Identification of the Nucleocytoplasmic Shuttling Sequence of Heterogeneous Nuclear Ribonucleoprotein D-like Protein JKTBP and Its Interaction with mRNA*

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JKTBP proteins are related to a family of heterogeneous nuclear ribonucleoproteins (hnRNPs) that function in mRNA biogenesis and mRNA metabolism. JKTBP proteins constitute of isoforms 1, 2, and 1αβl are localized in the nucleus. We show that the predominant form JKTBP1 shuttles between the nucleus and the cytoplasm and interacts with mRNA. Immunofluorescence microscopy and immunoblotting of the subcellular fractions and overexpression of JKTBP tagged with green fluorescent protein indicated that JKTBP1 and JKTBP1αβl, but not JKTBP2, accumulate in the cytoplasm upon polymerase II transcription inhibition. After release from inhibition, the return of accumulated cytoplasmic JKTBP to the nucleus was temperature-dependent. In heterokaryons, green fluorescent protein-tagged JKTBP1 and JKTBP1αβl migrated from the HeLa nucleus to the mouse nucleus, but JKTBP2 did not. Using various JKTBP deletion mutants, the 25-residue C-terminal tail was identified as a shuttling sequence like M9. It is conserved in the C-terminal tails of hnRNP D/AUF1 and type A/B hnRNP/ABBP-1. Analysis of its sequence-specific interacting protein indicated that JKTBP nuclear import is mediated by the receptor transportin 1/karyopherin β2. UV cross-linking revealed the increased occurrence of JKTBP1 directly interacting with poly(A)* RNA in the cytoplasm following actinomycin D treatment. We discuss a role of JKTBP in mRNA nuclear export.

Pre-mRNAs transcribed in the nucleus are processed by a variety of processes into mRNAs that are exported into the cytoplasm for translation. In these processes, including alternative splicing, mRNA nuclear export, translational regulation, and mRNA turnover, >20 different heterogeneous nuclear ribonucleoproteins (hnRNPs)† are associated with pre-mRNA and mRNA (1–3). hnRNPs are groups of proteins consisting of multiple RNA-binding domains, each containing conserved RNP-1 and RNP-2 or hnRNP K homology motifs, and of divergent amino- and carboxyl-terminal domains (1–3). Although they are primarily nuclear, hnRNPs A1, A2, D, E, I, and K shuttle between the nucleus and the cytoplasm, whereas hnRNPs C and U are always retained in the nucleus (4–6). The nuclear localization of hnRNPs A1 and A2 bearing the M9 shuttling sequence is mediated by the import receptor transportin 1 (Trn-1) and is polymerase II transcription-dependent (7–13). The nuclear localization of hnRNP C bearing a classical nuclear localization signal (NLS) is transcription-independent (14, 15). Differences in their subcellular movements are connected with their different roles in cells (2, 3, 15, 16). Cytoplasmic shuttling hnRNP A1 is strongly correlated with mRNA nuclear export, and nuclear persisting hnRNP C is associated with prevention of pre-mRNA from moving to the cytoplasm (5, 14, 15, 17).

We previously isolated JKTBP cDNAs by DNA affinity screening of human myeloid leukemia cDNA libraries using a cis-element (JKT41) in intron 9 of the human myeloperoxidase gene (18, 19). JKTBP proteins are composed of two RNA-binding domains arranged tandemly and a glycine- and tyrosine-rich carboxyl-terminal domain and are more closely related to hnRNP D/AUF1 and type A/B hnRNP/ABBP-1 and more distantly related to hnRNPs A1 and A2 (18, 19). There are three isoforms of JKTBP, 1 and 2 (major forms) and 1αβ (a minor form), which are abundant in HeLa and HL-60 cells and in tissues, especially in brains and testes (18–20). JKTBP is abundant in nuclei, but its nuclear localization pathway is not yet known (20). Recombinant JKTBP binds preferentially to poly(A) and poly(G) (18) and supposedly to an AU-rich element of the 3′-untranslated region of mRNA (21). This study reports that the predominant isoform JKTBP1 shuttles between the nucleus and the cytoplasm in a pathway consisting of a 25-residue shuttling sequence and Trn-1 and of the interaction of the cytoplasmic shuttling JKTBP with mRNA.

EXPEDIMENTAL PROCEDURES

Cell Cultures and Drug Treatments—HeLa cells were grown in RPMI 1640 medium at 37 °C as described (18). Balb/c 3T3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% calf bovine serum (Dusinippon Pharmaceutical) and 50 units/ml penicillin and 50 μg/ml streptomycin (Invitrogen). For inhibition of transcription and protein synthesis, HeLa cells were cultured with 5 μg/ml actinomycin D (Sigma) or 100 μg 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB; Calbiochem) for 3 h in the presence of 20 μg/ml cycloheximide (Sigma) (5).

DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; GST, glutathione S-transferase; DAPI, 4′,6-diamidino-2-phenylindole; EGFP, enhanced green fluorescent protein; GFP, green fluorescent protein.

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Indirect Immunofluorescence Microscopy—Approximately 10⁴ HeLa cells were seeded on 15-mm glass coverslips and grown for 1 day. The cells were then fixed with 4% paraformaldehyde in Mg²⁺- and Ca²⁺-free phosphate-buffered saline at 4 °C for 30 min. The cells were incubated with 0.5% Triton X-100 for 3 min. After preincubation with Mg²⁺- and Ca²⁺-free phosphate-buffered saline for 5 min, 2% normal goat serum, and 0.1% Triton X-100, the cells were incubated with 4000-fold diluted rabbit anti-GST-JKTBP1 serum in the same buffer overnight at 4 °C (19). The cells were incubated with 200-fold diluted goat anti-rabbit IgG (H + L)-biotin conjugate (Wako Pure Chemicals) in the same buffer and with 500-fold diluted streptavidin-Cy3 conjugate (Amersham Biosciences) for 30 min at room temperature. Protein concentrations were determined by the method of Bradford (25) with bovine serum albumin as a standard.

In Vivo Nuclear Import Assay and Heterokaryon Assay—HeLa cells (4 × 10⁴) suspended in 0.5 ml of RPMI 1640 medium were transfected with EGFFP-JKTBP plasmids (50 μg) by electroporation (200 V, 1180-microfarad capacitance, and low ohm in a Cell-Portar, Invitrogen). About 1 × 10⁴ cells were grown on a 15-mm glass coverslip, and the rest were grown for analysis of expression of EGFFP-JKTBP fusion proteins. For in vivo import assay, after 24 h, the subcellular distributions of EGFFP-JKTBP fusion proteins were studied by fluorescence microscopy. Heterokaryon assay was performed as described by Cáceres et al. (26). The above-cultured cells were overlaid with 8 × 10⁴ NIH3T3 fibroblasts and were cultured for 3 h. After that, 10 μl of 50% (v/v) polyethylene glycol 3400 (Polyscience) in RPMI 1640 medium for 2 min at 37 °C and, after washing, incubated in Dulbecco’s modified Eagle’s medium for 1 h in the presence of cycloheximide added to the culture medium 15 min before cell fusion. The cells were fixed and stained with 25 μg/ml Hoechst 33342 (Sigma) and studied by fluorescence microscopy. Fluorescent signals and cell images recorded with a cooled CCD camera (SenSys 1400, Photometrics Ltd.) were pseudo-colored.

Expression of EGFFP-JKTBP fusion proteins was checked by immuno- blotting using rabbit anti-GFP serum (CLONTECH).

In Vitro Nuclear Import Assay—HeLa cells on a 15-mm coverslip were permeabilized by treatment with 40 μg/ml digitonin transport buffer for 5 min as described by Adam et al. (27). The import reaction mixture (20 μl) containing 8 μl of rabbit reticulocyte lysate (Promega), 0.1 μg GST-JKTBP (226–420) as an import substrate with or without 3 μM competitor, and 5 μl of 4× transport buffer (80 mM HEPES (pH 7.3), 8 mM magnesium acetate, 20 mM sodium acetate, 440 mM potassium acetate, and 4 mM EGTA) containing 2 μg/ml each aprotinin, leupeptin, and pepstatin was overlaid on the cells and incubated at 30 °C for 30 min. After the reaction, the cells were fixed and probed with 4 μl of anti-GST IgG rabbit hybridoma IgG rabbit antibody (1:500) as an import substrate with or without 3 μM competitor, and 5 μl of 4× transport buffer (80 mM HEPES (pH 7.3), 8 mM magnesium acetate, 20 mM sodium acetate, 440 mM potassium acetate, and 4 mM EGTA) containing 2 μg/ml each aprotinin, leupeptin, and pepstatin was overlaid on the cells and incubated at 30 °C for 30 min. After the reaction, the cells were fixed and probed with 4 μl of anti-GST IgG rabbit antibody (1:500) as an import substrate with or without 3 μM competitor, and 5 μl of 4× transport buffer (80 mM HEPES (pH 7.3), 8 mM magnesium acetate, 20 mM sodium acetate, 440 mM potassium acetate, and 4 mM EGTA) containing 2 μg/ml each aprotinin, leupeptin, and pepstatin was overlaid on the cells and incubated at 30 °C for 30 min. After the reaction, the cells were fixed and probed with 4 μl of anti-GST IgG rabbit antibody (1:500) as an import substrate with or without 3 μM competitor, and 5 μl of 4× transport buffer (80 mM HEPES (pH 7.3), 8 mM magnesium acetate, 20 mM sodium acetate, 440 mM potassium acetate, and 4 mM EGTA) containing 2 μg/ml each aprotinin, leupeptin, and pepstatin was overlaid on the cells and incubated at 30 °C for 30 min. After the reaction, the cells were fixed and probed with 4 μl of anti-GST IgG rabbit antibody (1:500) as an import substrate with or without 3 μM competitor, and 5 μl of 4× transport buffer (80 mM HEPES (pH 7.3), 8 mM magnesium acetate, 20 mM sodium acetate, 440 mM potassium acetate, and 4 mM EGTA) containing 2 μg/ml each aprotinin, leupeptin, and pepstatin was overlaid on the cells and incubated at 30 °C for 30 min. After the reaction, the cells were fixed and probed with 4 μl of anti-GST IgG rabbit antibody (1:500) as an import substrate with or without 3 μM competitor, and 5 μl of 4× transport buffer (80 mM HEPES (pH 7.3), 8 mM magnesium acetate, 20 mM sodium acetate, 440 mM potassium acetate, and 4 mM EGTA) containing 2 μg/ml each aprotinin, leupeptin, and pepstatin was overlaid on the cells and incubated at 30 °C for 30 min. After the reaction, the cells were fixed and probed with 4 μl of anti-GST IgG rabbit antibody (1:500) as an import substrate with or without 3 μM competitor, and 5 μl of 4× transport buffer (80 mM HEPES (pH 7.3), 8 mM magnesium acetate, 20 mM sodium acetate, 440 mM potassium acetate, and 4 mM EGTA) containing 2 μg/ml each aprotinin, leupeptin, and pepstatin was overlaid on the cells and incubated at 30 °C for 30 min.
Fig. 1. Cytoplasmic accumulation of JKTBP in HeLa cells treated with RNA synthesis inhibitors. A, effect of actinomycin D on the subcellular localization of JKTBP. HeLa cells were treated with or without 5 μg/ml actinomycin D (Act. D) in the presence of 20 μg/ml cycloheximide for 3 h, and then the cells were fixed and stained with anti-JKTBP serum. Panels a and b, JKTBP localization in cells without and with actinomycin D, respectively; panels c and d, nuclear DNA stained with DAPI of the cells in the same views as in panels a and b, respectively. B, effect of DRB on the subcellular localization of JKTBP. HeLa cells were treated without or with 100 μM DRB as described for A (panels a and b, respectively). After removal of the DRB, the cell cultures were continued for another 1 h at either 37 or 4 °C, respectively; panels c and d, JKTBP localization in cells without and with DRB, respectively; panels e–h, nuclear DNA stained with DAPI of the cells in the same views as in panels a–d, respectively. C, effects of actinomycin D on the subcellular distributions of JKTBP1 and JKTBP2. HeLa cells treated as described for A were subcellularly fractionated into nuclear and cytosolic fractions. Protein blots loaded with 1 × 10⁶ cell eq samples of each fraction were probed with anti-JKTBP serum (upper panel) and reprobed with anti-hnRNP A1 monoclonal antibody 4B10 (middle panel) or reprobed with anti-hnRNPC monoclonal antibody 4F4 (lower panel). T, whole lysates; C, cytosolic fraction; N, nuclear fraction. Lanes 1–3 and 4–6, without and with actinomycin D, respectively. Molecular mass standards are shown on the left of the upper panel. Arrowheads on the right of the upper panel show the bands of 38-kDa JKTBP1 and 53-kDa JKTBP2. D, effects of DRB on the subcellular distributions of JKTBP1 and JKTBP2. HeLa cells were treated as described for B. JKTBP proteins in cytoplasmic and nuclear fractions were analyzed as described for C. Lanes 1–3 and 4–6, cells were treated without and with DRB, respectively. Lanes 7–9 and 10–12, after removal of DRB, the cells were cultured at 37 and 4 °C, respectively. The blots were exposed for 1 min (upper panel) or for 5 min (lower panel) to display JKTBP2 bands.

16,000 × g for 10 min. The resultant supernatant was adjusted to 0.5% SDS, 1% 2-mercaptoethanol, and 10 mm EDTA and called the nuclear fraction. Cytoplasmic and nuclear poly(A)⁺ RNAs were prepared by two repeats of oligo(dT)-cellulose column chromatography (0.18 g; Collaborative Research), and the precipitated RNAs were digested with a mixture of RNase A₁ (25 μg/ml) and RNase T₁ (0.25 μg/ml) at 37 °C for 30 min. The digests were analyzed by immunoblotting using anti-JKTBP serum.

RESULTS

Translocation of Nuclear JKTBP to the Cytoplasm—For examination of whether the nuclear localization of JKTBP is perturbed by RNA synthesis inhibition, HeLa cells were treated with or without actinomycin D for 3 h in the presence of cycloheximide and stained with anti-JKTBP serum. As shown in Fig. 1A, in the untreated cells, the JKTBP signal was mostly confined to the nucleus stained with DAPI (panel a), whereas in the treated cells, the JKTBP signal was found uniformly throughout the cells (panel b), indicating that RNA synthesis inhibition results in cytoplasmic accumulation of JKTBP. Next, we treated cells with a reversible inhibitor of RNA synthesis, DRB (Fig. 1B). Like actinomycin D, DRB also resulted in JKTBP accumulation in the cytoplasm (panels a and b). After removal of the drug by culturing cells at 37 °C for 1 h, the cytoplasmic JKTBP signal decreased, and the signal was found only in the nucleus, like that in the untreated cells, whereas at 4 °C, the cytoplasmic signal persisted (panels c and d). These results suggest that JKTBP shuttles between the cytoplasm and the nucleus.

To further examine the two JKTBP1 and JKTBP2 isoforms for subcellular localization in cells treated with or without actinomycin D, we prepared their cytoplasmic and nuclear fractions and analyzed them by immunoblotting using anti-JKTBP serum. On the blots of whole cell lysates, two major bands of ~53 kDa (JKTBP2) and 38 kDa (JKTBP1) and at least two minor bands of ~48 and 36 kDa were detected. No appreciable difference in the JKTBP contents between the untreated and treated cells was observed (Fig. 1C). In the untreated cells, the relative amounts of JKTBP1 recovered in the cytoplasmic and nuclear fractions were 25 and 75%, respectively (Fig. 1C, upper panel, lanes 1–3). In the treated cells, the percentage of JKTBP1 in the cytoplasmic fraction increased to 50% (lanes 4–6). Unlike JKTBP1, JKTBP2 was recovered only in the nuclear fractions from the untreated and treated cells. The minor proteins of 48 and 38 kDa became obscure in the subcellular fractionation. hnRNPs A₁ and C as controls for polymerase II transcription-dependent and -independent nuclear localization, respectively, were detected on the same blots. Upon actinomycin D treatment, hnRNP A₁ in the cytoplasm increased (middle panel), whereas hnRNP C was always recovered only...
in the nuclear fraction of the untreated and treated cells (lower panel), as reported previously (5). These results indicated that the increased amount of cytoplasmic JKTBP1 was not due to leakage from the nucleus. In the DRB-treated cells, cytoplasmic JKTBP increased compared with that in the untreated cells (Fig. 1D, lanes 1–6). Upon deprivation of the drug, the increased cytoplasmic JKTBP1 decreased to a low level at 37°C, but not at 4°C (lanes 8–12). This indicated that the accumulated cytoplasmic JKTBP1 was re-imported into the nucleus.

**Differential Effects of Actinomycin D on Nuclear Localization of the Three JKTBP Isoforms**—The three JKTBP isoforms are depicted in Fig. 2A. JKTBP1 and JKTBP2 have been characterized previously (18, 19), and JKTBP1Δ6 was characterized previously (18, 19), and JKTBP1 depicted in Fig. 2 of the Three JKTBP Isoforms accumulated cytoplasmic JKTBP1 was re-imported into the nucleus before and after treatment (Fig. 2, C and D, panel a). The signals of EGFP-tagged JKTBP1 and JKTBP1Δ6 were confined solely to the nucleus in the untreated cells (Fig. 2C, panels c and d), whereas following actinomycin D treatment, the JKTBP1 and JKTBP1Δ6 signals appeared to be distributed evenly over the cells (Fig. 2D, panels c and d). The JKTBP2 signal was always retained in the nucleus before and after treatment (Fig. 2, C and D, panel b). These results indicated that JKTBP1 and JKTBP1Δ6 differed in nucleocytoplasmic movement from JKTBP2.

**Identification of Nuclear Localization and Nuclear Export Signals of JKTBP Proteins**—First, we searched for potential NLSs of JKTBP by sequence homology. Sequence homologous to neither basic type NLSs nor the nucleocytoplasmic shuttling sequence of M9 was present in JKTBP. To delineate an NLS sequence of JKTBP, N- and C-terminal deletion mutants were prepared by PCR with parent JKTBP cDNAs as a template. All of the mutants are represented according to JKTBP2 number—signals lacking the exon 6-encoded 57-amino acid sequence indicated a.a. (Fig. 3A). The mutant cDNAs were fused to the 3′-end of an EGFP-GST gene encoding an EGFP-GST fusion protein. In these constructs, a tripartite fusion gene of EGFP, GST, and mutant genes rather than a bipartite fusion gene of EGFP and mutant genes was constructed because the smallest mutant gene examined encoded a 23-residue peptide. These constructs were used to transfect HeLa cells and were expressed for 22 h. Expression of all EGFP-GST mutant fusion proteins with the expected sizes was confirmed by immunoblotting using anti-GFP serum (Fig. 3B). In parallel cultures, the subcellular localization of mutant proteins was studied by fluorescence microscopy. Panels a–d, fluorescent signals of cells transfected with the plasmids shown at the top of each panel; panels e–h, nuclear DNA stained with DAPI of the cells in the same views as in a–d, respectively. D, expression of three EGFP-JKTBP fusion proteins. HeLa cells transfected with plasmids carrying EGFP-JKTBP fusion genes were cultured for 22 h. Blots loaded with 10 μg of protein were probed with anti-GFP serum. Lane 1, pEGFP-C vector; lane 2, JKTBP2; lane 3, JKTBP1; lane 4, JKTBP1Δ6.

**Fig. 2. Differential effects of actinomycin D on the subcellular localization of the three EGFP-tagged JKTBP isoforms.** A, structural relationship among isoforms. JKTBP is identical to amino acids (a.a.) 120–420 of JKTBP2. JKTBP1Δ6 is identical to the JKTBP1 sequence lacking the exon 6-encoded 57-amino acid sequence indicated by the wavy thin line. RBD, RNA-binding domain. B, expression of three EGFP-JKTBP fusion proteins. HeLa cells transfected with plasmids carrying EGFP-GST-JKTBP fusion genes were cultured for 22 h. Blots loaded with 10 μg of protein were probed with anti-GFP serum. Lane 1, pEGFP-C vector; lane 2, JKTBP2; lane 3, JKTBP1; lane 4, JKTBP1Δ6. C, subcellular localization of EGFP-JKTBP proteins. HeLa cells were studied by fluorescence microscopy. Panels a–d, fluorescent signals of cells transfected with the plasmids shown at the top of each panel; panels e–h, nuclear DNA stained with DAPI of the cells in the same views as in panels a–d, respectively.
nuclei (Fig. 4, 6 were found in both the HeLa and mouse/H9004 persisted in the HeLa nucleus (Fig. 4 mate outlines of the heterokaryon). Like hnRNP C, JKTBP2 lane 3 341; pEGFP-GST vector; Lane 1 immunoblotting using anti-EGFP serum. bearing a JKTBP deletion mutant cDNA cells transfected with plasmid constructs determined. A EGFP-tagged JKTBP deletion mu- nters. AEGFP-GST, and their amino acid num- ration mutants. Mutants are represented as a protein fused to a C-terminal end in EGFP-GST, and was weakly homologous to M9 (see Fig. 6 mutation. Of the deletion mutants examined, represent- JKTBP-(398 – 420), JKTBP-(323 – 420), JKTBP-(396 – 420), and JKTBP-(398 – 420) were all found in both HeLa and mouse nuclei (Fig. 3A). Of the deletion mutants examined, representa- tive JKTBP-(396 – 420) and JKTBP-(398 – 420) are shown in Fig. 4 e and f). JKTBP-(396 – 420) migrated between the two nuclei in all heterokaryons examined, but JKTBP-(398 – 420) migrated in only 40% of the heterokaryons examined. These results indicate that a 25-residue C-terminal tail is necessary and sufficient for nuclear import and export of JKTBP1 and JKTBP1Δ6. Although JKTBP2 has the same C-terminal sequence as JKTBP1, it did not shuttle between the nucleus and the cytoplasm. The 25-residue shuttling sequence 396 – 420 contains 5 glycine, 4 glutamine, 3 asparagine, 4 tyrosine, and 3 serine residues. This carboxy-terminal tail sequence was 72 and 60% identical to the 24-residue carboxy-terminal tails of hnRNP DAU1 and type A/B hnRNP/ABBP-1, respectively, and was weakly homologous to M9 (see Fig. 6C).

Interaction between JKTBP and Trn-1—First, we examined whether JKTBP and hnRNP A1 can compete for in vitro nuclear import into digitonin-permeabilized HeLa cells. The cells supplemented with reticulocyte lysate were incubated at 30 °C for 30 min with the GST-JKTBP-(226 – 420) fusion protein as a transport substrate in the presence or absence of 30-fold molar excess of hnRNP A1 or other competitors. The nuclear localization of GST-JKTBP-(226 – 420) was detected by anti-GST IgG staining. In the absence of competitor, its incorpora-
TBP2, was UV-cross-linked to the cytoplasmic RNA (lane 3), confluent cells, a measurable amount of JKTBP1, but not JK-serum. As shown in Fig. 7, in the actinomycin D-treated TBP proteins in the digests were detected using anti-JKTBP localization of GST-JKTBP1-(226–30° in the same views as in aabilized HeLa cells were incubated with 0.1 mM GST-JKTBP1-(226–420) by hnRNP A1 in digitonin-permeabilized HeLa cells. A mixture (20 of the competitors indicated at the top of each panel in a nuclear import 420) supplemented with reticulocyte lysates for 30 min at 30 °C, and the cells were stained with anti-GST IgG. a–d, nuclear localization of GST-JKTBP1-(226–420); e–h, nuclei stained with DAPI in the same views as in a–d, respectively.

binding of JKTBP1 to Poly(A)+ RNA—To understand the role of nucleocytoplasmic shuttling of JKTBP in cells, we studied the in vivo association of JKTBP with poly(A)+ RNA by UV cross-linking. Confluent and sparse HeLa cells were treated with or without actinomycin D in the presence of cycloheximide. a–f, fluorescent signals from heterokaryons; m–r, images of heterokaryons treated with DAPI; l, images of heterokaryons merged upon nuclear staining (approximate outlines are shown by dashed lines). A heterokaryon in a column is in the same view.

JKTBP extracts (6 mg of proteins in 0.5 ml) were incubated with 20 µl of GST- or GST-JKTBP-bound glutathione-Sepharose beads at 4 °C for 4 h. Bound proteins were analyzed by immunoblotting. A, blots stained with Amido Black 10B; B, blots probed with monoclonal antibody to JKTBP. Lane 1, 10 µg of whole cell lysates; lanes 2–12, GST- or GST-JKTBP fusion proteins interacting with protein isolated from 450 µg of whole cell lysates. Lane 2, GST-JKTBP; lane 3, GST-JKTBP1; lane 4, GST-JKTBP1Δ6; lane 5, GST-JKTBP-(226–420); lane 6, GST-JKTBP-(227–398); lane 7, GST-JKTBP-(227–341); lane 8, GST-JKTBP-(396–420); lane 9, GST; lane 10, GST; lane 11, GST-JKTBP-(396–420); lane 12, GST-JKTBP-(398–420). Lanes 1–9 and 10–12 were prepared separately. C, alignment of the JKTBP nucleocytoplasmic shuttle sequence with other hnRNP proteins. The carboxyl-terminal 25 residues of JKTBP1 are aligned with the carboxyl-terminal tails of the indicated hnRNPs, a 38-amino acid sequence of M9, and a consensus transportin interaction motif in M9 (36). The asterisk indicates the amino acid substitution of Gly with Ala.

JKTBP was significantly UV-cross-linked to the nuclear RNA, but not at all to the cytoplasmic RNA (lanes 1, 2, and 5). The amount of poly(A)+ RNA-cross-linked JKTBP1 in the cytoplasm of sparse cells was about twice that in the cytoplasm of the confluent cells, suggesting a difference in the poly(A)+ RNA contents in the two cultures. These results indicate that actinomycin D-induced cytoplasmic shuttling JKTBP1 is associated with poly(A)+ RNA.
Discussion

This work presents evidence that the predominant isoform JKTB1 shuttles between the nucleus and the cytoplasm and that the shuttling protein can interact directly with mRNA. Shuttling JKTB1 was demonstrated in two ways: polymerase II transcription inhibition and heterokaryon formation. Actinomycin D or DRB induced marked translocation of nuclear JKTB from the nucleus to the cytoplasm in the presence of new protein synthesis. This cytoplasmic accumulation could account for the decreased nuclear import caused by transcription inhibition, as found with hnRNP A1 and other pre-mRNA- and mRNA-binding proteins (4, 12, 26). Nuclear protein import in cells is regulated by post-translational modifications such as methylation and phosphorylation of the nuclear import machinery and import proteins, which may be sensitive to transcription inhibition (31–33). However, the mechanism of the transcription-dependent nuclear import is still unclear. Non-shuttling JKTB2, unlike the JKTB1 and JKTB1Δα sequences, has a 119-residue N-terminal extension that has no homology to nuclear retention sequences of hnRNP C and D (14, 34). Its nuclear retention mechanism remains unknown. The findings obtained by transcription inhibition and with heterokaryons indicate that JKTB1 and JKTB1Δα, but not JKTB2, shuttle between the nucleus and the cytoplasm. Such an isoform-specific nucleocytoplasmic movement may be associated with different roles in cells, as known for other hnRNPs with multiple functions in the nucleus and cytoplasm (2, 3, 15, 16).

Nuclear localization of hnRNP A1 bearing the 38-residue shuttling sequence M9 is mediated by the import receptor Trn-1, and the activities of the NLS and nuclear export signal of M9 are not separated (7, 9, 10, 15, 35). Analysis of JKTB deletion mutants for subcellular localization and their heterokaryon assays indicated that the 25-residue carboxy-terminal sequence has both NLS and nuclear export signal activities, indicating that it is a shuttling sequence. In this sequence, the amino-terminal 15-residue sequence (positions 396–410) is partly homologous to the amino-terminal portion in M9 and also to a 12-residue consensus transportin interaction motif in M9 (Fig. 6C) (35). Single amino acid substitution of JKTB (G404A) revealed a weak but significant reduction of its nuclear import. This seems similar to a characteristic of M9, although a single mutation of M9 (G274A) almost completely abolished the both the NLS and nuclear export signal activities (9). These 25- and 23-residue NLS sequences of JKTB interacted with Trn-1. Moreover, JKTB1 could compete for Trn-1 with hnRNP A1, but not with UP1 lacking M9. These findings give convincing evidence that the nuclear localization of JKTB is mediated by an M9-transportin pathway. The JKTB shuttling sequence is highly homologous to the carboxy-terminal tails of nucleocytoplasmic shuttling hnRNP D/AUF1 and ABBP-1, a nuclear component of apoB mRNA-editing complexes (36). This suggests that their carboxy-terminal tails are shuttling sequences. JKTB1 and its N-terminal deletion mutants, when tagged at the carboxy-terminal end with EGFP, were partially or severely hindered for in vivo nuclear localization.2 This suggests that the position of a shuttling sequence in molecules is important for interaction with the Trn-1 molecule whose carboxy-terminal domain is involved in their interaction (10, 13). Examination of the structural relationship between Trn-1 and M9 as revealed by structural studies of Trn-1/karyopherin β2 complexed with Ran-GTP (13) will be required for precise understanding.

The occurrence of shuttling JKTB suggested that a possible role of nuclear JKTB is in mRNA nuclear export. UV cross-linking of JKTB1 to poly(A)+ RNA showed that, under the transcription inhibition conditions resulting in JKTB1 cytoplasmic accumulation, the JKTB1-poly(A)+ RNA complex was found at higher levels in the cytoplasm than in the nucleus, whereas under transcriptional conditions, the JKTB1-poly(A)+ RNA complex was found in the nucleus, but not in the cytoplasm. These results suggest that shuttling JKTB1 may carry mRNA from the nucleus to the cytoplasm. The estimated amount of the cytoplasmic JKTB1-poly(A)+ RNA complex was far lower than the amount of cytoplasmic JKTB1. This may primarily account for the low efficiency of UV cross-linking. The cytoplasmic accumulation of the JKTB1-poly(A)+ RNA complex could be due to either increased stability of mRNAs or decreased nuclear re-import of JKTB1, or both. In addition to hnRNP A1, the shuttling mRNA-binding proteins TAP, Aly, Y14, SRp20, and 9G8 have been shown to be involved in mRNA nuclear export (37–41). To understand the meaning of JKTB1-poly(A)+ RNA association, further studies on JKTB1-RNA sequence specificity, compositions of JKTB1-mRNA complexes, and relationships to messenger ribonucleoprotein and hnRNP complexes are needed. In addition to nuclear export, various roles of cytoplasmic hnRNPs in translational regulation, mRNA stability, and local localization of mRNA have been uncovered (16, 42–44). A possible role of JKTB1 in the cytoplasm also needs to be studied. JKTB1 shown as a shuttling mRNA-binding protein is a candidate for mRNA nuclear export in cells.

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Identification of the Nucleocytoplasmic Shuttling Sequence of Heterogeneous Nuclear Ribonucleoprotein D-like Protein JKTBP and Its Interaction with mRNA
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