent compilation of nuclear localization sequences which allows for the classification of more than six different subtypes (La Casse and Lefebvre, 1995) that could interact with different types of NLS receptors. Similarly, other factors involved in 5S RNP import, which we also find to be insensitive towards p(lys)-BSA, remain to be identified. It will be important to assay for an overlap with the import pathways of other RNPs, which is very likely to exist.

Finally, results reported in this communication add information to the events that lead to 5S RNA incorporation into ribosomes during oogenesis in *X.laevis*. 5S RNA that is made in excess over other ribosomal components during early stages of oogenesis is exported from the nucleus primarily in a complex with TFIIIA. This 7S RNP is sequestered in the cytoplasm. Upon increased synthesis of ribosomal protein L5, which is paralleled by a significant reduction in TFIIIA concentration towards late stages of oogenesis (Shastry *et al.*, 1984; Wormington, 1989), 5S RNA could be exchanged from 7S RNP to 5S RNP and thereby return to the nucleus for incorporation into ribosomes, as proposed by Allison *et al.* (1991).

## Material and methods

### *Plasmids and cloning procedures*

Plasmids encoding the class III genes used for microinjection have been described previously. References are Pieler *et al.* (1985) for the WT 5S RNA gene, Guddat *et al.* (1990) for the M2 gene, Theunissen *et al.* (1992) for the CA67 gene, Bogenhagen and Brown (1981) for the Maxi gene and Clarkson *et al.* (1978) for the t-$RNA^{Met}$ gene.

The T7-5SXls clone is a M13mp18 derivative containing the WT 5S gene flanked by the T7 promoter and a *Dra*I restriction site. The T7 promoter and the *Dra*I site have been introduced by site-directed mutagenesis using synthetic oligonucleotides following the procedure described by Kunkel (1985).

The plasmid T3myc/L5 N16 was kindly provided by Mike Wormington (Charlottesville, USA). Due to cloning procedures this clone lacks the sequence element coding for the first 16 N-terminal amino acids of the WT L5b cDNA (Wormington, 1989).

For the construction of the T3mycTFIIIA and the SP6-WT-TFIIIA clones, plasmid M13mp11-TFIIIA was used as a source for TFIIIA cDNA fragments. M13mp11-TFIIIA contains the TFIIIA cDNA (Ginsberg *et al.*, 1984) cloned as *Eco*RI–*Hpa*I-fragment into the *Eco*RI/*Hinc*II sites of M13mp11. The *Eco*RI–*Bam*HI-fragment of M13mp11-TFIIIA was cloned into the same sites of pSPT19 in order to generate the SP6-WT-TFIIIA clone, from which TFIIIA can be expressed *in vitro*. For the construction of the T3mycTFIIIA clone the M13mp11-TFIIIA DNA was digested with Cfr10I which cuts once at the 5′-end of the TFIIIA coding region. The recessed 3′-termini were filled using Klenow polymerase, followed by the ligation of an 8mer *Eco*RI-Linker (New England Biolabs) to the blunt end. This step was followed by a re-cutting with *Eco*RI and an additional cut with *Bam*HI, generating a fragment which contains the TFIIIA cDNA shortened by 18 bp at the 5′-end. This *Eco*RI–*Bam*HI-fragment was cloned into the *Eco*RI/*Bam*HI sites of the plasmid T3myc/L5 N16, thereby substituting the L5 cDNA by the modified TFIIIA cDNA. When compared with the *in vitro* translated WT-TFIIIA protein, the TFIIIA-*tag* protein showed no significant differences in nucleocytoplasmic transport (data not shown) or in 5S RNA binding (Figure 2B). To generate T3mycTFIIIAΔ5-6 and T3mycTFIIIAΔ2 the *Pst*I–*Sph*I-fragment of T3mycTFIIIA was substituted by the appropriate fragment from either pAX4c+TFIIIAΔ5-6 or pAX4c+TFIIIAΔ2 (Theunissen *et al.*, 1992), respectively. The change of the amino acid sequence at the N-terminus of the TFIIIA-*tag* proteins compared with the WT-TFIIIA is shown below. The underlined letters denote the c-myc-epitope, the proline represents the beginning of the amino acid identity.

WT-TFIIIA: MGEKALP
TFIIIA-*tag*: MEQKLISEEDLNSP

Immunoprecipitations with the myc-Ab are not very efficient under the conditions used in our experiments. In order to increase immunoprecipitation efficiency, clones were generated which carry six repeats of the c-myc epitope (*ptag*). In these constructs, which encode L5-*ptag* and TFIIIA-*ptag*, the *Cla*I–*Nco*I-fragment of L5-*tag* and TFIIIA-*tag* were substituted by the *Cla*I–*Nco*I-fragment of the plasmid CS2+MT (Rupp *et al.*, 1994; Turner and Weintraub, 1994). The immunoprecipitation efficiency with the *ptag*-proteins was ~3- to 4-fold higher compared with the corresponding *tag*-proteins. The *ptag* has no influence on the nucleocytoplasmic transport behaviour of these proteins, when compared with the corresponding *tag*-proteins (data not shown).

To generate the SV40-NLS/myc-TFIIIA clone the TFIIIA cDNA containing *Eco*RI–*Spe*I-fragment of T3mycTFIIIA was inserted into the *Eco*RI/*Xba*I sites of the plasmid CS2+NLS-MT, which is a derivative of CS2+MT. In CS2+NLS-MT a SV40 large T antigen nuclear localization sequence (NLS) was introduced in front of the myc-tag repeats. Except for the NLS, the vector is identical to CS2+MT.

### *Expression of proteins in vitro*

*In vitro* translations were performed using cDNAs cloned into vectors that allow transcription by T3 or SP6 polymerases. Proteins were expressed from these cDNAs in the coupled transcription/translation ($T_NT$) system from Promega. Reactions were performed following the Promega ($T_NT$) protocol and [$^{35}$S]methionine or [$^{35}$S]cysteine (Amersham Corporation) were used for radiolabelling. *In vitro* translation products were dialysed against injection buffer (10 mM PIPES, pH 7.4, 40 mM KCl, 10 mM NaCl) and analysed by SDS–PAGE and phosphoimaging (Molecular Dynamics).

### *Preparation of radiolabelled RNAs*

For all *in vitro* transcriptions the RNA Transkription Kit from Stratagene was used. *In vitro* transcription and radiolabelling of WT 5S RNA was performed following the standard reaction protocol supplied by Stratagene. One microgram of *Dra*I-digested T7-5SXls DNA was added to this reaction and [α-$^{32}$P]UTP (Amersham) was used for the radiolabelling of the RNA. To remove unincorporated nucleotides the reaction was applied to a Sephadex G-25 spin column (Boehringer) and the eluted RNA was used directly for microinjection or binding assays.

The 5S RNA mutants Maxi, CA67, M2 and the t-RNA could not be transcribed *in vitro*. For radiolabelling of these RNAs, plasmids containing the appropriate genes were injected together with [α-$^{32}$P]GTP into oocyte nuclei. For each RNA to be transcribed 100 oocytes were injected and incubated for 16 h at 19°C. Newly transcribed RNAs were extracted as described below and separated on native 8% polyacrylamide gels. Gel pieces containing the radiolabelled RNAs were cut out, incubated in 400 μl RNA elution buffer (500 mM ammonium acetate, 10 mM magnesium acetate, 100 μM EDTA–NaOH, pH 8.0, 0.1% SDS) for 24 h at room temperature. The eluate was ethanol-precipitated, dissolved in water and used for microinjections.

### *Preparation of 5′-capped mRNAs*

For *in vitro* synthesis of 5′-capped mRNAs the RNA Transcription Kit from Stratagene was used but supplemented with the RNA cap structure analogue [$m^7$G(5′)ppp(5′)G, New England Biolabs] according to the standard reaction protocol for the synthesis of *in vitro* capped RNA transcripts (supplied with the manual of the Rabbit Reticulocyte Lysate System, Promega).

### *Isolation of Xenopus 5S RNA*

In order to isolate *Xenopus* 5S RNA, crude ovary homogenates were phenol-extracted, the clear phase ethanol-precipitated and the precipitated molecules were separated on a native 8% polyacrylamide gel. RNA molecules were visualized by UV shadowing and the gel pieces containing the 5S RNA were cut out, incubated in 400 μl RNA elution buffer (500 mM ammonium acetate, 10 mM magnesium acetate, 100 μM EDTA–NaOH, pH 8.0, 0.1% SDS) for 24 h at room temperature. The eluate was ethanol-precipitated and dissolved in water.

### *Microinjection experiments*

Stage V/VI oocytes were isolated by manual dissection of mature *X.laevis* ovaries and maintained in 1× MBSH at 19°C (Gurdon, 1977). Samples of 10–20 nl were injected into the nucleus, or 40–50 nl into the cytoplasm of the oocytes. Microinjected oocytes were maintained in 1× MBSH for the indicated time at 19°C. The nucleus and cytoplasm from each oocyte were separated manually at 4°C in 1× MBSH and the fractions homogenized. From each injection 10–20 nuclei and cytoplasm were prepared. When $^{35}$S-labelled proteins were extracted, the nuclear and cytoplasmic fractions were homogenized in NET-2 (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% NP-40) supplemented with 1 mM PMSF and 1 μg/ml pepstatin. The homogenates were centrifuged for 3 min (maximum speed in Eppendorf table centrifuge) and the supernatant used for an immunoprecipitation (see below). The immunopellets were dried, dissolved in SDS-sample buffer and aliquots of 5–10 oocytes were analysed on 8% SDS–polyacrylamide gels followed by phosphoimaging (Molecular Dynamics). When $^{32}$P-labelled RNAs were extracted, the fractions were homogenized in TNES (20 mM Tris–HCl, pH 7.6, 380 mM NaCl, 2 mM EDTA, 0.1% SDS), the homogenates centrifuged and the supernatant extracted with phenol and phenol/chloroform. After ethanol-precipitation, 5–10 oocyte equivalents of the RNAs were analysed on 8% polyacrylamide–7 M urea gels followed by phosphoimaging (Molecular Dynamics).

### *Immunoprecipitations*

The α-TFIIIA-Ab or the myc-Ab (c-myc Ab-1/clone 9E10, Oncogene Science, Inc.) (Evan *et al.*, 1985), were bound to Protein A–Sepharose (Pharmacia) or to GammaBind Plus Sepharose (Pharmacia), respectively, for 2 h at room temperature in NET-2 buffer. The PAS- or PGS-antibody-pellets were washed three times with 1 ml NET-2. Subsequently, *in vitro* translated proteins or supernatants of homogenized cytoplasmic or nuclear fractions of injected oocytes (see above) were added and incubated for at least 1 h at 4°C. The immunoprecipitates were washed three times with NET-2 buffer, dried and dissolved in SDS-sample buffer. The samples were heated to 96°C and aliquots loaded on a SDS–polyacrylamide gel.

### *Binding of 5S RNA to in vitro translated proteins*

Proteins were *in vitro* translated in the coupled transcription/translation ($T_NT$) system of Promega and $^{32}$P-labelled 5S RNA and [$^{35}$S]cysteine were added to the reaction. The samples were incubated for 90 min at

30°C and subsequently added to PAS-TFIIIA-Ab-pellets or PGS-myc-Ab-pellets. The immunoprecipitation was performed in a final volume of 500 μl for 1 h at 4°C. The immunoprecipitates were washed three times with NET-2 and divided into two fractions. From one fraction the co-precipitated $^{32}$P-labelled 5S RNA was phenol-extracted, ethanol-precipitated and analysed on a 8% polyacrylamide–7 M urea gel followed by phosphoimaging. The immunoprecipitate of the other fraction was dried, dissolved in SDS-sample buffer and aliquots loaded on SDS–polyacrylamide gels in order to analyse the amounts of the $^{35}$S-labelled proteins that were synthesized in the binding assay reaction. After SDS–PAGE the proteins were visualized by phosphoimaging.

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