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Subunits of the Heterotrimeric Transcription Factor NF-Y Are Imported into the Nucleus by Distinct Pathways Involving Importin β and Importin 13

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The transcriptional activator NF-Y is a heterotrimeric complex composed of NF-YA, NF-YB, and NF-YC, which specifically binds the CCAAT consensus present in about 30% of eukaryotic promoters. All three subunits contain evolutionarily conserved core regions, which comprise a histone fold motif (HFM) in the case of NF-YB and NF-YC. Our results of in vitro binding studies and nuclear import assays reveal two different transport mechanisms for NF-Y subunits. While NF-YA is imported by an importin β-mediated pathway, the NF-YB/NF-YC heterodimer is translocated into the nucleus in an importin 13-dependent manner. We define a nonclassical nuclear localization signal (ncNLS) in NF-YA, and mutational analysis indicates that positively charged amino acid residues in the ncNLS are required for nuclear targeting of NF-YA. Importin β binding is restricted to the monomeric, uncomplexed NF-YA subunit. In contrast, the nuclear import of NF-YB and NF-YC requires dimer formation. Only the NF-YB/NF-YC dimer, but not the monomeric components, are recognized by importin 13 and are imported into the nucleus. Importin 13 competes with NF-YA for binding to the NF-YB/NF-YC dimer. Our data suggest that a distinct binding platform derived from the HFM of both subunits, NF-YB/NF-YC, mediates those interactions.

The existence of a cell nucleus in eukaryotes implies the spatial separation of transcription and translation and therefore requires bidirectional intracellular trafficking of macromolecules. The site of exchange is the nuclear pore complex (NPC), one of the largest macromolecular assemblies in a eukaryotic cell, which can be traversed in a passive or a facilitated manner. The passive mode applies for small molecules but becomes ineffective for proteins with a molecular mass greater than 40 kDa. In addition, molecules which may potentially passively diffuse are often actively translocated, since this allows a more efficient and regulated transport (for reviews, see references 17, 25, 47, 64, and 77).

Most nuclear transport processes are mediated by soluble transport receptors that recognize specific sequences or structural characteristics of their cargoes and facilitate the passage of receptor-cargo complexes through the NPC. Transport receptors constantly shuttle between the nucleus and cytoplasm, thereby rapidly crossing the permeability barrier of nuclear pores (59). The largest class of nuclear transport receptors is the superfamily of importin β-like factors (also named karyopherins) that can be classified as importins (import karyopherin) and exportins (export karyopherin) depending on the direction in which they transport the cargo (reviewed in references 25, 32, 43, 72, and 80). Cargo binding and release of importins and exportins is controlled by a steep RanGTP gradient, which is maintained across the nuclear envelope through the asymmetric distribution of factors that regulate the guanine nucleotide-bound state of Ran (25, 41, 43, 47, 76). The exchange factor, RanGEF (also called RCC1), is exclusively nuclear, while the GTPase-activating protein, RanGAP, is cytoplasmic. Importins load cargoes in the absence of Ran in the cytoplasm and release their cargo upon RanGTP binding in the nucleus (27, 33, 58). In contrast, exportins bind substrates only in the presence of RanGTP in the nucleus and cargo release is accomplished when the Ran-bound GTP molecule is hydrolyzed in the cytoplasm (10, 22, 39). In these transport cycles GTP hydrolysis constitutes the sole input of metabolic energy, which allows import and export cargoes to accumulate against gradients of chemical activities (21, 29, 38, 60, 67, 78).

Proteins bearing a classical nuclear localization signal (cNLS) are imported into the nucleus by the importin α/β heterodimer (26, 49, 55). cNLSs consist of short stretches of positively charged amino acids. They can be monopartite, as in the simian virus 40 (SV40) large T antigen that consists of a heptapeptide containing five basic amino acids (35), or bipartite, as in nucleoplasmin. The NLS in nucleoplasmin consists of two short basic clusters separated by a spacer of 10 amino acids (19, 62). In addition to the cNLS-dependent pathway, importins can also function in the absence of adapter molecules like importin α. In this alternative pathway the cargoes contain a nonclassical NLS (ncNLS), which is in general longer than the cNLS (15). Proteins bearing ncNLSs directly bind to one of the approximately 20 members of the importin β family present in higher eukaryotes (72). The list of adapter-independent cargoes is constantly increasing and includes, for instance, the transcription factors CREB, Jun, Fos (23), Smad-3 (81), the retroviral proteins Rev and Tat in human immunodeficiency virus type 1 (HIV-1) (74), the ribosomal proteins L5 and L23a (34), and the core histones (3, 51–53) that directly bind to importin β. The paired-type homeodomain transcription factors Pax6, Pax3, and Crx (56), the SUMO-1/sentrin-conjugat-

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ing enzyme hUBC9, and MGN binding protein RBM8 (Y14) (50) bind directly to importin 13, whereas transportin mediates the nuclear import of M9-containing proteins such as hnRNP A1 (57).

We have studied the nuclear transport of the mammalian CCAAT-specific transcription factor NF-Y (also termed CBF). In higher eukaryotes, the CCAAT box is found in about 30% of the promoters (12) and is generally localized in close vicinity of other promoter elements. Genes controlled by NF-Y include cell cycle-regulated genes, such as *CDC2*, *CDC25A*/*B*/*C*, *cyclin A2*, *cyclin B1*/*B2*, and *E2F1* (14), and NF-Y mediated transcription is required for cell proliferation and viability (8). NF-Y can be considered as the major CCAAT-binding activator (44, 45), and its key role is to act synergistically with other transcription factors for activation. NF-Y is a heterotrimeric complex composed of three subunits, A, B, and C, which are all essential for CCAAT binding (48, 69). Each subunit contains a core region that has been highly conserved throughout evolution. The conserved segments of the two subunits NF-YB and NF-YC contain a histone fold motif (HFM) as a main structural feature (2, 6) and tightly dimerize head to tail via their HFMs, which offer a complex surface necessary for NF-YA association. NF-YA is not able to interact with either NF-YB or NF-YC alone (37, 68, 69), and the mode of protein interactions between the subunits appears to be similar in higher and lower eukaryotes. The resulting trimeric complex has a high affinity and sequence specificity for the CCAAT boxes (9, 20, 36).

Presently, we report that the three subunits of NF-Y are imported into the nucleus in a signal-dependent, energy-dependent, and receptor-mediated pathway. It could be shown that NF-YA contains a ncNLS in the evolutionarily conserved region at the C terminus of the protein and mediates nuclear import by importin β. NF-YB and NF-YC do not carry an independent NLS. In principle, they could passively diffuse through the NPCs. However, we show that active nuclear transport requires heterodimerization and depends on importin 13. Binding is controlled by the RanGTPase system in the typical importin-like fashion, e.g., the NF-YB/NF-YC heterodimer can bind importin 13 at low RanGTP levels in the cytoplasm and becomes displaced upon RanGTP binding in the nucleus.

MATERIALS AND METHODS

Cell culture. HeLa cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ no. ACC57). The cells were grown in modified Eagle's medium (MEM; Biochrom) containing 10% (vol/vol) fetal calf serum (FCS; Biochrom) and maintained in a humidified incubator with 5% CO_2 atmosphere at 37°C.

Expression constructs. The coding regions of the respective proteins were first amplified from plasmid DNA (the cDNA of the NF-Y subunits was a kind gift from Roberto Mantovani, Dipartimento di Biologia Animale, Università di Modena e Reggio Emilia, Modena, Italy) using specific primer pairs with appropriate restriction sites.

The various coding regions for the glutathione *S*-transferase (GST) fusion proteins were cloned as follows: murine wild-type NF-YA and mutant NF-YA (developed by site-directed mutagenesis and characterized in Fig. 4A) were inserted as EcoRI-XhoI fragments, and human NF-YB was inserted as a BamHI-SmaI fragment into the respective sites of pGEX4T-1 (Amersham; N-GST-tagged); human NF-YC was inserted as a SpeI-SacI fragment into the SpeI-SacI sites of pET41a (Novagen; N-GST tagged).

The expression constructs for the zz/6z fusion proteins were generated as follows: murine NF-YA was inserted as an EcoRV-SpeI fragment and human NF-YB and human NF-YC were inserted as SmaI-NheI fragments from the corresponding pKS vector of the transfection system (described in reference 66) into the EcoRV-SpeI sites of p6z70. p6z70 is a modified pQE70 (QIAGEN) vector in which 6 z-tags (immunoglobulin G-binding domain from *Staphylococcus aureus* protein A) were inserted at the N terminus in addition to the C-terminal His tag. Human NF-YB was inserted as a SmaI-NheI fragment from the corresponding pKS vector of the transfection system (described in reference 66) into the EcoRV-SpeI sites of p2z70 (modified pQE70 in which 2 z-tags were inserted at the N terminus in addition to the C-terminal His tag).

The expression constructs for 6myc fusion proteins were generated as follows: murine NF-YA, human NF-YB, and human NF-YC were inserted as EcoRI-StuI fragments into the EcoRI-StuI sites of pCS2+MT (65, 75) containing 6 myc tags at the N terminus.

The expression constructs for β-galactosidase (β-gal) fusion proteins were generated as follows: the corresponding coding regions or gene fragments of the different NF-Y subunits were cloned via PCR into the modified pSVβ (Clontech) mammalian expression vector pKS10 (4, 66) using the unique restriction sites BglII and NheI. Deletion constructs were generated by combined insertion of two PCR-amplified gene fragments (PCR products) cloned into the BglII-NheI and NheI-NruI restrictions sites.

Hemagglutinin (HA)–NF-Y fusion protein expression constructs were generated as follows: murine NF-YA was inserted as an EcoRI-XhoI fragment and human NF-YB and human NF-YC were inserted as BamHI-XhoI fragments into the respective sites of pcHA (modified pcDNA3 [Invitrogen] in which the N-terminal HA tag was inserted as a KpnI-BamHI fragment).

Enhanced green fluorescent protein (EGFP)–NF-Y fusion protein expression constructs were generated as follows: murine NF-YA, human NF-YB, and human NF-YC were inserted as XhoI-ApaI fragments into the XhoI-ApaI sites of pcEGFP (modified pcDNA3 in which the N-terminal EGFP was inserted as an EcoRV-XhoI fragment).

The expression construct for the NF-YA–His fusion protein was generated as follows: murine NF-YA was inserted as an NdeI-SacI fragment into the NdeI-SacI sites of pJK45 (C-His-tagged construct, pJK45 is modified pET21b in which the PstI-NruI fragment of pACYC177 was inserted into the PstI-PshAI-digested pET21b).

The expression constructs for the GST-EGFP-Pax6 fusion protein was generated as follows: *Xenopus* Pax6 was inserted as an EcoRI-XhoI fragment into the EcoRI-XhoI sites of pGEX-EGFP (modified pGEX4T-1 in which EGFP was inserted as a BamHI-EcoRI fragment).

All constructs were verified by DNA sequencing (Andreas Nolte and Marco Winkler, Department of Developmental Biochemistry, University of Göttingen).

Site-directed mutagenesis. Nucleotide exchanges in pJK103, pJK104, pJK105, pJK106, pJK130, and pJK131 were done by site-directed mutagenesis as described by Ho et al. (31). The following primers were used to generate the various mutations: 5′-CTGTATGTGAATGCCGCACAGTATCACCGCAT C-3′ (sense) and 5′-GATGCGGTGATACTGTGCGGCATTCACATACAG-3′ (antisense) for pJK103, 5′-AAACAGTATCACCGCATCCTTAATCTGGGAC AAGCACGGGCTAAGCTAGA G-3′ (sense) and 5′-CTCTAGCTTAGCCCG TGCTTGTCCCAGATTAAGGATGCGGTGATACTGTTT-3′ (antisense) for pJK104, 5′-GAAGGGAAAATCCCAAAGGAACTAGGGCAATACCTCCAT GAGTCTCGGCATCGG-3′ (sense) and 5′-CCGATGCCGAGACTCATGGA GGTATTGCCCTAGTTCCTTTGGGATTTTCCCTTC-3′ (antisense) for pJK105, 5′-CGGCATCGGCACGCCATGGCACTGGCGGGTGGGGAAGGGGG C C GCTTCTTC-3′ (sense) and 5′-GAAGAA GCGGCCCCCTTCCCCACCCGCC AGTGCCATGGCGTGCCGATGCCG-3′ (antisense) for pJK106. To generate pJK130 the same primers were used as for pJK104 and pJK105. In the case of pJK131 the same primers were used as for pJK104 and pJK106.

Transfection and immunofluorescence. Transfection experiments and immunofluorescence were done as previously described (4, 66). Briefly, 1×10^5 HeLa cells/ml MEM were seeded on 10-mm glass coverslips 24 h prior to transfection. Chemical transfection was done with 400 ng DNA of each construct using the Effectene Transfection Reagent (QIAGEN) according to the manufacturer's instructions. Either after 24 h or 48 h of incubation at 37°C, the cells were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min, permeabilized with 0.5% Triton X-100 in PBS for 10 min, and blocked with 3% bovine serum albumin (BSA) in PBS. The nuclei were visualized with 4′6′-diamidino-2-phenylindole (DAPI) (Hoechst), and the subcellular localization of the gene products, endogenous NF-Y subunits, and nonfluorescent transport substrates was detected by indirect immunofluorescence. For that purpose, monoclonal mouse anti-NF-YA (BD Pharmingen), polyclonal rabbit anti-CBF-A (Santa Cruz), polyclonal rabbit anti-CBF-C (Santa Cruz), monoclonal mouse anti-β-galactosidase (Sigma), monoclonal mouse anti-HA (Santa Cruz), monoclonal mouse anti-Myc (Invitrogen), monoclonal mouse anti-GST (Santa Cruz) antibodies (primary), and Cy3-labeled goat anti-mouse (Dianova), Alexa-

Fluor488-labeled goat anti-mouse (Molecular Probes), AlexaFluor555-labeled goat anti-mouse (Molecular Probes), and AlexaFluor488-labeled goat anti-rabbit (Molecular Probes) antibodies (secondary) were used.

Recombinant protein expression and purification. The NF-Y proteins were expressed in BL21(DE3) strains (Novagen) as follows: cultures were grown at 37°C to an optical density at 600 nm of ~1.0 and then were shifted to 25°C. After the temperature was equilibrated, the cultures were induced with isopropyl-β-D-thiogalactopyranoside (IPTG) (0.2 mM for single NF-YA, NF-YB, and NF-YC cultures, 0.4 mM for the NF-YB/NF-YC coexpression culture) and were grown for 3 h with shaking at 230 rpm. Cells were then collected by low-speed centrifugation, resuspended in buffer A (50 mM Tris-HCl, pH 8.0, 400 mM NaCl, and 5 mM β-mercaptoethanol), and lysed by sonication. The recombinant proteins were purified on nickel nitrilotriacetic (NTA) agarose (QIAGEN) or glutathione-Sepharose 4B (Amersham) and GST-Bind resin (Novagen) according to the manufacturer's instructions.

To preassemble the trimeric NF-Y holocomplex, recombinant NF-YA protein was immobilized on nickel NTA-agarose (QIAGEN) and used as bait to fish out the NF-YB/NF-YC dimeric complex from the soluble fraction recovered by high-speed centrifugation of the coexpression lysate. After 3 h of incubation, the supernatant was removed and the resin was washed extensively with buffer A. The formed NF-Y trimer was eluted with imidazole and was applied onto a gel filtration column Hiload 16/60 Superdex 200 (Amersham) equilibrated with buffer A. The purified complex was concentrated on Vivaspin (Viva Science) to a final concentration of ~2 mg/ml.

The following import factors were expressed in *Escherichia coli* JM109 as described in the literature indicated and were purified on nickel NTA-agarose, followed by chromatography on Superdex 200: *Xenopus* importin α (28), human importin β (40), transportin (33), *Xenopus* importin 7, importin 5 (34), and importin 13 (50). Expression and purification (including affinity and ion exchange chromatography) of the following proteins was performed as described previously: NTF2 (40, 61), M9-GST (57), Ran and RanQ69L(GTP) (61), and 4z-rpL23a (34). GST-nucleoplasmin was expressed in *E. coli* BL21(DE3) for 3 h at 25°C and purified on glutathione-Sepharose 4B (Amersham).

GST pulldown assays. Bacteria expressing the recombinant GST fusion proteins were lysed in buffer A (50 mM Tris-HCl, pH 8.0, 400 mM NaCl, 5 mM β-mercaptoethanol) and centrifuged, and subsequently the supernatant was incubated with glutathione-Sepharose 4B beads or GST-Bind resin, respectively. Bound proteins (~0.5 mg/ml of resin) were washed three times in binding buffer B (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 5 mM MgCl, 5 mM β-mercaptoethanol) and used as affinity matrix for the binding experiments. For each binding experiment appropriate amounts of affinity matrix (0.2 μM final concentration of bound protein) were incubated with either precleared bacterial lysates containing expressed import receptors (~200 μl) or the corresponding purified recombinant import factor in buffer B (0.2 μM final concentration) at 4°C for 3 h in the presence or absence of 2 μM RanQ69L(GTP). Supernatants were removed and the beads were washed four times with ice-cold buffer B. Proteins were eluted with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, boiled for 10 min, and analyzed by SDS-PAGE.

Preparation of fluorescence-labeled import substrates. Cargo substrates that did not contain GST or EGFP were fluorescence labeled as previously described (5). Briefly, purified 6z fusion proteins expressed in *E. coli* were dialyzed against 20 mM HEPES-KOH, pH 7.4, 110 mM potassium acetate, 5 mM magnesium acetate, and incubated overnight at 4°C with a 10-fold molar excess of fluorescein isothiocyanate (Sigma) or alternatively with a 10-fold molar excess of FLUOS (Roche). Labeling of 4z-rpL23a with fluorescein 5′-maleimide (Pierce) through the engineered cysteine residue was performed in 50 mM HEPES-KOH, pH 7.5, 300 mM potassium acetate, 5 mM magnesium acetate. The labeled reaction mixtures were passed over NAP 5 columns (Pharmacia) equilibrated in 20 mM HEPES, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM dithiothreitol, and 250 mM sucrose to remove unincorporated fluorochrome.

Import assays. Import reactions were performed at 37°C essentially as described previously (34) based on the method established by Adam et al. (1). This assay allows reconstitution of nuclear import events by using recombinant purified transport factors without the interference of endogenous importins. Briefly, HeLa cells were grown on 10-mm glass coverslips to 40 to 80% confluence. Permeabilization was done with 40 μg/ml digitonin (Calbiochem) for 10 min on ice. The permeabilized cells were incubated for 25 min with 20 μl of a transport reaction mix consisting of cargo (2 μM) and either recombinant importin (2 μM) or 10 μl of reticulocyte lysate (Promega) in transport buffer (20 mM HEPES-KOH, pH 7.4, 110 mM potassium acetate, 5 mM magnesium acetate, 0.5 mM EGTA, 2 mM dithiothreitol, and 250 mM sucrose) along with an energy-regenerating system (0.5 mM ATP, 0.5 mM GTP, 10 mM creatine phosphate, and 50 μg/ml creatine kinase). In case of reconstitution experiments with recombinant transport factors, 3 μM Ran(GDP) and 0.5 μM NTF2 (called Ran mix) were added to the transport reaction mix to create a RanGTP gradient across the nuclear membrane. Cells were fixed by using 3% paraformaldehyde for 15 min and were mounted by using Vectashield with DAPI (Vector Laboratories). For the negative controls the assay was done in the absence of any transport factors, i.e., without reticulocyte lysate and recombinant transport factors. For competition experiments, 10 times the concentration of the actual cargo of nonfluorescent competitor protein was added to the import reaction. Import reactions were visualized by fluorescence microscopy with a Zeiss microscope (Axioskop 20) using a 40× objective lens (Plan Neofluar).

In vitro transcription-translation. In vitro transcription-translation of NF-YA, NF-YB, NF-YC, transcription factor IIIA (TFIIIA), and GST was performed from the corresponding SP6 promoter constructs (pCS2+MT). Using the T_NT-coupled reticulocyte lysate system (Promega) according to the manufacturer's instructions, the proteins were labeled with [^{35}S]methionine (Amersham). Reactions were performed at 30°C for 2 h in a 25-μl volume, and the samples were then either frozen or directly used for oocyte microinjections.

Oocyte microinjection and analysis. Preparation of oocytes, microinjection, and immunoprecipitation was performed as described before (30, 79). Briefly, collagenase-treated *Xenopus laevis* oocytes (stage V/VI) were kept over night in MBSH (88 mM NaCl, 2.4 mM $NaHCO_3$, 1 mM KCl, 10 mM HEPES, pH 7.6, 0.8 mM $MgSO_4$, 2 mM $CaCl_2$, 3.3 mM KNO_3) for recovery. Samples of 10 to 20 nl of the in vitro translation reaction were injected into the cytoplasm of oocytes. At the indicated time points, 10 to 15 oocytes were dissected manually and homogenized in NET-2 buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% NP-40). Proteins of the nuclear and cytoplasmic fractions were precipitated using antibodies against the myc tag (Santa Cruz) and finally analyzed by SDS-PAGE and phosphorimaging (Amersham).

RESULTS

NF-Y subunits are predominantly nuclear at steady state. The subcellular localization of endogenous NF-Y subunits and differently tagged, overexpressed versions of the three NF-Y subunits was examined in HeLa cells. In this assay, β-galactosidase-tagged fusion constructs were under the control of an SV40 promoter causing moderate overexpression of the β-gal fusion proteins. In contrast, EGFP-, HA-, and myc-tagged fusion proteins were strongly overexpressed in HeLa cells, because their genes were under the control of a cytomegalovirus (CMV) promoter. Dominant nuclear localization patterns were evident for the endogenous and β-galactosidase-tagged NF-YA, NF-YB, and NF-YC subunits. In contrast, strong overexpression influenced the distribution of NF-YB and, significantly, of NF-YC, while the dominant nuclear localization pattern of NF-YA remained unchanged (Fig. 1A, lower panels). The fluorescent staining of EGFP-tagged NF-YB (EGFP–NF-YC) and HA-tagged NF-YB (HA–NF-YC) became more cytoplasmic compared to the β-galactosidase fusion protein, and EGFP– and HA–NF-YC remained essentially in the cytoplasm (Fig. 1A). When EGFP–NF-YC was overexpressed together with myc-tagged NF-YB and vice versa the subcellular localization changed, becoming largely nuclear (Fig. 1B). This behavior could also be observed coexpressing HA–NF-YC and myc-tagged NF-YB (Fig. 1B lowest panel), which further indicates that the nuclear localization of NF-YC depends on the presence of its histone fold partner NF-YB, as previously shown by Frontini et al. (24). Taken together, these results point toward NF-YA being imported into the nucleus independently of NF-YB and NF-YC, whereas these HFM-containing subunits mediate the nuclear localization of the corresponding histone fold partner.

Sequence motifs necessary for nuclear localization of NF-Y subunits correspond to their conserved domains. To identify

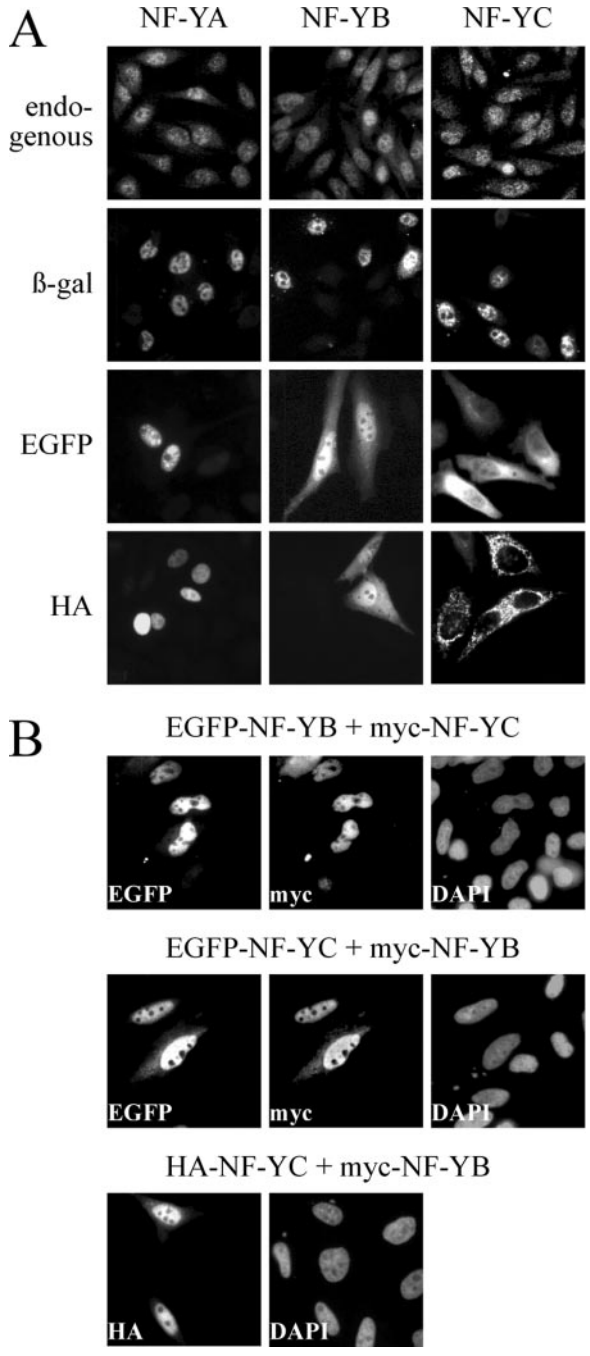

FIG. 1. Subcellular localization of NF-Y subunits in HeLa cells. (A) The endogenous NF-Y subunits stained with anti-NF-YA, anti-CBF-A (NF-YB), and anti-CBF-C (NF-YC) antibodies and moderately overexpressed (SV40 promoter) β-galactosidase-tagged NF-Y subunits are predominantly nuclear at steady state in HeLa cells. Subcellular localization of strongly overexpressed (CMV promoter) EGFP-tagged and HA-tagged NF-Y subunits differs in HeLa cells. (B) EGFP–NF-YB, EGFP–NF-YC, and HA–NF-YC were cotransfected with the corresponding histone fold partner (myc tagged) and costained as indicated. In all cases the subcellular localization of the tagged fusion proteins was examined 24 h after transient transfection, either by direct (EGFP) or indirect (β-gal, HA, myc) fluorescence microscopy. Nuclei were stained with DAPI.

the regions essential for the nuclear localization of NF-Y subunits, we constructed a series of proteins which contained fragments of the individual NF-Y subunits fused to bacterial β-galactosidase as reporter protein and expressed the gene constructs in HeLa cells. The results of these in vivo transfection experiments, summarized in Fig. 2 and Fig. 3, revealed that the nuclear targeting signal of each subunit corresponded to its conserved domain. In particular, the region required for nuclear localization of NF-YB and NF-YC consists of their known protein-protein interaction domain termed histone fold motif (HFM). Constructs containing the N or C terminus of the protein or only parts of the HFM were not imported into the nucleus (except for the partial nuclear staining observed for pMB117; Fig. 2B) but remained in the cytoplasm (Fig. 2A and B). In NF-YA two overlapping regions within the conserved part containing either the subunit interaction domain (labeled BC) or the DNA-binding domain (labeled DNA) together with the linker region confer nuclear import when fused to β-galactosidase (Fig. 3, lower panel, pJK68 and pJK69). Surprisingly, deletion constructs bearing either of these two minimal regions targeting β-galactosidase into the nucleus were deficient in mediating nuclear uptake (Fig. 3, upper panel, pJK84 and pJK100). These results showed that the two minimal regions were not sufficient to confer nuclear import in the context of (nearly) the entire protein. In conclusion, NF-YA contains a nonclassical NLS (ncNLS) in the 56 amino acid long evolutionarily conserved region at the C terminus of the protein (amino acids 262 to 317). This is not unexpected, since NLS- and DNA-binding domains overlap in 90% of the proteins for which both the NLS- and DNA-binding regions are known (16).

Positively charged amino acids are required for nuclear targeting of NF-YA. The ncNLS of NF-YA contains 11 arginine and 7 lysine residues within the conserved region of 56 amino acids. The number of positively charged amino acids is very similar to the BIB domain of rpL23a that contains nine arginine and eight lysine residues (43 amino acids long) (34). To analyze the function of the basic amino acid residues in the NLS sequence of NF-YA, we generated mutants substituting some of the positively charged amino acid residues (Fig. 4A). These NF-YA mutants, along with wild-type NF-YA, all of them fused to β-galactosidase, were expressed in HeLa cells, and the subcellular localization was detected by indirect immunofluorescence. Based on the fluorescence intensity the nucleocytoplasmic distribution of the overexpressed NF-YA mutants was quantified, and the results of the in vivo transfection studies are summarized in Fig. 4B. Nuclear localization of NF-YA was strongly reduced when the basic cluster (KRR) in the subunit interaction domain (BC) was mutated (pJK104, 46% nuclear uptake corresponding to an equal distribution of fluorescence in nucleus and cytoplasm). In contrast, the mutation of other positively charged amino acid residues and basic clusters did not (pJK103, pJK106) or just moderately (pJK105) reduce the nuclear localization of (mutant) NF-YA (Fig. 4B). However, mutation of the three basic amino acid residues (KRR) in the subunit interaction domain in addition to the mutations as described in pJK105 or pJK106, respectively (6 out of the 18 positively charged amino acid residues mutated in total), led to a significant loss of nuclear accumulation (Fig. 4B; pJK130, 21% nuclear localization; pJK131, 17% nuclear local-

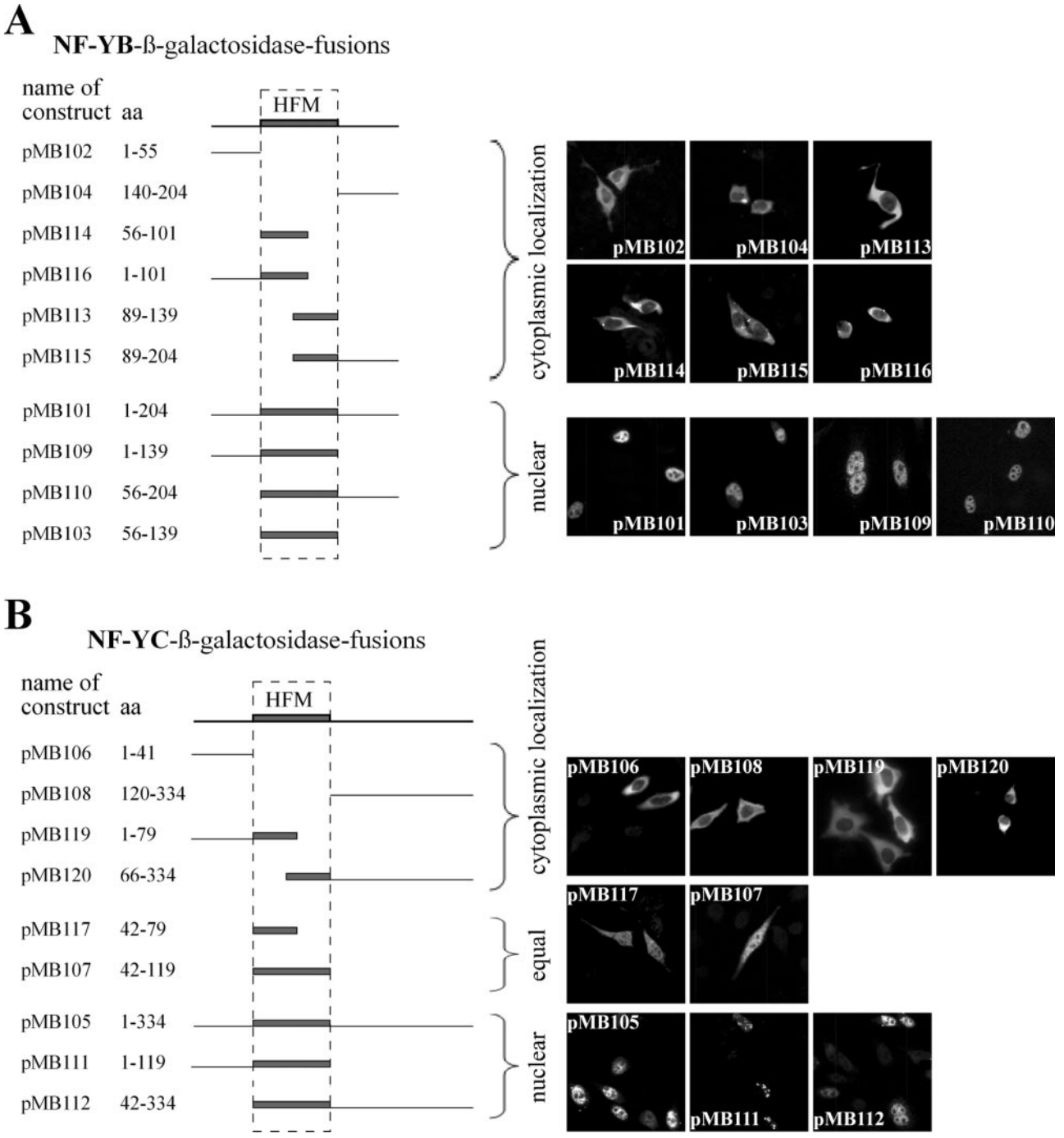

FIG. 2. Nuclear transport of NF-YB and NF-YC fragments fused to β-galactosidase. The regions or sequence motifs that are essential for the nuclear localization of the histone fold motif (HFM)-containing subunits were determined by overexpressing fragments of NF-YB (A) and NF-YC (B). HeLa cells were transiently transfected with gene fragments of the subunits fused to bacterial β-galactosidase as reporter gene, and the subcellular localization of the gene products was examined by indirect immunofluorescence 24 h later. Nuclei were stained with DAPI. On the left, the names of the expression constructs used in this study and the amino acids contained in the constructs are listed. Lines indicate the protein regions, and gray bars represent the histone fold motif (HFM) or parts of it. On the right, representative photographs of the subcellular localization of the constructs. The regions required for nuclear localization of NF-YB and NF-YC correspond to the evolutionarily conserved HFM. aa, amino acid.

ization). Two conclusions can be drawn from these observations. Firstly, a certain number of positively charged amino acid residues in the NLS sequence of NF-YA is required for nuclear uptake. Secondly, some of the basic amino acid residues in the region essential for subunit interactions (46) are also crucial for nuclear localization (for example, amino acids mutated in pJK104; Fig. 4A). Additionally, the mutant NF-YA constructs were expressed as GST fusion proteins in *E. coli* and

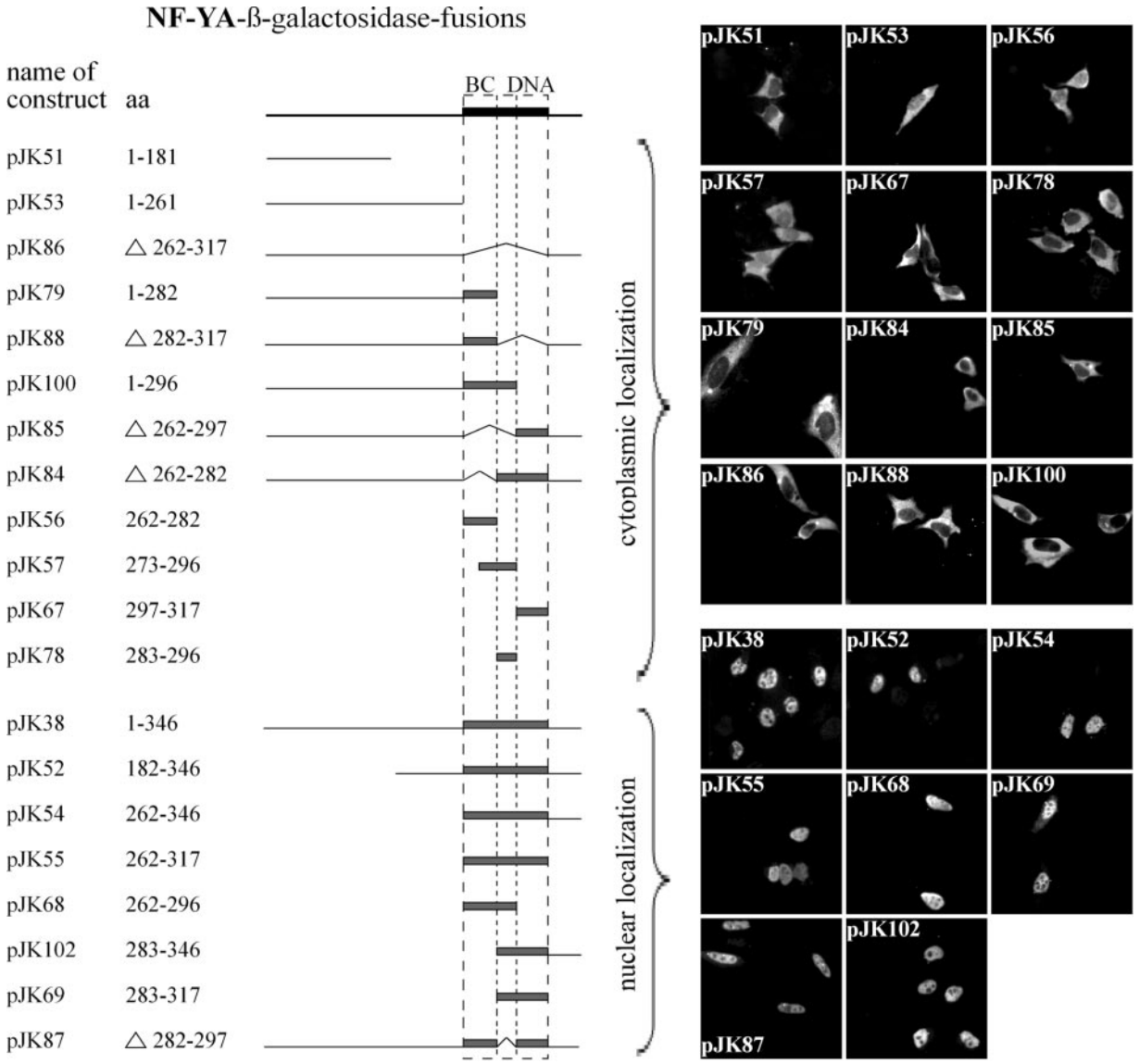

FIG. 3. NF-YA contains an NLS in the evolutionarily conserved domain. The NLS was mapped essentially as described in the legend to Fig. 2. On the left, the names of the expression constructs used in this study and the amino acids contained (or deleted) in the constructs are listed. Lines indicate the protein regions, bent lines indicate deleted regions, and gray bars represent the conserved domain including subunit interaction domain (BC) and DNA binding domain (DNA) or parts of it. On the right are representative photographs of the subcellular localization of the constructs. The 56-amino-acid-long conserved region at the C terminus of NF-YA contains a nonclassical NLS. aa, amino acid.

tested for binding to importin β (Fig. 4C). The results of these in vitro binding studies are described and discussed below in detail (in the context of the identification of importin β as import receptor for NF-YA).

NF-Y subunits are actively transported in *Xenopus* oocytes. To characterize and compare the nuclear transport of the different NF-Y subunits, in vitro transcribed and translated, ^{35}S-labeled, myc-tagged fusion proteins were injected into the cytoplasm of *Xenopus* oocytes. Nuclear and cytoplasmic fractions were separated manually at the indicated time points, homogenized, immunoprecipitated using antibodies against the myc tag, and analyzed by SDS-PAGE with subsequent phosphorimaging. As shown in Fig. 5A, 24 h after microinjection the majority of the cytoplasmically injected NF-Y subunits were detected in the nuclear fractions. However, the kinetics of nuclear translocation differed among the three NF-Y subunits. While NF-YA was imported rapidly into the nucleus of *Xenopus* oocytes, NF-YB and NF-YC were imported much more slowly (for better illustration, see the graphic in Fig. 5B). One explanation for the different import kinetics would be that the interaction with the endogenous histone fold partner, which is abundant in *Xenopus* oocytes (42), is a prerequisite for nuclear uptake. When the temperature was lowered to 4°C the nuclear import of the three subunits was nearly blocked (Fig. 5C). This strong temperature dependence points to an active nuclear transport of the NF-Y subunits rather than passive diffusion. Furthermore, the relative amount of nuclear import of NF-YB and NF-YC depended on the quantity of injected protein. The

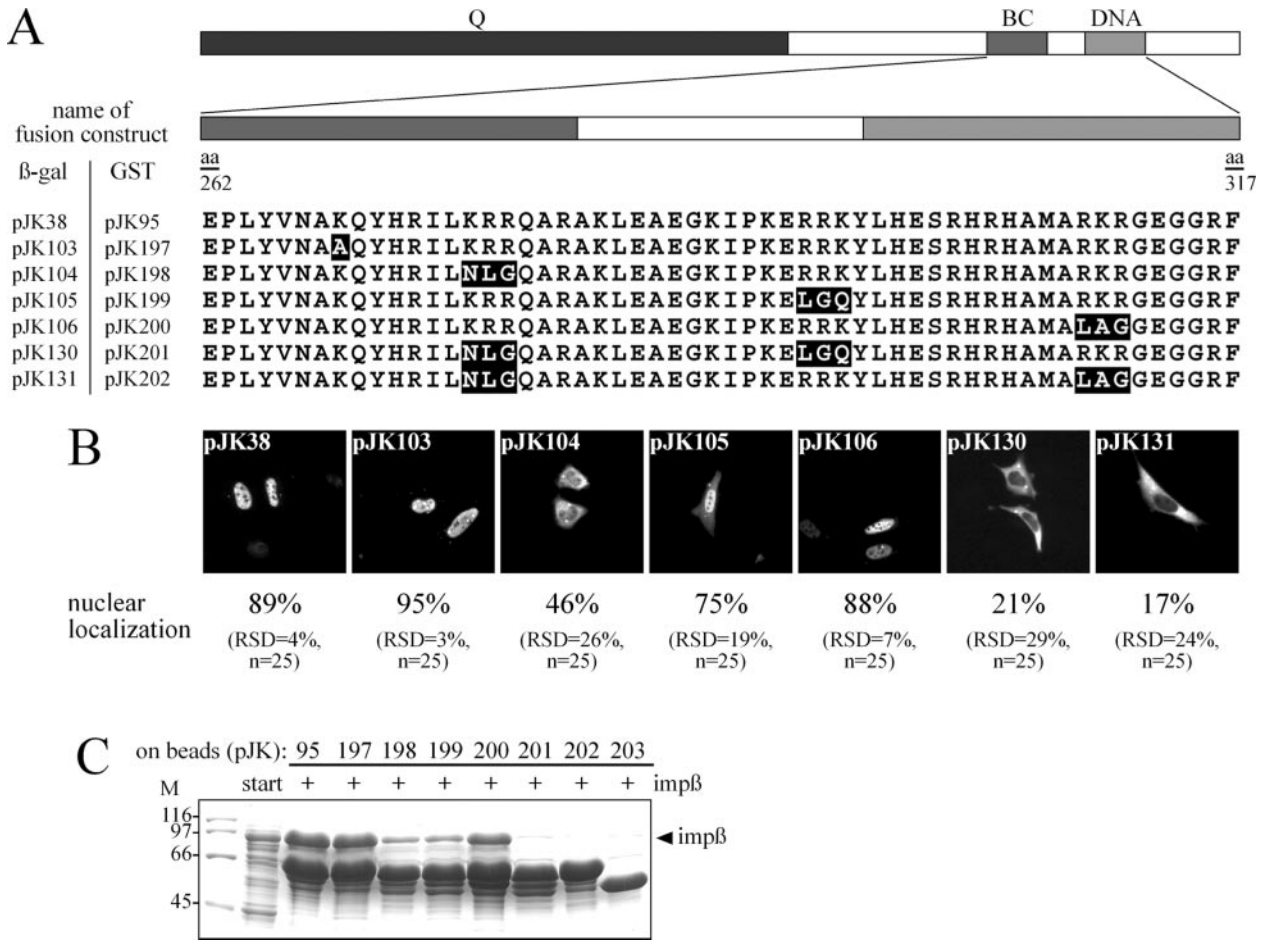

FIG. 4. Characterization of the ncNLS of NF-YA by mutagenesis of basic clusters; positive charge is required for nuclear targeting. (A) Wild-type and mutant NF-YA were fused to either β-galactosidase or GST (construct names are listed on the left). The amino acids that were substituted for the basic amino acid residues are indicated by a black background. (B) The β-galactosidase fusion constructs were overexpressed in HeLa cells, and the subcellular localization of the NLS mutants was detected by indirect immunofluorescence as described in the legend to Fig. 2. The nucleocytoplasmic distribution of the overexpressed NF-YA mutants was quantified with the program ImageJ (nuclear uptake in percentages, written below the representative photographs; RSD, relative standard deviation). The results indicate that nuclear import of NF-YA was reduced when the basic cluster (KRR) in the subunit interaction domain (BC) was mutated (pJK104). Mutation of 6 out of 18 basic amino acids inside the conserved domain (ncNLS) almost completely blocked nuclear uptake (pJK130, pJK131). (C) In addition, binding studies with importin β were done using GST fusion constructs carrying the same mutations in the ncNLS of NF-YA as used in the in vivo import experiments described in panel B. The names of these constructs (pJK197 to pJK202) are included in panel A. The GST fusion constructs were expressed in *E. coli*, immobilized on glutathione-Sepharose, and incubated with importin β from bacterial lysate. The bound fractions were analyzed by SDS-PAGE followed by Coomassie staining. The basic cluster (KRR) in the subunit interaction domain proved to be crucial for importin β binding, and the additional mutation of three more positively charged amino acid residues caused a complete loss of importin β binding (pJK201, pJK202). The gene product pJK203 (1 to 269 amino acids), which resembles a C-terminal NF-YA deletion construct lacking nearly the entire conserved domain (harboring the ncNLS), was used as negative binding control. DNA, DNA binding domain; Q, glutamine-rich *trans*-activation domain; aa, amino acid; M, molecular mass in kilodaltons.

more protein that was injected, the lower the percentage of nuclear uptake (data not shown). These results are in accordance with the differences observed between strong and moderate overexpression of NF-YB and NF-YC in HeLa cells and further indicate that nuclear accumulation presumably relies on interaction/dimerization of the two HFM-containing subunits.

NF-Y subunits interact with different import receptors. Assuming that the NF-Y subunits were actively imported into the nucleus, their nuclear transport would have been mediated most likely by transport receptors of the importin β superfamily. The NLS of NF-YA resembles a nonclassical (importin α-independent) arginine-rich type similar to the BIB domain of rpL23a that confers import by importin β, importin 5, importin 7, and transportin. Thus, it was obvious to investigate the role of these transport factors with regard to the nuclear import of NF-YA. In addition, importin 13 was included, since binding of the NF-YB-like protein YBL1 (11) to immobilized importin 13 had been reported (50). YBL1 shows a strong NF-YB homology covering the histone fold motif, but despite their similarity YBL1 differs from NF-YB in respect to specific functions (11). In order to identify potential import receptors, GST pulldown assays were performed with NF-Y subunits. The three NF-Y subunits were expressed as GST fusions in *E.*

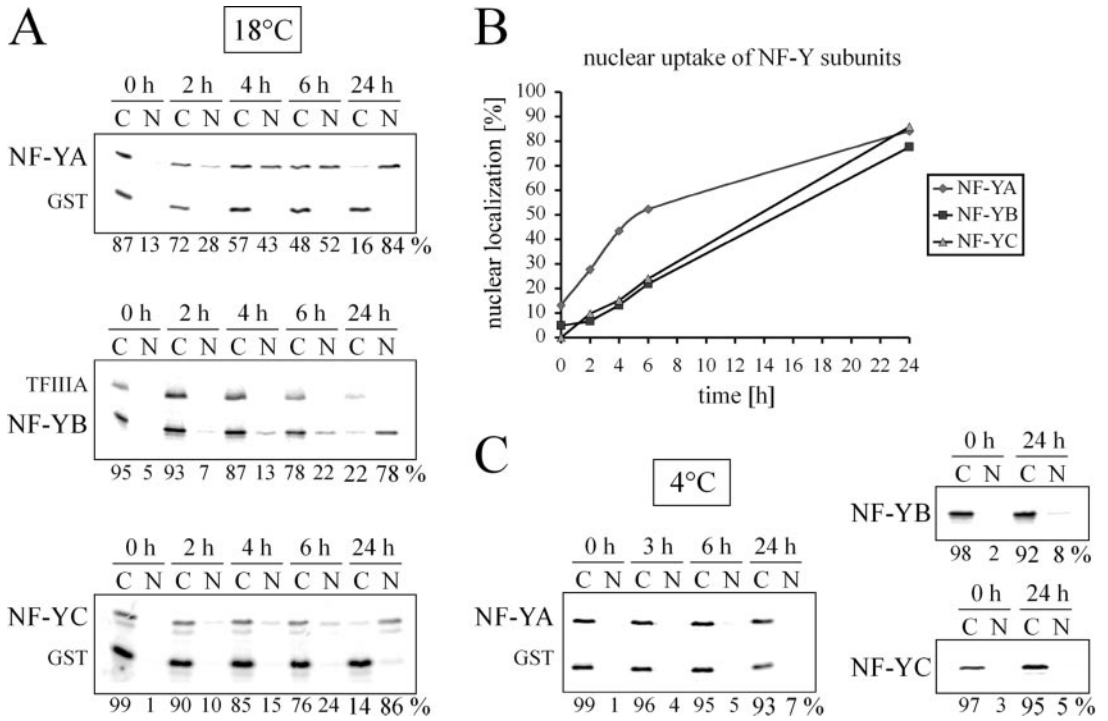

FIG. 5. Active nuclear transport of NF-Y subunits in *Xenopus laevis* oocytes. ^{35}S-labeled NF-YA, NF-YB, NF-YC myc-tagged fusion proteins were injected into the cytoplasm of *Xenopus* oocytes; nuclear (N) and cytoplasmic (C) fractions were separated manually, either immediately (0 h) or at the indicated time points (up to 24 h of incubation), and were analyzed for the nucleocytoplasmic distribution of the different subunits by SDS-PAGE. To control the injection/separation of nuclear and cytoplasmic fractions, coinjection of either transcription factor IIIA (TFIIIA) or GST protein was used. The nucleocytoplasmic distribution of the injected proteins was quantified with the program ImageQuant 5.2 after phosphorimaging (in percentages, written below the gels). (A) Twenty-four hours after microinjection the majority of the cytoplasmically injected NF-Y subunits were detected in the nuclear fractions. (B) The kinetics of nuclear translocation differ among the three subunits. NF-YA was imported rapidly, while the histone fold motif subunits NF-YB and NF-YC were imported much slower. (C) When the temperature was lowered to 4°C the nuclear import of the subunits was blocked. This strong temperature dependence points to an active nuclear transport of the NF-Y subunits rather than passive diffusion.

coli and immobilized on glutathione-Sepharose beads. In addition, the HFM-containing subunits were coexpressed in *E. coli* as either GST–NF-YB/GST–NF-YC or zz–NF-YB/GST–NF-YC complex and also used as bait after immobilization on glutathione-Sepharose beads. The beads were incubated with an importin α/β heterodimer, importin β, transportin, importin 5, and importin 13, all from bacterial lysates, and were subsequently washed. The retained proteins were analyzed by SDS-PAGE followed by Coomassie staining. Importin β, transportin, and importin 5 showed binding to NF-YA, whereas under the same conditions none of the import factors bound efficiently to the monomeric NF-YB and NF-YC subunits. Surprisingly, importin 13 only bound to the NF-YB/NF-YC dimer, while binding of other transport receptors to the preformed complex was insignificant (Fig. 6).

Importins bind specifically to NF-Y subunits. In order to verify that binding of the different members of the importin β family to NF-YA and binding of importin 13 to the NF-YB/NF-YC complex is specific, GST pulldown assays were performed in the presence and absence of RanGTP, which was used to simulate nuclear Ran conditions. Immobilized GST–NF-YA was incubated with purified recombinant importin β, importin 7, transportin, and importin 5 (Fig. 7A). All these import receptors were bound specifically to NF-YA, and this binding was reduced by the GTP-bound form of RanQ69L. Among the four importins that bind specifically to NF-YA, importin β showed the highest binding competence (Fig. 7B). On the other hand, the immobilized NF-YB/NF-YC complex showed binding of recombinant importin 13 from a bacterial lysate. The retained proteins were analyzed by SDS-PAGE, and indeed importin 13 was bound specifically to the histone fold dimer and this binding was abolished by RanGTP, as expected for an import cargo (Fig. 7C). Since importin β had shown the highest binding competence to GST–NF-YA, these in vitro binding studies were extended to the different NLS mutants characterized in Fig. 4A and B. The mutant NF-YA GST-fusion constructs were expressed in *E. coli*, immobilized on glutathione-Sepharose, and incubated with importin β (from bacterial lysate), and bound fractions were analyzed by SDS-PAGE followed by Coomassie staining. As shown in Fig. 4C, in vitro binding assays indicate that the basic clusters in the subunit interaction domain (KRR, pJK198) and linker region (RRK, pJK199) are crucial for importin β binding. The additional mutation of three more positively charged amino acid residues caused a complete loss of importin β binding (pJK201, pJK202). These binding data fit well with the results from the in vivo transfection studies (Fig. 4B) and strongly support our conclusion that a certain number of positively charged amino acid residues in the NLS sequence of NF-YA are required for nuclear targeting.

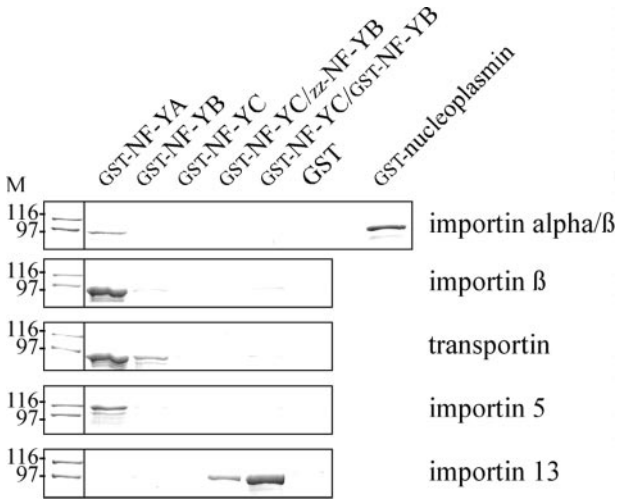

FIG. 6. Interaction of NF-Y subunits with different import receptors. The three NF-Y subunits were expressed as GST fusions in *E. coli* and immobilized on glutathione-Sepharose. In addition, the HFM-containing subunits were coexpressed in *E. coli* as GST–NF-YB/GST–NF-YC complex and zz–NF-YB/GST–NF-YC complex and used as bait after immobilization on glutathione-Sepharose. The immobilized fusion proteins were incubated with either importin α/β, importin β, transportin, importin 5, or importin 13, all from bacterial lysates. The bound fractions were analyzed by SDS-PAGE followed by Coomassie staining. Note that importin β, transportin, and importin 5 were bound to NF-YA, whereas under the same conditions none of the used import factors bound efficiently to single HFM-containing subunits NF-YB and NF-YC. Surprisingly, importin 13 only bound the dimerized NF-YB/NF-YC complex while binding of other transport receptors to the preformed HMF-dimer was insignificant. Further, the importin α/β heterodimer just bound efficiently to the control cargo nucleoplasmin and importins did not bind to GST alone. M, molecular mass in kilodaltons.

Binding sites in the NF-YB/NF-YC complex for NF-YA and importin 13 overlap. Since the minimal regions essential for nuclear localization of NF-YB and NF-YC correspond to their histone fold motifs (as shown by the in vivo transfection experiments in Fig. 2) and only the preformed NF-YB/NF-YC dimer was able to bind to importin 13, it is reasonable to assume that the binding site in the NF-YB/NF-YC complex for importin 13 consists of the dimerized histone fold motifs. To further test this assumption, binding of recombinant importin 13 to an immobilized zz–NF-YB/GST–NF-YC complex was performed in the presence of an excess of NF-YA, which is thought to bind to a groove formed by the dimerized histone fold motifs (63). This approach showed that importin 13 interacts with the NF-YB/NF-YC complex in an NF-YA sensitive manner. Importin 13 was bound specifically to the histone fold dimer, and this binding was abolished by excess of NF-YA (Fig. 8A, note the binding of NF-YA instead of importin 13). To test the specificity of the competition, GST–NF-YB/GST–NF-YC complex on beads was exposed to purified recombinant importin 13 and increasing amounts of NF-YA. Again, the results show that importin 13 competes with NF-YA for binding to the NF-YB/NF-YC dimer (Fig. 8B). Addition of equimolar concentrations of importin 13 and NF-YA to the NF-YB/NF-YC complex results in weaker binding of importin 13 (Fig. 8B, lane 7), indicating a higher affinity of NF-YA than importin 13 for the NF-YB/NF-YC dimer.

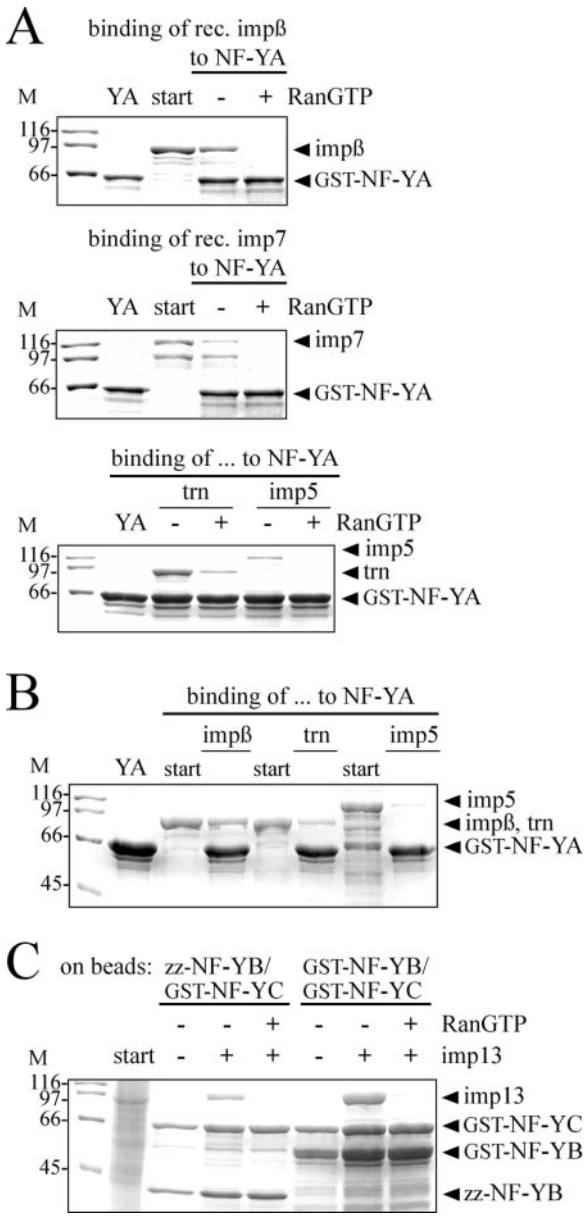

FIG. 7. Specific binding of importins to NF-Y subunits. GST pull-down assays were performed as described in the legend to Fig. 6. (A) Immobilized GST–NF-YA was incubated with purified recombinant importin β, importin 7, transportin (trn), and importin 5. Starting material (10% of the importins that were used) and bound fractions were analyzed by SDS-PAGE followed by Coomassie staining. As shown in panel A, the import receptors were bound specifically to immobilized NF-YA, and this binding was reduced by the GTP-bound form of RanQ69L (2 μM) which was used to simulate nuclear Ran conditions. (B) Among the transport factors binding to NF-YA, importin β showed the highest binding competence. (C) Immobilized zz–NF-YB/GST–NF-YC complex and GST–NF-YB/GST–NF-YC complex (see also arrows on the right) were used to bind recombinant importin 13 from a bacterial lysate. Importin 13 was bound specifically to the immobilized NF-YB/NF-YC dimer, and this binding was abolished by RanGTP. Hence, the NF-YB/NF-YC dimer behaves as an importin 13-specific import substrate. M, molecular mass in kilodaltons; rec. imp, recombinant importin.

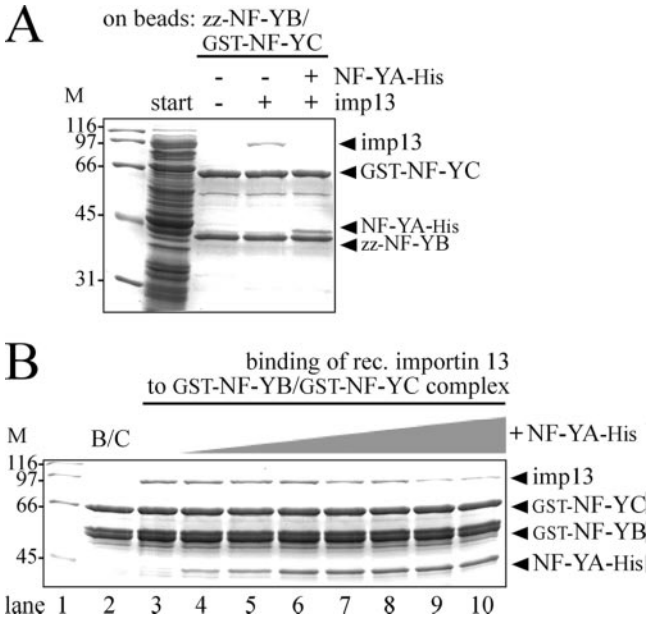

FIG. 8. Binding sites in the NF-YB/NF-YC complex for NF-YA and importin 13 overlap. Importin 13 interacts with the NF-YB/NF-YC complex in a NF-YA sensitive manner. (A) Importin 13 was bound specifically to the immobilized zz–NF-YB/GST–NF-YC complex, and this binding was abolished by a 10-fold molar excess of NF-YA. As can be seen in the last lane, NF-YA was bound to the histone fold dimer instead of importin 13. (B) GST–NF-YB/GST–NF-YC complex on beads (also labeled B/C) was exposed to purified recombinant importin 13 (1 μM, lane 3 to 10) and increasing amounts of His-tagged NF-YA (lane 4, 0.2 μM; lane 5, 0.4 μM; lane 6, 0.8 μM; lane 7, 1 μM; lane 8, 1.25 μM; lane 9, 2.5 μM; lane 10, 5 μM). The results show that importin 13 competes with NF-YA for binding to the NF-YB/NF-YC dimer. Addition of equimolar concentrations of importin 13 and NF-YA to the NF-YB/NF-YC complex (lane 7) results in weaker binding of importin 13. M, molecular mass in kilodaltons; rec., recombinant; imp, importin.

NLS in NF-YA is not recognized by importins once the subunit is assembled into the NF-Y complex. Since the identified NLS of NF-YA also contains the known subunit interaction domain, we wished to determine if NF-YA can bind import receptors once it is assembled into the trimeric NF-Y complex. To address this question, two GST pulldown experiments were performed. Firstly, a GST–NF-YB/GST–NF-YC complex was used as bait and exposed to recombinant importin β and/or NF-YA (Fig. 9A). NF-YA and importin β were added either at the same time (lane 7) or stepwise, NF-YA first and importin β 1 h later (Fig. 9A, lane 8). Again, bound fractions were analyzed by SDS-PAGE followed by Coomassie staining. When NF-YA was prebound to the NF-YB/NF-YC complex, no binding of importin β could be observed, showing that NF-YA was unable to mediate the binding of the entire heterotrimeric NF-Y complex to importin β. In the presence of an importin β excess, NF-YA showed reduced binding to the histone fold dimer (data not shown), also indicating that the importin β/NF-YA complex cannot bind to the NF-YB/NF-YC dimer. Secondly, a preassembled NF-Y holocomplex (see Materials and Methods regarding recombinant protein expression and purification) was immobilized on glutathione-Sepharose beads and incubated with recombinant importin β, transportin, and importin 5 (Fig. 9B). None of the import factors bound efficiently to the preassembled NF-Y holocomplex. In conclusion, the NLS of NF-YA cannot be recognized by import receptors once the trimeric NF-Y complex is formed, which is probably due to the fact that the subunit interaction domain and NLS overlap.

NF-Y subunits are imported into nuclei of permeabilized cells by distinct pathways. Finally, we wanted to examine whether the differentially interacting importins represent functional import receptors for NF-YA or for the NF-YB/NF-YC complex. For that purpose, HeLa cells were grown on glass coverslips and the plasma membranes were selectively permeabilized with digitonin to reconstitute nuclear import by using recombinant purified import factors (1). The use of this system revealed that the nuclear import of

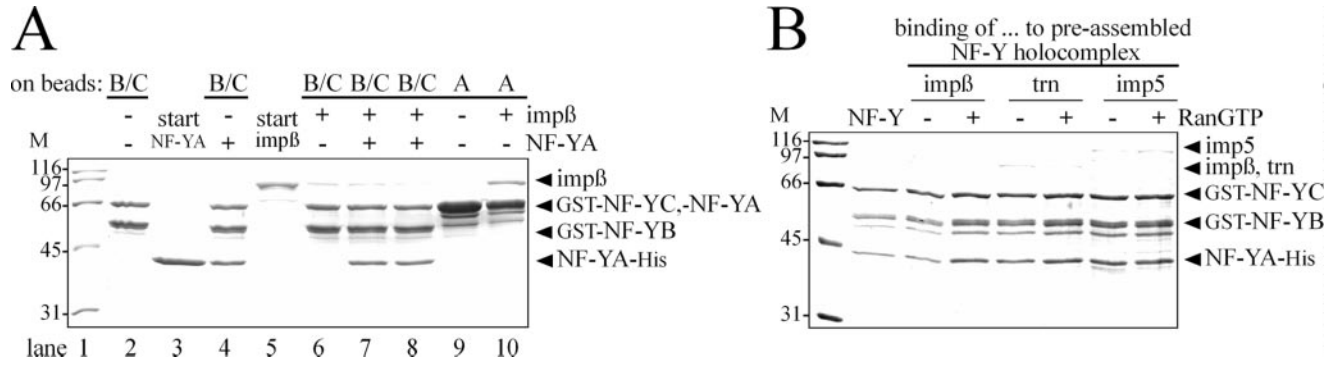

FIG. 9. NLS in NF-YA is not recognized by importins once the subunit is assembled into the NF-Y complex. (A) GST–NF-YB/GST–NF-YC (labeled B/C) on beads was exposed to recombinant importin β with and without addition of His-tagged NF-YA to determine whether NF-YA can bridge binding of importin β to the trimeric NF-Y complex. NF-YA and importin β were added either at the same time (lane 7) or stepwise, NF-YA first and importin β 1 h later (lane 8). The last lane (10) shows the control binding of importin β to bead-bound GST–NF-YA (labeled A) alone. (B) Preassembled NF-Y holocomplex (see Materials and Methods) consisting of NF-YA-His, GST–NF-YB, and GST–NF-YC was immobilized on glutathione-Sepharose beads and incubated with recombinant importin β, transportin (trn), and importin 5. None of the import receptors that bound to GST–NF-YA alone (see Fig. 7B) were bound to the preassembled NF-Y holocomplex. Starting material (20% of the recombinant importin β and NF-YA that were used; panel A) and bound fractions were analyzed by SDS-PAGE followed by Coomassie staining. M, molecular mass in kilodaltons; imp, importin.

fluorescently labeled 6z–NF-YA was dependent on the presence of rabbit reticulocyte lysate as a source of import factors (Fig. 10A). To analyze the transport factors involved in the nuclear import of NF-YA in vitro, import assays were performed in the presence of nonfluorescent core histones as potential import competitors. The addition of individual nonfluorescent core histones to the import mix completely blocked the nuclear import of NF-YA (shown for H2A and H2B in Fig. 10A). The competition by core histones indicated that the nuclear import of NF-YA depends entirely on at least one of the import receptors that mediate nuclear transport of the core histones, namely importin β, importin 5, importin 7, importin 9, or transportin (3, 53). Despite the fact that importin β, importin 5, importin 7, and transportin bound specifically to GST–NF-YA (see Fig. 7A), it was only imported into the nuclei of permeabilized cells when importin β together with Ran mix and an energy-regenerating system was present (Fig. 10C). While the monomeric HFM-containing subunits NF-YB and NF-YC (as 6z fusion protein) were not imported into nuclei of permeabilized cells (Fig. 10B), nuclear accumulation of the GST–NF-YB/GST–NF-YC complex was strictly importin 13 dependent and stimulated by Ran mix and an energy regenerating system (Fig. 10E). Other importins, such as importin β, had no significant effect. Addition of an NF-YA excess to the import mix led to a reduced nuclear import of the preformed NF-YB/NF-YC complex in the presence of importin 13 (data not shown), indicating that only the histone fold dimer can be recognized by importin 13 and that NF-YA competes with importin 13 for binding to the NF-YB/NF-YC dimer (see Fig. 8). Taken together, we can conclude that importin β functions as import receptor for NF-YA, whereas nuclear transport of the NF-YB/NF-YC complex indeed can be mediated by importin 13.

DISCUSSION

A major effort in the field of nucleocytoplasmic transport is the identification of cargoes for the individual importin β family members that mediate most of the transport events between nucleus and cytoplasm. Indeed, more than 17% of all eukaryotic proteins are nuclear (16) and therefore must be transported into the nucleus at some stage of their life. Among the classes of proteins whose role is clearly nuclear are the transcription factors. Surprisingly little is known regarding the pathway by which the ubiquitous CCAAT-specific transcription factor NF-Y translocates into the nucleus, and therefore our aim in the present study was to examine the nuclear transport of NF-Y in detail.

Here, we report that in higher eukaryotes the subunits of NF-Y are imported into the nucleus by distinct pathways. Importin β mediates nuclear import of NF-YA, whereas dimerization of the two HFM-containing subunits NF-YB and NF-YC is a prerequisite for nuclear accumulation conferred by importin 13. In contrast to known import and export cargoes of importin 13, i.e., the SUMO-conjugating enzyme hUBC9, RBM8 (Y14) (either alone or with MGN), translation initiation factor eIF1A (50), and paired-type homeodomain transcription factors Pax6, Pax3, and Crx (56), the NF-YB/NF-YC dimer represents the first example of an importin 13-specific substrate that solely depends on complex formation. The single HFM-containing subunits NF-YB and NF-YC do not contain an independent NLS and consequently were neither recognized (in vitro binding studies) nor imported (in vitro nuclear import assays) by import receptors. To identify the regions or sequence motifs that are essential for the nuclear localization of NF-YB and NF-YC, fragments of each HFM-containing subunit were moderately overexpressed in HeLa cells (in the presence of the corresponding endogenous histone fold partner). Thereby it was shown that the minimal sequence motifs essential for the nuclear localization of NF-YB and NF-YC correspond to their histone fold motifs. Since only the NF-YB/NF-YC dimer but not the monomeric components is translocated into the nucleus in an importin 13-dependent manner, the in vivo transfection experiments can also be considered as a first attempt to map the binding sites in the NF-YB/NF-YC complex for importin 13. Hence, the results showing that the histone fold motifs resemble the sequence region necessary for the nuclear localization of NF-YB and NF-YC also indicate that the binding site in the NF-YB/NF-YC dimer for importin 13 is located within the dimerized histone fold motifs. This assumption was further supported by the fact that importin 13 and NF-YA competed for binding to the NF-YB/NF-YC complex. Binding of importin 13 was completely abolished by an excess of NF-YA, which seems to have a higher affinity than importin 13 for the NF-YB/NF-YC dimer. It is known that NF-YB requires two conserved amino acids in helix α2 (E90 and S97), and NF-YC requires several residues in both helices α1 and αC for NF-YA association (37, 63, 68, 82). Whether these three elements of secondary structure that form a groove where NF-YA is supposed to bind are also recognized by importin 13 is not known but is currently under investigation. Additionally, clusters of basic amino acid residues created by dimerization of the histone fold motifs may promote the binding of importin 13.

With regard to NF-YA, the functional NLS is located in the evolutionarily conserved domain at the C terminus of the protein including the subunit interaction domain and DNA binding domain. Overlap of DNA binding region and NLS may aid release of NF-YA from its import receptor (importin β) after nuclear translocation, although specific DNA binding was observed only for the trimeric complex (reviewed in reference 45). The nonclassical NLS of NF-YA consists of up to 56 amino acids (not further characterized by N- or C-terminal deletions), including 11 arginine and 7 lysine residues. Mutation of 6 out of the 18 basic amino acid residues within that stretch caused a nearly complete loss of nuclear accumulation, indicating that a certain number of positively charged amino acids in the ncNLS of NF-YA are required for nuclear targeting.

Comparing the NLS of NF-YA to other ncNLSs, such as the IBB domain of importin α, the BIB domain of rpL23, and the amino-terminal tails of core histones, reveals no actual sequence homology. A striking similarity between these different import signals, however, is the large number of basic amino acid residues in the sequence (lysine and arginine residues comprise more than 30%). Hence, in the cases mentioned above a certain number of positively charged amino acids per total number of amino acids contributing to the nuclear targeting function may be required to be recognized by the rather acidic import receptors. A large number of positively charged

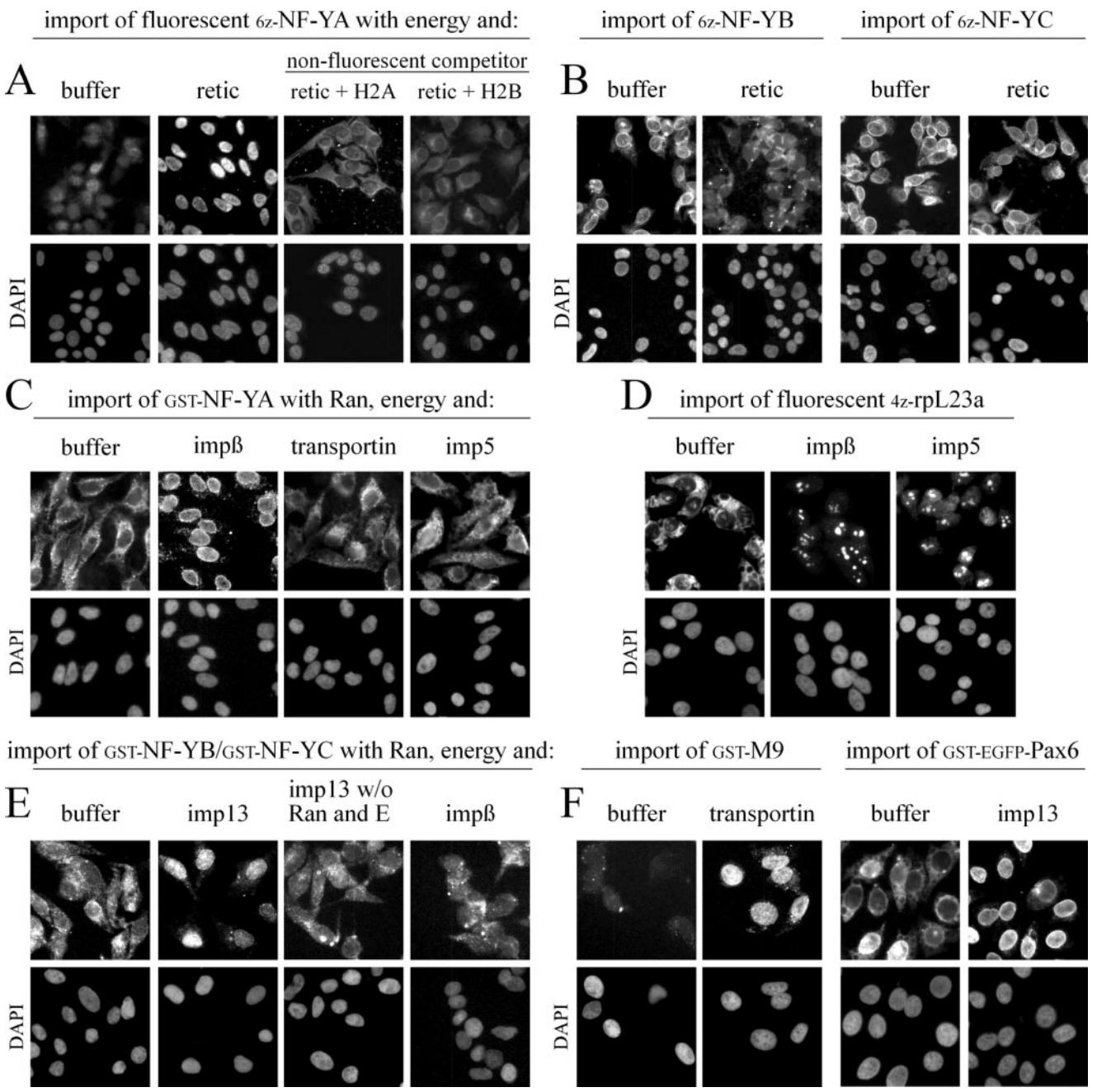

FIG. 10. NF-Y subunits are imported into nuclei of permeabilized cells by distinct pathways. Importin β mediates import of NF-YA, and importin 13 confers nuclear uptake of a preformed NF-YB/NF-YC dimer. Digitonin-permeabilized cells were incubated with 1 μM of each substrate, rabbit reticulocyte lysate (retic) or the indicated combinations of nuclear transport receptors (2 μM each), a Ran mix (including RanGDP and NTF2), and an energy regenerating system (energy [E]) for 25 min at 37°C. For a negative control, retic or the recombinant import receptor was replaced by transport buffer (buffer). Panels show the cargo distribution after import and fixation. Substrates carrying GST (except GST-EGFP-Pax6) were detected by indirect immunofluorescence, whereas fluorescently labeled cargoes (6z–NF-YA, 6z–NF-YB, 6z–NF-YC, and 4z-rpL23a) were detected by direct immunofluorescence. (A) 6z–NF-YA was imported into nuclei of permeabilized cells in the presence of retic and energy; however, 6z–NF-YA tended to slightly diffuse into nuclei in the absence of import receptors (buffer). Nuclear import reactions were also performed in the presence of retic, energy, and either 10 μM H2A or H2B (nonfluorescent) as indicated. Both core histones completely inhibited the nuclear uptake of 6z–NF-YA. (B) 6z–NF-YB and 6z–NF-YC were not imported into nuclei of permeabilized cells in the presence of retic and energy but remained in the cytoplasm in vicinity to the nuclear membrane. (C) Nuclear accumulation of GST–NF-YA was dependent on the presence of importin β, Ran mix, and an energy-regenerating system. However, GST–NF-YA remained in the cytoplasm in the presence of other importins such as transportin and importin 5. (E) The GST–NF-YB/GST–NF-YC complex was imported into nuclei of permeabilized cells in the presence of importin 13, Ran mix, and an energy regenerating system but was not imported in the absence of importin 13 or in the absence of Ran and the energy regenerating system. (D and F) In vitro nuclear import assay of the reference substrates 4z-rpL23a, GST-M9, and GST-EGFP-Pax6 in the presence and absence of appropriate transport receptors. imp, importin.

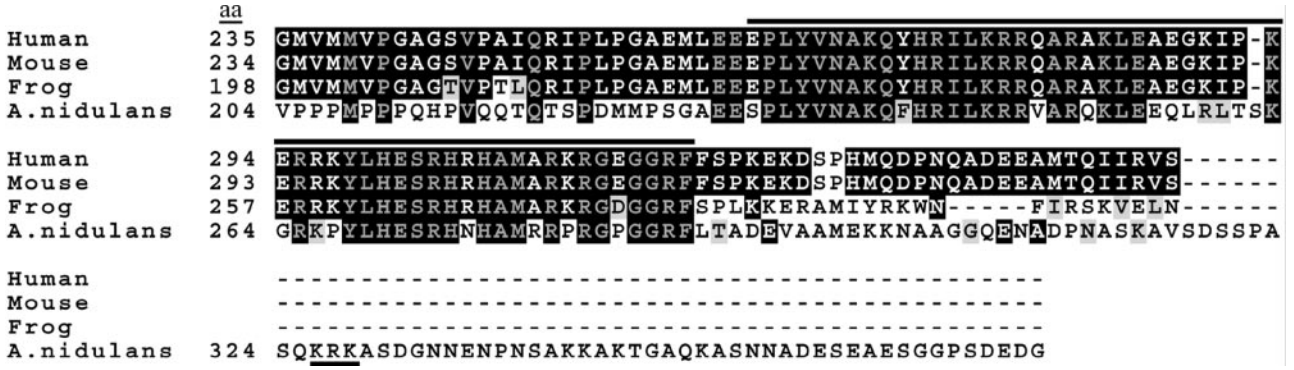

FIG. 11. Partial sequence alignment of NF-YA. Sequence alignment of the C-terminal end of NF-YA from human, *Mus musculus*, *Xenopus laevis*, and *Aspergillus nidulans*. Identical amino acids are indicated by a black background, whereas conserved amino acids (identical residues in all sequences) are additionally shaded in gray (aa, position of the amino acid in the respective organism). The bar on top of the sequence alignment represents the ncNLS of NF-YA which corresponds to the evolutionarily conserved region (for mapping and characterization of the ncNLS of NF-YA, see Fig. 3 and 4). The basic amino acid residues 326 to 328 (KRK, additionally underlined) are essential for the nuclear localization of HapB (NF-YA homologue) in *Aspergillus nidulans* (71).

amino acids in the sequence of potential cargo molecules certainly increase the probability to include a binding motif that meets the requirements given by the transport receptors eventually leading to cargo binding.

As mentioned above, the greater complexity compared with classical NLSs and the very basic nature of the ncNLS of NF-YA reminds one of the BIB domain of rpL23a. The 43-amino-acid BIB domain includes nine arginine and eight lysine residues (34) and may be considered an archetypical import signal that specifically binds to importin β, importin 5, importin 7, or transportin. We observed the same for NF-YA. However, in contrast to the BIB domain, binding of the last three import factors was not sufficient to confer nuclear uptake in vitro. NF-YA was only imported into the nuclei of permeabilized cells when importin β together with a Ran mix and an energy-regenerating system was present. Nevertheless, we do not exclude the possibility that the other three import factors also mediate nuclear transport of NF-YA in vivo, which perhaps could not be reconstituted because of lower affinity towards the cargo and the experimental conditions chosen. In that context, a weaker affinity of an NLS for other import receptors probably allows import factors to back up each other. This might help cells to respond to particularly high transport demands.

Since the identified NLS of NF-YA also contains its subunit interaction domain, indicating that the respective amino acids are of equal importance for subunit interaction and importin binding, it was of interest to find out whether NF-YA can still bind import receptors (particularly importin β) once it is assembled into the NF-Y complex. The results of GST pulldown experiments showed that NF-YA cannot mediate the interaction of importin β with the functional NF-Y holocomplex. Hence, our data suggest that binding and subsequent nuclear transport mediated by importin β is restricted to the single NF-YA subunit and cannot be applied to the trimeric NF-Y complex. However, the in vitro preassembled NF-Y complex consisted of subunits carrying GST tags that might have interfered with importin binding. Thus, it cannot be completely excluded that NF-YA can mediate the interaction of importin β with an untagged NF-Y complex in vivo. On the other hand, we have shown that NLS and subunit interaction domains overlap.

The nuclear import of *Aspergillus nidulans* CCAAT binding factor AnCF, the fungal homologue of the evolutionarily conserved heterotrimeric NF-Y complex, recently has been studied in this filamentous fungus. AnCF consists of three subunits (HapB, HapC, and HapE), and an NLS was identified in HapB (NF-YA homologue) located at the C terminus of the protein outside of the evolutionarily conserved domain (71). In contrast, we mapped the functional NLS of mammalian NF-YA to the 56-amino-acid-long conserved region (Fig. 3). In analogy to our findings in the human homologues NF-YB and NF-YC, no independent NLSs were identified in the HFM-containing subunits HapC and HapE. Since the identified NLS in HapB (amino acid residues 326 to 328, KRK) is not present in higher eukaryotes (Fig. 11), the proposed piggyback transport of the two HFM-containing subunits via HapB as a preassembled trimeric complex cannot be generally applied but appears to be rather unique for the *A. nidulans* CCAAT-binding complex AnCF. In addition, the physiological significance of NF-Y seems to be different in higher and lower eukaryotes. *A. nidulans* lacking HapB (Δ*hapB* strain) showed a phenotype of slow growth, poor conidiation, and reduced expression of genes containing a CCAAT box (70, 71) but was viable, whereas in higher eukaryotes NF-Y-mediated transcription is essential for cell proliferation and viability. This was, for instance, demonstrated by a conditional deletion of both NF-YA alleles in primary cultures of mouse embryonic fibroblast cells, which led to a complete block in cell cycle progression and subsequent initiation of apoptosis (8).

In general, NF-Y is required to organize the chromatin in proximity of transcriptional start sites, thereby enabling recruitment of coactivators (42, 45). In the literature there are contradictory statements regarding the nature of NF-Y interactions with cell cycle (regulated) promoters, varying from highly dynamic (14) to totally constant through the cell cycle (73, 83). In this context, Frontini et al. showed recently that the nuclear localization of NF-YC is cell cycle regulated and depends on the presence of its histone fold partner NF-YB (24).

The results of our coexpression studies are in accordance with these observations and indicate that NF-YB and NF-YC mediate nuclear localization of its corresponding histone fold partner (Fig. 1B). Frontini et al. interpreted the cytoplasmic localization of strongly overexpressed NF-YC by a mechanism in which NF-YC first enters the nucleus and is then exported to the cytoplasm via a CRM1-dependent pathway (24). However, our results rather point to a scenario where NF-YC has to dimerize with NF-YB (and vice versa) to be imported into the nucleus. Since dimerization of the HFM-containing subunits depends on a reduced form of NF-YB (54), the cellular redox environment of mammalian cells might regulate the nuclear levels of NF-YC.

If nuclear import of NF-YB and NF-YC entirely depends on dimer formation and subsequent nuclear translocation mediated by importin 13, one would expect that both HFM-containing subunits behave analogously if strongly overexpressed. However, strong overexpression of NF-YB did not as dramatically alter nuclear localization as observed for NF-YC. How can that be explained? Since the monomeric NF-YB subunit was neither bound to importin 13 nor imported into the nucleus of permeabilized cells, it is rather unlikely that NF-YB contains an independent NLS which is exposed by NF-YC and enhances binding of importin 13 to NF-YB. We assume that NF-YB in contrast to NF-YC is able to interact (in the cytoplasm) with another nuclear protein and then is piggyback transported into the nucleus. While H2A-like proteins such as YCL1 and NF-YC are incapable of interacting directly with core histones, H2B-like proteins such as YBL1 and NF-YB are able to interact with H3 or H4, respectively (11, 13). Thus, piggyback transport of NF-YB into the nucleus may be achieved via the ncNLSs in the amino-terminal tails of the core histones H3/H4.

Further, it was shown that NF-YB/NF-YC dimers can efficiently associate with DNA during nucleosome formation and that these complexes have an intrinsic affinity for H3-H4 (13). Activation assays with HFM-containing subunits NF-YB and NF-YC fused to GAL4 also indicated that they are sufficient to activate transcription robustly, two- to fourfold better than the NF-Y trimer (18), and biochemical evidence suggests that NF-YB and NF-YC are associated with different complexes of high molecular weight in the absence of NF-YA (7). Our findings that the HFM-containing subunits NF-YB and NF-YC were imported into the nucleus as heterodimeric complex by a pathway distinct from NF-YA are in line with the observation that the HFM-containing subunits can fulfill different nuclear functions independent of NF-YA. Regulated traffic of cargoes between nucleus and cytoplasm is rather the rule than the exception, and the efficiency of nuclear transport of a protein depends on its interaction with the components of the transport machinery. In the case of the NF-Y subunits this is obviously controlled by complex formation of NF-YB and NF-YC. Transport of the dimerized complex mediated by importin 13 ensures proper nuclear localization of equimolar concentrations of NF-YB and NF-YC.

Finally, the histone fold pair of NF-Y is closely related to other members of the histone fold family. Further work will be directed towards the question of whether histone fold pairs in general can be recognized by importin 13.

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REFERENCES

1. **Adam, S. A., R. S. Marr, and L. Gerace.** 1990. Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. J. Cell Biol. **111:**807–816.
2. **Arents, G., and E. N. Moudrianakis.** 1995. The histone fold: a ubiquitous architectural motif utilized in DNA compaction and protein dimerization. Proc. Natl. Acad. Sci. USA **92:**11170–11174.
3. **Baake, M., M. Bauerle, D. Doenecke, and W. Albig.** 2001. Core histones and linker histones are imported into the nucleus by different pathways. Eur. J. Cell Biol. **80:**669–677.
4. **Baake, M., D. Doenecke, and W. Albig.** 2001. Characterisation of nuclear localisation signals of the four human core histones. J. Cell Biochem. **81:** 333–346.
5. **Bauerle, M., D. Doenecke, and W. Albig.** 2002. The requirement of H1 histones for a heterodimeric nuclear import receptor. J. Biol. Chem. **277:** 32480–32489.
6. **Baxevanis, A. D., G. Arents, E. N. Moudrianakis, and D. Landsman.** 1995. A variety of DNA-binding and multimeric proteins contain the histone fold motif. Nucleic Acids Res. **23:**2685–2691.
7. **Bellorini, M., D. K. Lee, J. C. Dantonel, K. Zemzoumi, R. G. Roeder, L. Tora, and R. Mantovani.** 1997. CCAAT binding NF-Y-TBP interactions: NF-YB and NF-YC require short domains adjacent to their histone fold motifs for association with TBP basic residues. Nucleic Acids Res. **25:**2174–2181.
8. **Bhattacharya, A., J. M. Deng, Z. Zhang, R. Behringer, B. de Crombrugghe, and S. N. Maity.** 2003. The B subunit of the CCAAT box binding transcription factor complex (CBF/NF-Y) is essential for early mouse development and cell proliferation. Cancer Res. **63:**8167–8172.
9. **Bi, W., L. Wu, F. Coustry, B. de Crombrugghe, and S. N. Maity.** 1997. DNA binding specificity of the CCAAT-binding factor CBF/NF-Y. J. Biol. Chem. **272:**26562–26572.
10. **Bischoff, F. R., and D. Gorlich.** 1997. RanBP1 is crucial for the release of RanGTP from importin beta-related nuclear transport factors. FEBS Lett. **419:**249–254.
11. **Bolognese, F., C. Imbriano, G. Caretti, and R. Mantovani.** 2000. Cloning and characterization of the histone-fold proteins YBL1 and YCL1. Nucleic Acids Res. **28:**3830–3838.
12. **Bucher, P.** 1990. Weight matrix descriptions of four eukaryotic RNA polymerase II promoter elements derived from 502 unrelated promoter sequences. J. Mol. Biol. **212:**563–578.
13. **Caretti, G., M. C. Motta, and R. Mantovani.** 1999. NF-Y associates with H3–H4 tetramers and octamers by multiple mechanisms. Mol. Cell. Biol. **19:**8591–8603.
14. **Caretti, G., V. Salsi, C. Vecchi, C. Imbriano, and R. Mantovani.** 2003. Dynamic recruitment of NF-Y and histone acetyltransferases on cell-cycle promoters. J. Biol. Chem. **278:**30435–30440.
15. **Christophe, D., C. Christophe-Hobertus, and B. Pichon.** 2000. Nuclear targeting of proteins: how many different signals? Cell Signal. **12:**337–341.
16. **Cokol, M., R. Nair, and B. Rost.** 2000. Finding nuclear localization signals. EMBO Rep. **1:**411–415.
17. **Conti, E., and E. Izaurralde.** 2001. Nucleocytoplasmic transport enters the atomic age. Curr. Opin. Cell Biol. **13:**310–319.
18. **de Silvio, A., C. Imbriano, and R. Mantovani.** 1999. Dissection of the NF-Y transcriptional activation potential. Nucleic Acids Res. **27:**2578–2584.
19. **Dingwall, C., and R. A. Laskey.** 1991. Nuclear targeting sequences–a consensus? Trends Biochem. Sci. **16:**478–481.
20. **Dorn, A., J. Bollekens, A. Staub, C. Benoist, and D. Mathis.** 1987. A multiplicity of CCAAT box-binding proteins. Cell **50:**863–872.
21. **Englmeier, L., J. C. Olivo, and I. W. Mattaj.** 1999. Receptor-mediated

substrate translocation through the nuclear pore complex without nucleotide triphosphate hydrolysis. Curr. Biol. **9:**30–41.
22. **Fornerod, M., M. Ohno, M. Yoshida, and I. W. Mattaj.** 1997. CRM1 is an export receptor for leucine-rich nuclear export signals. Cell **90:**1051–1060.
23. **Forwood, J. K., M. H. Lam, and D. A. Jans.** 2001. Nuclear import of Creb and AP-1 transcription factors requires importin-beta 1 and Ran but is independent of importin-alpha. Biochemistry **40:**5208–5217.
24. **Frontini, M., C. Imbriano, I. Manni, and R. Mantovani.** 2004. Cell cycle regulation of NF-YC nuclear localization. Cell Cycle **3:**217–222.
25. **Gorlich, D., and U. Kutay.** 1999. Transport between the cell nucleus and the cytoplasm. Annu. Rev. Cell Dev. Biol. **15:**607–660.
26. **Gorlich, D., and I. W. Mattaj.** 1996. Nucleocytoplasmic transport. Science **271:**1513–1518.
27. **Gorlich, D., N. Pante, U. Kutay, U. Aebi, and F. R. Bischoff.** 1996. Identification of different roles for RanGDP and RanGTP in nuclear protein import. EMBO J. **15:**5584–5594.
28. **Gorlich, D., S. Prehn, R. A. Laskey, and E. Hartmann.** 1994. Isolation of a protein that is essential for the first step of nuclear protein import. Cell **79:**767–778.
29. **Gorlich, D., M. J. Seewald, and K. Ribbeck.** 2003. Characterization of Ran-driven cargo transport and the RanGTPase system by kinetic measurements and computer simulation. EMBO J. **22:**1088–1100.
30. **Gurdon, J. B.** 1977. Methods for nuclear transplantation in amphibia. Methods Cell Biol. **16:**125–139.
31. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:**51–59.
32. **Imamoto, N.** 2000. Diversity in nucleocytoplasmic transport pathways. Cell Struct. Funct. **25:**207–216.
33. **Izaurralde, E., U. Kutay, C. von Kobbe, I. W. Mattaj, and D. Gorlich.** 1997. The asymmetric distribution of the constituents of the Ran system is essential for transport into and out of the nucleus. EMBO J. **16:**6535–6547.
34. **Jakel, S., and D. Gorlich.** 1998. Importin beta, transportin, RanBP5 and RanBP7 mediate nuclear import of ribosomal proteins in mammalian cells. EMBO J. **17:**4491–4502.
35. **Kalderon, D., B. L. Roberts, W. D. Richardson, and A. E. Smith.** 1984. A short amino acid sequence able to specify nuclear location. Cell **39:**499–509.
36. **Kim, C. G., and M. Sheffery.** 1990. Physical characterization of the purified CCAAT transcription factor, alpha-CP1. J. Biol. Chem. **265:**13362–13369.
37. **Kim, I. S., S. Sinha, B. de Crombrugghe, and S. N. Maity.** 1996. Determination of functional domains in the C subunit of the CCAAT-binding factor (CBF) necessary for formation of a CBF-DNA complex: CBF-B interacts simultaneously with both the CBF-A and CBF-C subunits to form a heterotrimeric CBF molecule. Mol. Cell. Biol. **16:**4003–4013.
38. **Kose, S., N. Imamoto, T. Tachibana, T. Shimamoto, and Y. Yoneda.** 1997. Ran-unassisted nuclear migration of a 97-kD component of nuclear pore-targeting complex. J. Cell Biol. **139:**841–849.
39. **Kutay, U., F. R. Bischoff, S. Kostka, R. Kraft, and D. Gorlich.** 1997. Export of importin alpha from the nucleus is mediated by a specific nuclear transport factor. Cell **90:**1061–1071.
40. **Kutay, U., E. Izaurralde, F. R. Bischoff, I. W. Mattaj, and D. Gorlich.** 1997. Dominant-negative mutants of importin-beta block multiple pathways of import and export through the nuclear pore complex. EMBO J. **16:**1153–1163.
41. **Lei, E. P., and P. A. Silver.** 2002. Protein and RNA export from the nucleus. Dev. Cell. **2:**261–272.
42. **Li, Q., M. Herrler, N. Landsberger, N. Kaludov, V. V. Ogryzko, Y. Nakatani, and A. P. Wolffe.** 1998. Xenopus NF-Y pre-sets chromatin to potentiate p300 and acetylation-responsive transcription from the Xenopus hsp70 promoter in vivo. EMBO J. **17:**6300–6315.
43. **Macara, I. G.** 2001. Transport into and out of the nucleus. Microbiol. Mol. Biol. Rev. **65:**570–594.
44. **Maity, S. N., and B. de Crombrugghe.** 1998. Role of the CCAAT-binding protein CBF/NF-Y in transcription. Trends Biochem. Sci. **23:**174–178.
45. **Mantovani, R.** 1999. The molecular biology of the CCAAT-binding factor NF-Y. Gene **239:**15–27.
46. **Mantovani, R., X. Y. Li, U. Pessara, R. Hooft van Huisjduijnen, C. Benoist, and D. Mathis.** 1994. Dominant negative analogs of NF-YA. J. Biol. Chem. **269:**20340–20346.
47. **Mattaj, I. W., and L. Englmeier.** 1998. Nucleocytoplasmic transport: the soluble phase. Annu. Rev. Biochem. **67:**265–306.
48. **McNabb, D. S., Y. Xing, and L. Guarente.** 1995. Cloning of yeast HAP5: a novel subunit of a heterotrimeric complex required for CCAAT binding. Genes Dev. **9:**47–58.
49. **Melchior, F., and L. Gerace.** 1995. Mechanisms of nuclear protein import. Curr. Opin. Cell Biol. **7:**310–318.
50. **Mingot, J. M., S. Kostka, R. Kraft, E. Hartmann, and D. Gorlich.** 2001. Importin 13: a novel mediator of nuclear import and export. EMBO J. **20:**3685–3694.
51. **Mosammaparast, N., Y. Guo, J. Shabanowitz, D. F. Hunt, and L. F. Pemberton.** 2002. Pathways mediating the nuclear import of histones H3 and H4 in yeast. J. Biol. Chem. **277:**862–868.
52. **Mosammaparast, N., K. R. Jackson, Y. Guo, C. J. Brame, J. Shabanowitz, D. F. Hunt, and L. F. Pemberton.** 2001. Nuclear import of histone H2A and H2B is mediated by a network of karyopherins. J. Cell Biol. **153:**251–262.
53. **Muhlhausser, P., E. C. Muller, A. Otto, and U. Kutay.** 2001. Multiple pathways contribute to nuclear import of core histones. EMBO Rep. **2:**690–696.
54. **Nakshatri, H., P. Bhat-Nakshatri, and R. A. Currie.** 1996. Subunit association and DNA binding activity of the heterotrimeric transcription factor NF-Y is regulated by cellular redox. J. Biol. Chem. **271:**28784–28791.
55. **Nigg, E. A.** 1997. Nucleocytoplasmic transport: signals, mechanisms and regulation. Nature **386:**779–787.
56. **Ploski, J. E., M. K. Shamsher, and A. Radu.** 2004. Paired-type homeodomain transcription factors are imported into the nucleus by karyopherin 13. Mol. Cell. Biol. **24:**4824–4834.
57. **Pollard, V. W., W. M. Michael, S. Nakielny, M. C. Siomi, F. Wang, and G. Dreyfuss.** 1996. A novel receptor-mediated nuclear protein import pathway. Cell **86:**985–994.
58. **Rexach, M., and G. Blobel.** 1995. Protein import into nuclei: association and dissociation reactions involving transport substrate, transport factors, and nucleoporins. Cell **83:**683–692.
59. **Ribbeck, K., and D. Gorlich.** 2001. Kinetic analysis of translocation through nuclear pore complexes. EMBO J. **20:**1320–1330.
60. **Ribbeck, K., U. Kutay, E. Paraskeva, and D. Gorlich.** 1999. The translocation of transportin-cargo complexes through nuclear pores is independent of both Ran and energy. Curr. Biol. **9:**47–50.
61. **Ribbeck, K., G. Lipowsky, H. M. Kent, M. Stewart, and D. Gorlich.** 1998. NTF2 mediates nuclear import of Ran. EMBO J. **17:**6587–6598.
62. **Robbins, J., S. M. Dilworth, R. A. Laskey, and C. Dingwall.** 1991. Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. Cell **64:**615–623.
63. **Romier, C., F. Cocchiarella, R. Mantovani, and D. Moras.** 2003. The NF-YB/NF-YC structure gives insight into DNA binding and transcription regulation by CCAAT factor NF-Y. J. Biol. Chem. **278:**1336–1345.
64. **Rout, M. P., and J. D. Aitchison.** 2001. The nuclear pore complex as a transport machine. J. Biol. Chem. **276:**16593–16596.
65. **Rupp, R. A., L. Snider, and H. Weintraub.** 1994. Xenopus embryos regulate the nuclear localization of XMyoD. Genes Dev. **8:**1311–1323.
66. **Schwamborn, K., W. Albig, and D. Doenecke.** 1998. The histone H1(0) contains multiple sequence elements for nuclear targeting. Exp. Cell Res. **244:**206–217.
67. **Schwoebel, E. D., B. Talcott, I. Cushman, and M. S. Moore.** 1998. Ran-dependent signal-mediated nuclear import does not require GTP hydrolysis by Ran. J. Biol. Chem. **273:**35170–35175.
68. **Sinha, S., I. S. Kim, K. Y. Sohn, B. de Crombrugghe, and S. N. Maity.** 1996. Three classes of mutations in the A subunit of the CCAAT-binding factor CBF delineate functional domains involved in the three-step assembly of the CBF-DNA complex. Mol. Cell. Biol. **16:**328–337.
69. **Sinha, S., S. N. Maity, J. Lu, and B. de Crombrugghe.** 1995. Recombinant rat CBF-C, the third subunit of CBF/NFY, allows formation of a protein-DNA complex with CBF-A and CBF-B and with yeast HAP2 and HAP3. Proc. Natl. Acad. Sci. USA **92:**1624–1628.
70. **Steidl, S., P. Papagiannopoulos, O. Litzka, A. Andrianopoulos, M. A. Davis, A. A. Brakhage, and M. J. Hynes.** 1999. AnCF, the CCAAT binding complex of *Aspergillus nidulans*, contains products of the *hapB*, *hapC*, and *hapE* genes and is required for activation by the pathway-specific regulatory gene *amdR*. Mol. Cell. Biol. **19:**99–106.
71. **Steidl, S., A. Tuncher, H. Goda, C. Guder, N. Papadopoulou, T. Kobayashi, N. Tsukagoshi, M. Kato, and A. A. Brakhage.** 2004. A single subunit of a heterotrimeric CCAAT-binding complex carries a nuclear localization signal: piggy back transport of the pre-assembled complex to the nucleus. J. Mol. Biol. **342:**515–524.
72. **Strom, A. C., and K. Weis.** 2001. Importin-beta-like nuclear transport receptors. Genome Biol. **2:**3008.1–3008.9.
73. **Tommasi, S., and G. P. Pfeifer.** 1995. In vivo structure of the human *cdc2* promoter: release of a p130-E2F-4 complex from sequences immediately upstream of the transcription initiation site coincides with induction of *cdc2* expression. Mol. Cell. Biol. **15:**6901–6913.
74. **Truant, R., and B. R. Cullen.** 1999. The arginine-rich domains present in human immunodeficiency virus type 1 Tat and Rev function as direct importin beta-dependent nuclear localization signals. Mol. Cell. Biol. **19:**1210–1217.
75. **Turner, D. L., and H. Weintraub.** 1994. Expression of achaete-scute homolog 3 in Xenopus embryos converts ectodermal cells to a neural fate. Genes Dev. **8:**1434–1447.

76. **Weis, K.** 2002. Nucleocytoplasmic transport: cargo trafficking across the border. Curr. Opin. Cell Biol. **14:**328–335.
77. **Weis, K.** 2003. Regulating access to the genome: nucleocytoplasmic transport throughout the cell cycle. Cell **112:**441–451.
78. **Weis, K., C. Dingwall, and A. I. Lamond.** 1996. Characterization of the nuclear protein import mechanism using Ran mutants with altered nucleotide binding specificities. EMBO J. **15:**7120–7128.
79. **Wischnewski, J., F. Rudt, and T. Pieler.** 2004. Signals and receptors for the nuclear transport of TFIIIA in Xenopus oocytes. Eur. J. Cell Biol. **83:**55–66.
80. **Wozniak, R. W., M. P. Rout, and J. D. Aitchison.** 1998. Karyopherins and kissing cousins. Trends Cell Biol. **8:**184–188.
81. **Xiao, Z., X. Liu, and H. F. Lodish.** 2000. Importin beta mediates nuclear translocation of Smad 3. J. Biol. Chem. **275:**23425–23428.
82. **Xing, Y., J. D. Fikes, and L. Guarente.** 1993. Mutations in yeast HAP2/HAP3 define a hybrid CCAAT box binding domain. EMBO J. **12:**4647–4655.
83. **Zhu, W., P. H. Giangrande, and J. R. Nevins.** 2004. E2Fs link the control of G1/S and G2/M transcription. EMBO J. **23:**4615–4626.