Analysis of a Developmentally Regulated Nuclear Localization Signal in *Xenopus*

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Abstract. The 289 residue nuclear oncoprotein encoded by the adenovirus 5 Ela gene contains two peptide sequences that behave as nuclear localization signals (NLS). One signal, located at the carboxy terminus, is like many other known NLSs in that it consists of a short stretch of basic residues (KRPRP) and is constitutively active in cells. The second signal resides within an internal 45 residue region of Ela that contains few basic residues or sequences that resemble other known NLSs. Moreover, this internal signal functions in injected *Xenopus* oocytes, but not in transfected *Xenopus* A6 cells, suggesting that it could be regulated developmentally (Slavicek et al. 1989. *J. Virol.* 63:4047). In this study, we show that the activity of this signal is sensitive to ATP depletion in vivo, efficiently directs the import of a 50 kD fusion protein and can compete with the Ela carboxy-terminal NLS for nuclear import. In addition, we have delineated the precise amino acid residues that comprise the second Ela NLS, and have assessed its utilization during *Xenopus* embryogenesis. Using amino acid deletion and substitution analyses, we show that the signal consists of the sequence $FV(X)_{7\text{-}20}MXSLXYM(X)_4MF$. By expressing in *Xenopus* embryos a truncated Ela protein that contains only the second NLS and by monitoring its cytoplasmic/nuclear distribution during development with indirect immunofluorescence, we find that the second NLS is utilized up to the early neurula stage. In addition, there appears to be a hierarchy among the embryonic germ layers as to when the second NLS becomes nonfunctional. For this reason, we refer to this NLS as the developmentally regulated nuclear localization signal (drNLS). The implications of these findings for early development are discussed.

Most proteins that are selectively transported into the cell nucleus contain a discrete peptide sequence that functions as a nuclear localization signal (NLS)[1]. Many NLSs are similar in residue composition to the archetypically simple SV-40 large T antigen NLS, which consists of the short stretch of basic amino acids, PPKKKRKV (22; reviewed in 14, 43, 56). Other NLSs, such as that present in nucleoplasmin, are more complex in character and have a bipartite structure with import-requiring basic residues separated by a spacer region (48).

While the exact sequence and structure among NLSs varies, it has been shown that nuclear transport proceeds through the nuclear pore complex (2, 12), that it requires ATP hydrolysis (35) and that it occurs as a two-step process; the binding of proteins to the pore complex, and the transport through the central pore complex channel (2, 34). It has also been shown that the rate of nuclear import is directly related to the number of NLSs present in a protein (11), that the kinetics of nuclear import are saturatable (16) and that NLSs from different proteins can compete with each other for import (16, 66). These data suggest that nuclear transport is receptor mediated and, further, that there is a common pathway used by many different proteins for nuclear import. Indeed, proteins that can recognize a variety of NLS sequences have been identified from a number of organisms (1, 61, 66).

Although nuclear localization signals often function constitutively, their activity can also be regulated through the phosphorylation of residues residing within or flanking the NLS (31, 46, 47), or through distant peptide regions that can either "mask" the NLS or anchor the protein in the cytoplasm (18, 19, 53). This is the case for a number of proteins including cyclins A and B (41), the transcription factor NF-kB (53), and the c-*fos* protein (50). One particularly dramatic example of regulated nuclear import and its utility in the control of gene expression is the *Drosophila dorsal* protein (49, 51, 60). Dorsal, the morphogen that determines the dorsoventral polarity of the embryo, is found in the cytoplasm during early embryogenesis. During cleavage, however, dorsal becomes localized to the nucleus only in the ventral portion of the embryo, where it appears to activate the transcription of the ventralizing genes, *twist* and *snail* (24, 62). The failure of regulated nuclear localization in this case results in aberrant development and demonstrates the importance of regulated nuclear transport in the control of gene expression.

One example of a protein with both a constitutive and

1. *Abbreviations used in this paper*: drNLS, developmentally regulated NLS; MBP, maltose binding protein; MBS, modified Barth's solution; NLS, nuclear localization signal.

regulated nuclear localization signal is the 289 residue nuclear oncoprotein encoded by the adenovirus type 5 Ela gene. In this protein, the constitutive carboxy-terminal NLS is T-antigen NLS-like in composition and consists of the sequence KRPRP (25). An apparently regulated NLS resides within residues 140–185 (59) and while this region contains a zinc-finger structure important for Ela-mediated transactivation activity (5, 64), there are few basic residues and no similarities to other known nuclear localization signals. This region is thought to be regulated because it can direct nuclear import in injected *Xenopus* oocytes, but not transfected *Xenopus* A6 cells or Hela cells.

In this report, we show that nuclear import via the second, regulated Ela NLS is ATP dependent and that the signal can compete with the Ela carboxy terminus NLS for nuclear import, demonstrating that the signal directs nuclear import using selective import pathways and not through diffuse and bind mechanisms (38, 39). We have also defined the amino acid residues that constitute this NLS and have assessed its use during early *Xenopus* development. We find the structure of the signal to be complex in that nuclear import requiring residues are separated by spacer regions. In addition, this signal is very hydrophobic, contains no basic residues and, therefore, bears no resemblance to other known NLSs. In *Xenopus* embryos derived from DNA-injected fertilized eggs, we find that this NLS directs nuclear import up until the early neurula stage in all embryonic tissues. Subsequently, the use of this NLS is turned off in specific tissues at specific times. By the tailbud stage of development, the NLS does not function in any tissue. We discuss the implication of this finding for the regulated nuclear localization of proteins during early development.

Materials and Methods

Plasmid Construction

Plasmid pCE, which contains the genomic copy of the adenovirus type 5 Ela gene, including the promoter region, has been described (54). This plasmid was digested with XbaI, filled-in and religated to give pCER that lacks 66 carboxy-terminal residues, thus eliminating the COOH terminus NLS (59). Plasmids encoding Ela deletion mutants, pDL1110, pDL1112, pDL1113, and pDL1114 (gifts of S. Bayley, McMaster University) (20) were treated as described for pCER to give carboxy-terminal truncations that lack the COOH-terminal NLS. Plasmids pSPElaX, pSP12S, and pSP12SX have been described previously (59).

In vivo ATP Depletion

Manually defolliculated stage VI oocytes were injected with 30 nl of apyrase (Sigma Type I) at 100 U/ml, incubated for 1 h, then injected with 30 nl of rabbit reticulocyte lysate (Promega Corp., Madison, WI) that were primed with either SPElaX or SP12S mRNAs and [^{35}S]methionine. Oocytes were held for times indicated in 1× modified Barth's solution (MBS) (26) supplemented with 50 μg/ml cycloheximide and germinal vesicle and cytoplasmic fractions then were manually separated. Cytoplasmic fractions were homogenized in 0.02% Triton X-100, centrifuged at 10,000 *g* for 1 min and supernatant was collected and combined with 4 vol of ethanol and held at −70°C for 30 min to precipitate proteins. Proteins from germinal vesicle fractions were directly precipitated with 4 vol of ethanol at −70°C. Proteins were resuspended in Laemmli sample buffer (23), boiled for 5 min and 1.5 oocyte equivalents were loaded onto 10% SDS–polyacrylamide gels. After electrophoresis, gels were fixed, enhanced (EnHance, Amersham Corp., Arlington Heights, IL), dried and exposed to x-ray film for 3–7 d. Bands on the autoradiographs were then scanned and quantitated densitometrically.

Site Directed Mutagenesis and Synthesis of Deletion Mutants

Oligonucleotide directed mutagenesis was done essentially as described (57) except that ssDNA was made from pBSSK$^-$ CER, which was constructed by placing the SacII–SacI fragment of pCER into the SacII–SacI sites of pBlueScriptSK$^-$ (Stratagene Inc., La Jolla, CA). Constructs were confirmed by restriction enzyme digestion and DNA sequencing according to Sanger et al. (52). The following oligonucleotides were used to generate mutant sequences (newly inserted restriction sites are noted in bold): pH172 (NsiI)- GCGAATGCATAATATCTGG; pF2- CATATAGAAAAGCGAAAACATAATATC; pH3 (HindIII)- GTAGACAAGCTTGCCACAGG; p846 (EcoRI)- CCTCATATAGCAGAATTCACACATAATATCTGG; p847 (EcoRI)- CCACAGGTCCTGAATTCGCAAAGCGAACAC; p848 (EcoRI)- GCGAACAGAATTCATCTGGGTCCC.

The clone pΔ20 was produced by first cutting pH171 in the polylinker with XhoI and BamHI, filling in with Klenow and religating; this makes the SmaI site of the Ela coding region unique. This clone was cut with SmaI and NsiI and the adapter oligonucleotide, ATGCA, was ligated to the NsiI 5′ cut site, filled in with Klenow and religated. Deletions p4153 and p4157 were constructed by opening the pH171 clone with SmaI and digesting with BalI, filling in with Klenow and religating. In-frame deletions were determined by DNA sequencing. Clones p4153-NH3 and p4157-NH3 were constructed by replacing the NsiI–SacI fragment of p4153 and p4157 with the NsiI–SacI fragment from pCER-H3.

Oocyte Injection and Immunoprecipitation

For nuclear transport studies, manually defolliculated stage VI oocytes (10) were injected intranuclearly with ~5 nl of DNA (0.1 μg/μl), cultured overnight in 1× MBS (26) and then placed in 100 uCi/ml [^{35}S]methionine (New England Nuclear, Boston, MA) in 1× MBS. Oocytes were labeled for 4 h before germinal vesicles and cytoplasms were manually separated. Typically, 20 oocytes per construct were used. Cytoplasms were homogenized in 1× MBS containing 0.02% Triton X-100 and centrifuged at 10,000 *g* for 1 min. The supernatant was collected and adjusted to 1× radioimmunoprecipitation assay buffer (RIPA) (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS). Germinal vesicles were directly homogenized in 1× RIPA buffer. Immunoprecipitations were done using either monoclonal (M73, Oncogene Sciences, Inc., Manhasset, NY) or rabbit antiserum directed against bacterially expressed Ela protein. Antibodies were diluted to 1:50 and incubated with homogenates for 1–3 h at 4°C, which was followed by the addition of 15 μl of protein A–Sepharose beads suspended in 1× RIPA. After incubation for 1 h at 4°C with constant agitation, the beads were centrifuged, washed 3× with RIPA buffer, resuspended in Laemmli sample buffer (23), boiled for 5 min and applied to a 12% SDS–polyacrylamide gel. After electrophoresis, gels were fixed, enhanced (EnHance, Amersham Corp.), dried and exposed to x-ray film for 0.5–7 d. Several exposures of autoradiographs were scanned densitometrically, areas under appropriate bands were determined, and the percent nuclear localization for each relevant protein was determined. The amount nuclear of each mutant protein was standardized relative to the amount nuclear of the ElaX protein, which was determined empirically in each experiment.

Injections and Immunofluorescence

Fertilized, dejellied eggs were injected with ~5 nls (0.1 μg/μl) of either pCE, which codes for the wt Ela protein, or pCER, which encodes a truncated protein that lack the carboxy-terminal 66 residues and, hence, the COOH-terminal NLS. Embryos were maintained in 5% Ficoll, 0.1× MBS until gastrulation then transferred to 0.1× MBS. They were allowed to develop until the stages indicated in the Figures and then fixed in 4% paraformaldehyde, 0.1× MBS, pH 7.5, for 12 h at 4°C and prepared for paraffin histology. Alternatively, some fixed embryos were embedded in plastic according to the manufacturers directions (Immunobed, Polysciences, Warrington, PA). Wax sections were taken at 8–10 μm and plastic sections were taken at 5 μm. Sections were blocked against nonspecific binding using 5% BSA in TBS, pH 7.5, and immunostaining used either monoclonal (M73) or rabbit anti-serum diluted into blocking solution at ratios of 1:100–1:500. Appropriate fluoresceinated secondary antibodies were applied at 1:500 dilution. Hoechst dye (0.05 μg/ml) was used as a nuclear stain. Embryos were staged according to Nieuwkoop and Faber (37).

Fusion Proteins

To study specifically the NLS activity of Ela residues 140–185, fusion proteins were constructed that placed these residues at the carboxy terminus of the maltose binding protein (MBP) using the procedures and materials described in the pMAL kit (New England Biolabs, Beverly, MA). For insertion into the pMAL-c vector, an EcoRI restriction endonuclease site was placed at nucleotide 968 in the pCER plasmid using site-directed mutagenesis as described above with the oligonucleotide- CTCCTCACCGAATTC-ATCCTCGTC. This construct, pCER-845, was then digested with EcoRI and HincII to release a 604 nucleotide fragment that was inserted into the pMAL-c vector which had been digested with XbaI, filled in with Klenow and then digested with EcoRI. The resulting fusion protein (14085-MBP) contains Ela residues 140–185 at the carboxy terminus of the MBP. A translational stop codon (TGA) is positioned two codons downstream of Ela 13S codon 185 so that the fusion protein would contain Ela residues 140–185 plus an additional lysine at the carboxy terminus. An additional fusion protein was also constructed by cleaving the pSP12S clone with XbaI and HindIII and ligating this fragment into the pMAL-c vector that had also been digested with XbaI and HindIII. This clone produces a fusion protein (CNLS-MBP) that includes the carboxy-terminal 66 residues of Ela and, therefore, contains the carboxy-terminal NLS. Purified fusion proteins were concentrated and buffers were exchanged using Centricon 30 microconcentrators (Amicon, Beverly, MA).

For use in rate determinations, 5 nl of a 20 μg/μl solution of fusion proteins in TBS were injected into oocytes, groups of three were taken at the times indicated and cytoplasms and germinal vesicles were manually separated. Cytoplasms were homogenized in 0.2% Triton X-100, centrifuged at 10,000 *g* for 3 min, supernatants were collected, combined with 4 vol of ethanol and placed at −70°C for 30 min to precipitate proteins. Germinal vesicle fraction proteins were precipitated directly with 4 vol of ethanol and incubation at −70°C. Proteins from both fractions were resuspended in sample buffer and, typically 0.5 oocyte equivalents were resolved on 10% SDS-polyacrylamide gels. Proteins were then transferred to nitrocellulose which was then blocked for nonspecific binding with 5% dried milk in 200 mM NaCl, 20 mM Tris, pH 7.5, 0.1% Tween-20, for 1 h and probed with anti-MBP antibodies (New England Biolabs) diluted 1:10,000 into blocking buffer. The blots were washed in 200 mM NaCl, 20 mM Tris, 0.1% Tween-20, which was followed by the application of a HRP-conjugated goat anti-rabbit secondary antibody (Vector Labs, Inc., Burlingame, CA) diluted 1:5,000 into blocking buffer. Blots were washed extensively, developed using enhanced chemiluminescence (ECL, Amersham Corp.) and exposed to x-ray film. Resulting images were quantitated densitometrically.

For competition studies, we first determined that the intraoocyte concentration of the CNLS-MBP fusion protein that could effectively compete with the Ela 12S protein without increasing oocyte mortality was ~25–30 μM (~30 nl of a 20 μg/μl solution injected assuming a 50-kD protein and 0.5 μl yolk-free volume in the oocyte). All proteins were made to 20 μg/ul in TBS and 30 nl of each were injected into oocytes, which were then incubated in 1× MBS for 1 h. 30 nl of reticulocyte lysate containing [^{35}S]methionine-labeled Ela 12S protein were then injected and oocytes were held in 1× MBS supplemented with 50 μg/ml cycloheximide. At indicated times groups of three oocytes were collected, cytoplasmic and germinal vesicles manually separated, and then prepared as above for ATP depletion studies. Protein concentrations were made using the Bradford assay with BSA as the standard.

Results

General Characterization of Ela Nuclear Localization Signals

Using injected *Xenopus* oocytes, Slavicek et al. (59), localized an Ela NLS to residues 140–185. To confirm and extend these data, we injected DNAs or mRNAs encoding wild-type

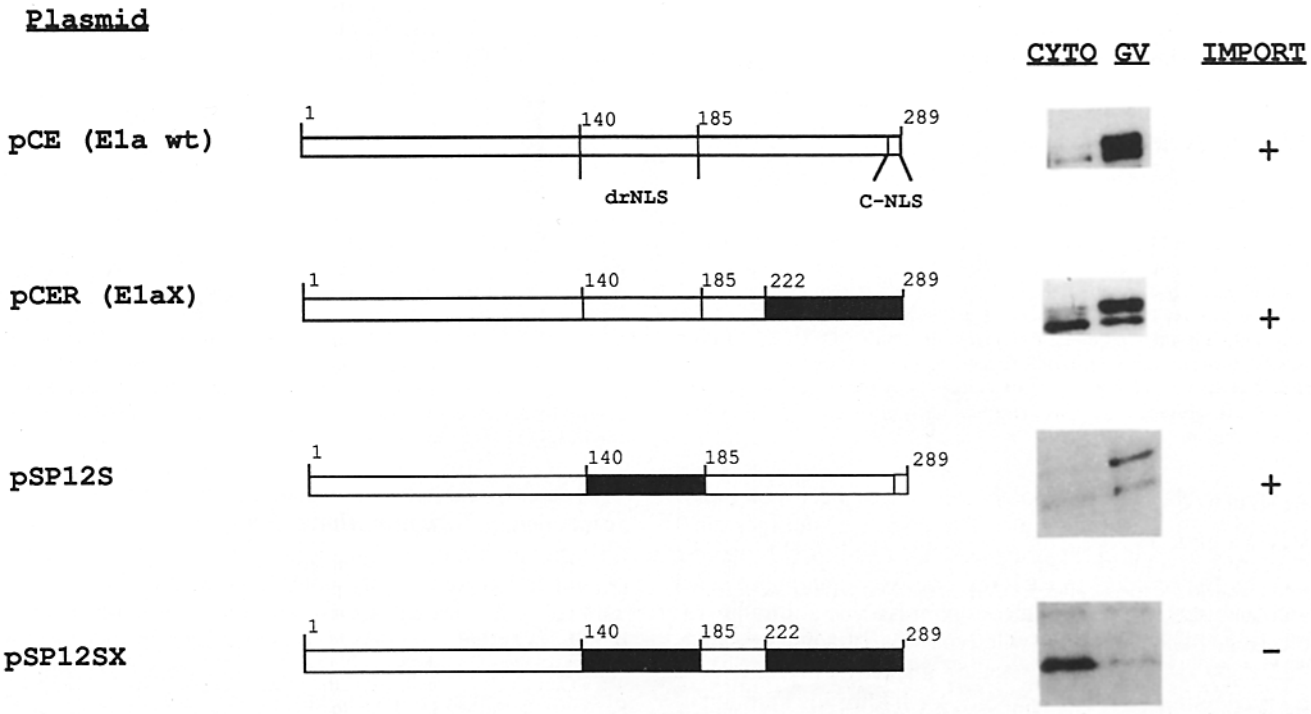

Figure 1. The 289-residue Ela protein contains two nuclear localization signals. DNAs (*pCE* and *pCER*) and RNAs (*pSP12S* and *pSP12SX*) encoding wild-type or mutant Ela proteins were injected into oocytes that were then incubated with [^{35}S]methionine. Cytoplasms (*CYTO*) and nuclei (germinal vesicles or *GV*) were manually separated and Ela proteins were then immunoselected from each fraction and analyzed with SDS-PAGE and autoradiography. The distribution of the Ela wild type, ElaX (lacking residues 222–289), Ela12S (SP12S: lacking residues 140–185), and 12SX protein (SP12SX: lacking residues 140–185 and 222–289) were based on quantitative densitometry. The amount nuclear for all proteins in Figs. 1–4 is expressed as a percentage of the amount nuclear for the ElaX protein, which was determined empirically within each experiment. The relative values are expressed symbolically as: +, 90–100% of +/−, 30–90% of ElaX; −, <30% of ElaX.

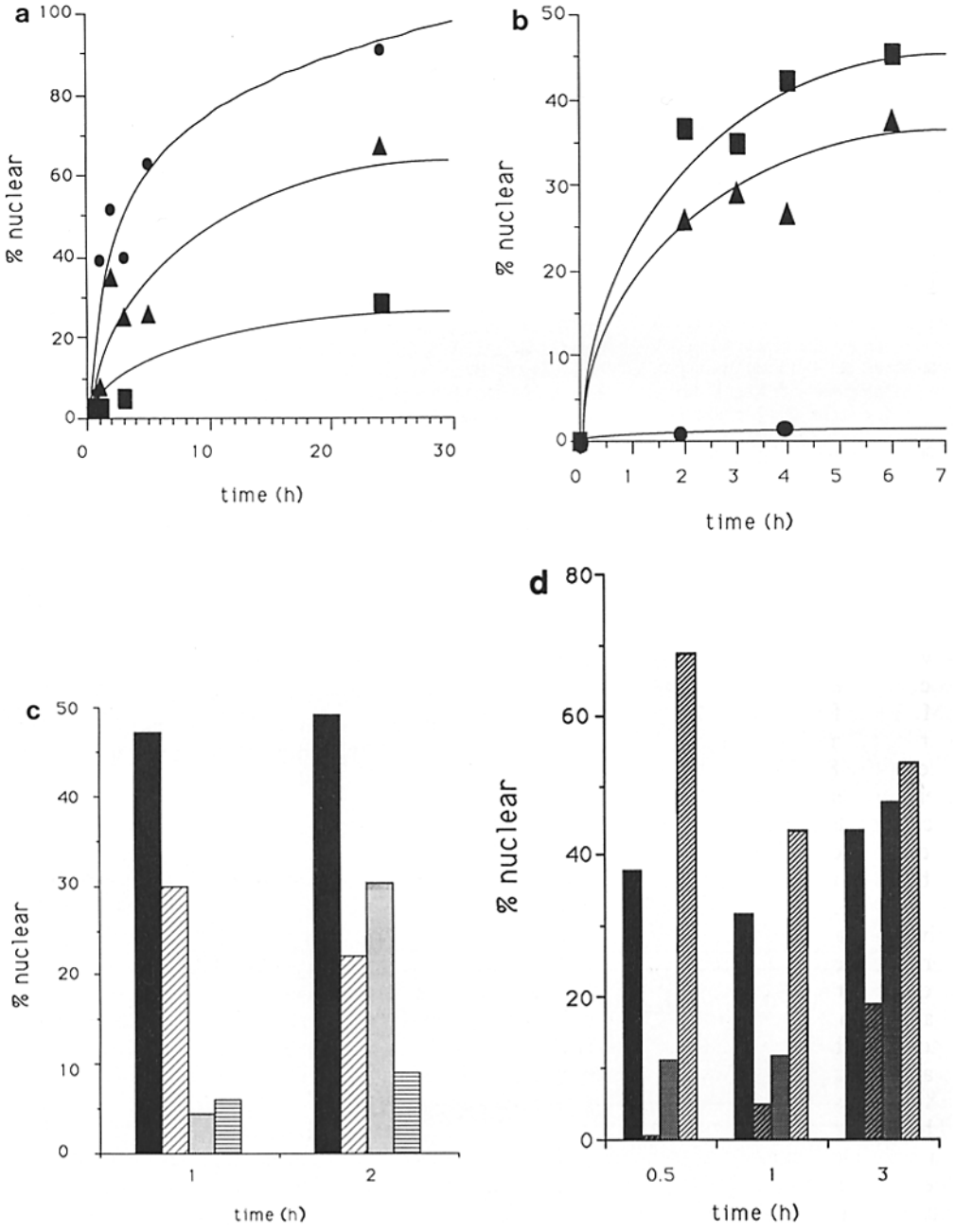

Figure 2. Functional characterization of the Ela second NLS. (*a*) Typical profile for nuclear accumulation of reticulocyte lysate-synthesized [^{35}S]methionine-labeled, ElaX (▲), 12S (●), and 12SX (■) proteins in oocytes after cytoplasmic injection. (*b*) Time course for nuclear accumulation of fusions proteins; 14085-MBP (▲), CNLS-MBP (■), and MBP (●) after cytoplasmic injection. Proteins were detected by Western blotting using anti-MBP antibodies. (*c*) In vivo ATP depletion by apyrase enzyme inhibits the nuclear accumulation of ElaX and 12S proteins. A graphic representation of the distribution of the 12S and ElaX proteins in the presence (▨12S + Apyrase; ▤ElaX + Apyrase) or absence (■12S; ▨ElaX) of the apyrase enzyme (0.3 U/oocyte) at 1 or 2 h after injection of [^{35}S]methionine-labeled, reticulocyte lysate generated proteins. (*d*) Competition studies between the Ela carboxy terminus NLS and drNLS for nuclear import. [^{35}S]methionine-labeled, reticulocyte lysate synthesized 12S protein (■) was injected into the cytoplasm of oocytes previously injected with 0.5 μg of CNLS-MBP (▨), 14085-MBP (▨), or MBP (▨). After incubation, the amount of labeled 12S protein in the nucleus was determined by SDS-PAGE of cytoplasmic and nuclear protein, autoradiography, and densitometry of resulting bands.

or mutant Ela proteins into oocytes, which were then radiolabeled with [^{35}S]methionine. The oocyte nuclei (germinal vesicle or gv) and cytoplasms were then manually separated and the Ela proteins in each fraction were immunoselected and analyzed by SDS-gel electrophoresis and autoradiography. Fig. 1 shows that Ela wild type, ElaX (lacking residues 222–289) and Ela SP12S (lacking residues 140–185), were all predominantly nuclear (>50%) 4 h after the addition of label. Because Ela SP12SX (lacking residues 140–185 and 222–289) was not transported to the nucleus to any significant extent, (<10% nuclear), this experiment suggests the presence of two NLSs in Ela, one residing between residues 140 and 185, and one residing between residues 222 and 289. Finally, we note that the multiple Ela molecular weight variants observed in Figs. 1 and 3–5 are due to differential phosphorylation, which probably does not play a role in nuclear localization (45).

To determine whether Ela residues 140–185 direct nuclear accumulation through selective import pathways or through "diffuse and bind" mechanisms, we first compared the rate of import of the signal contained within Ela residues 140–185 with the Ela COOH-terminal NLS, the latter of which is similar to the SV-40 T-antigen NLS in structure and function (25). As shown in Fig. 2 *a*, in vitro synthesized 12S protein, which contains only the COOH-terminal NLS, and the ElaX, which contains only the putative NLS within residues 140–185, accumulate rapidly in oocyte nuclei after cytoplasmic injection. By comparison, 12SX, which contains no nuclear localization signal, does not localize in the nucleus. We also note that because Ela 12S has a more rapid accumulation relative to ElaX, that protein probably contains the more effective NLS.

Next, we have determined whether the putative Ela NLS residing between residues 140–185 can direct nuclear import

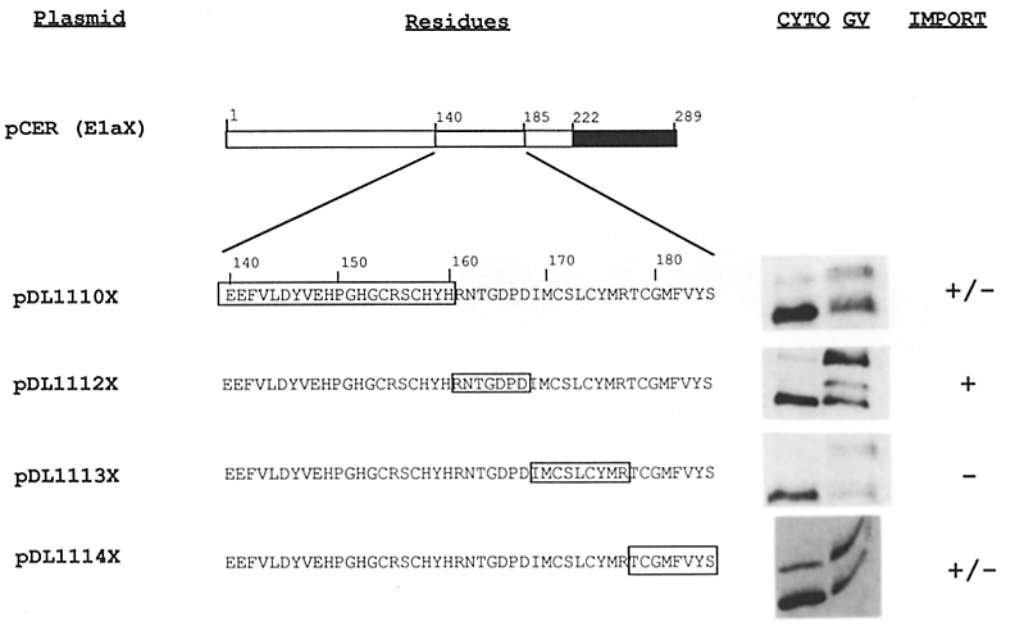

Figure 3. Deletion analysis of Ela residues 140–185. Four consecutive deletions were made within residues 140–185 of the ElaX protein and are denoted with a box. Ela proteins were detected and quantitated as in Fig. 1.

of a heterologous protein. This was necessary since the Ela residues 140–185 globin construct reported previously (59) was shown to be incorrect (D. M. Standiford; personal observation). When fused to the carboxy terminus of the maltose binding protein, Ela residues 140–185 stimulated nuclear import of the 50-kD chimeric protein (Fig. 2 *b*). For comparison, we show that the carboxy-terminal Ela NLS also directs nuclear import of the maltose binding protein, but the maltose binding protein by itself remains predominantly cytoplasmic (Fig. 2 *b*).

An important feature of selective nuclear import is its dependence on ATP for the transport of proteins across the nuclear pore (34, 35, 42). We have tested the effects of ATP depletion on the second Ela signal in vivo using the apyrase enzyme, which has been shown to block the import of other karyophilic proteins both in vivo and in vitro (34, 35). When in vitro–synthesized 12S or ElaX proteins are injected into oocytes previously injected with apyrase (0.3 U/oocyte), both show a marked reduction in their nuclear import compared with that of nonapyrase-injected oocytes (Fig. 2 *c*), indicating that both signals are dependent on ATP for function. When effects of apyrase were tested using the CNLS- and 14085-MBP fusion proteins, both were also found to be inhibited for nuclear import (not shown).

It has been shown recently that a number of different nuclear targeting pathways exist in the cell (13, 28) and that macromolecules using these pathways do not compete with each other for import. For instance the m_3GpppN-Sm protein containing UsnRNPs do not compete with T-antigen–like NLSs for import (27). To determine whether the two Ela NLSs operate using like or different import pathways, competition studies were performed by challenging the radiolabeled reticulocyte lysate synthesized 12S protein for nuclear import with either the CNLS-MBP, 14085-MBP, or MBP proteins (Fig. 2 *d*). From these experiments, it was found that the nuclear accumulation of the 12S protein is effectively reduced by the presence of both the 14085-MBP and CNLS-MBP protein, but is not affected by the maltose binding protein alone, indicating that the two signals are using similar components of the nuclear import pathway. The increase in import of the 12S protein in the presence of MBP is likely due to experimental variation. Interestingly, the CNLS-MBP appears to be a better competitor than does 14085-MBP which may be related to the observation that the Ela COOH terminus NLS is the more effective NLS.

Determination of the drNLS Primary Structure

To begin defining the exact residues that comprise the internal NLS, a series of deletion mutations through the 140–185 domain was used. Fig. 3 shows that deletion mutant DL1110X, which lacks residues 140–160 in the internal domain, was reduced in the nucleus by 20–30% relative to ElaX after 4 h of radiolabeling. Deletion of residues 161–168 (DL1112X) had no effect on nuclear transport, but the removal of residues 169–177 (DL1113X) had a severe effect on transport, reducing the amount of Ela in the nucleus by 75% compared with ElaX. The final deletion of residues 178–184 (DL1114X) also reduced transport, but only by 20–30% relative to ElaX. These data show that residues 169–177 are critical for nuclear import, but also that residues 140–160 and 177–184 have some influence on nuclear localization.

To identify the precise nuclear localizing residues defined by mutant DL1113X, a further series of mutations was constructed (Fig. 4). The mutant Ela protein encoded by p308 substitutes ala.tyr for met^{170}.cys and deletes ser^{172}-met^{176}. When tested for nuclear localization in oocytes as described above, this protein was found to be <25% nuclear after 4 h, showing that Ela residues met^{170}-met^{176} are important components of the NLS. Inspection of residues 170–176 reveals the presence of two cysteine residues that have been shown previously to participate in a zinc finger structure (5). To ascertain if this cysteine-mediated structure was important for drNLS function, cys^{171} was substituted with histidine, pH171, or both cys^{171} and cys^{174} were substituted with phenylalanine, pF2. Neither of these substitutions had any effect on transport indicating that the zinc finger is not important for signal function.

Further pair-wise amino acid substitutions were then made

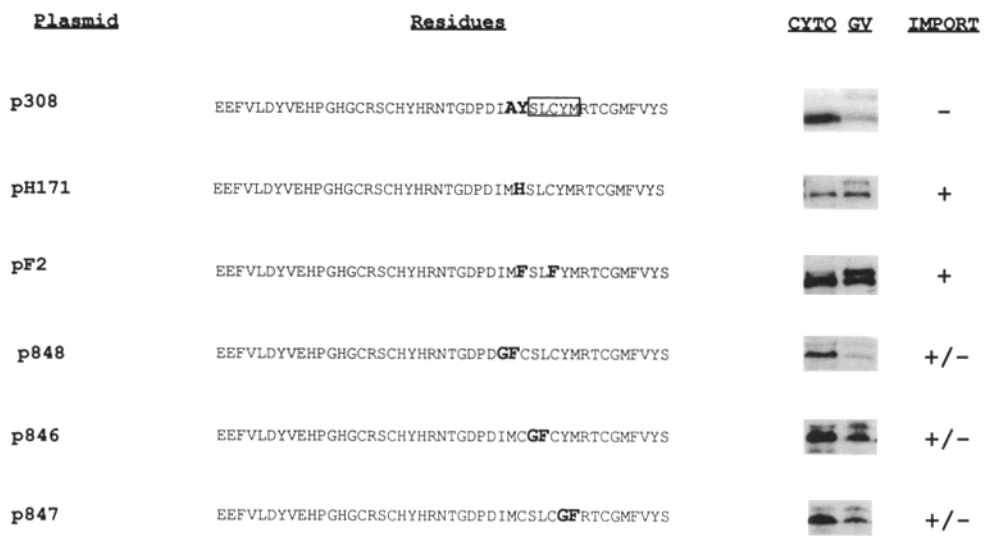

Figure 4. Fine mutational analysis of Ela residues 169–177. Plasmids encoding Ela proteins with amino acid substitutions (*bold*) or a deletion (*boxed*) were injected into oocytes, followed by radiolabeling with [^{35}S]methionine. The detection of Ela in nuclei and cytoplasms and the calculation of their relative amounts in the nuclei, were as described in Fig. 1.

through residues 169–176, replacing the wild-type residues with glu.phe. Each of these mutations; p848 (ile^{170}met→glu.phe), p846 (ser^{172}leu→glu.phe), and p847 (tyr^{175}met→glu.phe), resulted in the reduction of nuclear transport. The mutation in p848, however, was found to have the strongest effect, reducing transport by >60% relative to ElaX. These data show that Ela residues met^{170}.X.ser.leu.X.tyr.met are important for signal function.

As shown in Fig. 3, the removal of residues 140–160 (DL1110X) or 178–184 (DL1114X) reduced nuclear localization of Ela. To further explore these regions and their effects on transport, an additional set of mutations were constructed and are shown in Fig. 5.

To determine which amino acids among residues 178–185 are important for transport, met^{181}.phe were substituted with lys.leu by the placement of a HindIII restriction site in the coding sequence, pH 3. These substitutions reduced Ela transport by ~30% compared with ElaX, which is similar to that caused by the entire deletion in DL1114X (Fig. 3). Thus met^{181}.phe comprise a portion of this Ela NLS.

Residues in the 140–160 region were first examined with the pΔ20 deletion, which removes Ela residues 150–170 (Fig. 5). This mutant was found to be equal to that of ElaX in its nuclear/cytoplasmic distribution (>50%), indicating that these residues are not important for transport. Two further deletions of leu^{144}-his^{160} (p4157) and phe^{142}-his^{160} (p4153) were then constructed and when tested in oocytes, were found to have different effects on nuclear localization. The protein encoded by p4157 retained ElaX levels of nuclear transport, whereas the protein encoded by p4153 was reduced in the nucleus by ~30% relative to ElaX. These results show that phe^{142}.val are important for signal function.

Next we combined the amino acid deletions of p4153 and p4157 with the met^{181}.phe represented in the pH 3 substitution (p4153-H3 and p4157-H3, respectively) (Fig. 5). The protein encoded by the p4157-H3 was reduced in nuclear localization by ~30% compared with ElaX. Nuclear transport of the protein encoded by p4153-H3, on the other hand, was essentially eliminated. Taken together, the above data demonstrate that the second Ela NLS has the structure phe.val(x)$_{7-20}$.met.x.ser.leu.x.tyr.-met.(x)$_4$.met.phe.

Analysis of Ela Nuclear Localization During Xenopus Embryogenesis

Slavicek et al. (59) have shown that an Ela containing only

Figure 5. Identification of a minimal Ela NLS. Plasmids encoding proteins containing amino acid substitutions (*bold*) or deletions (*boxed*) were injected into oocytes. The detection and quantification of Ela in nuclei were as described in Fig. 1.

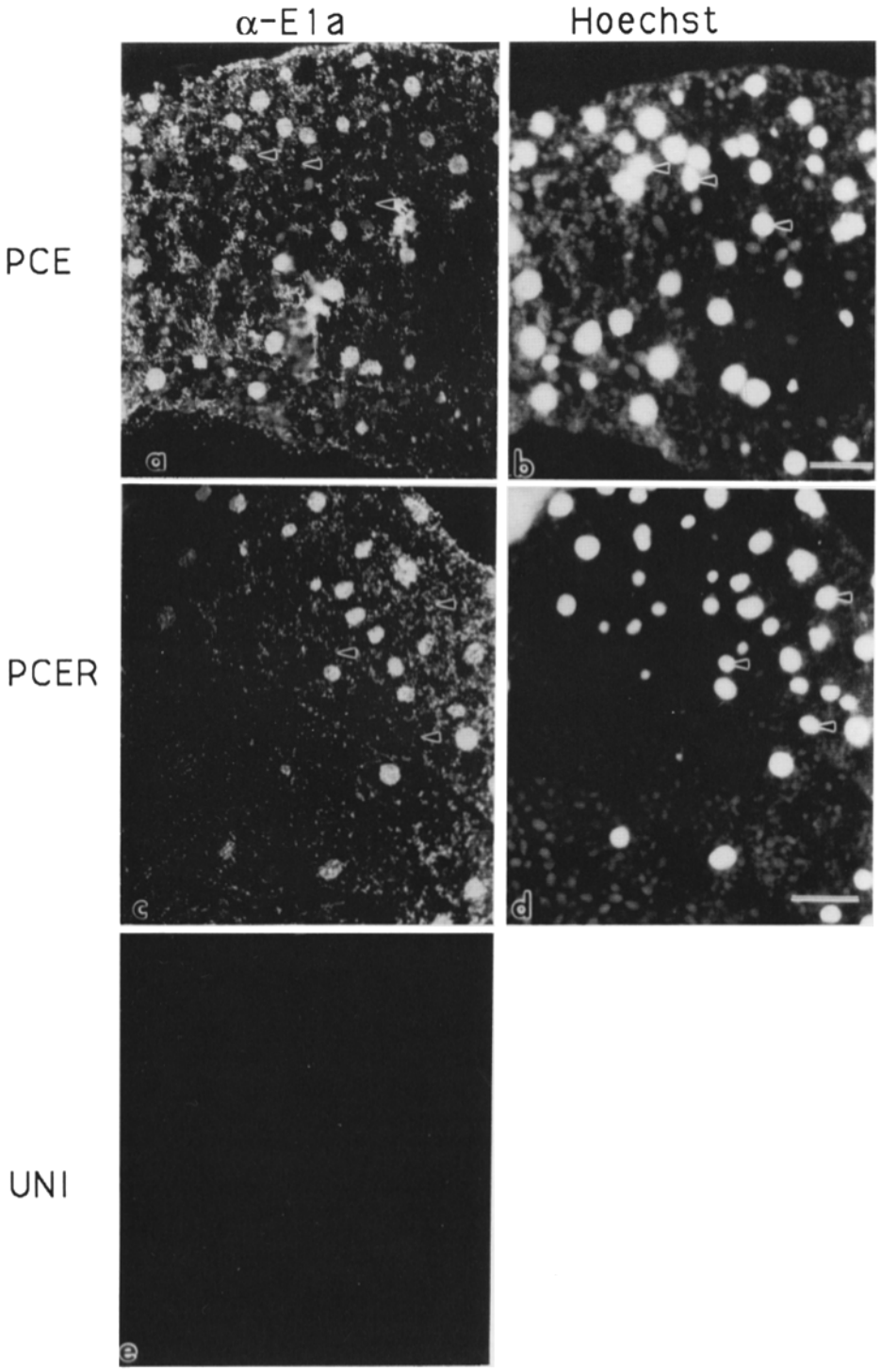

Figure 6. The second Ela NLS functions during the late gastrula stage of development. Fertilized eggs were injected with DNA coding for the wild-type Ela (*pCE*), which contains both Ela NLSs, or DNA coding for a truncated Ela (*pCER*), which contains only the NLS located within residues 140–185 (the drNLS). The embryos were allowed to develop to the late gastrula stage when they were fixed, embedded in plastic resin, sectioned, and stained for Ela protein using anti-Ela antibodies or DNA using Hoechst dye. *a* and *b* show a section of the dorsal region of a pCE injected embryo stained with anti-Ela antibodies or Hoechst, respectively. Arrowheads denote nuclei that did not stain for Ela because of mosaicism. *c* and *d* show a section of the dorsal region of a pCER-injected embryo stained with anti-Ela antibodies or Hoechst, respectively. *e* shows an uninjected late gastrula stage embryo treated with anti-Ela antibodies. Bars, 25 μm.

the NLS located between residues 140 and 185 becomes localized in nuclei of injected *Xenopus* oocytes, but not transfected *Xenopus* A6 cells or Hela cells. This might suggest that the function of this NLS is regulated during *Xenopus* development. To determine whether this is the case, DNAs encoding wild-type or a carboxy-truncated Ela (ElaX) were injected into fertilized *Xenopus* eggs, which were then allowed to develop into embryos of various stages. The embryos were fixed, embedded, sectioned, and reacted with Ela antibodies and fluorescein-conjugated secondary antibodies.

Fig. 6 shows the pattern of nuclear localization of these proteins in late gastrula stage embryos. *a* and *b* show immunostaining and DNA staining (Hoechst's dye) of the dorsal part of a sectioned embryo injected with pCE (encoding wild-type Ela). While not all cells show immunostaining due to mosaicism (*arrowheads*), those cells that do react with Ela antibody show fluorescence over nuclei. Similarly, the truncated protein encoded by pCER (ElaX) shows nuclear localization (compare *c* and *d*). *e* demonstrates a lack of fluorescence when noninjected embryos were treated with the same antibodies as in *a* and *c*.

Sections from a pCER injected embryo taken at the early

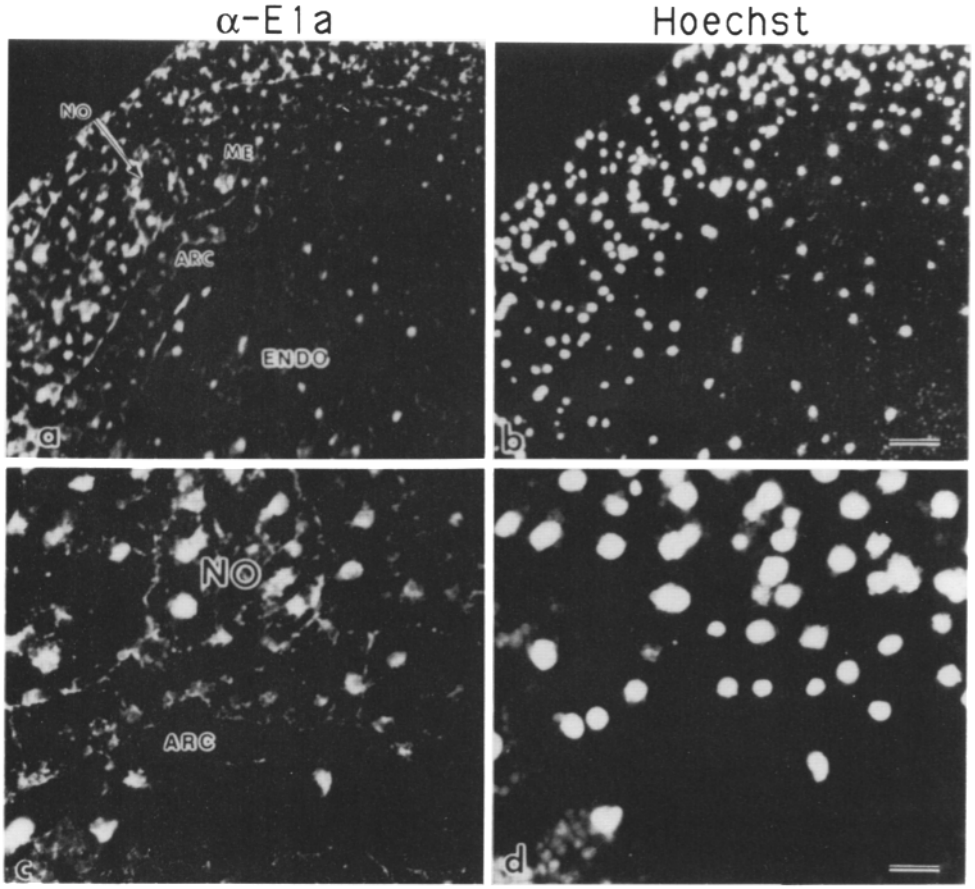

Figure 7. The second Ela NLS functions during the early neurula stage of development. Fertilized eggs were injected with pCER, allowed to develop until the early neurula stage, embedded in paraffin, sectioned, and stained with either anti-Ela antibodies (*a* and *c*) or with Hoechst (*b* and *d*). In *a* and *b* is a low magnification view of a section through an embryo showing various embryonic tissues while *c* and *d* show a higher magnification of the notochord and surrounding tissues. *ARC*, archentron; *ENDO*, endoderm; *ME*, mesoderm; *NO*, notochord. Bars: (*b*) 50 μm; (*d*) 25 μm.

neurula stage of development are shown in Fig. 7. In this embryo, the ElaX protein is still localized to the nuclei of cells throughout (compare *a* and *b*). This is particularly evident in the notochord (*c* and *d*). By the mid-neurula stage, however, the ability of the embryo to localize ElaX protein to the nucleus is beginning to abate. Fig. 8 shows an embryo injected with pCE in which the wild-type Ela protein continues to localize to the nuclei of cells in all mid-neurula stage tissues (*a* and *b*). At this same stage in embryos injected with pCER, however, the truncated ElaX protein has become primarily cytoplasmic in the notochord, mesoderm, and neural ectoderm, while remaining nuclear in other tissues such as endoderm (*c* and *d*). This difference is most evident under closer inspection of the notochord. While wild-type Ela is clearly nuclear in this tissue (*e* and *f*), the truncated ElaX protein is cytoplasmic (*g* and *h*). Therefore, the ability of Ela to be localized to the nucleus via the residues within 140–185 changes during mid-neurulation.

By the late neurula stage, ElaX is cytoplasmic in all tissues except for the epidermal ectoderm (Fig. 9, *a–d*). In tailbud stage embryos, ElaX protein is now cytoplasmic in all tissues including epidermal ectoderm (*e* and *f*). For comparison, a tailbud stage embryo that is expressing wild-type Ela continues to localize the protein to the nuclei of cells (compare *g* and *h*). We also show that noninjected embryos taken at the tailbud stage do not offer a fluorescent signal when reacted with the same antibodies as in Fig. 8 *e*.

The data in Figs. 5–8 show that ElaX localizes to the nucleus until the neurula stage of development. Because the sole NLS in this protein is that which we have defined as the residues within residues 140–185, we now define this signal as the Ela developmentally regulated NLS (drNLS).

Discussion

We have presented evidence to show that the adenovirus Ela protein contains two NLSs. The first signal is located at the Ela carboxy terminus and corresponds to the pentapeptide KRPRP, which was identified initially by Lyons et al. (25). This sequence, which is reminiscent of many other NLSs in that it is a short peptide composed of basic residues, acts constitutively to direct nuclear import in several cell lines as well as in injected *Xenopus* oocytes (25, 59; see also Fig. 1). The second Ela NLS resides internally in the protein and is composed of the sequence $FVX_{7\text{-}20}MXSLXYMX_4MF$. This sequence directs nuclear localization only in oocytes and early embryos; hence, it is referred to as the Ela drNLS.

Because the drNLS is located in a small protein (35 kD) that could potentially diffuse into the nucleus and bind preferentially to some nuclear component via the drNLS, we performed several experiments to test whether the import and accumulation of the ElaX protein is through a diffuse and bind pathway, or through selective import pathways. We first showed that the drNLS can efficiently direct the nuclear import of a large (50 kD) fusion protein (14085-MBP; Fig. 2 *c*) to the nucleus and does so with kinetics approximating that of the ElaX protein (26 kD), suggesting that import of ElaX and 14085-MBP is NLS dependent. We should note that a previous report showed that the Ela residues 1–120 also accumulated in the oocyte nucleus after a 24-h incubation

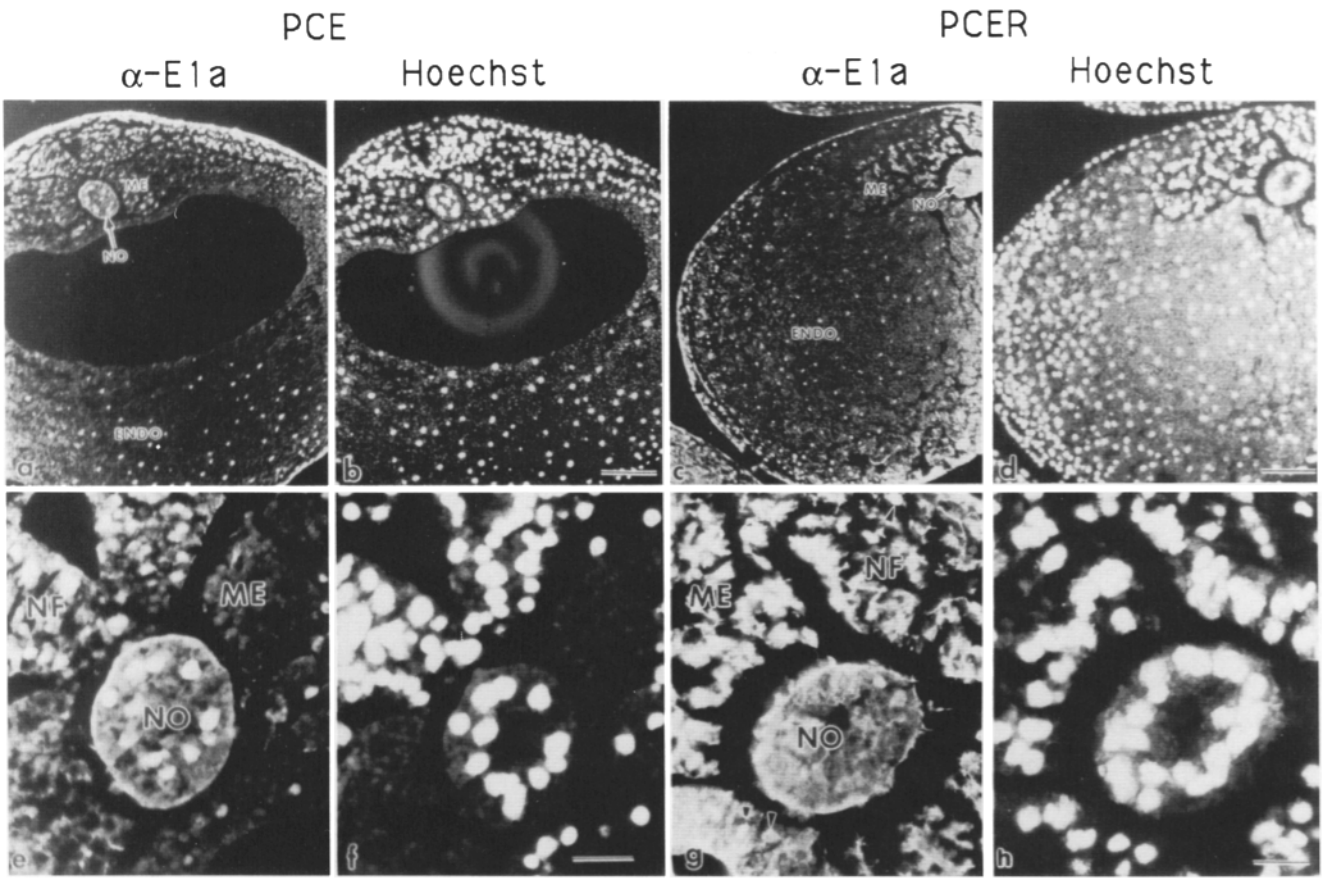

Figure 8. The activity of the second Ela NLS begins to abate during neurulation. Fertilized eggs, injected with either the pCE (*a*, *b* and *e*, *f*) or pCER (*c*, *d* and *g*, *h*) DNAs were allowed to develop until the mid-neurula stage, then fixed, embedded in paraffin, sectioned, and stained with anti-Ela antibodies (*a*, *c*, *e*, and *g*) or Hoechst (*b*, *d*, *f*, and *h*). Low magnification views in *a–d* show the distribution of Ela proteins in various tissues, while at a higher magnification (*e–h*), the distribution of proteins in notochord and surrounding tissues can be compared. Arrowheads in *g* indicate continued nuclear staining of Ela in endodermal and epidermal ectoderm. *ENDO*, endoderm; *EPI*, epidermal ectoderm; *ME*, mesoderm; *NF*, neural fold; *NO*, notochord. Bars: (*b*) 75 μm; (*d*) 25 μm.

(44). Given the small size of this peptide, we attribute this to diffusion, although it is unclear why it was retained in the nucleus.

It has been shown by several investigators that the NLS-directed movement of proteins across the nuclear pore is an energy-dependent process (4, 34–36). The injection of the apyrase enzyme into oocytes, shown to reduce ATP and nuclear transport both in vivo and in vitro (35), was found to reduce nuclear import for both the Ela carboxy terminus and drNLSs (Fig. 2 *c*). In addition, we find that both the 12S and ElaX proteins are excluded from the nucleus when oocytes were incubated at 0°C (not shown). This latter observation also suggests the presence of an NLS within Ela residues 140–185 because Breeuwer and Goldfarb (4) have found that small proteins that do not contain an NLS freely diffuse into nuclei, while small NLS-containing proteins are retained in the cytoplasm of chilled cells.

The existence of several distinct nuclear targeting pathways has recently been recognized (13, 27, 28). We have begun to identify the pathway used by the drNLS for nuclear import by examining its ability to compete with the Ela carboxy terminus NLS (a large T-antigen–like NLS). When the nuclear import of the 12S protein was challenged with the drNLS– and CNLS–MBP fusion proteins, it was found that both could efficiently compete for nuclear import (Fig. 2 *d*), while MBP had no effect. These data indicate that the two signals use similar components of the nuclear import pathway.

Mechanisms of Nuclear Localization

There are several models through which the Ela drNLS might direct nuclear localization. First, the drNLS could act by binding directly to a NLS-receptor protein similar to that used by the SV-40 large T-antigen–like NLSs (1, 61), and thus would use the same nuclear import mechanisms as these NLSs (56). However, because drNLS function is regulated during development, one would have to imagine that there are several isotypes of the NLS receptor protein available and that the particular receptor protein bound to the drNLS is itself developmentally regulated, or that the drNLS is "anchored" in the cytoplasm during development by additional proteins not present in the oocyte. A second possibility is that the drNLS binds to a soluble protein that itself contains a T-antigen–like NLS, thus promoting the import of the drNLS via a piggy-back mechanism; a situation that has been observed elsewhere (3). This model could account for the developmental regulation of drNLS-mediated nuclear import if the NLS-containing protein is itself present in a developmentally regulated fashion. However, the observation

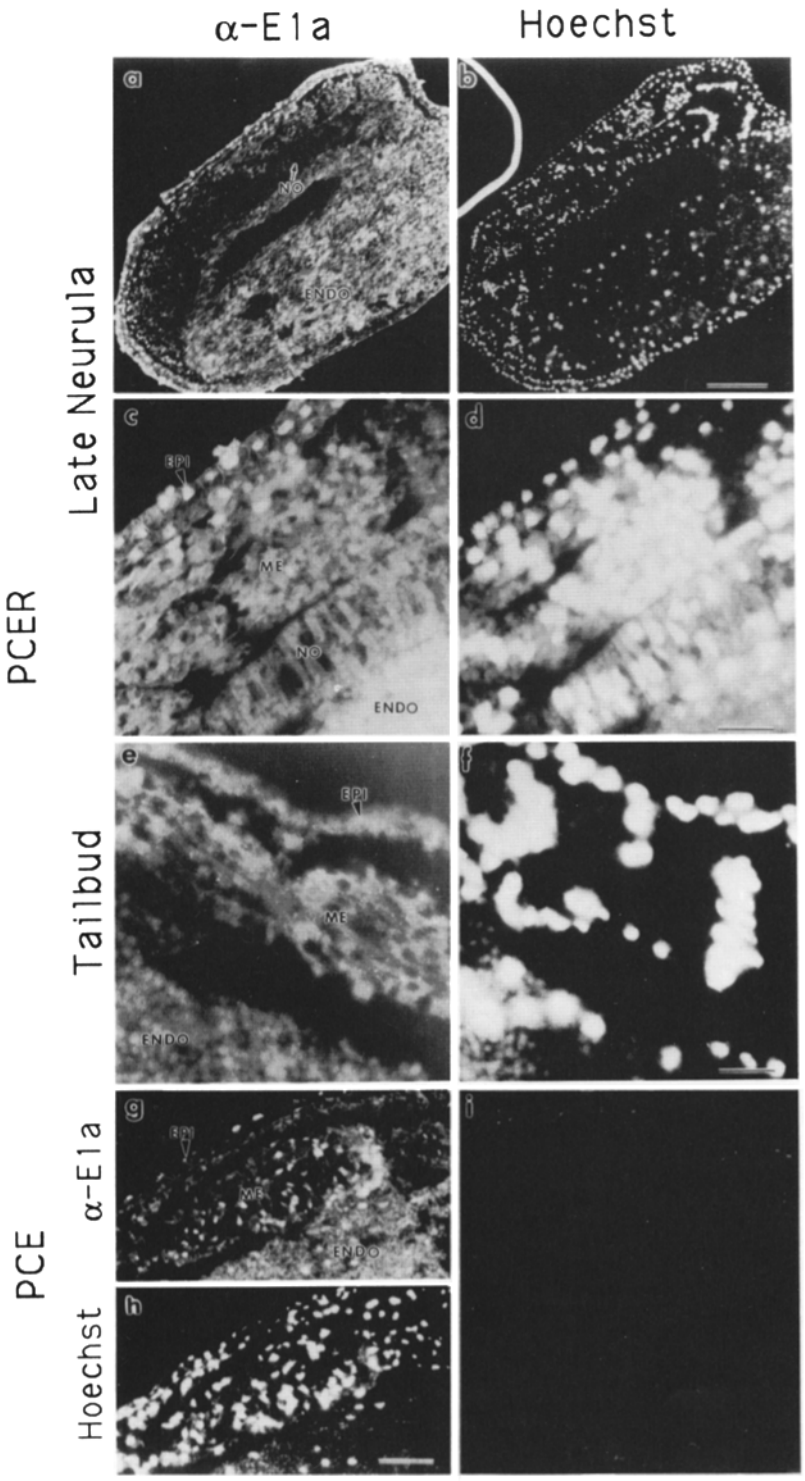

Figure 9. The second Ela is nonfunctional in all tissues at the end of neurulation. Embryos injected with pCER were allowed to develop until the late neurula (*a–d*) or tailbud (*e* and *f*) stage of development, then prepared as described in Fig. 7. In *a* and *b* is a low magnification view of an embryo stained with anti-Ela antibodies or Hoechst, respectively, showing the distribution of the truncated Ela across the embryo. *c* and *d* show a higher magnification view of the dorsal region of the same embryo. A pCER injected embryo allowed to develop until the tailbud stage before sectioning and staining with anti-Ela or Hoechst is shown in *e* and *f*, respectively. In *g* and *h* is a pCE-injected embryo at the tailbud stage of development stained with either anti-Ela or Hoechst, respectively, and in *i*, a noninjected tailbud stage embryo stained for Ela is shown. *ENDO*, endoderm; *EPI*, epidermal ectoderm; *ME*, mesoderm; *NO*, notochord. Bars: (*b* and *h*) 75 μm; (*d* and *f*) 30 μm.

that the 14085-MBP protein can compete with the 12S protein for nuclear import would suggest that the pool of drNLS binding proteins is large and that import does not occur in the absence of drNLS binding. A third possibility is that the drNLS might have affinity for some component of the transport machinery found at the nuclear pore complex, such as a pore receptor that serves to recognize the cytoplasmic NLS-receptor protein.

Distinctive Features of the drNLS

If it is the case that the Ela drNLS represents a new type of nuclear localization signal, then two features distinguish it from other known NLSs. First, functionally important amino acids are separated by apparently nonspecific spacer residues. While complex NLSs with this general character have been described previously (reviewed in 56), they usu-

ally are composed of two groups of basic residues separated by nonspecific spacer residues (48). In contrast, the Ela drNLS is composed of three distinct groups that are separated by spacer residues.

The second distinctive feature of the Ela drNLS is that it is remarkably hydrophobic; indeed, five of seven important residues are classified as such. This might suggest that the activity of the drNLS depends upon its ability to interact hydrophobically with other proteins. These types of interactions seem important and would perhaps account for the observed context dependency of the drNLS for function, a feature influencing the behavior of various other NLSs (15, 33, 63). This was implied when a minimal drNLS sequence represented by the Δ20 protein (Fig. 5) was placed before the *Xenopus* β-globin protein where it functioned very inefficiently (not shown). Because of its wild-type levels of transport in the Ela background, the minimal drNLS could require a certain number of flanking residues, likely of hydrophobic character, for it to function efficiently.

Assessment of an Ela drNLS-like Sequence in Other Proteins

We have searched the entire SwissProt data base for proteins with sequences similar to the Ela drNLS using the GCG Findpatterns program (7). Because we have not determined all the parameters of spacing between the drNLS functional groups, we conducted the search using the somewhat broadened search sequence $FVX_{6\text{-}30}MXSLXYMX_{1\text{-}10}MF$ and allowed for up to three mismatches. Among the more than 2×10^4 protein sequences searched many contained sequences similar to the Ela drNLS. Several of these were nuclear proteins, perhaps the most intriguing of which was that of the glucocorticoid receptor from rat, mouse, and human. The rat receptor (29) contains the sequence- $a^{594}V(x)_{17}$MxlLxYsxMF, (residues matching the drNLS are in capital); similar sequences also exist in the mouse (6) and human (17) receptor proteins as well. Interestingly, this sequence lies within the steroid binding domain of the receptor, which is also the region shown to contain an as yet undefined NLS that is regulated through the binding of steroid hormone (32, 40). It is possible that this sequence is the glucocorticoid receptor hormone-regulated NLS.

The Ela drNLS Functions Only during Early Development

We have demonstrated that the Ela drNLS directs nuclear import in a temporal and tissue-specific manner during *Xenopus* embryogenesis. Fig. 6 shows that an Ela protein truncated at residue 222, whose sole NLS is that which we have defined as the drNLS, directs nuclear import in gastrula stage cells. While the truncated protein continues to accumulate in the nucleus in the early neurula before the formation of the neural tissues (Fig. 7 *c*), by the mid-neurula stage when neural ectoderm is well developed, the drNLS is nonfunctional in notochord, mesoderm and neural ectoderm as evidenced by clear cytoplasmic immunostaining (Fig. 8, *g* and *h*). Importantly, full length Ela, which carries the constitutive carboxy-terminal NLS as well as the drNLS, is nuclear at this same stage (Fig. 8, *e* and *f*). At late-neurula, the truncated Ela has become cytoplasmic in location everywhere but in the epidermal ectoderm (Fig. 9, *a–d*) and the drNLS is completely nonfunctional in the embryo in tailbud stage embryos (Fig. 9, *e* and *f*).

We believe that cellular proteins contain drNLSs that are similar in structure too that which is fortuitously present in Ela. Moreover, it is not unreasonable to suspect that normal development depends upon cellular proteins whose nuclear/cytoplasmic distribution changes in a manner similar to that displayed by ElaX. Thus, it is possible that the redistribution of a protein from nucleus to cytoplasm could have important consequences for neurulation. Clearly, regulated nuclear transport controls developmental events in *Drosophila* (see introduction) and possibly in *Xenopus* as well. For example, fibroblast growth factors (FGFs), which are thought to be important for mesoderm induction in *Xenopus* (58), have been shown by Shibura et al. (55) to be positioned in the cytoplasm until the blastula stage. At this point, which coincides with the start of mesoderm induction (21), FGF relocalizes to the cell nucleus. Whether this time-dependent nuclear localization of FGF is critical for mesoderm induction is unclear, but the provocative nature of these results suggest that it could. Given this example and the large number of additional proteins whose cellular location shifts during *Xenopus* development (8, 9, 30, 65), it seems that regulated nuclear import could potentially be a major mechanism controlling certain developmental events.

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