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# The Carboxyl-Terminus Directs $TAF_I48$ to the Nucleus and Nucleolus and Associates with Multiple Nuclear Import Receptors

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**The protein complex Selectivity Factor 1, composed of TBP, $TAF_I48$, $TAF_I63$ and $TAF_I110$, is required for rRNA transcription by RNA polymerase I in the nucleolus. The steps involved in targeting Selectivity Factor 1 will be dependent on the transport pathways that are used and the localization signals that direct this trafficking. In order to investigate these issues, we characterized human $TAF_I48$, a subunit of Selectivity Factor 1. By domain analysis of $TAF_I48$, the carboxyl-terminal 51 residues were found to be required for the localization of $TAF_I48$, as well as sufficient to direct Green Fluorescent Protein to the nucleus and nucleolus. The carboxyl-terminus of $TAF_I48$ also has the ability to associate with multiple members of the β-karyopherin family of nuclear import receptors, including importin β (karyopherin β1), transportin (karyopherin β2) and RanBP5 (karyopherin β3), in a Ran-dependent manner. This property of interacting with multiple β-karyopherins has been previously reported for the nuclear localization signals of some ribosomal proteins that are likewise directed to the nucleolus. This study identifies the first nuclear import sequence identified within the TBP-Associated Factor subunits of Selectivity Factor 1.**

**Key words: nuclear, nucleolar, Selectivity Factor 1 (SL1), TBP-associated factors (TAFs), transcription.**

In human cells, RNA polymerase I transcription requires a protein complex called Selectivity Factor 1 (SL1). Selectivity Factor 1 is composed of the TATA-box binding protein (TBP) and three TBP-associated factors ($TAF_I48$, $TAF_I63$ and $TAF_I110$) (*1*) and plays a central role in assembly of the RNA polymerase I preinitiation complex (reviewed in Refs. *2–5*). For proper activity, Selectivity Factor 1 must be translocated to its site of action in the nucleolus. The first step in nucleolar localization is the crossing of the nuclear membrane, which relies upon nuclear localization signals (NLS) encoded in the transported protein. While the nuclear import pathway of TBP has been studied in *S. cerevisiae* (*6*), the nuclear trafficking of the various protein complexes containing TBP is poorly characterized. For example, with the exception of TBP, nuclear localization signals have not been identified within the subunits of Selectivity Factor 1, as well as TFIID and TFIIIB (the TBP-containing complexes involved in RNA polymerase II and III transcription, respectively).

Classical nuclear localization signals (NLS) consist of either a single cluster of basic amino acids or a bipartite sequence composed of two sets of two or three basic residues separated by approximately 10–12 amino acids (reviewed in Refs. *7* and *8*). Ribosomal proteins such as rpL23a (*9*), rpS6 (*10*) and rpL7a (*11*) contain atypical nuclear localization signals that are more basic and complex than classical nuclear localization signals (*9*). During nuclear entry, the nuclear localization signal is bound directly by either a member of the importin α or importin β families of nuclear import receptors (reviewed in Ref. *8*). The importin β family includes importin β (*12*), transportin (*13*), RanBP5 (*9*) and RanBP7. (These are also called karyopherin β1, β2, β3 and β4, respectively.) Nuclear import pathways typically terminate with release into the nucleus mediated by Ran, a GTP-binding protein (reviewed in Ref. *14*). In contrast, nucleolar localization is less well characterized. Consensus nucleolar localization signals have not been well defined, although they often contain a high proportion of basic residues. In some cases, nucleolar localization signals overlap with the NLS (*15*, *16*), and in others, they are distinct (*17*, *18*).

The signals responsible for targeting the proteins that make up the RNA polymerase I (pol I) transcription machinery to the nucleus and nucleolus are poorly characterized. While these translocation events contribute to the assembly of the RNA polymerase I transcription complex, they also offer potential points of regulatory control (reviewed in Refs. *8* and *19*). Unique among the Selectivity Factor 1 subunits, TBP is shared among all three cellular RNA polymerase systems and is partitioned between the nucleoplasm and the nucleolus. For this reason, the remaining TAF subunits of Selectivity Factor 1 likely direct it to its ultimate site of action in the nucleolus. In order to elucidate the steps involved in Selectivity Factor 1 localization, and help provide a framework for

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understanding the targeting and assembly of the RNA polymerase I transcription complex, we investigated the subcellular trafficking of $TAF_I48$, then identified and characterized the region within $TAF_I48$ that directs it. We observe that $TAF_I48$ fusion proteins are localized in the nucleolus. In addition, we detect them in puncta distributed throughout the nucleoplasm. We identify a single domain necessary for nuclear targeting of $TAF_I48$. When fused to a heterologous protein, such as Green Fluorescent Protein, this region is sufficient to direct it to both the nucleus and nucleolus. This domain, composed of the carboxyl-terminal 51 residues of $TAF_I48$, also binds multiple nuclear import receptors, similar to the nuclear localization signals of some ribosomal proteins that are likewise directed to the nucleolus.

## MATERIALS AND METHODS

*Molecular Biology*—The vectors expressing the various $TAF_I48$ fusion proteins were engineered using the polymerase chain reaction (PCR) to generate DNA fragments encoding the appropriate residues with restriction sites on the termini. In the case of the HA3, β-galactosidase (β-gal) and glutathione-S-transferase (GST) fusions, the fragments were inserted into the *Bam*HI site of pKH3 (*20*), the *Bgl*II and *Xba*I sites of pCS2+C-β-gal (*21*), or the *Bam*HI and *Xba*I sites of pGEX-KG (*22*), respectively, and sequenced. In the case of the various GFP-$TAF_I48$ fusion proteins, the fragments were generated by PCR, sequenced, and then inserted between the *Bgl*II and *Xba*I sites of either pEGFP-N1 or pEGFP-C1 (Clontech, Palo Alto, CA) depending on whether the GFP was on the amino- or carboxyl-terminus, respectively. DNA sequencing was performed by the Hartwell Center at St. Jude Children's Research Hospital (Memphis, TN) or the Molecular Resource Center of Excellence at the University of Tennessee Health Science Center (Memphis, TN). All restriction and DNA modifying enzymes were purchased from New England Biolabs (Beverly, MA) or Promega (Madison, WI). Taq DNA polymerase and PCR kits were purchased from either Promega or Epicentre (Madison, WI). All plasmids were screened and amplified in the TOP10 strain (Invitrogen, Carlsbad, CA) of *E. coli*.

*Cell Culture and Transfections*—HeLa cells were grown in DMEM containing glucose (Mediatech, Herndon, VA) and 10% Fetal Bovine Serum (Mediatech). For transfections, cells were plated onto either 35 mm plates with loose coverslips (for immunofluorescence and β-gal assays) or 35 mm plates with integrated coverslip (Mattek Corporation, Ashland, MA) designed for use with an inverted microscope (for GFP). When the cells reached 30–50% confluence, they were transfected with each expression vector using FuGene6 (Roche Biochemicals, Indianapolis, IN), as specified by the manufacturer.

*Immunofluorescence*—Cells grown on coverslips were harvested one day post-transfection and fixed using 4% paraformaldehyde. The fixed cells were permeabilized with PBS and 0.5% Triton X-100 for 5 min at room temperature and blocked with 10% Fetal Bovine Serum in PBS for 30 min at room temperature. The cells were then incubated with anti-HA (Roche Biochemicals) and anti-fibrillarin (Sigma, St. Louis, MO) antibodies and washed three times with 1xPBS. Subsequently, cells were incubated with Texas Red-conjugated anti-mouse IgG2b (Southern Biotechnology Associates, Inc., Birmingham, AL) and FITC-conjugated anti-human (Pierce, Rockford, IL) secondary antibodies for 30 min at 37°C, and washed five times with 1xPBS. The coverslips were then rinsed with $H_2O$ and mounted onto slides using Vectashield mounting media (Vector Labs, Burlingame, CA) with or without DAPI. The cells were imaged on an Olympus BMX50 (Tokyo) fluorescence microscope with a 60× (NA 1.40) oil-immersion objective, using a Photometrics Sensys 14000 CCD camera (Tuscon, AZ) and V++ Precision Digital Imaging System (Digital Optics, Auckland, New Zealand). For quantitation, the slides were scanned and a total of at least 100 transfected cells in at least 20 different fields were examined. Images were processed in Photoshop 5.5 (Adobe, San Jose, CA) and index colored using the color table.

*Fluorescence Imaging of Living Cells*—Living HeLa cells were imaged one to two days post-transfection in the same media used for growth in 35-mm petri dishes with integrated coverslips (Mattek Corporation). Fluorescent confocal and transmitted light images were obtained using an Olympus Fluoview laser scanning confocal microscope with Argon and krypton lasers and both epifluorescence and transmitted light detectors. The qualitative nature of the expression for each $TAF_I48$ derivative was unchanged over an approximate a 10-fold range of fluorescence intensity. Both 40x UPlanApo (0.85 N.A.) and 100x PlanApo (1.4 N.A.) objectives were used. Images were processed in Photoshop 5.5 (Adobe). The contrast of the transmitted light images was increased using an unsharp mask filter. Composite fluorescence-transmitted light images were made by merging separate images using the "screen" blending mode in Adobe Photoshop. For quantitation, the culture dishes were scanned progressively, starting at one side and counting the first 100 transfected cells within the field of view. The distribution of $TAF_I48$-GFP was not quantitated because there was an approximate two order of magnitude reduction in the detectable number of cells expressing this protein due to either reduced transfection efficiency and/or reduced expression levels. The cells expressing $TAF_I48$-GFP displayed a consistent nuclear distribution, not observed in the GFP control, in approximately 20 cells examined over several different transfection experiments.

*Protein Expression and Purification*—The GST, GST-$TAF_I48$ 400–450 and GST-RNPA1 (a generous gift of Sanjay Vasu, UCSD) affinity resins were obtained by expressing the protein in the BL21(DE3) strain of *E. coli* (Novagen, Madison, WI). After induction of the protein(s) with 0.5 mM IPTG, the cells were lysed in PBS by sonication, and centrifuged at high speed. The supernatant was incubated at 4°C with glutathione-agarose (Sigma), after which the resin was isolated by centrifugation and washed extensively with PBS. The resulting affinity resins were separated on an SDS-polyacrylamide gel alongside known amounts of BSA, stained using Coomassie Brilliant Blue, and subsequently destained. The stained proteins on the gel were used to quantitate the absolute amount present on the various resins and to monitor the amount of degradation. $His_6$-tagged Ran Q69L (a generous gift of Sanjay Vasu) was induced in the BL21(DE3)

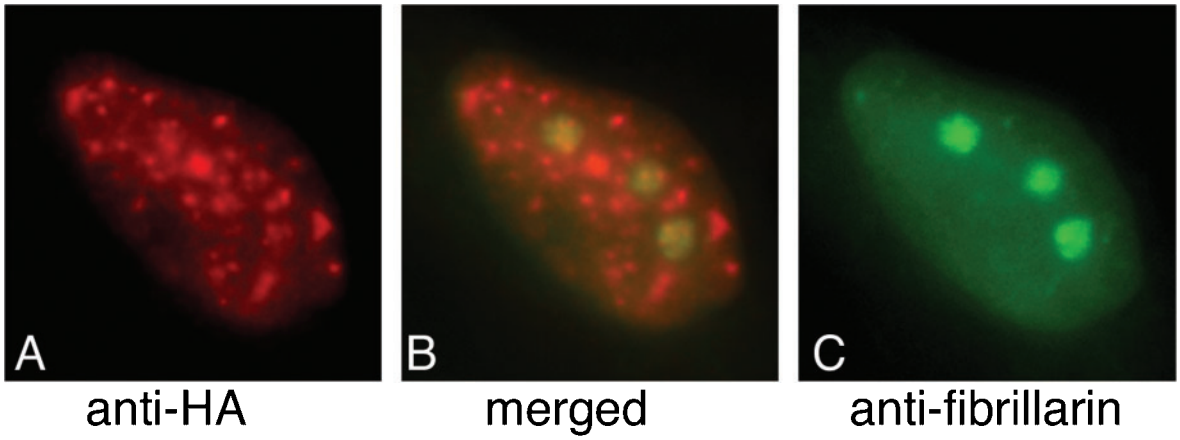


Fig. 1. **Distribution of HA$_3$-TAF$_I$48 in HeLa cells.** HeLa cells transiently expressing HA$_3$-TAF$_I$48 were fixed and stained with (A) an anti-hemagglutinin antibody (12CA5) or (C) an anti-fibrillarin antibody which identifies the nucleoli, and to a lesser extent the nucleoplasm. The co-localization of these proteins is defined by (B) merging the two images.

strain of *E. coli* using 0.5 mM IPTG. The cells were lysed in 1xPBS by sonication and centrifuged at 10,000 rpm for 15 min in a GSA rotor. The supernatant was incubated with 1-ml of Ni-affinity resin (Qiagen, Valencia, CA) and loaded into a column. After washing with 20 column volumes of 1× PBS containing 10 mM imidazole, the protein was eluted with 1× PBS containing 200 mM imidazole. The fractions were analyzed on an SDS-polyacrylamide gel, and the concentrations of those containing Ran Q69L were quantitated (Bio-Rad Laboratories, Hercules, CA). RanQ69L was initially in a GDP-bound state and was converted to the GTP-bound state in the following manner. RanQ69L-GDP at 1 mg/ml was incubated for 25 min at room temperature, then 10 min on ice in the presence of 10 mM EDTA, 1 mM DTT and a 50-fold excess of GTP. Finally, $Mg_2Cl$ was added to a final concentration of 30 mM, and the sample was dialyzed against 1× PBS containing 10% glycerol.

*Protein-Protein Interaction Assays*—Thirty microliters of rabbit reticulocyte extracts (Promega) were incubated in 300 μl of 1× PBS at room temperature or 4°C for 60 min or for the indicated times with equivalent amounts of the affinity matrix displaying 5 μg total of either GST or GST-fusion protein. The samples were then washed three times with 0.5 ml of 1× PBS. The GST-TAF$_I$48 400–450 fusion protein was approximately 30% full-length and the remainder of this protein was degraded to the size of GST alone (unpublished results). In all experiments, the bound fraction was separated on an SDS-polyacrylamide gel and transferred to Hybond-C (Amersham, Piscataway, NJ). The membranes were then incubated with either a monoclonal antibody against importin β or transportin (BD Biosciences, Palo Alto, CA) or a rabbit polyclonal antibody against RanBP5 (Santa Cruz Biotechnology, Santa Cruz, CA) and washed with PBS containing 0.1% Tween-20. The membranes were then incubated with either HRP-conjugated anti-mouse or HRP-conjugated anti-rabbit antibody (Bio-Rad) and subsequently developed using Super Signal West Dura (Pierce, Rockford, IL) and exposed to CL-Xposure Film (Pierce).

## RESULTS

While the components of Selectivity Factor 1 are synthesized in the cytoplasm, the assembled complex ultimately functions in the nucleolus. The steps involved in the assembly and trafficking of Selectivity Factor 1 are poorly understood. This study characterizes the distribution of human TAF$_I$48, a 450-amino acid subunit of Selectivity Factor 1, within interphase cells and the mechanisms that target it to the nucleus and nucleolus.

*Full-Length TAF$_I$48 Is Directed to the Nucleolus and the Nucleoplasm*—In order to examine the distribution of TAF$_I$48, it was fused to readily visualized "tags," and these fusion proteins were transiently expressed using the constitutively active cytomegalovirus immediate early promoter. Previously, it has been difficult to detect the endogenous subunits of Selectivity Factor 1 in interphase cells. For example, the distribution of TAF$_I$63, TAF$_I$48, TBP and UBF, but not TAF$_I$48, could be detected during either mitosis or in the presence of Actinomycin D

Table 1. **Cellular localization of HA$_3$- and GFP-TAF$_I$48 constructs.**

| TAF$_I$ 48 derivative | Nuclear[a] | Cytoplasmic | Nuclear and cytoplasmic |
|---|---|---|---|
| HA$_3$-1–450 | 96% | 0% | 4% |
| HA$_3$-1–343 | 0% | 81% | 19% |
| HA$_3$-1–400 | 0% | 97% | 3% |
| HA$_3$-217–450 | ND | ND | ND |
| HA$_3$-343–450 | 100% | 0% | b |
| HA$_3$-400–450 | 92% | 2.7% | 5.3% |
| HA$_3$-217–343 | 0% | 93%[C] | 7%[C] |
| N-GFP-1–450 | 94% | 0% | 6% |
| 1-450-GFP-C | d | d | d |
| N-GFP-400–450 | 91%[b] | 0% | 9% |

[a]Nucleoplasmic and/or nucleolar. [b]>80% of cells have extemely low levels of cytoplasmic staining. [c]Speckled pattern in 44% of cells with cytoplasmic staining and all cells with nuclear staining, but does not co-localize with the nucleoli. [d]Quantitative values were not obtained because the number of cells with detectable expression was approximately two orders of magnitude below that of N-GFP-TAF48 1-450.

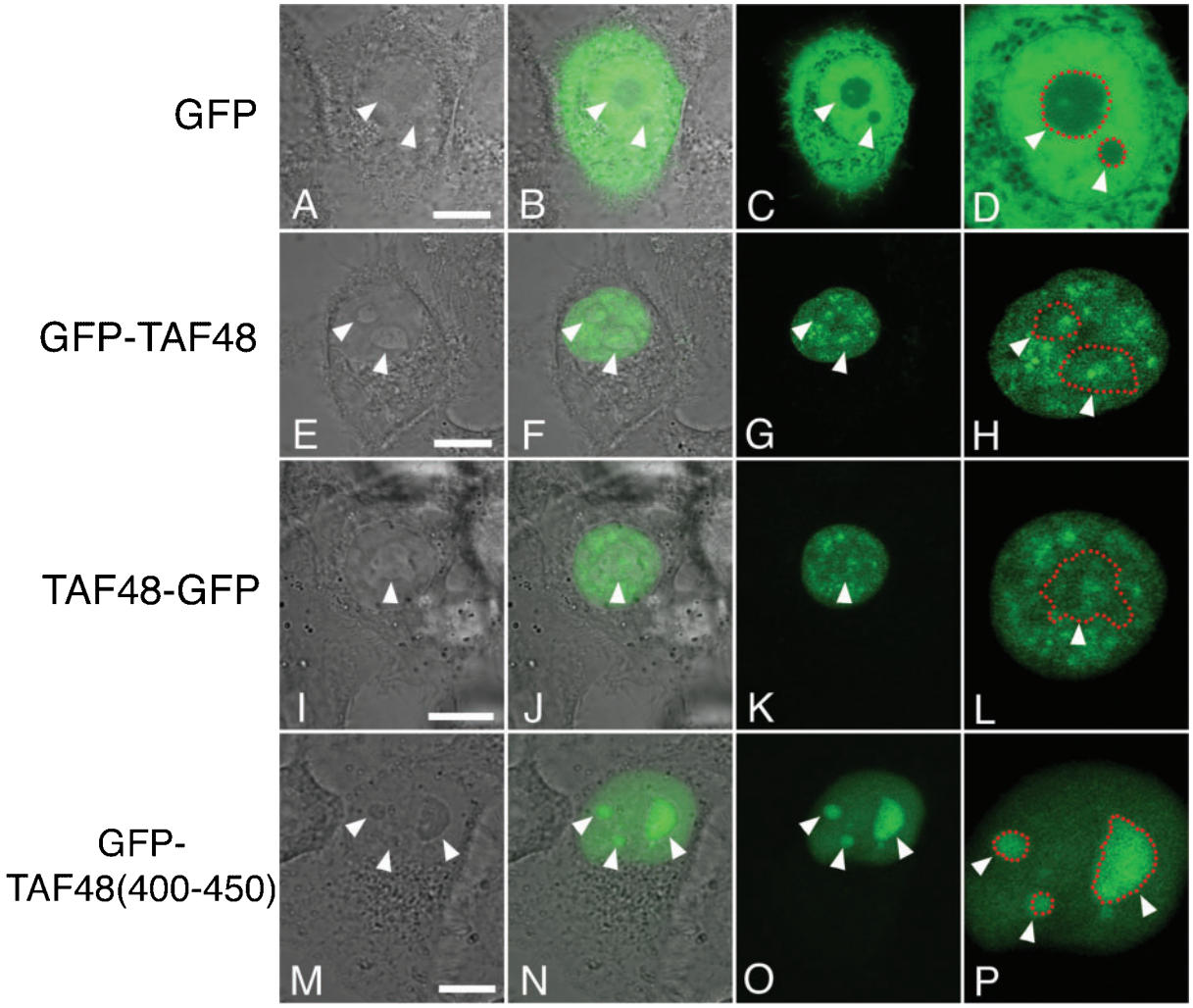


Fig. 2. **Distribution of GFP fused to $TAF_I48$ or the $TAF_I48$ carboxyl-terminus in HeLa cells.** HeLa cells transiently expressing either (A–D) GFP, (E–H) GFP-$TAF_I48$, (I–L) $TAF_I48$-GFP or (M–P) $TAF_I48$ 400–450-GFP were examined using laser scanning confocal microscopy. Panels A, E, I and M are light images and C, G, K and O are fluorescence images. Panels B, F, J and N merge the transmitted light and fluorescence images. In panels D, H, L and P, the fluorescence images have been enlarged by a factor of two and the nucleoli are outlined in red dots. The positions of nucleoli are indicated by arrowheads. The scale bars in panels A, E, I and M are 10 microns.

by immunofluorescence (*23*, *24*). In contrast, none of the Selectivity Factor 1 subunits could be detected within interphase cells in those studies. However, a recent study has successfully used overexpressed RNA polymerase I preinitiation factors to kinetically model the assembly of the pol I transcription complex (*25*). Thus, this approach should likewise be useful for localization studies. In our experiments, the subcellular distribution of full-length $TAF_I48$ (1–450) was investigated by localizing protein fusions with either three copies of the hemagglutinin tag (HA3) or the Green Fluorescent Protein (GFP). When expressed transiently, the fusion protein HA3-$TAF_I48$ was detected exclusively in the nucleus with a primarily punctate distribution (Fig. 1A). Upon quantitation, 96% of the cells expressing HA3-$TAF_I48$ 1–450 displayed this pattern (Table 1). The nucleoli were defined by double-label immunofluorescence using an antibody against the nucleolar protein fibrillarin (*26–28*). This antibody sometimes displays a weaker pattern of nucleoplasmic staining as well (Figs. 1C, 3, and 4). When the images are merged, HA3-$TAF_I48$ is found within the nucleoli, and the remainder show a punctate distribution in the nucleoplasm (Fig. 1B). This pattern was observed in 87% of the cells expressing HA3-$TAF_I48$. This distribution is consistent with a recent study that found 68% of TAFI48 in the nucleoplasm and 15% in the nucleolus [(*25*)-*Supplementary Table S1*].

The distribution of full-length $TAF_I48$ was investigated in parallel using a fusion to a different tag (Green Fluorescent Protein) and examined in living, rather than fixed, HeLa cells using laser scanning confocal microscopy. In these experiments, nucleoli (labeled by arrowheads in Fig. 2) and nuclei can be identified by morphology in images simultaneously acquired using the confocal transmitted light detector (Fig. 2, A, E and I). When expressed transiently, GFP alone is distributed in a roughly homogeneous pattern in both the cytoplasm and nucleoplasm, but largely excluded from the nucleolus and some sites in the cytoplasm, which are probably membrane-bounded organelles such as mitochondria (Fig. 2,

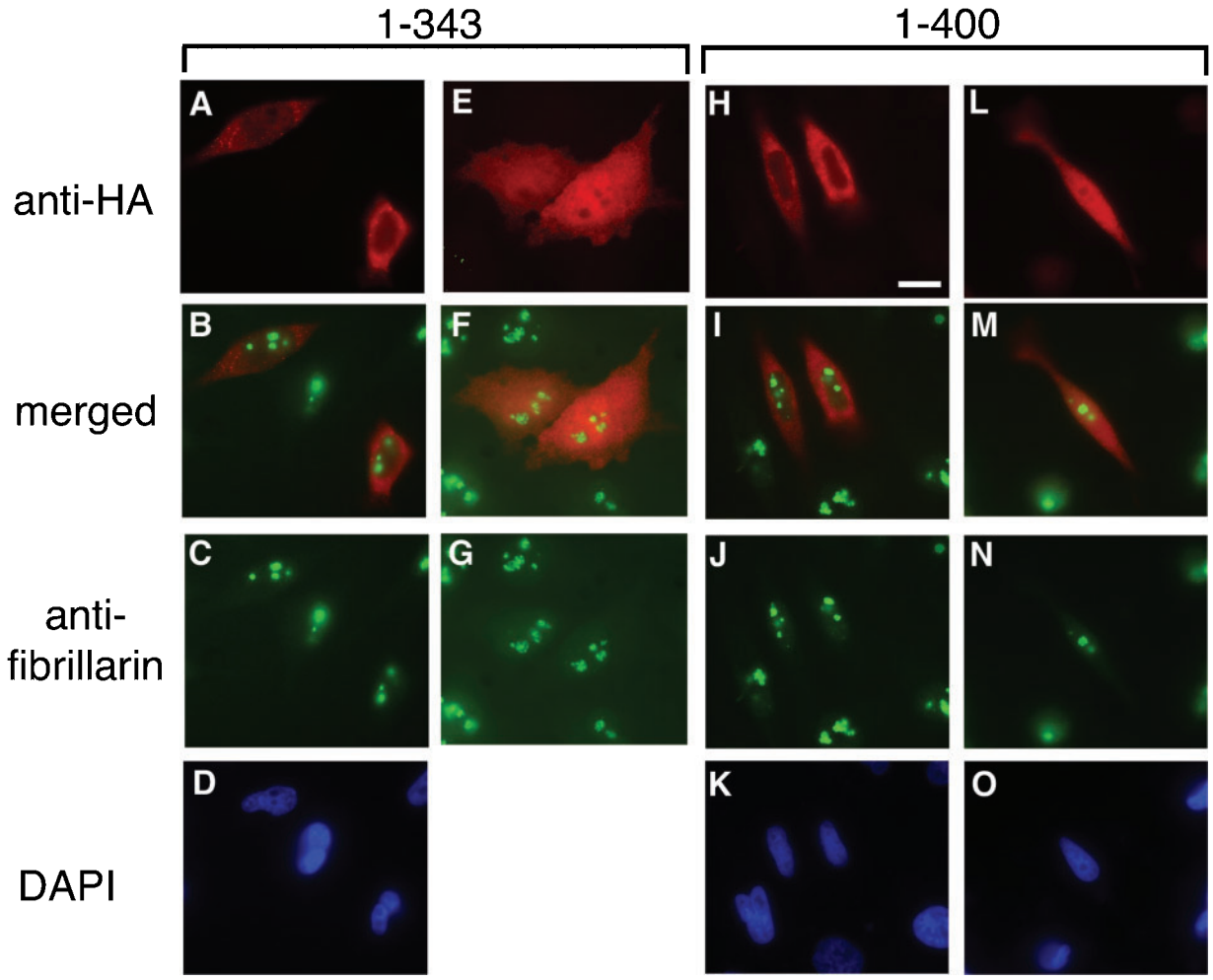


Fig. 3. **The carboxyl-terminus of TAF$_I$48 is required for proper localization.** The localization of TAF$_I$48 derivatives truncated from the carboxyl-terminus was examined. (A–G) TAF$_I$48 1–343 or (H–O) TAF$_I$48 1–400, fused to HA$_3$, were stained with (A, E, H and L) an anti-hemagglutinin antibody or (C, G, J and N) an anti-fibrillarin antibody, which identifies the nucleoli. The co-localization of these proteins is defined by merging the two images (B, F, I and M). In panels D, K and O, the pattern of DAPI staining is shown. The scale bar in panel H is 20 microns.

A–D). When Green Fluorescent Protein (GFP) was fused to the amino-terminus of TAF$_I$48 (GFP-TAF$_I$48) and examined by laser scanning confocal microscopy, GFP-TAF$_I$48 was targeted exclusively to the nucleus in 94% of the cells expressing it (Fig. 2G; Table 1). The distribution of TAF$_I$48-GFP (GFP fused to the carboxyl-terminus of TAF$_I$48) was also exclusively nuclear (Fig. 2K), but was not quantitated because there was a dramatic reduction in the number of detectable cells expressing this protein. When the positions of the nucleoli are identified by either merging the fluorescence and transmitted light images (Fig. 2, B, F and J), or outlining the borders of the nucleoli in enlarged images (Fig. 2, D, H and L), the TAF$_I$48 fusion proteins were found to be expressed at similar levels in both the nucleoli and the nucleoplasm (Fig. 2, F, H, J and L). In contrast, GFP alone is largely excluded from the nucleoli (Fig. 2, B–D). Using several experimental regimens that included live and fixed cells, small (triple HA-tag – ~4 kDa) and large (GFP-tag – ~28 kDa) protein tags, and amino and carboxyl sites of fusion, TAF$_I$48 expression was observed in both the nucleolus, the site of SL1 function, and, unexpectedly, in the nucleoplasm, as will be discussed below.

*The Carboxyl-Terminus of TAF$_I$48 Is Required for Its Nucleolar Targeting*—Since the HA-tagged version of TAF$_I$48 is localized to the nucleolus and nucleoplasm, various derivatives of TAF$_I$48 were engineered to identify the sequences responsible for directing it to these locations. Initially, a deletion (1–343), that removed the carboxyl-terminal 107 residues, was engineered to contain an HA3-tag on the amino-terminus, then expressed transiently in HeLa cells and examined by immunofluorescence. This protein displays a smooth distribution pattern throughout much of the cell, but is absent from a central region (Fig. 3A). When this pattern was compared to that of fibrillarin and DAPI counter-staining (Fig. 3, B, C and D), the regions lacking TAF$_I$48 corresponded to the nucleus. When quantitated, 81% of the cells expressed HA3-TAF48 1–343 exclusively in the cytoplasm (Table 1). In order to examine the carboxyl-terminus of TAF$_I$48 further, a smaller deletion (1–400) that removed only the carboxyl-terminal 50 residues was expressed as an HA3-

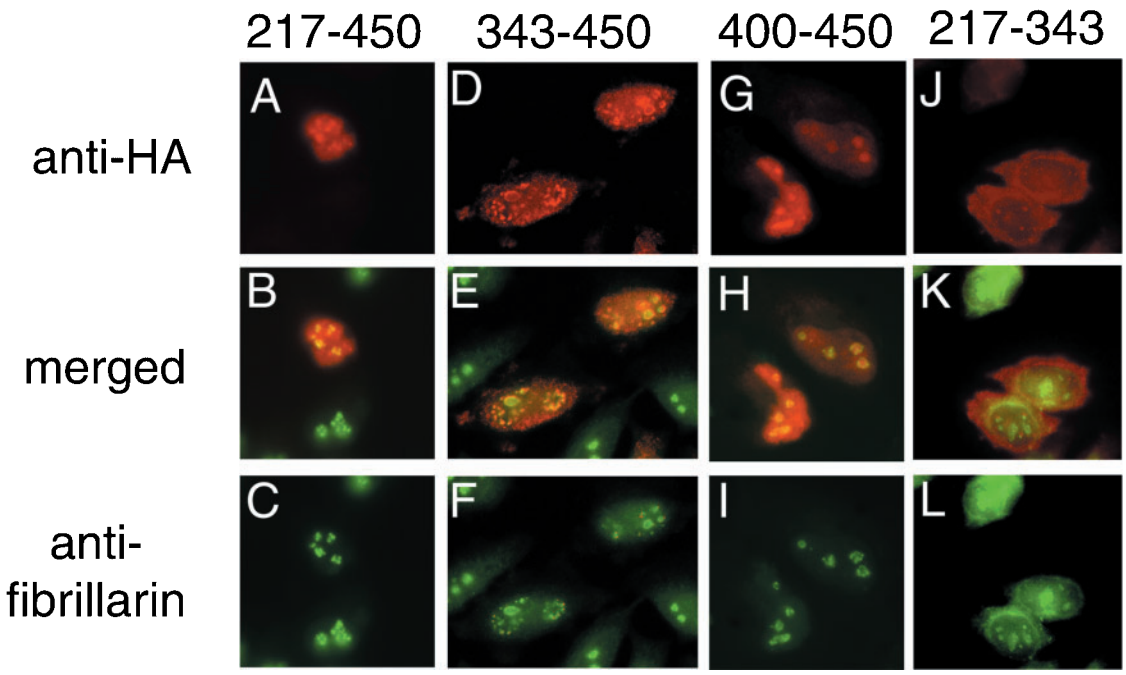


Fig. 4. **The carboxyl-terminus of TAF$_I$48 directs itself to the nucleolus.** The distribution of either carboxyl-terminal or internal regions of TAF$_I$48 was determined. (A–C) TAF$_I$48 217–450, (D–F) TAF$_I$48 343–450, (G–I) TAF$_I$48 400–450, and (J–L) TAF$_I$48 217–450, fused to the HA$_3$-tag, were expressed transiently and stained with (A, D, G and J) an anti-hemagglutinin antibody or (C, F, I and L) an anti-fibrillarin antibody, which identifies the nucleoli. The co-localization of these proteins is defined by merging the two images (B, E, H and K).

fusion protein. This protein also exhibited a smooth distribution throughout the cytoplasm that was excluded from the nucleus in 97% of the expressing cells (Fig. 3, H–K; Table 1). In a smaller percentage of cells (19% and 3%, respectively), HA3-TAF$_I$48 1–343 and HA3-TAF$_I$48 1–400 displayed a relatively smooth distribution throughout the cytoplasm and nucleus, but were excluded from several prominent regions. When compared to the fibrillarin stained images, the regions lacking TAF$_I$48 in those cases corresponded to the nucleolus (Fig. 3, E–G and L–O). Since these fusion proteins are ~40 and 45 kDa, respectively, which is close to the exclusion limit of the nuclear pore (*29*), their ability to passively diffuse through the nuclear pore may account for this variation in nuclear entry. Of interest, even when these proteins are able to enter the nucleus, they are not targeted to the nucleolus. Taken together, these results suggest the carboxyl-terminus of TAF$_I$48 is involved in nuclear and nucleolar targeting.

*The Carboxyl-Terminus of TAF$_I$48 Is Sufficient to Direct Nucleolar Targeting*—In order to examine TAF$_I$48 for additional regions required for nucleolar and/or nuclear localization, amino-terminal deletions were constructed. These deletions retain amino acids 217–450, 343–450, and 400–450 of TAF$_I$48 and contain the HA3-tag on the amino-terminus. Upon transient expression in HeLa cells, each of the TAF$_I$48 fusion proteins exhibited exclusive nuclear expression with enrichment within the nucleoli (Fig. 4, A–I). When quantitated, 100% and 92% of cells expressing TAF$_I$48 343–450 and 400–450, respectively, displayed exclusively nuclear staining (Table 1). They also exhibited staining that co-localized with nucleoli (*i.e.*, fibrillarin staining) in 60% and 70% of cells, respectively. As a control, an internal ~14 kDa fragment (residues 217–343) lacking the carboxyl-terminus was also expressed as an HA3-tag fusion (Fig. 4, J–L). Upon inspection, this protein exhibited cytoplasmic staining in 93% of cells. In 7% of cells, this protein exhibited homogeneous staining throughout the cell that was not enriched in the nucleolus. These results are consistent with the conclusion that the carboxyl-terminus of TAF$_I$48 is required for nuclear import. In addition, these experiments suggest that the carboxyl-terminal 51 residues also have the ability to target TAF$_I$48 to the nucleolus.

The ability of TAF$_I$48 400–450 to target heterologous proteins to the nucleus and nucleolus was investigated by fusing it to the carboxyl-terminus of GFP (GFP-TAF$_I$48 400–450). When the distribution of GFP-TAF$_I$48 400–450 was examined in live HeLa cells, it was enriched in the nucleoli and displayed a weaker, but relatively homogeneous, distribution in the nucleoplam, similar to the distribution of HA3-TAF$_I$48 400–450 (compare Fig. 2, M–P to Fig. 4, G–I). When quantitated, 91% of the cells expressing this protein displayed this pattern (Table 1). Thus, the carboxyl-terminus of TAF$_I$48 is also sufficient to direct GFP to the nucleus and nucleolus.

*The TAF$_I$48 Carboxyl Terminus Binds to Multiple β-Karyopherins*—Nuclear import involves various members of the β-karyopherin family of nuclear import receptors (reviewed in Refs. *29* and *30*). In this process, either the β-karyopherin, or proteins associated with them, such as importin α, bind directly to the signal sequence. The specific import receptor(s) that bind depends upon the signal sequence present (reviewed in Ref. *8*). Because

some ribosomal subunits, such as rpL23a, are directed to the nucleolus and interact with multiple β-karyopherin family members, we decided to determine whether TAF$_I$48 has a similar ability to associate with multiple nuclear import receptors. The interaction of three β-karyopherins—importin β, transportin and Ran BP5—for TAF$_I$48 400–450 was examined in rabbit reticulocyte lysates (*31*) using "GST pull-down" experiments. Transportin was found to interact specifically with an affinity matrix displaying GST-TAF$_I$48 400–450 (residues 400–450 of TAF$_I$48 fused to the carboxyl-terminus of GST). Background levels were defined by the amount of transportin bound to an affinity matrix displaying an equivalent amount of GST (compare lanes 4–5 to 2–3 in Fig. 5A). Since the GTP-bound form of Ran typically promotes the release of cargo from karyopherins, the effect of Ran GTP was examined. Ran Q69L, a mutant version of Ran with greatly diminished ability to convert bound GTP to GDP (*32*), was utilized to obtain the GTP-bound species in a stable form. The addition of Ran Q69L GTP inhibited the ability of transportin to interact stably with the carboxyl-terminus of TAF$_I$48 (compare lanes 6–7 to 2–5 in Fig. 5A). In contrast, the addition of RanQ69L GDP had no effect (compare lanes 8–9 to 2–5 in Fig. 5A), most likely due to the high amounts of Ran GDP in the extracts (*30*). As controls for the interaction and the role of Ran GTP, the affinity of transportin for GST-RNPA1, which is the initial molecule shown to bind transportin (*13*), was tested. This experiment illustrates that both TAF$_I$48 and RNPA1 associate with transportin in a manner that is inhibited by Ran GTP (compare lanes 6–7 and 10 to lanes 4–5 and 8–9, respectively in Fig. 5B). However, less transportin associates with TAF$_I$48 than RNP A1 (compare lanes 4–5 and 8–9 in Fig. 5B).

Next, the ability of TAF$_I$48 400–450 to associate specifically with importin β and RanBP5 (other β-karyopherins) was examined and demonstrated to be specific (see Fig. 5, C and D). Importin β is associated with GST-TAF$_I$48 400–450, but not GST (compare lanes 8 and 5 in Fig. 5C), and the interaction is completely abolished by the presence of RanQ69L GTP; there is no affect of RanQ69L GDP (compare lanes 8–10 in Fig. 5C). Similarly, RanBP5 is associated with GST-TAF$_I$48 400–450 and not GST (compare lanes 5 and 8 in Fig. 5D). This interaction is entirely eliminated by RanQ69L GTP while RanQ69L GDP has only a small effect on the interaction between TAF$_I$48 and RanBP5 (compare lanes 8–10 in Fig. 5D).

The ability of a nuclear localization signal to bind multiple import receptors has been documented previously (*9*). In order to characterize further the association between TAF$_I$48 and these β-karyopherins, and to determine which complex forms initially, time-courses for each interaction were compared. Since the formation of a complex between TAF$_I$48 400–450 and each of the β-karyopherins could be observed at five min, there was little or no difference in the initial time of their formation. While the interaction between importin β and RanBP5 continued to increase at 10, 20 and 80 min of incubation, the

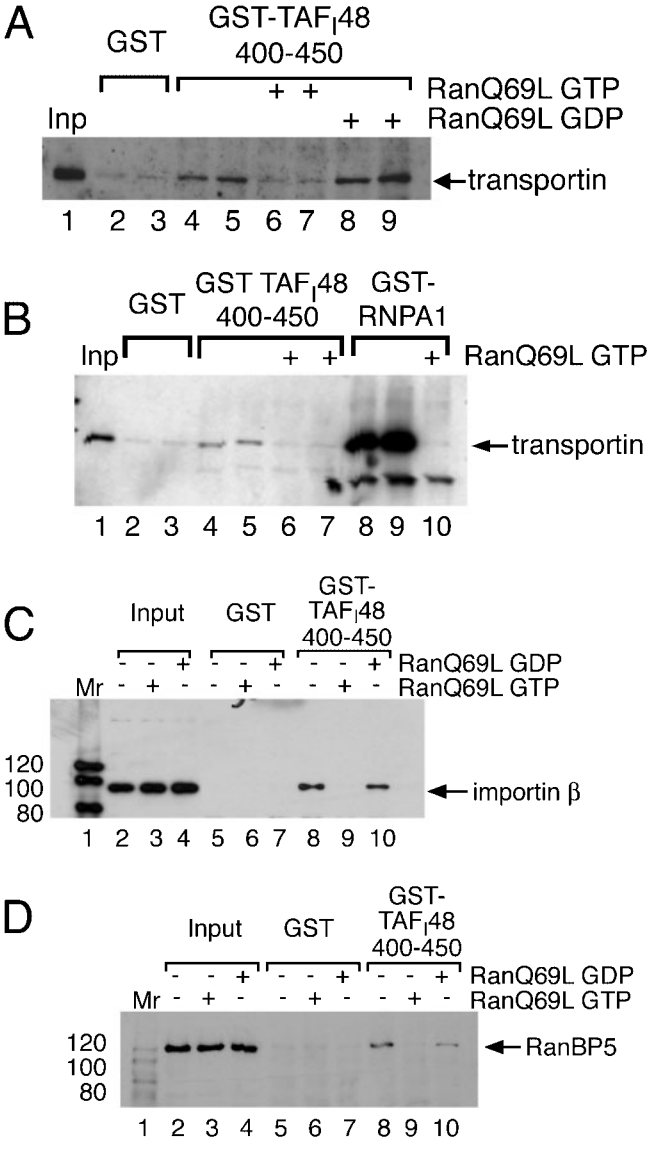


Fig. 5. **The carboxyl-terminus of TAF$_I$48 binds to transportin, RanBP5 and importin β in a Ran-dependent manner.** (A) TAF$_I$48 binds transportin. The fractions bound to GST (lanes 2–3) and GST-TAF$_I$48 400–450 (lanes 4–9) were assayed for transportin using an immunoblot. Some samples were pre-incubated with RanQ69L-GTP (lanes 6–7) or RanQ69L-GDP (lanes 8–9). Lane 1 contains 4% of the input. (B) Both TAF$_I$48 400–450 and RNP A1 associate with transportin in a Ran-dependent manner. The fractions bound to GST (lanes 2–3), GST-TAF$_I$48 400–450 (lanes 4–7) and GST-RNP A1 (lanes 8–10) were assayed for transportin using an immunoblot. Some samples were pre-incubated with RanQ69L-GTP (lanes 6, 7 and 10). Lane 1 contains 4% of the input. (C) TAF$_I$48 400–450 associates with importin β in a Ran-dependent manner. The fractions bound to GST (lanes 5–7) and GST-TAF$_I$48 400–450 (lanes 8–10) were assayed for importin β in an immunoblot. The samples were either untreated (lanes 5 and 8), pre-incubated with RanQ69L-GTP (lanes 6 and 9) or pre-incubated with RanQ69L-GDP (lanes 7 and 10). Lanes 2–4 contain 5% of the input from each respective reaction, and lane 1 contains molecular mass standards. (D) TAF$_I$48 400–450 associates with RanBP5 in a Ran-dependent manner. The fractions bound to GST (lanes 5–7) and GST-TAF$_I$48 400–450 (lanes 8–10) were assayed for RanBP5 in an immunoblot. The samples were either untreated (lanes 5 and 8), pre-incubated with RanQ69L-GTP (lanes 6 and 9) or pre-incubated with RanQ69L-GDP (lanes 7 and 10). Lanes 2–4 contain 5% of the input from each respective reaction and lane 1 contains molecular mass standards.

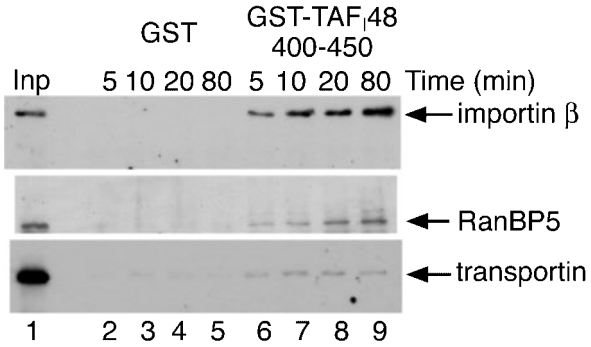


Fig. 6. **Kinetics of the association between TAF$_I$48 and three β-karyopherins.** The amounts of importin β (top panel), RanBP5 (middle panel), and transportin (bottom panel) associated with GST (lanes 2–5) and TAF$_I$48 400–450 (lanes 6–9) after 5, 10, 20 and 80 min of incubation were determined by immunoblotting. Lane 1 contains 5% of the input importin β and transportin, and 3% of the input RanBP5. The position of each β-karyopherin is denoted by an arrow.

association of transportin reached a plateau at 10 min (compare lanes 6–9 in each panel of Fig. 6). There was no association with GST during these reactions (see lanes 2–5 in each panel of Fig. 6).

## DISCUSSION

In this study, we examined the subcellular distribution of exogenously expressed TAF$_I$48 in interphase cells and the mechanisms that target it to the nucleus and nucleolus. To our knowledge, this is the first study of the pathways that direct Selectivity Factor 1 or its subunits to the nucleus and nucleolus. Exogenously expressed TAF$_I$48 was distributed in both the nucleolus and nucleoplasm, and the carboxyl-terminal 51 residues were found to be required for targeting HA-tagged TAF$_I$48 to the nucleus. This region was sufficient to direct transport to the nucleus and nucleolus when fused to a heterologous protein such as GFP. Furthermore, the carboxyl-terminal 51 residues also have the ability to associate with multiple nuclear import factors (transportin, RanBP5 and importin β), similar to some ribosomal proteins.

TAF$_I$48 possesses carboxyl-terminal sequences that are necessary and sufficient to direct transport into the nucleus. As an additional test of this activity, we examined the subcellular distribution of beta-galactosidase fused to either full-length TAF$_I$48 or residues 400–450 of TAF$_I$48. These fusion proteins were localized to the nucleus while beta-galactosidase alone was in the cytoplasm (Xu and Hori, unpublished observation). The carboxyl-terminus of TAF$_I$48 also mediates nucleolar localization. This domain possesses one or multiple sites that can interact with three different nuclear-import receptors. Taken together, the simplest explanation for these data is that at least one of these nuclear-import receptors associates with the carboxyl-terminus of newly synthesized TAF$_I$48 in the cytoplasm and mediates its import into the nucleus.

Because nuclear entry does not require the association of multiple import receptors, the association of TAF$_I$48 with a minimum of three different nuclear import receptors is somewhat surprising. However, other proteins have also been found to bind multiple karyopherins. The NLS of ribosomal protein rpL23a binds with similar affinity to importin β, transportin, RanBP5 and RanBP7, and each interaction can support nuclear import (*9*). There are other similarities between TAF$_I$48 and rpL23a: both proteins are transported to the nucleolus, and neither protein competes with RNP A1 for transportin binding [(*9*); (Xu and Hori, unpublished observation)]. In Fig. 7, the primary amino acid sequence of human TAF$_I$48 carboxyl-terminus, the beta-like importin binding domain of rpL23a (*9*), the monopartite nuclear localization signal of SV40 large T antigen (*33*), and the bipartite nuclear localization signal of nucleoplasmin (*34*) are compared. (Note: The internal basic residues of the nucleoplasmin bipartite NLS are not necessary for nuclear import.) The TAF$_I$48 and rpL23a sequences contain a more complex region of basic residues than these typical monopartitite and bipartite nuclear localization signals. This is similar to other nucleolar proteins that are also targeted by sequences that are more basic than classical nuclear localization signals, although there is no identified consensus sequence. In part, this may reflect that localization is stabilized by interaction with other components of the RNA polymerase I transcription machinery.

Another curious property of the TAF$_I$48 carboxyl-terminus is its relatively weak affinity for β-karyopherins. For example, the affinity of the TAF$_I$48 carboxyl-terminus for transportin is much lower than that of RNP A1 (Fig. 5B). However, localization signals with weaker affinities for β-karyopherins are functional. In one study, nuclear transport was similar between proteins containing a consensus transportin binding site and mutants with affinities as low as 2–7% of the consensus site (*35*). The ability to bind multiple β-karyopherins may help to compensate for weak affinities.

The steps involved in the genesis of Selectivity Factor 1 will depend to some degree on the properties of the individual SL1 subunits. Proteins containing full-length TAF$_I$48 were identified not only in the nucleolus, but also found at discrete positions throughout the nucleoplasm (Figs. 1 and 2). It is possible that this nucleoplasmic staining is an artifact of over-expression. Alternatively, since exogenous expression of TAF$_I$48 may significantly change its abundance relative to the other components of

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hTAF_I48          400-ACEKAFVAGLLLGKGCRYFRYILKQDHQILGKKIKRMKRSVKKYSIVNPRL-450
rpL23a             32-VHSHKKKKIRTSPTFRRPKTLRLRRQPKYPRKSAPRRNKLDHY-74
Monopartite NLS   126-PKKKRKV-132
Bipartite NLS     155-KRPAAIKKAGQAKKKK-170
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Fig. 7. **Comparison of human TAF$_I$48 400–450 with the beta-like import receptor binding site of human rpL23a, the monopartite NLS of SV40 large T antigen, and the bipartite NLS of *Xenopus* nucleoplasmin.** The basic residues are shown in bold. (Note: The two internal basic residues in the bipartite classical NLS are not necessary for nuclear import.)

Selectivity Factor 1, intermediate structures potentially involved in the assembly or sequestration of SL1 may be enriched. A recent study has demonstrated $TAF_I48$ and other components of the RNA polymerase I machinery are rapidly shuttled between the nucleoplasm and the sites of rRNA transcription in the nucleolus (*25*). While these studies examine the distribution of $TAF_I48$ and identify a nuclear and nucleolar signal sequence within it, additional characterization of $TAF_I63$ and $TAF_I110$ will facilitate the interpretation of these observations in the context of Selectivity Factor 1 assembly.

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REFERENCES

1. Comai, L., Zomerdijk, J.C., Beckmann, H., Zhou, S., Admon, A., and Tjian, R. (1994) Reconstitution of transcription factor SL1: exclusive binding of TBP by SL1 or TFIID subunits. *Science* **266**, 1966–1972
2. Paule, M.R. and White, R.J. (2000) Survey and summary: transcription by RNA polymerases I and III. *Nucleic Acids Res.* **28**, 1283–1298
3. Hannan, K.M., Hannan, R.D., and Rothblum, L.I. (1998) Transcription by RNA polymerase I. *Front Biosci.* **3**, 376–398
4. Grummt, I. (1999) Regulation of mammalian ribosomal gene transcription by RNA polymerase I. *Prog. Nucleic. Acid Res. Mol. Biol.* **62**, 109–154
5. Jacob, S.T. and Ghosh, A.K. (1999) Control of RNA polymerase I-directed transcription: recent trends. *J. Cell. Biochem. Suppl.* 41–50
6. Pemberton, L.F., Rosenblum, J.S., and Blobel, G. (1999) Nuclear import of the TATA-binding protein: mediation by the karyopherin Kap114p and a possible mechanism for intranuclear targeting. *J. Cell Biol.* **145**, 1407–1417
7. Koepp, D.M. and Silver, P.A. (1998) Nucleocytoplasmic transport and cell proliferation. *Biochim. Biophys. Acta* **1377**, M39–47
8. Jans, D.A., Xiao, C.Y., and Lam, M.H. (2000) Nuclear targeting signal recognition: a key control point in nuclear transport? *Bioessays* **22**, 532–544
9. Jakel, S. and Gorlich, D. (1998) Importin beta, transportin, RanBP5 and RanBP7 mediate nuclear import of ribosomal proteins in mammalian cells. *EMBO J.* **17**, 4491–4502
10. Schmidt, C., Lipsius, E., and Kruppa, J. (1995) Nuclear and nucleolar targeting of human ribosomal protein S6. *Mol. Biol. Cell* **6**, 1875–1885
11. Russo, G., Ricciardelli, G., and Pietropaolo, C. (1997) Different domains cooperate to target the human ribosomal L7a protein to the nucleus and to the nucleoli. *J. Biol. Chem.* **272**, 5229–5235
12. Gorlich, D., Vogel, F., Mills, A.D., Hartmann, E., and Laskey, R.A. (1995) Distinct functions for the two importin subunits in nuclear protein import. *Nature* **377**, 246–248
13. Pollard, V.W., Michael, W.M., Nakielny, S., Siomi, M.C., Wang, F., and Dreyfuss, G. (1996) A novel receptor-mediated nuclear protein import pathway. *Cell* **86**, 985–994
14. Adam, S.A. (1999) Transport pathways of macromolecules between the nucleus and the cytoplasm. *Curr. Opin. Cell Biol.* **11**, 402–406
15. Rosorius, O., Fries, B., Stauber, R.H., Hirschmann, N., Bevec, D., and Hauber, J. (2000) Human ribosomal protein L5 contains defined nuclear localization and export signals. *J. Biol. Chem.* **275**, 12061–12068
16. Das, A., Park, J.H., Hagen, C.B., and Parsons, M. (1998) Distinct domains of a nucleolar protein mediate protein kinase binding, interaction with nucleic acids and nucleolar localization. *J. Cell Sci.* **111**, 2615–2623
17. Creancier, L., Prats, H., Zanibellato, C., Amalric, F., and Bugler, B. (1993) Determination of the functional domains involved in nucleolar targeting of nucleolin. *Mol. Biol. Cell* **4**, 1239–1250
18. Yan, C. and Melese, T. (1993) Multiple regions of NSR1 are sufficient for accumulation of a fusion protein within the nucleolus. *J. Cell Biol.* **123**, 1081–1091
19. Stein, G.S., van Wijnen, A.J., Stein, J.L., Lian, J.B., Montecino, M., Choi, J., Zaidi, K., and Javed, A. (2000) Intranuclear trafficking of transcription factors: implications for biological control. *J. Cell Sci.* **113**, 2527–2533
20. Mattingly, R.R., Sorisky, A., Brann, M.R., and Macara, I.G. (1994) Muscarinic receptors transform NIH 3T3 cells through a Ras-dependent signalling pathway inhibited by the Ras-GTPase-activating protein SH3 domain. *Mol. Cell. Biol.* **14**, 7943–7952
21. Turner, D.L. and Weintraub, H. (1994) Expression of achaetescute homolog 3 in Xenopus embryos converts ectodermal cells to a neural fate *Genes Dev.* **8**, 1434–1447
22. Li, R. and Zheng, Y. (1997) Residues of the Rho family GTPases Rho and Cdc42 that specify sensitivity to Dbl-like guanine nucleotide exchange factors. *J. Biol. Chem.* **272**, 4671–4679
23. Roussel, P., Andre, C., Comai, L., and Hernandez-Verdun, D. (1996) The rDNA transcription machinery is assembled during mitosis in active NORs and absent in inactive NORs. *J. Cell Biol.* **133**, 235–246
24. Jordan, P., Mannervik, M., Tora, L., and Carmo-Fonseca, M. (1996) *In vivo* evidence that TATA-binding protein/SL1 colocalizes with UBF and RNA polymerase I when rRNA synthesis is either active or inactive. *J. Cell Biol.* **133**, 225–234
25. Dundr, M., Hoffmann-Rohrer, U., Hu, Q., Grummt, I., Rothblum, L.I., Phair, R.D., and Misteli, T. (2002) A kinetic framework for a mammalian RNA polymerase *in vivo*. *Science* **298**, 1623–1626
26. Aris, J.P. and Blobel, G. (1991) cDNA cloning and sequencing of human fibrillarin, a conserved nucleolar protein recognized by autoimmune antisera. *Proc. Natl Acad. Sci. USA* **88**, 931–935
27. Biggiogera, M., Malatesta, M., Abolhassani-Dadras, S., Amalric, F., Rothblum, L.I., and Fakan, S. (2001) Revealing the unseen: the organizer region of the nucleolus. *J. Cell Sci.* **114**, 3199–3205
28. Masson, C., Andre, C., Arnoult, J., Geraud, G., and Hernandez-Verdun, D. (1990) A 116, 000 Mr nucleolar antigen specific for the dense fibrillar component of the nucleoli. *J. Cell Sci.* **95**, 371–381
29. Gorlich, D. and Kutay, U. (1999) Transport between the cell nucleus and the cytoplasm. *Annu. Rev. Cell. Dev. Biol.* **15**, 607–660
30. Macara, I.G. (2001) Transport into and out of the nucleus. *Microbiol. Rev.* **65**, 570–594
31. Shah, S. and Forbes, D.J. (1998) Separate nuclear import pathways converge on the nucleoporin Nup153 and can be dissected with dominant-negative inhibitors. *Curr. Biol.* **8**, 1376–1386
32. Klebe, C., Bischoff, F.R., Ponstingl, H., and Wittinghofer, A. (1995) Interaction of the nuclear GTP-binding protein Ran with its regulatory proteins RCC1 and RanGAP1. *Biochemistry* **34**, 639–647

33. Kalderon, D., Roberts, B.L., Richardson, W.D., Smith, A.E., Robbins, J., Dilworth, S.M., Laskey, R.A., and Dingwall, C. (1984) A short amino acid sequence able to specify nuclear location: Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. *Cell* **39**, 499–509
34. Robbins, J., Dilworth, S.M., Laskey, R.A., and Dingwall, C. (1991) Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. *Cell* **64**, 615–623
35. Bogerd, H.P., Benson, R.E., Truant, R., Herold, A., Phingbodhipakkiya, M., and Cullen, B.R. (1999) Definition of a consensus transportin-specific nucleocytoplasmic transport signal. *J. Biol. Chem.* **274**, 9771–9777