

# Synergy of importin $\alpha$ recognition and DNA binding by the yeast transcriptional activator GAL4

Chee-Kai Chan, David A. Jans\*

Nuclear Signalling Laboratory, Division of Biochemistry and Molecular Biology, John Curtin School of Medical Research, Australian National University, 0200 Canberra, A.C.T., Australia

Received 1 October 1999; received in revised form 25 October 1999

**Abstract** The N-terminus of the yeast transcriptional activator GAL4 contains partially overlapping nuclear targeting and DNA binding functions. We have previously shown that GAL4 is recognised with high affinity by importin  $\beta$  and not by the conventional nuclear localisation sequence binding importin  $\alpha$  subunit of the importin  $\alpha/\beta$  heterodimer. The present study uses ELISA-based binding and electrophoretic mobility shift assays to show that recognition of GAL4 by importin  $\alpha$  can occur, but only when GAL4 is bound to its specific DNA recognition sequence. Intriguingly, binding by importin  $\alpha$  enhances DNA binding on the part of GAL4, implying a synergistic co-operation between these two functions. The results implicate a possible role for importin  $\alpha$  in the nucleus additional to its established role in nuclear transport, as well as having implications for the use of GAL4 as a DNA carrier in gene therapy applications.

© 1999 Federation of European Biochemical Societies.

**Key words:** Importin; GAL4; DNA binding protein; Nuclear targeting signal; Transcription factor

## 1. Introduction

The 881 amino acid transcription factor GAL4 regulates galactose catabolism in *Saccharomyces cerevisiae* in concert with GAL80 [1]. GAL4 contains several functional domains including two transcriptional activating domains encompassing amino acids 149–238 and 768–881 [2,3], a central region comprising inhibitory and glucose response domains [4] and a zinc requiring DNA binding domain at the amino terminus [5–7]. In particular, the amino-terminal 147 amino acids of GAL4 bind specifically to a pseudo palindromic 17 bp (CGGN<sub>11</sub>CCG) nucleotide consensus recognition sequence [8,9]. Significantly, the amino-terminal 74 amino acids of the DNA binding region have nuclear targeting activity, being sufficient to localise the large heterologous  $\beta$ -galactosidase protein in the nucleus [10].

GAL4's ability to be targeted to the nucleus and to bind to a specific DNA sequence makes it a promising candidate as a gene delivery vehicle [11–14]. We have previously shown, however, that the nuclear targeting and DNA binding activities are mutually exclusive [14]. Intriguingly, we found that GAL4 was found to be recognised directly by importin  $\beta$ 1 rather than the conventional nuclear localisation sequence (NLS) binding importin  $\alpha$  subunit. This implied that nuclear import of GAL4 was unlikely to be mediated through the conventional nuclear import pathway, the first step of which involves the recognition of the NLS-containing protein by the importin

$\alpha/\beta$  heterodimer [15], mediated by binding of importin  $\alpha$  to the NLS directly. Importin  $\beta$  does not normally interact with conventional NLS-containing proteins directly, but can bind importin  $\alpha$  and dock the importin-transport substrate complex to the nuclear pore. Subsequent energy-dependent translocation into the nucleus requires the GTP binding protein Ran in its GDP form [16,17] and NTF2 [18]. Nuclear targeting of GAL4 was thus concluded to be through a novel importin  $\beta$ -mediated pathway, with release within the nucleus possibly assisted by DNA binding triggering GAL4 release from importin  $\beta$  at the nuclear pore [14].

In the present study, we show that GAL4 can be recognised by importin  $\alpha$ , but only when GAL4 is bound to its specific DNA recognition sequence. We also show that importin  $\alpha$  itself enhances GAL4-DNA binding, suggesting a synergistic relationship between GAL4-DNA binding and importin  $\alpha$  recognition. The results suggest that importin  $\alpha$  may have a role in the nucleus additional to that in mediating conventional NLS-dependent nuclear protein import. They also have implications for the use of GAL4 as a DNA carrier in gene therapy applications.

## 2. Materials and methods

### 2.1. Expression and purification of HisGAL4(1–147)

The induction of the hexahistidine tagged fusion protein HisGAL4(1–147) containing the N-terminal 1–147 amino acids of GAL4 protein in *Escherichia coli* using isopropylthio- $\beta$ -D-galactoside and protein purification using nickel affinity chromatography were carried out as described [14,19].

### 2.2. Expression of importin $\alpha$ and $\beta$ fusion proteins

Expression and purification of mouse (m) and yeast (y) importin (IMP)  $\alpha$  and  $\beta$  glutathione-S-transferase (GST) fusion proteins were performed as described previously [20,21]. GST free importin  $\alpha$  was prepared by thrombin cleavage as described [20–23].

### 2.3. ELISA-based binding assay

The recognition and binding affinity of GAL4 by importin  $\alpha$  and  $\beta$  when bound and not bound to its specific DNA recognition sequence was assessed using an ELISA-based assay [14,20]. Briefly, 96-well microtitre plates were coated with HisGAL4(1–147), with and without preincubation with DNA, and hybridised with increasing concentrations of importins. Detection was carried out using goat anti-GST primary antibody and alkaline phosphatase-coupled rabbit anti-goat secondary antibodies and *p*-nitro-phenyl phosphate (*p*-NPP) as the substrate. Absorbance was measured over 90 min with a plate reader (Molecular Devices) with correction by subtracting absorbance at 0 min and in wells incubated without importin. As a control, the conventional importin  $\alpha/\beta$ -recognised NLS-containing peptide P101, comprising SV40 large tumour antigen (T-ag) amino acids 111–132 [24,25], was used.

### 2.4. Preparation and purification of oligonucleotides

The 44 bp oligonucleotide 5'-GATCGGAAGACTCTCCTCCGAGCGCTCGGAAGACTCTCCTCCGC-3' with tandem repeats of the 17-mer that is recognised specifically by GAL4 DNA binding

\*Corresponding author. Fax: (61) (6) 62490415.  
E-mail: david.jans@anu.edu.au

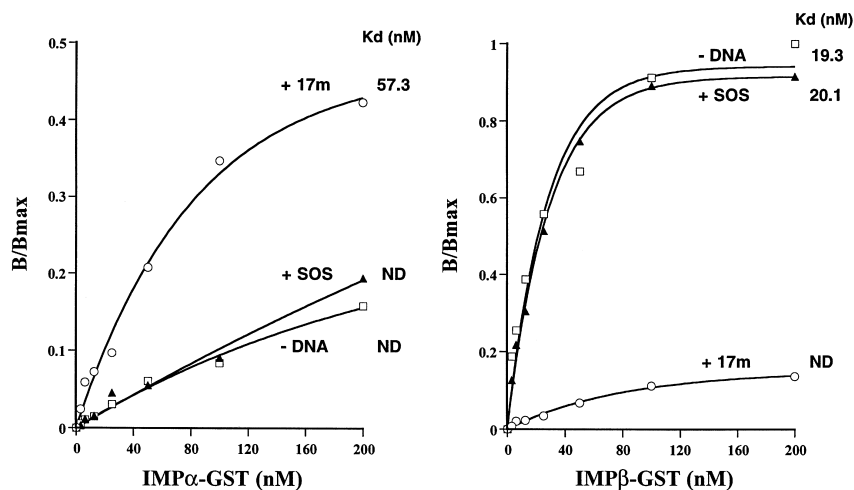


Fig. 1. Specific DNA binding enhances recognition of HisGAL4(1–147) by yeast importin  $\alpha$  (left panel) but diminishes recognition by importin  $\beta$  (right panel) as shown by an ELISA binding assay. Microtitre plates were coated with 0.5  $\mu$ g per well of HisGAL4(1–47) preincubated with or without oligonucleotides 17m or SOS as indicated and hybridised (triplicates) with increasing concentrations of yeast importins as described in Section 2. Curves were fitted for the function  $B(x) = B_{\max} (1 - e^{-kx})$  where  $x$  is the concentration of importin. Results for binding by importin  $\alpha$ -GST to DNA-bound HisGAL4(1–147) were compared to those for importin  $\beta$ -GST and importin  $\alpha$ -GST. The  $K_d$ s, which represent the concentration of importin yielding half maximal binding, are indicated, where correlation coefficients for the curve fits were not less than 0.98. Pooled data are presented in Table 1. ND, not able to be determined due to low importin binding.

domain and a 46 bp oligonucleotide 5'-GATCTGCTGTATATATA-TACAGCGCTACTGTATATACACCCAGGGC-3' which contains the tandem repeat sequences recognised by the DNA binding protein *lexA* were annealed to their respective complementary oligonucleotides to yield 17m and SOS respectively. They were purified using polyacrylamide gel electrophoresis followed by electro-elution and ethanol precipitation [14].

### 2.5. Electrophoretic mobility gel shift assay

Purified double stranded oligonucleotides 17m and SOS (see above) were labelled with [ $\gamma^{32}$ P]ATP using T4 polynucleotide kinase and unincorporated radionucleotide removed using a Sepharose Nick spin column. 0.2 ng of the radiolabelled oligomer in binding buffer BB1 (10  $\mu$ M ZnCl<sub>2</sub>, 25 mM HEPES-KOH pH 7.9, 4 mM MgCl<sub>2</sub>, 7% v/v glycerol, 2 mM DTT, 1 mg/ml BSA, 80 mM KCl) was incubated with 0.12 pmol of HisGAL4(1–147) in the absence or presence of increasing amounts of importin  $\alpha$  or  $\beta$  or  $\alpha/\beta$  at room temperature for 20 min. Samples were loaded onto a 5% polyacrylamide gel and electrophoresis performed in 0.5X Tris-borate buffer-EDTA (25 mM Tris, 25 mM boric acid, 0.5 mM EDTA, 1 mM MgCl<sub>2</sub>). The dried gel was exposed to radiographic film (Eastman Kodak) or quantitation by phosphorimaging (Molecular Dynamics) as described [14].

## 3. Results

### 3.1. Enhancement of importin $\alpha$ recognition of GAL4 by DNA binding

An ELISA based binding assay was used to analyse the binding affinities of importin  $\alpha$  and  $\beta$  for HisGAL4(1–147) with or without preincubation with either 17m, which contains specific binding sites for GAL4, or the unrelated SOS oligonucleotide which lacks a GAL4 binding site. Results indicated that, as previously [14], HisGAL4(1–147) was recognised with high affinity (an apparent dissociation constant ( $K_d$ ) of 19.5 nM) by yeast importin  $\beta$  (yIMP $\beta$ ), but not by yeast importin  $\alpha$  (yIMP $\alpha$ , Fig. 1). Significantly, incubation of HisGAL4(1–147) with 17m at a molar ratio of 1:1 increased binding by yIMP $\alpha$  about three-fold, the  $K_d$  being significantly increased to 57.3 nM (see Fig. 1, left panel; Table 1). This enhancement of yIMP $\alpha$  recognition of HisGAL4(1–147) in the presence of 17m was not observed when HisGAL4(1–

147) was incubated with SOS, indicating that enhancement was due to specific DNA binding. DNA binding thus appeared concomitantly to reduce recognition by IMP $\beta$  and increase recognition by IMP $\alpha$ . Despite the enhancement of recognition by yIMP $\alpha$  of HisGAL4(1–147) when bound specifically to DNA, the affinity of the interaction was still weaker than that of GAL4 with yIMP $\beta$  in the absence of DNA (compare left and right panels of Fig. 1; see Table 1).

As a control for both the ELISA assay, and the effect of the presence of DNA on importin binding, a peptide comprising the conventional NLS of SV40 large tumour antigen (T-ag) was assessed in the presence or absence of 17m, results indicating a  $K_d$  for recognition by IMP $\alpha/\beta$  of  $9.0 \pm 1.0$  nM ( $n = 2$ ), in either the presence or absence of 17m; binding of the T-ag peptide by IMP $\beta$  was negligible (data not shown), as observed previously [24–26].

Table 1  
HisGAL4(1–147) binding by importin subunits

| Protein      | Condition <sup>b</sup> | HisGAL4(1–147) binding parameters <sup>a</sup> |                 |     |
|--------------|------------------------|--|-----------------|-----|
|              |                        | $B_{\max}$ (%)                                 | $K_d$ (nM)      | $n$ |
| IMP $\alpha$ | –DNA                   | $8.7 \pm 0.6$                                  | LB <sup>+</sup> | 3   |
|              | +17m                   | $40.5 \pm 0.9$                                 | $52.2 \pm 3.5$  | 4   |
|              | +SOS                   | $10.8 \pm 0.7$                                 | LB <sup>+</sup> | 4   |
| IMP $\beta$  | –DNA                   | 100  | $19.8 \pm 1.7$  | 6   |
|              | +17m                   | $13.6 \pm 1.3$                                 | LB <sup>+</sup> | 3   |
|              | +SOS                   | $98.1 \pm 0.4$                                 | $20.1 \pm 1.4$  | 3   |

Results are for the mean  $\pm$  S.E.M. for  $n$  separate determinations for maximal binding (expressed as a percentage of the binding of yIMP $\beta$ -GST) and the apparent dissociation constant ( $K_d$ ) as indicated; LB<sup>+</sup>, low binding  $K_d$ , not able to be determined.

<sup>a</sup>Measurements were made using yeast GST-fusion proteins and an ELISA-based binding assay as described in Section 2 [14,20]

<sup>b</sup>Determinations performed in the absence or presence of the indicated oligomer at a ratio of 1:1.

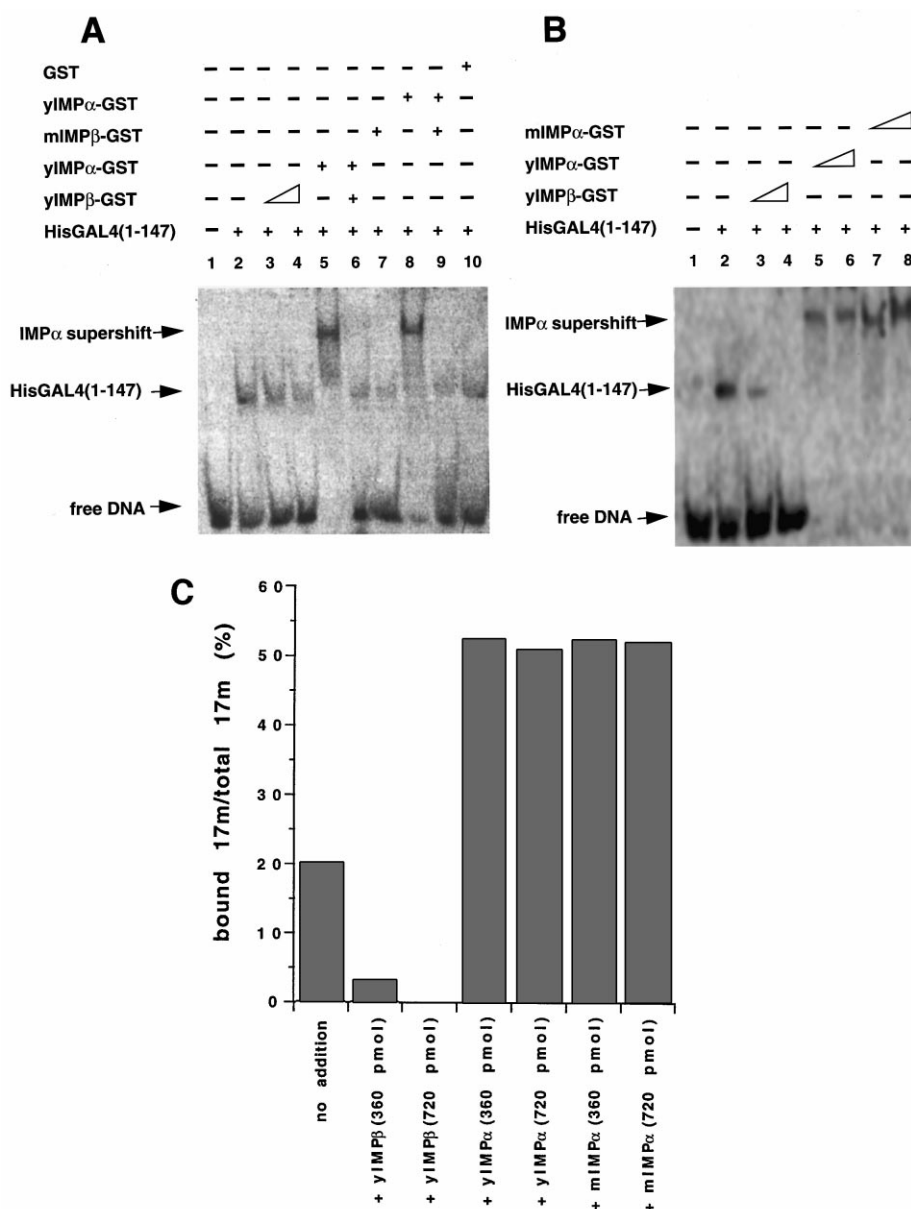


Fig. 2. Effect on HisGAL4(1-147)-DNA binding of importins as shown by electrophoretic mobility shift assays. A: NLS binding proteins or GST (720 pmol) were incubated as described in Section 2 with 1 pmol of HisGAL4(1-147) per lane except in lane 3 (360 pmol of yIMP $\beta$ -GST). B: Increasing amounts of importin (360 and 720 pmol) were incubated with 1 pmol of HisGAL4(1-147) per lane. The supershifted band due to importin  $\alpha$  binding is indicated. C: The gel shown in Fig. 2B was exposed onto a phosphorimager and quantitated as previously [14]. Results are expressed as the percentage of the total amount of radiolabelled 17m bound to HisGAL4(1-147). Results were typical of a series of three similar experiments.

### 3.2. IMP $\alpha$ -mediated enhancement of GAL4-DNA binding

To examine the effect of importins on specific DNA binding by HisGAL4(1-147), mouse importins  $\alpha$  or  $\beta$  (mIMP $\alpha$  and mIMP $\beta$ ) or the yeast correlates were added to HisGAL4(1-147) and incubated together with  $\gamma^{32}$ P-labelled 17m for 20 min at room temperature before loading onto a native 5% polyacrylamide gel. As previously [14], specific DNA binding was competed by mIMP $\beta$  and yIMP $\beta$  (lanes 3 and 4, Fig. 2A,B), and there was no evidence of supershifting of the GAL4-17m band, indicating that binding of IMP $\beta$  to DNA-bound GAL4 was negligible. In contrast, a supershift (lanes 5-8, Fig. 2B) of the GAL4-17m band was observed when IMP $\alpha$  was added, clearly indicating that IMP $\alpha$  is able to bind to DNA-bound

GAL4. Coincubation with IMP $\beta$  (lanes 6 and 9 of Fig. 2A) abolished the IMP $\alpha$ -mediated supershift, indicating the dominant effect of IMP $\beta$ , consistent with its higher affinity for GAL4 compared to that of importin  $\alpha$  (Fig. 1 and Table 1).

Significantly, the gel mobility shift experiments also indicated that IMP $\alpha$  specifically enhanced DNA binding by HisGAL4(1-147). In particular, the amount of specifically bound DNA appeared to be greater in the presence of IMP $\alpha$  compared to in its absence, as indicated by the clear reduction in the levels of unbound (free) DNA (compare lane 2 of Fig. 2A,B, with lanes 5 and 8, and 5-8 in Fig. 2A,B, respectively). Quantitation of the relative band intensities, performed using phosphorimaging, revealed an up to 2.5-fold enhancement of

HisGAL4(1–147)–17m binding in the presence of either mouse or yeast IMP $\alpha$ , compared to that in its absence (Fig. 2C). This was in contrast to the results in the presence of IMP $\beta$  HisGAL4(1–147) where DNA binding was markedly diminished and in some cases abolished (Fig. 2C). Thus, not only did DNA binding on the part of GAL4 enable it to be recognised with high affinity by IMP $\alpha$ , but binding by IMP $\alpha$  was able to increase specific DNA binding activity on the part of GAL4.

#### 4. Discussion

The results here show that GAL4 can be recognised with high affinity by importin  $\alpha$  but only when specifically bound to DNA. In addition, GAL4-DNA binding appears to be enhanced by importin  $\alpha$  binding, implying a co-operative effect between the two. We have previously shown [14] that high affinity importin  $\beta$  binding to GAL4 is incompatible with specific DNA binding and vice versa, and hypothesised that this may be due either to direct masking effects, or to conformational changes induced by DNA binding which prevent GAL4 recognition by importin  $\beta$ . The present study indicates that DNA binding by GAL4 enables recognition by importin  $\alpha$  which does not normally bind to GAL4 in the absence of specific DNA binding, implying that analogous unmasking effects or conformational changes must occur with respect to the importin  $\alpha$  binding site. Thus, whereas importin  $\beta$  binding must mask the GAL4 DNA binding domain either directly or through the induction of conformational changes [14], the same changes clearly facilitate importin  $\alpha$  binding. Our previous work [14] implied that nuclear transport of GAL4 involves recognition and docking to the nuclear pore complex by importin  $\beta$  in the absence of importin  $\alpha$ , with the conventional NLS-mediated nuclear import pathway, where importin  $\beta$  remains bound to the nuclear pore and importin  $\alpha$  enters the nucleus bound to the NLS-containing protein, clearly not in operation. Based on the observation here that importin  $\alpha$  can recognise GAL4 in its DNA-bound form, an intriguing possibility is that, following transport through the nuclear pore, DNA binding could play an important role in triggering release of GAL4 from the  $\beta$ -subunit and into the nucleoplasm, with importin  $\alpha$  facilitating this whole process. Whether, subsequent to nuclear import, importin  $\alpha$  enhancement of GAL4-DNA binding in the nucleus has a physiological role is unclear, but an intriguing possibility is that it may have a role in enhancing the activation of transcription by GAL4 at specific promoters.

The results here suggest that the suitability of GAL4 as a non-viral DNA delivery vehicle [11–14] needs to be reassessed; in the cytoplasm, the GAL4-DNA complex will be recognised poorly by importin  $\beta$ , which seems to be responsible for targeting GAL4 to the nucleus in the absence of DNA [14], but does appear able to be recognised by importin  $\alpha$ . Whether displacement of DNA from GAL4 by importin  $\beta$  is dominant over the enhancement of GAL4-DNA binding by importin  $\alpha$  (see Fig. 2A) or vice versa in the context of the living cell has yet to be ascertained, but the fact that the concentration of IMP $\alpha$  in the cytosol (6 $\mu$ M) is double that of IMP $\beta$  [27], implies that enhancement of GAL4-DNA binding through importin  $\alpha$  recognition may indeed occur. This may in fact represent the basis for the moderate increase in gene transfer effected by GAL4 observed in our previous

study [14] and in those of others [11–13]. Results from experiments in which the addition of 17m to fluorescently labelled GAL4 fusion protein abolished nuclear transport (data not shown) confirm that GAL4, when bound to its specific DNA recognition sequence, can no longer be efficiently targeted to the nucleus. Thus, if GAL4 is to be used successfully as a gene delivery vehicle, an additional functional NLS, such as that of T-ag, would appear to be required. Experiments to investigate this possibility are currently underway in this laboratory.

**Acknowledgements:** The authors gratefully acknowledge Wei Hu and Chong-Yun Xiao for assistance in purifying yeast and mouse importins.

#### References

- [1] Johnston, S.A., Zavortink, M.J., Debouck, C. and Hopper, J.E. (1986) *Proc. Natl. Acad. Sci. USA* 83, 6553–6557.
- [2] Ma, J. and Ptashne, M. (1987) *Cell* 51, 113–119.
- [3] Gill, G., Sadowski, I. and Ptashne, M. (1990) *Proc. Natl. Acad. Sci. USA* 87, 2127–2131.
- [4] Stone, G. and Sadowski, I. (1993) *EMBO J.* 12, 1375–1385.
- [5] Keegan, L., Gill, G. and Ptashne, M. (1986) *Science* 231, 699–704.
- [6] Lohr, D., Venkov, P. and Zlatanova, J. (1995) *FASEB J.* 9, 777–787.
- [7] Marmorstein, R., Carey, M., Ptashne, M. and Harrison, S.C. (1992) *Nature* 356, 408–414.
- [8] Liang, S.D., Marmorstein, R., Harrison, S.C. and Ptashne, M. (1996) *Mol. Cell. Biol.* 16, 3773–3780.
- [9] Carey, M., Kakidani, H., Leatherwood, J., Mostashari, F. and Ptashne, M. (1989) *J. Mol. Biol.* 209, 423–432.
- [10] Silver, P.A., Keegan, L.P. and Ptashne, M. (1984) *Proc. Natl. Acad. Sci. USA* 81, 5951–5955.
- [11] Paul, R.W., Weissner, K.E., Loomis, A., Sloane, D.L., LaFoe, D., Atkinson, E.M. and Overell, R.W. (1997) *Hum. Gene Ther.* 8, 1253–1262.
- [12] Fominaya, J. and Wels, W. (1996) *J. Biol. Chem.* 271, 10560–10568.
- [13] Uherek, C., Fominaya, J. and Wels, W. (1998) *J. Biol. Chem.* 273, 8835–8841.
- [14] Chan, C.K., Hubner, S., Hu, W. and Jans, D.A. (1998) *Gene Ther.* 5, 1204–1212.
- [15] Gorlich, D., Vogel, F., Mills, A.D., Hartmann, E. and Laskey, R.A. (1995) *Nature* 377, 246–248.
- [16] Moore, M.S. and Blobel, G. (1994) *Proc. Natl. Acad. Sci. USA* 91, 10212–10216.
- [17] Melchior, F., Paschal, B., Evans, J. and Gerace, L. (1993) *J. Cell Biol.* 123, 1649–1659.
- [18] Paschal, B.M. and Gerace, L. (1995) *J. Cell Biol.* 129, 925–937.
- [19] Schmitz, M.L. and Baeuerle, P.A. (1994) *Biotechniques* 17, 714–718.
- [20] Hubner, S., Xiao, C.Y. and Jans, D.A. (1997) *J. Biol. Chem.* 272, 17191–17195.
- [21] Hu, W. and Jans, D.A. (1999) *J. Biol. Chem.* 274, 15820–15827.
- [22] Rexach, M. and Blobel, G. (1995) *Cell* 83, 683–692.
- [23] Imamoto, N., Shimamoto, T., Takao, T., Tachibana, T., Kose, S., Matsubae, M., Sekimoto, T., Shimonishi, Y. and Yoneda, Y. (1995) *EMBO J.* 14, 3617–3626.
- [24] Akhlynina, T.V., Jans, D.A., Rosenkranz, A.A., Statsyuk, N.V., Balashova, I.Y., Toth, G., Pavo, I., Rubin, A.B. and Sobolev, A.S. (1997) *J. Biol. Chem.* 272, 20328–20331.
- [25] Chan, C.K. and Jans, D.A. (1999) *Hum. Gene Ther.* 10, 1695–1702.
- [26] Hübner, S., Smith, H.M.S., Hu, W., Chan, C.K., Rihs, H.-P., Paschal, B., Raikhel, N.V. and Jans, D.A. (1999) *J. Biol. Chem.* 274, 22610–22617.
- [27] Ribbeck, K., Lipowsky, G., Kent, H.M., Stewart, M. and Goerlich, D. (1998) *EMBO J.* 17, 6587–6598.