

A short region of its homeodomain is necessary for Engrailed nuclear export and secretion

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SUMMARY

Engrailed homeoprotein, a transcription factor involved in midbrain/hindbrain patterning, primarily localizes to the cell nucleus. However, significant amounts of the protein are also found in the cell cytoplasm or associated with membrane microdomains enriched in cholesterol and glycosphingolipids (Joliot, A., Trembleau, A., Raposo, G., Calvet, S., Volovitch, M. and Prochiantz, A. (1997) *Development* 124, 1865-1875). This non-nuclear localization, observed *in vitro* and *in vivo*, led us to investigate the possibility that Engrailed be transferred between nuclear and non-nuclear compartments. Monkey COS-7 cells expressing chick Engrailed-2 (cEN2) were fused with 3T3 mouse fibroblasts and the passage of cEN2 from COS-7 to 3T3 nuclei was followed in the interspecies heterokaryons. We find that, 10 minutes following cell fusion, cEN2 is detected in the 3T3 nuclei of 80% of the heterokaryons demonstrating rapid cEN2 nuclear export. Export from donor nuclei can be saturated and is strongly reduced after deletion of a 11 amino acid-long $\Delta 1$ sequence present within a slightly larger domain that extends

between helices 2 and 3 of the homeodomain and shows strong similarities with leucine-rich nuclear export signals (NES). This putative NES, when fused with a nuclear reporter protein, allows its nuclear export, demonstrating that it is not only necessary but also sufficient for nuclear export and can therefore be considered as a true nuclear export sequence. In an earlier report (Joliot, A., Maizel, A., Rosenberg, D., Trembleau, A., Dupas, S., Volovitch, M. and Prochiantz, A. (1998) *Current Biology* 8, 856-863), we demonstrated that the $\Delta 1$ sequence is necessary for the access of cEN2 to the lumen of a membrane compartment and for its intercellular transfer. The present study thus strongly suggests that the regulation of Engrailed nuclear export could play a role not only in Engrailed transcriptional activity but also in its ability to gain access to a secretory compartment.

Key words: Homeoprotein, Engrailed, Nuclear Export, Engrailed secretion

INTRODUCTION

Homeoproteins are a class of transcription factors involved in the development of all metazoans. They share a highly conserved 60 amino acid long DNA-binding domain, the homeodomain, composed of three alpha helices. In accordance with their role as transcriptional regulators, homeoproteins predominantly localize in the nucleus. However, they can also be found outside of the nucleus. For example, EMX-1 accumulates in the axons and the nerve terminals of olfactory receptors (Briata et al., 1996).

In addition to their nuclear localization, endogenous rat Engrailed homeoproteins expressed in the embryonic midbrain/hindbrain area are detected in cytosolic and membrane fractions (Joliot et al., 1997). The latter membranes, enriched in cholesterol and glycosphingolipids, share several biochemical and biophysical traits with caveolae or caveolae-like microdomains (Parton, 1996). In particular, they are not solubilized by high concentrations of non-ionic detergent (1% Triton X-100) and have a low density on saccharose

gradients. The specific association of Engrailed with caveolae was confirmed *ex vivo* in transiently transfected COS-7 cells (Joliot et al., 1997).

A significant percentage (5%) of Engrailed gains access to an intraluminal compartment both *in vivo* and in transiently transfected COS-7 cells. Using cEN2-expressing COS-7 cells, we have shown that the protein is secreted into the culture medium and subsequently internalized by abutting neurons with no apparent degradation (Joliot et al., 1998).

The presence of Engrailed homeoprotein in multiple intracellular compartments raises the possibility that it can move between these compartments. In this study, we have focused on the export from the nucleus, which is the major intracellular reservoir of Engrailed protein. Nucleocytoplasmic transport was measured using an interspecies heterokaryons assay in which the transfer of a protein from a donor nucleus to acceptor nuclei can be easily followed (Borer et al., 1989). We show that cEN2 is actively exported from the nucleus and we characterize a sequence, in the homeodomain, which acts as an autonomous nuclear export sequence (NES; for review

see Görlich and Mattaj, 1996). This finding suggests a new regulatory mechanism for cEN2 transcriptional activity.

The sequence necessary and sufficient for nuclear export encompasses the 11 amino acid long $\Delta 1$ sequence necessary for cEN2 non-conventional secretion (Joliot et al., 1998). In fact, deleting $\Delta 1$ sequence impairs both cEN2 nuclear export and secretion, therefore raising the possibility that the two processes are closely related.

MATERIALS AND METHODS

Plasmids constructs

The different proteins used in this report are described in Fig. 1B. All plasmids derive from pTLcEN2 (Joliot et al., 1997), pcDNA-PKNLS-I κ B-NES (Arenzana-Seisdedos et al., 1997) or pCL9Tm. Oligonucleotides encoding MYC tags (EQKLISEEDL) or cEN2-NES (RQSLAQELGLNESQIKIWFQ) have been inserted into appropriate restriction sites. Details on plasmids constructs are available upon request.

Interspecies heterokaryons

The protocol used to estimate nuclear export is as in Borer et al. (1989) with slight modifications. All cells were cultured in modified Dulbecco's modified Eagles medium (DMEM)/F12 medium (Gibco) supplemented with 10% fetal calf serum (FCS). COS-7 cells ($10^6/300 \mu\text{l}$) were electroporated as previously described (Joliot et al., 1998) and 5×10^4 transfected cells were seeded with 3T3 fibroblasts (5×10^4) on polyornithine coated (15 $\mu\text{g}/\text{ml}$, Sigma) 13 mm \varnothing glass coverslips and cultured for 16 hours. After two washes in serum-free DMEM/F12 medium (SFM), cells were incubated 1 hour in SFM containing 50 μM cycloheximide (Sigma). The medium was removed and cell fusion was induced by adding 50% polyethyleneglycol (PEG1000, w/v in SFM) for 90 seconds. The cells were intensively washed by immersion in cycloheximide-containing SFM and cultured in the same medium for the periods of time indicated.

Immunocytochemistry

After 2 washes with ice-cold phosphate buffer (PBS), the cells were fixed with 4% paraformaldehyde (25 minutes, room temperature) permeabilized (PBS-0.1% Triton X-100, 10 minutes, room temperature), and incubated in PBS-10% FCS for 1 hour at 37°C (saturation step). Double immunostaining was performed with the 9E10 monoclonal antibody (anti-MYC tag) and an anti- β -galactosidase (β -gal) rabbit polyclonal antiserum. Antibodies were visualized with biotinylated anti-mouse (1/200, Vector ABC-kit) plus streptavidin-FITC (1/800, Vector) and Cy3-conjugated anti-rabbit antibodies (1/400, Jackson laboratories), respectively. Coverslips were mounted in DAPI-containing Vectashield (Vector). Observations and pictures were made on a Leitz DMB-RBE microscope (40 \times /1.0 lens). Cy3 and FITC stainings were numerized independently and superimposed (Photoshop 4.01, Adobe). Alternatively (Fig. 6), pictures were taken on a Princeton instruments cooled-CCD camera.

Quantitative analysis

For each experiment and for each condition, 100 heterokaryons with only one expressing COS-7 nucleus were chosen at random and the percentage of fusion events positive for internuclear transfer was calculated. Fluorescence quantification was made on filtered confocal sections (Molecular Dynamics Sarastro 2000) with the IMAGESPACE program. The sum of fluorescence in recipient (R) and donor (D) nuclei was calculated and the R/(R+D) ratio was used as an index of protein transfer per positive heterokaryon. Distances between nuclei (from center to center) were evaluated with the same program. Cytoplasmic and nuclear signals in non-fused COS-7 cells were quantified with Metamorph 3.5 program.

Western blot analysis

10^6 transfected cells were seeded on culture plates and scraped with a rubber policeman. Cells were centrifuged (200 g, 10 minutes) and resuspended in Laemmli buffer. Proteins present in 1/50 of the cells extracts were separated by SDS-PAGE and transferred onto nitrocellulose (BAS 85, Schleicher & Shuell). Immunological detection was performed as described (Joliot et al., 1997).

Pulse-chase experiments

Transfected COS-7 cells (10^6) were cultured for 24 hours on polyornithine-coated 80 mm culture dishes, washed and preincubated for 20 minutes in methionine-cysteine-free medium plus 0.2 % FCS and 10 $\mu\text{g}/\text{ml}$ DNase I (Sigma). Labelling was performed in 1 ml methionine-free medium plus 0.2 % FCS, 25 $\mu\text{g}/\text{ml}$ heparin (Sigma) and 1 mCi [^{35}S]methionine/cysteine mix (1000 Ci/mmol; Promix, Amersham) for 25 minutes. Chase medium (2 ml) containing 1 mM methionine, 1 mM cysteine, 0.2% FCS, 25 $\mu\text{g}/\text{ml}$ heparin, 1 mM CaCl_2 and protease inhibitors was added for 30 minutes. The medium was collected and centrifuged (6000 g, 15 minutes, 4°C) before immunoprecipitation with the 9E10 anti-MYC antibody. Cells were rinsed twice in ice-cold PBS, scraped, pelleted and lysed in 1% boiling SDS. Extracts were then immunoprecipitated (Rousselet et al., 1988) and analyzed by SDS-PAGE.

RESULTS

Experimental model

The interspecies heterokaryons protocol, schematized in Fig. 1A, was originally designed to test the rapid shuttling of proteins between cytoplasmic and nuclear compartments. In this protocol, "donor" COS-7 cells expressing the studied nuclear protein are fused with non-expressing "recipient" 3T3 cells with a distinct nuclear morphology easily visualized by DAPI staining. Accumulation of the protein in 3T3 nuclei is used as an index of export from "donor" nuclei. Constitutive β -gal expression by 3T3 cells allows a simple immunocytochemical detection and delimitation of the syncytia (Fig. 2A,C,D). The two cell types are co-cultured for 16 hours and protein synthesis is blocked with cycloheximide (50 μM) 1 hour before polyethyleneglycol (PEG)-induced fusion. The cells, maintained in cycloheximide, are fixed at different times after fusion and the nuclear protein and β -gal are visualized by immunocytochemistry. Unless otherwise specified, to homogenize the sensitivity of the immunodetection between the different constructs, all nuclear proteins tested are tagged in N-ter and/or C-ter with a MYC epitope (Fig. 1B). Nuclear export was estimated with two parameters. First, among interspecies heterokaryons (characterized by the presence of nuclei of both species surrounded by β -gal-positive cytoplasm), we calculated the percentage of heterokaryons presenting detectable amounts of the protein in recipient nuclei (thereafter referred as "positive heterokaryons"). Second, within each positive heterokaryon, the signal accumulated in recipient 3T3 nuclei was quantified by confocal microscopy and normalised to that measured in the COS-7 donor nucleus.

cEN2 is exported from COS-7 nuclei

1 hour after fusion, 90% of the heterokaryons show cEN2^m in 3T3 nuclei (Fig. 2A,B). This is in strong contrast with the absence of staining of the 3T3 nuclei when SV40 large T-antigen^m (SV40 T^m), instead of cEN2^m, is overexpressed in COS-7 cells (Fig. 2C). To define the kinetics of the process,

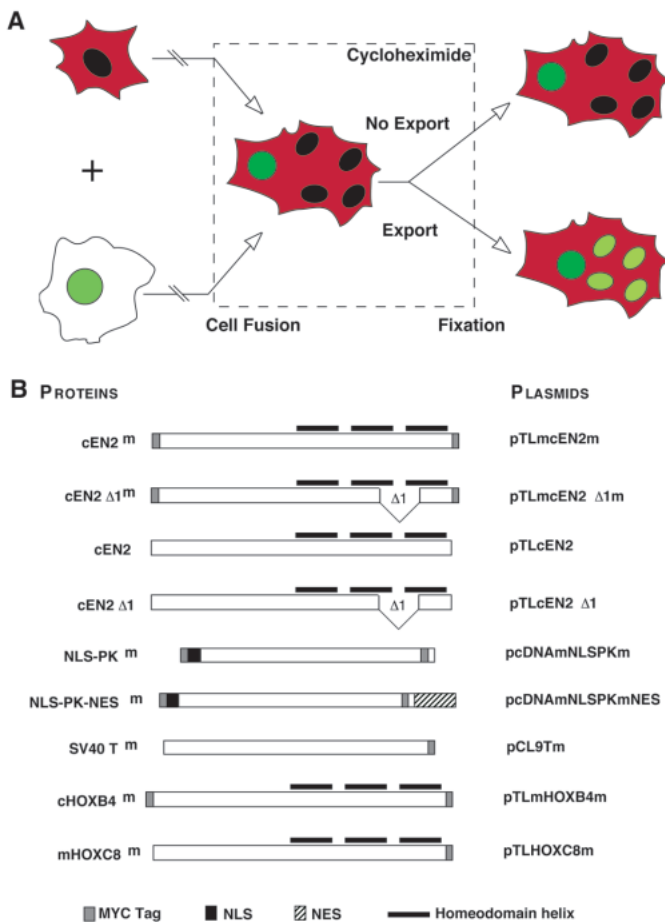


Fig. 1. Experimental approach. (A) Diagram of the experimental approach. Transiently transfected Simian COS-7 cells expressing a nuclear protein (green) are co-cultured for 16 hours with murine 3T3 fibroblasts constitutively expressing cytoplasmic β -gal (red). syncytium formation is induced by PEG treatment. Cells are fixed at different periods of time after fusion. Proteins exported from COS-7 nuclei are detected in 3T3 nuclei. Cycloheximide ($50 \mu\text{M}$) is present from one hour before fusion until fixation. (B) Constructs. Sequences added to the proteins are summarised in the central column; MYC-tags and SV40-nuclear localization sequences (NLS) are in grey and black, respectively. The hatched box in NLS-PK corresponds to the NES of either cEN2 or I κ B α . Names given to plasmids and proteins are indicated.

the cultures were fixed 0, 5, 10 or 60 minutes after fusion and stained with anti- β -gal and 9E10 (anti-MYC-tag) antibodies. At the time of fusion ($t=0$ minutes) between 3T3 fibroblasts and cEN2-expressing COS-7 cells, no cEN2^m staining is detectable in 3T3 nuclei (Fig. 2D). As plotted in Fig. 3A, the percentage of positive heterokaryons reaches a maximum 10 minutes after fusion. However, within each positive heterokaryon, the amount of cEN2^m in 3T3 nuclei increases at least up to 60 minutes (Fig. 3B,C). The parallel decrease in the cEN2^m content in COS-7 nuclei suggests that the latter nuclei are the main source of transferred protein.

Because the percentage of positive heterokaryons reaches a plateau 10 minutes after fusion, this time was chosen for most experiments. Due to the difference in cEN2^m concentrations between the 2 types of nuclei (see Fig. 3B), it was anticipated

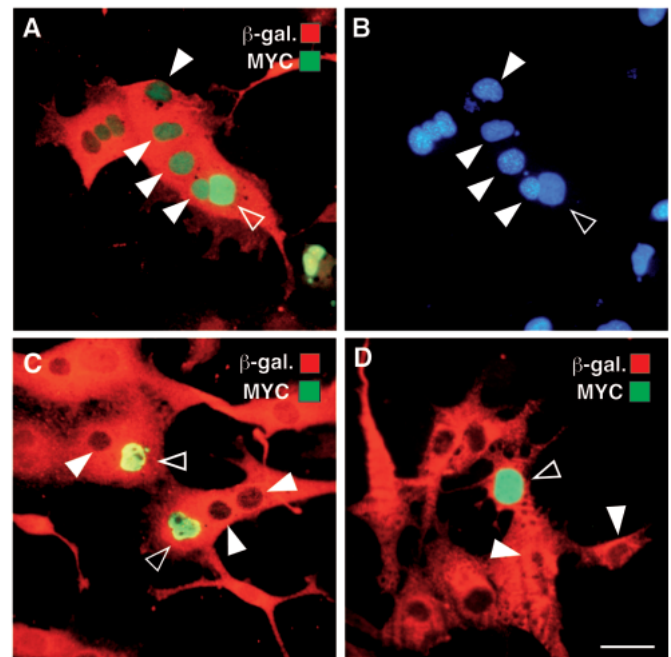


Fig. 2. cEN2 is transported from COS-7 to 3T3 nuclei. Cultures were fixed 60 minutes (A-C) or immediately (D) after fusion and double immunostained with polyclonal anti- β -gal (β -gal) and monoclonal anti-MYC (MYC) antibodies (A,C,D). DAPI was added to mounting medium to allow the distinction between COS (open arrowheads) and 3T3 (white arrowheads) nuclei (example in B). cEN2^m is absent in 3T3 nuclei at the time of cell fusion (D) but is detected in these nuclei 60 minutes after fusion (A). SV40 T^m is not detected in 3T3 fibroblasts 60 minutes after fusion (C). (Scale bar, $40 \mu\text{m}$).

that most of the transfer is unidirectional, going from COS-7 toward 3T3 nuclei. To confirm that cEN2^m accumulation in 3T3 nuclei actually reflects its export from COS-7 nuclei, the percentage of cEN2^m signal detected in a 3T3 nucleus 10 minutes after fusion was plotted as a function of the distance from the COS-7 nucleus. As illustrated in Fig. 3D, recipient nuclei show a proximodistal gradient in cEN2^m concentration. When cycloheximide is omitted, thus providing a continuous and more disperse cytosolic source of cEN2^m, the amount of the protein in recipient 3T3 nuclei after 10 minutes becomes independent of their distance from COS-7 donor nuclei (Fig. 3D). This establishes that, in the presence of cycloheximide, the accumulation of cEN2^m in 3T3 nuclei results from its export from COS-7 nuclei.

To investigate whether cEN2 nuclear export is an active process, cEN2^m (doubly tagged with the MYC epitope) was expressed in COS-7 cells with a 10-fold excess of non-tagged cEN2. Fig. 3E illustrates that, although the levels of tagged cEN2^m are similar in both conditions (Fig. 3E, upper panel), the percentage of heterokaryons showing cEN2^m export is reduced from 80% to 50% in the presence of a large excess of non-tagged cEN2 (Fig. 3E, lower panel). This competition experiment, which suggests that cEN2 nuclear export can be saturated, led us to search for a nuclear export signal.

Identification of a nuclear export sequence in cEN2 homeodomain

A 15 amino acid long sequence in cEN2, including parts of the

second and third helices of the homeodomain, shows significant analogies with a recently characterized class of Nuclear Export Signal (NES) referred to as “leucine-rich” NES (Fig. 4; Görlich and Mattaj (1996) for review). We compared the nuclear export capacity of the cEN2 “leucine-rich” sequence (thereafter called cEN2-NES) with that of the already characterized NES of IκBα (IκBα-NES) (Arenzana-Seisdedos et al., 1997). This was done by fusing either sequence to the C terminus of a nuclear protein, here a pyruvate-kinase containing the SV40 large T Nuclear Localization Signal (NLS-PK^m). 10 minutes after fusion, 30% of the heterokaryons show a basal nuclear export of NLS-PK^m and this export rises to >80% after the addition of either IκBα-NES or cEN2-NES (Fig. 5A). The same effect of the two NES is seen when the amount of fluorescence transferred between donor and acceptor nuclei within positive heterokaryons is quantified (Fig. 5B). We therefore conclude that cEN2-NES acts autonomously on nuclear export and that its activity is identical to that of prototypical IκBα-NES.

In order to test whether cEN2-NES stably affects the nuclear/cytoplasmic distribution of the nuclear reporter protein in non-fused cells, the accumulation in these two compartments of the three proteins (NLS-PK^m, NLS-PK^mcEN2-NES and NLS-PK^mIκBα-NES) was quantified. As illustrated in Fig. 6B-D and quantified in Fig. 6A, the addition of the NES from cEN2 or IκBα increases the cytoplasmic accumulation of the nuclear reporter protein up to 4-fold. Moreover, both NES modify the intranuclear distribution of NLS-PK^m from almost uniform to more peripheral (Fig. 6B-D).

Nuclear export of Hox class homeoproteins

Putative NES sequences similar to that of cEN2 are found in other homeoproteins (Fig. 7A; Duboule, 1994) suggesting that the nuclear export property of cEN2 could be generalized to other homeoproteins. To verify this point, nuclear export of mouse HOXC8 (mHOXC8) and chicken HOXB4 (cHOXB4) was tested in interspecies heterokaryons assay and compared to that of cEN2. Fig. 7B-D illustrates that 10 minutes after fusion the three proteins have accumulated similarly in the fibroblast nuclei.

A secretion-deficient mutant of cEN2 is also impaired in nuclear export

We have previously shown that cEN2 expressed in COS-7 cells can be secreted in the culture medium and internalized by abutting neurons. In

addition, a sequence of 11 amino acids (Δ 1 sequence) was identified which is necessary for cEN2 intercellular transfer (Joliot et al., 1998). This Δ 1 sequence (Fig. 4) is part of the cEN2-NES suggesting that cEN2 nuclear export and secretion could be related events. Intercellular transfer requires both secretion and internalization. To directly test the importance of Δ 1 in the secretion step, COS-7 cells expressing cEN2^m or cEN2 Δ 1^m (deletion of Δ 1) were metabolically labelled for 25 minutes. After 30 minutes of chase, immunoprecipitation of the culture medium with the 9E10 antibody demonstrates that, as opposed to cEN2^m, cEN2 Δ 1^m is not recovered in the culture medium (Fig. 8A) and, thus, that removing Δ 1 blocks cEN2 exocytosis.

Since Δ 1 (11 amino acids) only corresponds to a domain of the cEN2-NES (15 amino acids), we needed to investigate if cEN2 Δ 1 is exported from the nucleus. Fig. 8B illustrates that deleting the Δ 1 sequence strongly inhibits nuclear export as 20% of the heterokaryons now score positive instead of the

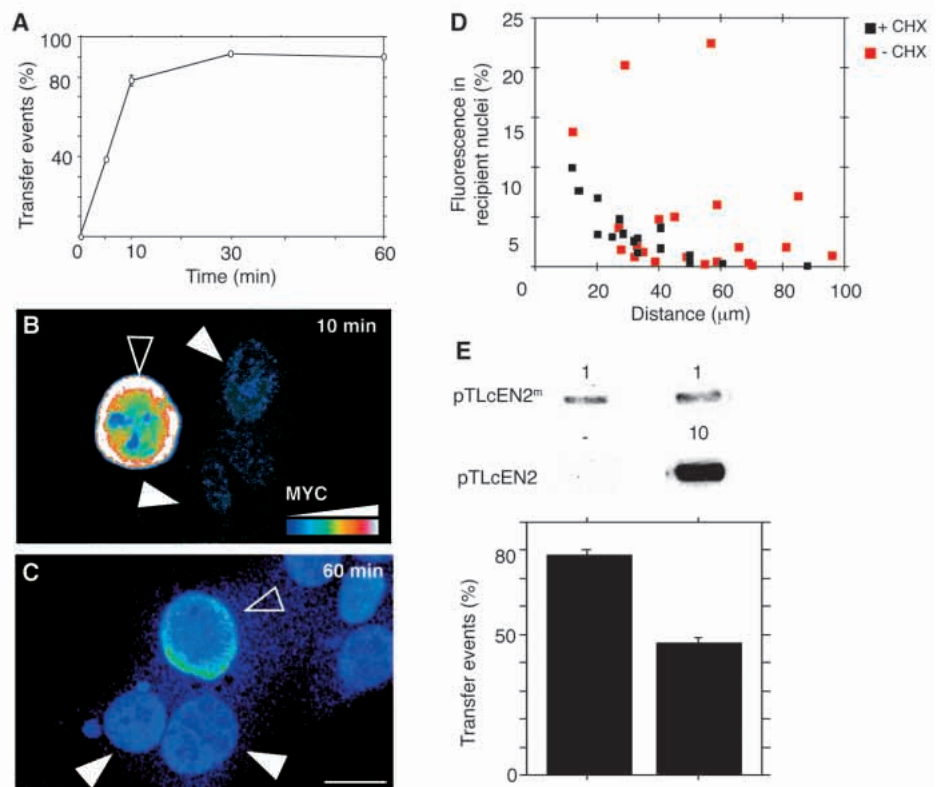


Fig. 3. cEN2 is exported from nucleus. (A) Kinetics of internuclear transfer of cEN2^m. Heterokaryons were fixed at various times after fusion and immunostained. The percentage of heterokaryons with internuclear transfer of cEN2^m (% transfer events) is plotted as a function of time. Each point is the mean of four independent experiments. 10 minutes after fusion, 80% of the heterokaryons show internuclear transfer. (B,C) Confocal sections illustrating that, with time, the amount of fluorescence in COS-7 (open arrowhead) and 3T3 nuclei (white arrowhead) decreases and increases, respectively. Scale bar, 30 μm. (D) COS-7 cells expressing cEN2^m were fused to 3T3 fibroblasts in presence (black) or in absence (red) of cycloheximide and cultured for 10 minutes. Fluorescence in 3T3 nuclei, plotted as a function of the distance from donor nuclei, points to the latter as a source in gradient formation (black). This effect is lost when the absence of cycloheximide allows the presence of a cytoplasmic newly synthesized pool of cEN2^m (red). (E) 1 μg of cEN2^m expression plasmid was cotransfected with or without 10 μg cEN2 expression plasmid. Although the amounts of cEN2^m protein is not modified by the presence of an excess of cEN2 (upper panel), its nuclear export is reduced (lower panel, $P < 0.01$, in Scheffe's test).

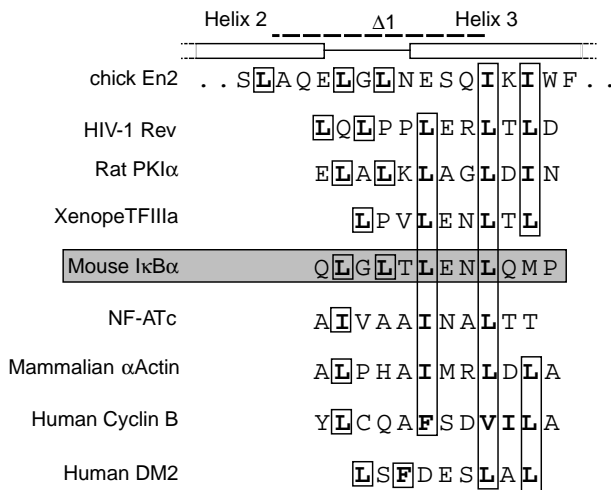


Fig. 4. Homology between a region of the cEN2 homeodomain and leucine-rich nuclear export sequences (NES). A sequence extending from within helix 2 into helix 3 in cEN2 homeodomain is compared to a series of identified leucine-rich NESs. Helices are schematized as open boxes, the turn as a line and the $\Delta 1$ sequence as a dashed line. Hydrophobic residues are in bold.

80% observed with the wild-type protein. This inhibition does not reflect a problem of import into 3T3 nuclei as the difference between cEN2 and cEN2 $\Delta 1$ is lost when the absence of cycloheximide allows protein accumulation in the cytoplasm (Fig. 8B).

Finally we tested if a 10-fold overexpression of cEN2 $\Delta 1$ could block the nuclear export of MYC-tagged cEN2^m. Fig. 8C shows that, as opposed to non-tagged cEN2 (Fig. 3E), non-tagged cEN2 $\Delta 1$ does not compete with the nuclear export of cEN2^m, strongly suggesting that nuclear export requires the association of a $\Delta 1$ -containing motif with unknown nuclear compounds.

DISCUSSION

In spite of the physiological importance of the nuclear or cytoplasmic localization of homeoprotein transcription factors (Casares and Mann, 1998, Hassan et al., 1997, Hirata et al., 1995, Mann and Abu-Shaar, 1996, Spit et al., 1998), reports on the regulation of their subcellular localization are still the exception and have almost only focused on nuclear import (Epps and Tanda, 1998, Zhao et al., 1996). We report here the identification, within the Engrailed homeodomain, of a short sequence of 15 amino acids necessary and sufficient for nuclear export. This cEN2-NES sequence bears strong structural similarities to other leucine-rich NES, suggesting that it is a true NES and that it might allow Engrailed export from the nucleus. Because similar sequences are found at the same position in different homeoproteins, we speculated that this finding could be extended to other homeoproteins and demonstrated that this is indeed the case for mHOXC8 and cHOXB4. In addition, we find that both cEN2 nuclear export and secretion in vitro are abolished after deletion of a sequence of 11 amino acids ($\Delta 1$) entirely comprised within the cEN2-NES. The latter result raises the possibility that nuclear export and secretion are related phenomena.

cEN2 is exported from the nucleus

Protein accumulation in recipient nuclei necessitates export and import from donor and recipient nuclei, respectively. However, in our experimental conditions, the transfer from COS-7 to 3T3 nuclei is a direct reflection of nuclear export. First, at the time of fusion, cEN2^m is exclusively in COS-7 nuclei and the presence of cycloheximide prevents new protein synthesis. Second, 10 minutes after fusion, a cEN2^m gradient has formed pointing toward COS-7 nuclei as the only source of cEN2^m. Third, allowing protein synthesis by omitting cycloheximide prevents gradient formation (Fig. 3D) and permits the translocation into recipient nuclei of a protein (cEN2 $\Delta 1$ ^m) that cannot be exported from the nucleus (Fig. 8B).

We have recently reported that cEN2, transiently expressed in COS-7 cells, is secreted intact and gains access to the nucleus of abutting neurons (Joliot et al., 1998). The conditions used in the present study preclude that detectable amounts of cEN2^m be internalized by the 3T3 cells before PEG-induced fusion (Fig. 2A). This was achieved by shortening the time of co-culture to 16 hours (24 hours are necessary to observe a significant exchange of cEN2) and by co-culturing transfected COS cell with 3T3 fibroblasts instead of neurons. Indeed, the uptake of homeodomains by alpha-2,8 polysialic acid-expressing cells, in particular young neurons, is four times that of fibroblasts (Joliot et al., 1991).

Finally, nuclear export is not due to the overexpression of cEN2^m as SV40 T^m, under the same conditions, is not exported. Nor is it due to the non-physiological presence of MYC-tags since non-tagged Engrailed, identified with the chick-specific 4D9 antibody, presents a behavior identical to that of the tagged protein (not shown) and competes with the latter in a nuclear export assay (Fig. 3E).

Identification of a leucine-rich NES in cEN2 homeodomain

The role of the cEN2 putative NES was assessed by the fusion of this sequence to an artificial nuclear protein, NLS-PK^m. The effect of cEN2-NES and of the already identified IκBα-NES (Arenzana-Seisdedos et al., 1997) were indistinguishable

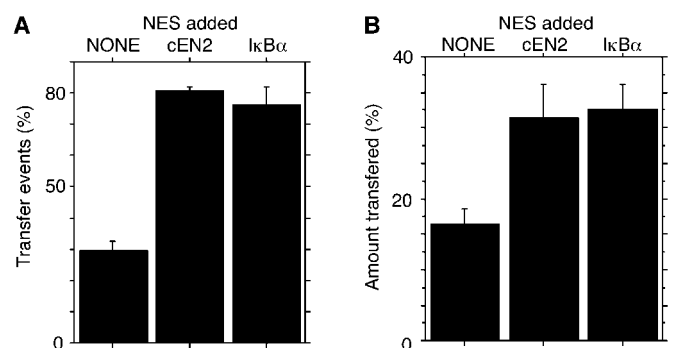


Fig. 5. Characterization of a cEN2 nuclear export signal. (A,B) PK-NLS^m alone (None), fused to cEN2 (cEN2) or IκBα (IκBα) NES was tested for nuclear export. (A) The percentage of positive heterokaryons increases from 30% to 80% when either NES is added to PK-NLS^m. (B) In positive heterokaryons, the amount of fluorescence transferred between nuclei is identical with both NESs. In A and B, the difference between NONE and either NES is significant ($P < 0.01$, in Scheffe's test).

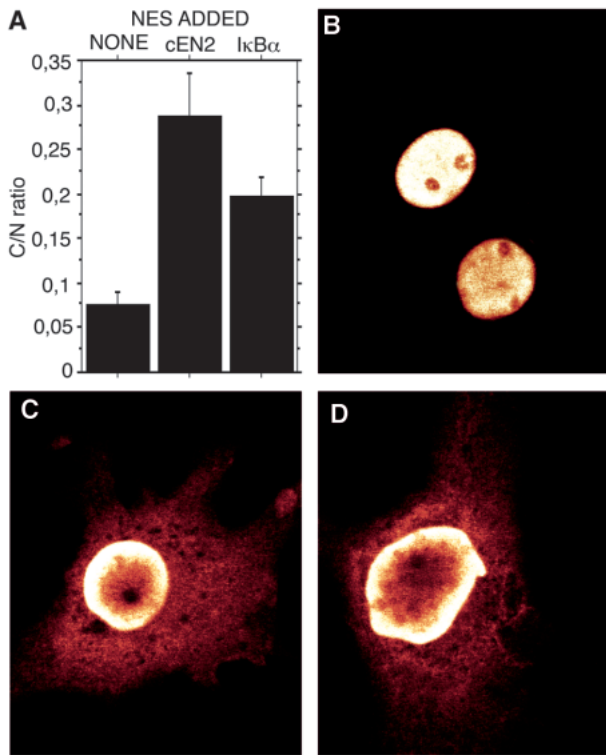


Fig. 6. NES-dependent cytoplasmic accumulation of PK-NLS. (A) The cytoplasmic/nuclear ratio (C/N) of PK-NLS^m alone (NONE), fused to cEN2 (cEN2) or IκBα (IκBα) NES was quantified. Even though the total cellular signal is equivalent for the three proteins (not shown), the addition of either NES increases the C/N ratio up to 4-fold. In contrast to the almost exclusive nuclear distribution of PK-NLS^m alone (B), the addition of cEN2 NES (C) or IκBα NES (D) induces the accumulation of the reporter protein in the cytoplasm. In addition, the nuclear distribution of PK-NLS (B) is modified from uniform to perinuclear by the presence of either NES (C,D).

qualitatively and quantitatively. Indeed, upon addition of either NES, both the percentage of positive heterokaryons and the amount of protein transferred between nuclei are increased. Because no direct effect of leucine-rich NES on nuclear import has ever been reported, the use of PK chimeric proteins has the advantage to clearly attribute to cEN2-NES a role in nuclear export.

The interspecies heterokaryons assay (Borer et al., 1989) does not directly inform about the intracellular distribution of the protein at equilibrium. In contrast, the increase of the cytoplasmic accumulation of NLS-PK^m in non-fused COS-7 cells upon addition of NES from IκBα or cEN2 (Fig. 7A) clearly demonstrates the stable effect of the latter sequences on protein compartmentalization.

One can also note that the addition of cEN2 or IκBα NES also modifies the intranuclear distribution of the reporter protein from uniform to more peripheral (Fig. 7B-D). A similar shift toward the periphery of the nucleus has been reported for gold particles linked to Rev-NES (Feldherr and Akin, 1997). It thus reinforces the idea that cEN2-NES is a true nuclear export sequence and, in addition, suggests the existence of a two-step mechanism in regulated nuclear export.

Within cEN2-NES, deleting Δ1, a shorter sequence of 11 amino acids which contains three of the five leucines or isoleucines of the NES, also impairs cEN2 nuclear export. This result demonstrates that cEN2-NES is functional not only when grafted to an exogenous protein, for example NLS-PK, but also in its natural protein context. We also observed that cEN2 but not cEN2Δ1 can compete with tagged cEN2^m for nuclear export. This indicates that at least one step of the process can be saturated and suggests the association of cEN2-NES with some components of the nuclear export machinery (Ossareh-Nazari et al., 1997). In conclusion, although a fine regulation of the export by other regions of cEN2 cannot be eliminated, it seems that cEN2-NES is necessary and sufficient for cEN2 nuclear export and that this export corresponds to an active mechanism.

The latter results suggest that Δ1 specifically interacts with molecules that either are in the nucleus or shuttle between the nucleus and the cytoplasm. The Δ1 sequence extending from the middle of helix 2 to that of helix 3 is prone to molecular interactions (Gehring et al., 1994). It is in particular noteworthy that, in addition to the fact that the third helix binds DNA, the Δ1 sequence corresponds exactly to the epitope recognized by the 4D9 monoclonal antibody (Patel et al., 1989).

Nuclear export can involve specific proteins and also RNA species. It is thus interesting that, in two examples, a non-nuclear localization of some homeoproteins has been linked to their ability to bind RNA: (i) Bicoid binds caudal mRNA through a domain overlapping the Δ1 sequence and represses its translation (Dubnau and Struhl, 1996, Rivera Pomar et al.,

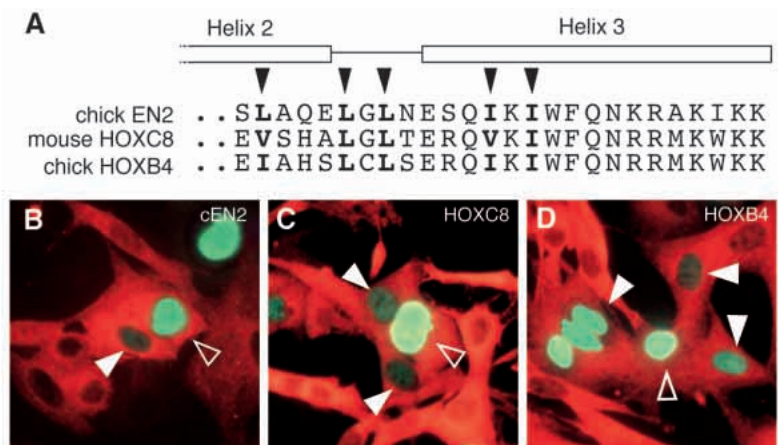


Fig. 7. Nuclear export of Hox homeoproteins. (A) Conservation of a putative NES in the homeodomain of the three homeoproteins. (B-D) Comparison of cEN2 (B), mHOXC8 (C) and cHOXB4 (D) nuclear export in the interspecies heterokaryons assay as in Fig. 3B.

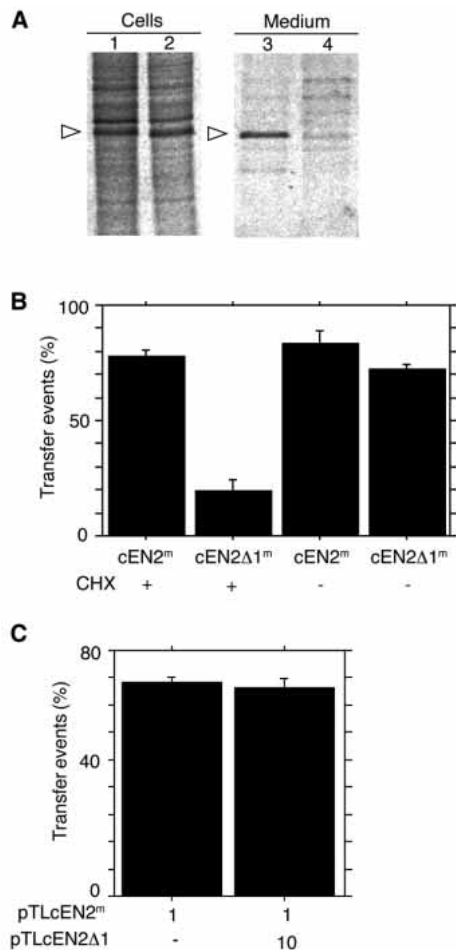


Fig. 8. The $\Delta 1$ sequence is necessary both for secretion and nuclear export. (A) COS-7 cells (10^6) expressing cEN2^m (lanes 1, 3) or cEN2 Δ 1^m (lanes 2, 4) were cultured for 24 hours, metabolically labeled for 25 minutes with [³⁵S]methionine, washed and chased for 30 minutes. Cells extracts (lanes 1, 2) and culture medium (lanes 3, 4) were collected and immunoprecipitated. cEN2^m and cEN2 Δ 1^m are expressed at the same levels (lanes 1, 2) but only cEN2^m is secreted (lanes 3, 4). Arrowheads indicate the mobility of cEN2^m protein. Respectively 100% and 2% of the medium and cell extract precipitates were loaded. (B) Nuclear transfer is strongly reduced by the $\Delta 1$ deletion ($P < 0.001$ in Scheffe's test). The presence of cEN2^m and cEN2 Δ 1^m in recipient nuclei in the absence of cycloheximide (–) demonstrates that $\Delta 1$ deletion blocks internuclear transfer at the level of nuclear export. (C) As opposed to cEN2 (Fig. 3E), a 10-fold excess of cEN2 Δ 1 does not antagonize cEN2^m nuclear export.

1996), and (ii) Knotted-1, a maize homeoprotein transported between cells through plasmodesmata is associated with its own mRNA (Lucas et al., 1995). We therefore do not exclude that their association with specific RNAs participates in the nuclear export of some homeoproteins.

Physiological implications of cEN2 nuclear export, links with unconventional secretion

The finding that Engrailed can shuttle between nuclear and non-nuclear compartments thanks to the presence of specific import and export sequences suggests a new mechanism for the regulation of homeoprotein transcriptional activity. Indeed, the

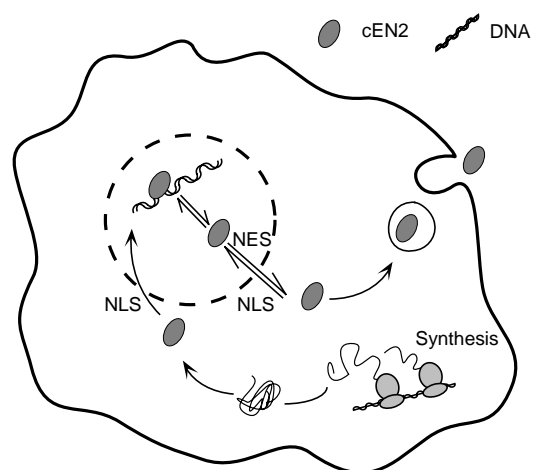


Fig. 9. Hypothetical model linking cEN2 nuclear export and secretion. After synthesis, cEN2 is imported into the nucleus due to its nuclear localisation sequence (NLS). Nuclear export of cEN2 allows its addressing to a membrane compartment enriched in cholesterol and glycosphingolipids, compatible with secretion (Joliot et al., 1997, 1998) and distinct from the classical secretory pathway. This model implies that Engrailed secretion could be regulated at the level of nuclear export.

intracellular concentration of homeoproteins, regulated at the level of nucleocytoplasmic transport, may have strong phenotypic consequences as suggested by gene dosage studies done in *Drosophila* (Scanga et al., 1995) and in the mouse (Gerlai et al., 1996).

A striking conclusion of this study is that $\Delta 1$ is necessary both for cEN2 secretion and nuclear export. Even though the $\Delta 1$ sequence is short, we cannot totally rule out that nuclear export and secretion involve distinct sequences directly or indirectly affected by $\Delta 1$ deletion. However, we favor the hypothesis that secreted cEN2 originates from the nuclei and, thus, that nuclear export is part of the secretion mechanism. It is noteworthy that cEN2, although secreted *in vitro* and present *in vivo* within a topologically extracellular compartment (Joliot et al., 1997, 1998), lacks a canonical secretion signal and is not targeted into the endoplasmic reticulum by a co-translational mechanism (unpublished results).

In several aspects, i.e., secretion, absence of signal sequence and nuclear localization, cEN2 resembles other proteins, for example HIV-TAT transcription factor and FGF-1. It is noteworthy that FGF-1, overexpressed in HeLa cells, first accumulates in the nucleus, then distributes uniformly between cytoplasm and nucleus and is finally secreted (Cao and Pettersson, 1993). The model schematized in Fig. 9 is based on the present report and on two previous studies demonstrating that Engrailed is secreted *in vitro* and present *in vivo* and *in vitro* in a membrane compartment enriched in cholesterol and glycosphingolipids (Joliot et al., 1997, 1998). It proposes that nuclear export plays an important regulatory role in Engrailed subcellular distribution, in its active transport to an unconventional secretory compartment and, therefore, in its actual secretion.

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