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An amino acid sequence motif sufficient for subnuclear localization of an arginine/serine-rich splicing factor

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ABSTRACT **We have identified an amino acid sequence in the *Drosophila* Transformer (Tra) protein that is capable of directing a heterologous protein to nuclear speckles, regions of the nucleus previously shown to contain high concentrations of spliceosomal small nuclear RNAs and splicing factors. This sequence contains a nucleoplasmin-like bipartite nuclear localization signal (NLS) and a repeating arginine/serine (RS) dipeptide sequence adjacent to a short stretch of basic amino acids. Sequence comparisons from a number of other splicing factors that colocalize to nuclear speckles reveal the presence of one or more copies of this motif. We propose a two-step subnuclear localization mechanism for splicing factors. The first step is transport across the nuclear envelope via the nucleoplasmin-like NLS, while the second step is association with components in the speckled domain via the RS dipeptide sequence.**

Eukaryotic pre-mRNA splicing takes place in spliceosomes, multicomponent nuclear complexes containing the small nuclear ribonucleoproteins (snRNPs) U1, U2, U4, U5, and U6, and a large number of splicing factors (for reviews, see refs. 1 and 2). Evidence that splicing components are nonrandomly distributed in the nucleus was first provided by intranuclear localization studies, which showed that snRNPs are preferentially associated with nuclear structures designated as perichromatin fibrils, interchromatin granules, and coiled bodies (3–6). These associations can be visualized in the light microscope by immunofluorescent staining and by *in situ* hybridization methods. The interchromatin granule regions appear as 20–50 heavily stained regions referred to as nuclear "speckles" (refs. 7 and 8; see ref. 9 for review).

The first indication that non-snRNP essential splicing factors are also associated with nuclear speckles was provided by immunofluorescent labeling studies with a monoclonal antibody (mAb) directed against the splicing factor SC35 (10). The SC35 protein (11) is a member of the SR family of general splicing factors (12), a group of proteins that also includes the splicing factor SF2/ASF (13–16). All of the proteins in this family are recognized by Mab104, and they contain one or more RNP binding domains and a region rich in arginine/serine dipeptides (RS domain; see ref. 12). Significantly, all of the SR family members examined to date colocalize with SC35 to nuclear speckles (10, 12, 17).

RS domains are also present in the *Drosophila* alternative splicing factors suppressor-of-white-apricot [Su(w^a)], Transformer (Tra), and Transformer 2 (Tra2), and these proteins localize to speckle domains in mammalian cells (ref. 17; this paper). In fact, the RS-rich domain is the only known sequence that is shared by all of the splicing factors that localize to speckles. Evidence that this domain may be involved in nuclear and/or subnuclear localization of splicing factors was provided by the observation that a 100-aa sequence of Tra, which includes the RS domain, is sufficient to direct a heterologous protein to the speckle domains (17). However, this study did not distinguish between sequence requirements for nuclear and subnuclear localization, nor did it define the minimal sequence necessary and sufficient for localization to the speckle domains.

To address these issues, we have identified the amino acid sequences required to direct the Tra protein to nuclear speckles. We find that Tra nuclear localization can be uncoupled from localization to the speckle domains. In addition, we show that a sequence that is necessary and sufficient for localization to the speckle domains contains a nucleoplasmin-type nuclear localization signal (NLS) and a stretch of basic residues adjacent to at least three contiguous RS dipeptides.

EXPERIMENTAL PROCEDURES

Cloning Procedures. The myc tag, MEQKLISEEDLN, was cloned in-frame in front of the Tra female-specific cDNA. All deletions were generated from this parent plasmid and subsequently cloned into the pXM vector for expression in mammalian cells. Tra2 constructs were generated by cloning the female-specific cDNA into the pXM vector either with or without the myc tag.

Transfections. COS and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). Plasmid DNA was transfected into COS cells using DEAE-dextran as described (18). Between 10 and 12 hr posttransfection, cells were fixed with 3% formaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature and subsequently permeabilized with 0.5% Triton X-100 for 10 min at room temperature.

Immunofluorescence. Cells were incubated in blocking buffer (PBS containing 20% FCS and 0.05% Tween 20) for 15 min. Blocking buffer was replaced with biotinylated anti-myc tag antibody, 9E10 (19), and the cells were incubated for 30 min. After a 15-min wash in PBS, fluorescein isothiocyanate (FITC)-conjugated streptavidin or FITC-conjugated goat anti-mouse (GAM) IgG was added, and incubation continued for 30 min at room temperature. Cells were washed and mounted with 70% (vol/vol) glycerol/2.5% 1,4-diazabicyclo[2.2.2]-octane. They were photographed with a ×64 objective, using Ektachrome 400 HC daylight film. For double staining, the cells were incubated sequentially with the following antibodies and washed after each incubation as indicated above: (*i*) 1:300 dilution of purified SC35 antibody (1 mg/ml) or culture supernatant containing 4D9 (a Tra2-specific mAb), (*ii*) rhodamine-conjugated GAM IgG (Cappel Laboratories), (*iii*) biotinylated 9E10, and (*iv*) FITC-conjugated streptavidin. When antibody in *i* was omitted, no red staining was observed, thus demonstrating that when this procedure

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Abbreviations: snRNP, small nuclear ribonucleoprotein; mAb, monoclonal antibody; NLS, nuclear localization signal; FITC, fluorescein isothiocyanate; GAM, goat anti-mouse; β-gal, β-galactosidase.

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was followed, the secondary rhodamine-conjugated GAM antibody did not react with the tertiary 9E10 antibody.

RESULTS

Tra and Tra2 Colocalize with SC35 in Mammalian Cell Nuclei. Previous studies have shown that the Tra protein localizes to nuclear speckles in mammalian cells, but direct colocalization with general splicing components was not demonstrated (17). We have found that overexpression of Tra can lead to formation of aggregates that are difficult to distinguish from normal speckle domains. Thus, in order to assay the effects of Tra mutations on nuclear localization it was necessary to analyze transfected cells no more than 12 hr posttransfection and to show that, in these cells, the Tra protein colocalizes with an endogenous splicing component, such as SC35. This was accomplished by introducing a myc epitope tag into the recombinant Tra protein and then carrying out double immunofluorescent staining with a mAb specific for the myc tag (9E10) and an anti-SC35 mAb. As shown in Fig. 1*A*, column 1, the myc-Tra protein displays a nuclear speckled staining pattern in COS cells transfected with the myc-Tra expression vector. Staining was not observed when the cells were transfected with vector alone (data not shown). Double staining of the transfected cells with a biotinylated 9E10 antibody and anti-SC35 mAb revealed that myc-Tra colocalizes with SC35 in nuclear speckles (Fig. 1*A*, column 1). Similar results were obtained in HeLa cells, where the transfected vector is not replicated to a high copy number (data not shown).

We also showed that the *Drosophila* splicing regulator Tra2 containing the myc epitope tag colocalizes with SC35 in mammalian cells (Fig. 1*A*, column 2). Because Tra and Tra2 localize to regions containing SC35 in mammalian cells, presumably they colocalize with each other. This prediction was confirmed by transfecting cells with cDNAs encoding myc-Tra and untagged Tra2 and double staining with anti-Tra2 mAb (4D9) and biotinylated 9E10 (Fig. 1*A*, column 3). We conclude that *Drosophila* splicing regulators Tra and Tra2, and the mammalian splicing factor SC35, colocalize in the speckle domains in mammalian cells.

Identification of the Tra Subnuclear Localization Signal. In a previous study a region between residues 19 and 119 of Tra was shown to direct a β-gal fusion protein to the nuclear speckles (17). To delineate more precisely the localization signal, we created a series of myc-Tra deletion mutants, transfected them into COS cells, and localized the mutant proteins by immunofluorescence staining. Localization to the speckle domains was scored positive only when the staining pattern of the Tra deletion mutant colocalized with that of endogenous SC35.

Deletion of the first 122 N-terminal residues of Tra results in production of a 75-aa C-terminal fragment that is distributed throughout the cytoplasm and nucleus (Δ4; Figs. 1*B* and 2*A*). A smaller deletion of the first 92 N-terminal amino acid residues creates a protein with a nuclear staining pattern, but this protein also fails to localize to nuclear speckles (Δ15; Figs. 1*B* and 2*A*). Finally, Δ16, which contains 9 additional residues (SRHRRHRQR; Fig. 3), 7 of which are basic, localizes to nuclear speckles (Δ16; Figs. 1*B* and 2*A*). Thus, aa 84–122 contain both a NLS and a motif required for subnuclear localization of Tra to speckle domains. Both signals are localized more precisely to the region aa 84–112 since Δ9, which lacks aa 113–197, localizes to the speckle domains (Δ9; Figs. 1*B* and 2*A*).

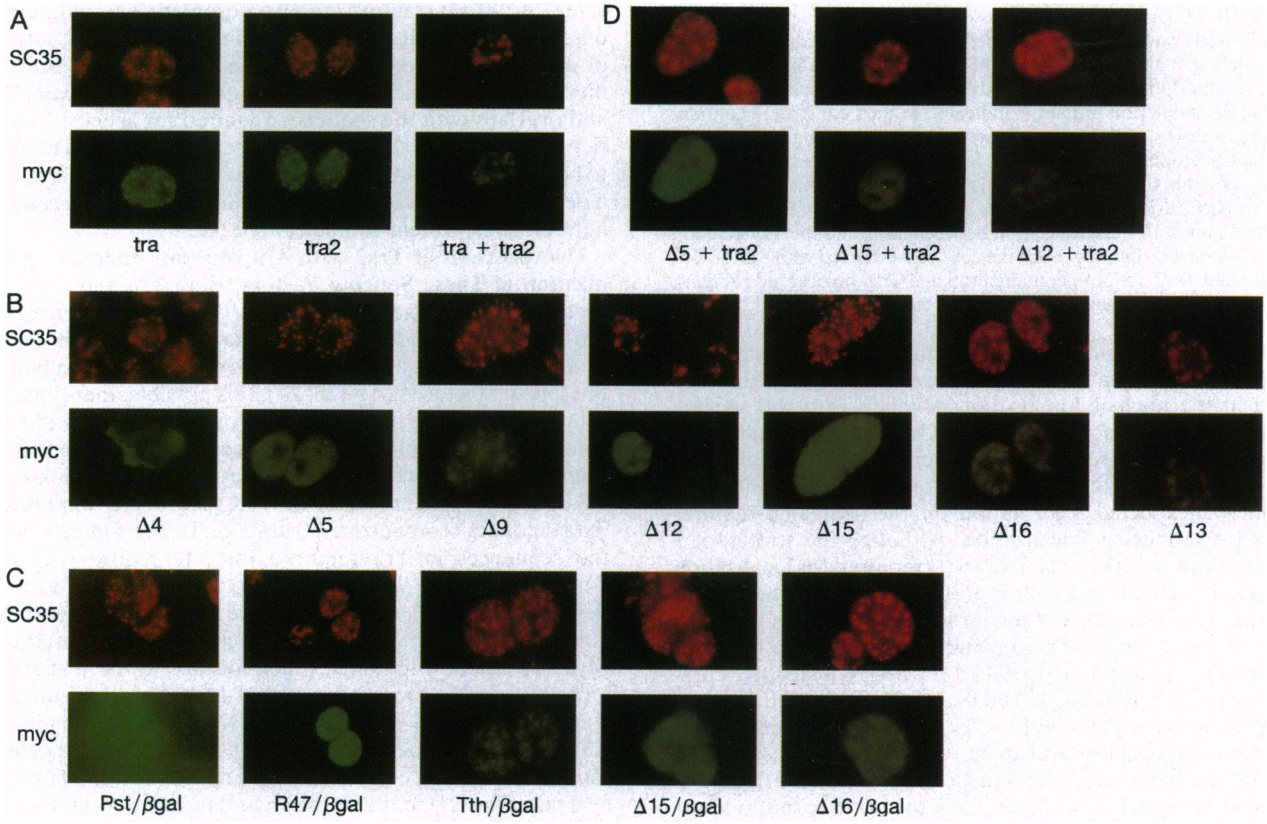

FIG. 1. Colocalization of splicing factors. (*A*) Columns 1 and 2, myc-Tra or myc-Tra2 transfected cells were stained sequentially with SC35, rhodamine-conjugated GAM, biotinylated 9E10, and FITC-conjugated streptavidin antibodies. Column 3, cells cotransfected with myc-Tra and Tra2 were stained sequentially with 4D9, rhodamine-conjugated GAM, biotinylated 9E10, and FITC-conjugated streptavidin antibodies. In this column, red staining indicates Tra2 and green staining represents Tra. (*B*) Cells were transfected with various myc-Tra deletions and double stained as in *A* (columns 1 and 2). (*C*) Cells were transfected with various myc-Tra/β-galactosidase (β-gal) fusion proteins and double stained as in *A* (columns 1 and 2). (*D*) Cells cotransfected with Tra2 and the indicated myc-Tra deletions were double stained as in *A* (column 3).

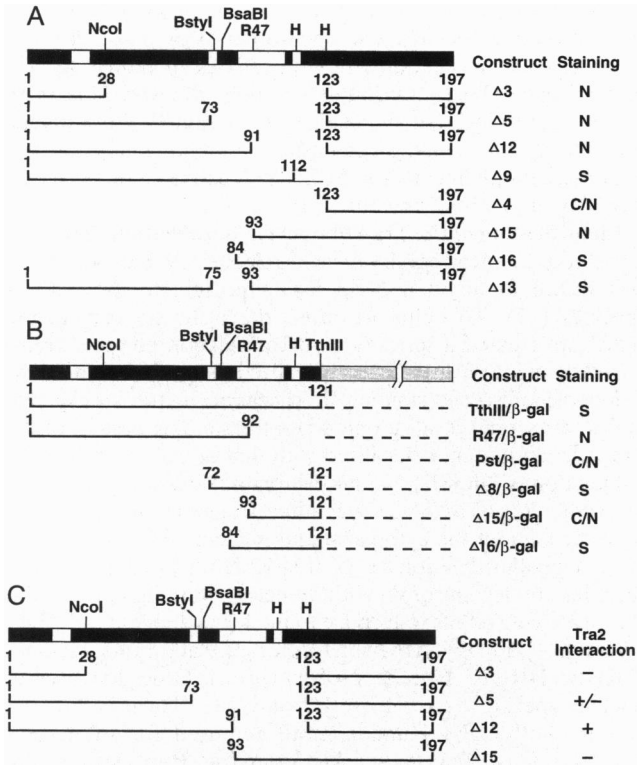

FIG. 2. Schematics of Tra mutants. The Tra coding sequence is indicated by a solid box, and regions containing at least two contiguous RS or SR dipeptides are indicated by an open box. Various restriction sites used to construct the deletions are indicated above the box (R47, *Eco*R47III; H, *Hin*cII). The regions of Tra present in each construct are indicated by a line. (*A*) Δ9 includes 25 additional residues not found in Tra before a stop codon is reached, indicated here by a thin line. These residues do not resemble a NLS or other sequences in Tra. The name of each deletion mutant is indicated in the "construct" column, and the staining pattern is indicated in the "staining" column as follows: C, cytoplasmic; N, nuclear; S, speckled. (*B*) Addition of β-gal coding sequences is indicated by a dashed line and a shaded box. The name of each fusion protein and the staining pattern are indicated as described above. (*C*) Colocalization between Tra2 and the Tra deletion mutant is indicated by + or −. Of the cells cotransfected with a given Tra deletion and Tra2, 25–50% contained Δ5 and Tra2 colocalized proteins, whereas 75–90% contained Δ12 and Tra2 colocalized proteins.

Three internal deletions that are missing most or all of the residues between aa 84 and 112 result in proteins that are found in the nucleus but do not associate with the speckle domains (Δ3, data not shown; Δ5 and Δ12, Figs. 1*B* and 2*A*). Comparison of the sequences between an additional internal deletion (Δ13) with the N-terminal truncations in Δ15 and Δ16 provides unexpected information on the sequence requirements for subnuclear localization. Although the stretch of 9 residues (aa 84–92) upstream of aa 93 required for localization to speckles is absent in Δ13, this protein localizes to the speckle domains. Interestingly, and similar to Δ16, construction of Δ13 brings an upstream basic region and a nucleoplasmin-like NLS immediately adjacent to aa 93 (Δ16, SRHRRHRQR, compared with Δ13, RRLRQRAHQSTRRTRSRS), and the mutant protein colocalizes with SC35 in the speckle domain.

Three main conclusions can be reached on the basis of this deletion analysis. First, the Tra protein contains a NLS between aa 93 and 122 (compare Δ15 to Δ4). Second, nuclear localization is necessary but not sufficient for directing Tra deletion mutants to the speckle domains (Δ3, Δ5, Δ12, and Δ15). Third, the minimal speckle localization signal appears to be a stretch of basic amino acids adjacent to a series of RS dipeptides (Δ13 and Δ16). In the case of Δ13, the basic amino acids normally present upstream of the RS region (aa 84–92) are deleted and fortuitously replaced by basic amino acids upstream from aa 75.

The Tra Speckle Localization Signal Is Sufficient for Subnuclear Localization of a Heterologous Protein. To identify the minimal Tra sequence sufficient for speckle localization, segments of the Tra cDNA were ligated to the N terminus of the *lacZ* gene coding sequence, and the fusion genes were transfected into COS cells. β-Gal is known to stain throughout the cell cytoplasm and nucleus (20, 21), and a protein with the myc tag fused to the N terminus of β-gal stains in a similar fashion (Pst/β-gal; Figs. 1*C* and 2*B*). Consistent with our deletion analysis, aa 1–92 of Tra are capable of directing the β-gal fusion protein to the nucleus but not to speckles (R47/β-gal; Figs. 1*C* and 2*B*). However, addition of aa 93–121 led to speckle localization of the fusion protein (TthIII/β-gal; Figs. 1*C* and 2*B*).

N-terminal deletions demonstrate that speckle localization was conferred on a fusion protein containing aa 72–121 (Δ8/β-gal; data not shown). However, a fusion protein containing aa 93–121 of Tra did not lead to speckle localization (Δ15/β-gal; Figs. 1*C* and 2*B*). Rather, this fusion protein displayed diffuse nuclear staining. In some cells transfected with this construct, nuclear and cytoplasmic staining was observed (data not shown). Although the aa 93–121 region of Tra contains a sequence that includes a NLS consensus (Fig. 3), which functions in Δ15, it does not function as efficiently in the context of the β-gal fusion protein. A NLS identified by deletion and mutational analysis will not always function in a heterologous protein (22), but this does not necessarily reflect on its role in the native protein. Consistent with the results of the deletion analysis, addition of aa 84–121 is sufficient for both nuclear and speckle localization of the β-gal fusion protein (Δ16/β-gal; Figs. 1*C* and 2*B*), although significant levels of diffuse staining are also observed. These data demonstrate that the NLS consensus reintroduced by the addition of aa 84–92 (NLS3; Fig. 3) to deletion Δ15/β-gal functions more efficiently than NLS4 (Δ15/β-gal) in the context of β-gal and overlaps with the sequence required for speckle localization. We conclude that the amino acid residues in Tra shown to be necessary for localization of Tra protein to speckles in the deletion analysis are sufficient to direct the localization of a heterologous protein to nuclear speckles.

Coexpression of Tra2 with Tra Mutants Induces Reorganization of These Splicing Factors. Both Tra and Tra2 bind specifically to doublesex pre-mRNA (23–25) and to each other (26). Moreover, both Tra and Tra2 interact with the SR proteins SF2/ASF and SC35 and with the 35-kDa subunit of the splicing factor U2AF (26, 27). It is possible, therefore, that subnuclear colocalization of Tra, Tra2, and SC35 is in part a consequence of protein–protein interactions through their RS domains. In support of this hypothesis, overexpression of the RS domain of splicing factor U1 70K (28) or Tra (unpublished data) induces reorganization of SC35. To determine whether the sequences in Tra required for interactions with Tra2 correspond to the Tra speckle signal or can occur independently of this signal, we examined the Tra deletion mutants for colocalization with Tra2 in the nucleus of transfected cells. Myc-Tra deletion mutants, which localize to the nucleus but lack the speckle localization signal (Δ3, Δ5, Δ12, and Δ15), were cotransfected with the Tra2 cDNA. Tra deletion mutant Δ3 did not colocalize with Tra2 (data not shown). Tra deletion mutant Δ5 did not colocalize with Tra2 in most instances (Δ5 + Tra2; Figs. 1*D* and 2*C*), whereas Tra deletion mutant Δ12 colocalized with Tra2 in the majority of cotransfectants (Δ12 + Tra2; Figs. 1*D* and 2*C*). Therefore, colocalization of Tra deletions lacking a speckle signal and Tra2 requires a region of Tra located between aa 28 and 91 (Fig. 3). The inability of Tra deletion Δ15 and Tra2 to colocalize is consistent with this hypothesis (Δ15 + Tra2; Figs. 1*D* and 2*C*).

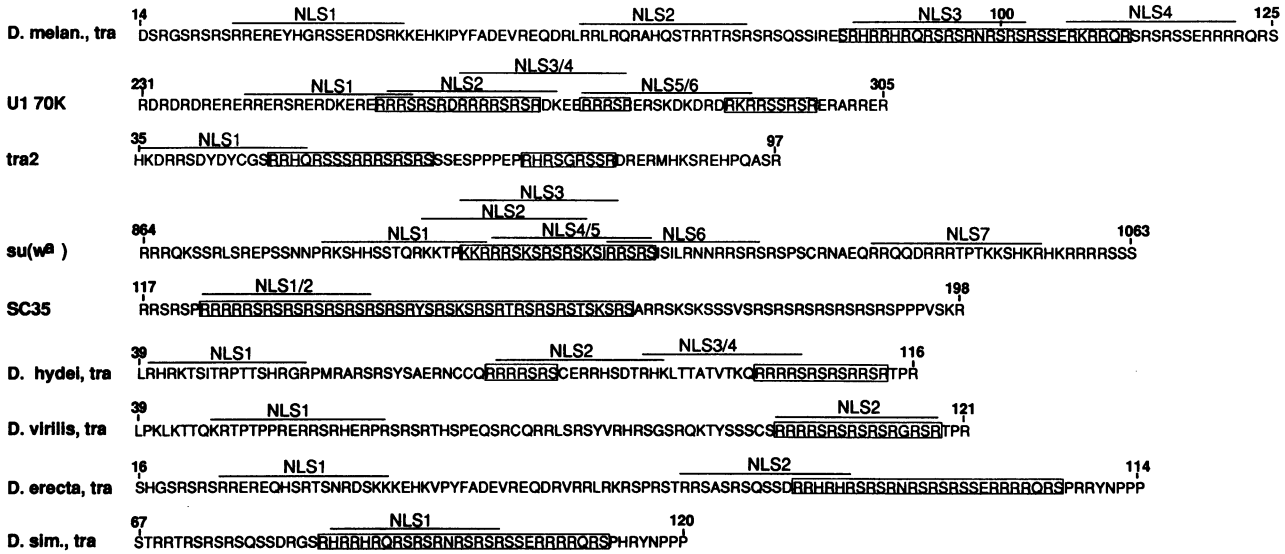

FIG. 3. Diagram showing potential nuclear and subnuclear localization signals in several splicing-related proteins. Shown is the amino acid sequence of a selected region of *Drosophila melanogaster* Tra, Tra2, and Su(w^a); human U1 70K, SC35, and Tra from *Drosophila hydei, Drosophila virilis, Drosophila erecta*, and *Drosophila simulans.* The sequence required for localization of Tra (*D. melanogaster*) is boxed, as are the sequences that most resemble this signal in the other proteins. The putative nucleoplasmin-like NLSs are indicated by lines above the protein sequences and are designated by numbers in sequential order. Signals that overlap significantly are designated with a slash (i.e., NLS 1/2).

These observations suggest that one region of Tra (aa 28–91) involved in interactions with Tra2 is distinct from the speckle localization signal, and they demonstrate that proteins lacking this signal (Tra deletion mutants) may localize to speckles through interactions with proteins (Tra2) that are normally found there. These data do not necessarily rule out a role for the contribution of the speckle localization signal to wild-type Tra–Tra2 interactions, but they do emphasize the importance of protein–protein interactions in speckle localization. In addition, these observations demonstrate that distinct protein–protein interaction domains can lead to localization to speckles.

A Sequence Similar to the Tra Speckle Localization Signal Is Present in Several Splicing Factors. A number of spliceosome components are known to localize to nuclear speckles. We therefore searched the amino acid sequences of these proteins for a sequence similar to the Tra speckle localization signal. As shown in Fig. 3, all of these proteins contain a sequence similar to the one found in Tra, which consists of three or four basic residues (generally arginine and histidine residues) directly adjacent to RS dipeptides. The Tra genes from several *Drosophila* species have recently been cloned and sequenced (29). A remarkable level of sequence conservation is observed in regions containing the signal required for localization to the speckle domain. Further evidence to support the generalization of this motif is that a deletion in Tra2, which includes the putative speckle localization signal, interferes with colocalization of Tra2 with SC35 in mammalian cells (M.L.H., unpublished data).

We also note that in all of the proteins examined, the putative speckle signals overlap with a nucleoplasmin-type NLS (Fig. 3). The importance of this arrangement is unclear, but it may be significant that (*i*) the speckle localization signal was never found in association with a simian virus 40-type NLS, and (*ii*) only Tra deletion mutants containing a nucleoplasmin-like NLS, which overlaps with the speckle signal (stretch of basic residues adjacent to RS dipeptides), were found in the speckle domains.

An individual speckle consensus signal within the RS region is likely to be required for speckle localization, but it may not always be sufficient. For example, a fragment of U1 70K containing the speckle consensus localizes to nuclear speckles. However, a smaller fragment containing this sequence localizes to the nucleus but not to speckles (28). This result suggests that factors such as sequence context, phosphorylation, and the number of signals present in the protein may affect subnuclear localization. A precedent for this possibility is provided by studies which show that all of these factors can affect the nuclear localization of other proteins (22, 30, 31). Sequence context may be especially important for localization to speckles since protein–protein interactions that require the RS region may be the driving force for speckle domain formation.

DISCUSSION

We have identified the minimal sequence in Tra that is necessary and sufficient for both nuclear and subnuclear localization. This sequence includes a nucleoplasmin-like NLS that overlaps with a stretch of basic residues adjacent to an RS repeat sequence. Nuclear transport is an active process involving specific cytoplasmic factors and interaction with components of the nuclear pores (for reviews, see refs. 32–34). By contrast, the process by which proteins become localized within the nucleus is poorly understood and could involve active transport via a distinct subnuclear localization signal or passive diffusion and binding to components attached to the nuclear matrix or to the nuclear matrix itself. Active splicing components, including SC35 (35) and the splicing regulators Tra and Tra2 (M. Tian and T.M., unpublished results), are tightly associated with the nuclear matrix (36–39), suggesting that the speckled domains are anchored directly or indirectly to the nuclear matrix.

We have shown that the RS domain of Tra is required for localization to the speckled domain, but our analysis of the colocalization of deletion mutants of Tra and intact Tra2 shows that Tra proteins lacking an RS domain can be directed to the speckles via their interaction with Tra2. A similar mechanism could be involved in the speckle localization of splicing factors lacking an RS domain. For example, the splicing factor PSF (40), which colocalizes with SC35, does not contain an RS domain. Thus, the same protein–protein interactions required for spliceosome assembly may be involved in the subnuclear localization of both protein and small nuclear RNA components of the spliceosome.

Previously, the RS domains of the splicing regulators Tra and Su(w^a) were implicated in subnuclear localization (17). We demonstrate that the sequence in Tra required for localization to nuclear speckle domains includes several RS dipeptides and lies between aa 84 and 112. While the stretch of RS dipeptides is required, it is not sufficient to induce subnuclear localization of Tra (Δ15). However, a run of basic residues and a functional nucleoplasmin NLS signal adjacent to or overlapping with the RS dipeptides are sufficient for speckle localization (Figs. 1*B* and 3). For example, the addition of either of two sequences to the N terminus of a single RS-rich region converts a Tra deletion mutant, which displays diffuse nuclear staining, to a protein that localizes to the speckle domains (Δ15 compared with Δ16 and Δ13). In both cases, the sequences are enriched for basic residues, and they add a nucleoplasmin-like NLS to the RS domain. The difference between the two proteins is the location of the RS region with respect to the NLS. In one case (Δ16), the RS dipeptides are an integral part of the NLS, whereas in Δ13 the RS dipeptides are located toward the end of the NLS. Since both of these deletions localize to the speckle domains, it seems that the position of the RS region with respect to the NLS is not critical. However, the proximity of a nucleoplasmin-like NLS to the speckle localizing signal could be of importance. Examination of the sequences of several RS-containing proteins revealed that they all contain a nucleoplasmin-like NLS that overlaps with a basic/RS-rich region (Fig. 3). The abundance of basic residues in the consensus NLS and the RS dipeptide suggests that the chances for overlap between the two types of signal are high, though it is striking to note the complete absence of a simian virus 40-type NLS [K(R/K)X(R/K)K] in nearly all of the speckle localizing proteins. Moreover, in a compiled list of several proteins that contain a nucleoplasmin NLS but are not involved in splicing, none has an overlapping RS-rich region (41). The overlap between the nuclear and subnuclear signals seen in the splicing-related proteins differs from the placement of signals in DNA methyltransferase, a nuclear protein that is targeted to subnuclear regions involved in DNA replication. In this protein, the two signals function independently and are found in different regions of the protein (42). While it is clear that a nucleoplasmin-like NLS is associated with the minimal sequence for Tra localization, and this relationship is conserved in other SR proteins, additional experiments are necessary to determine whether the overlap between the NLS and basic residue/SR-containing sequence is essential for nuclear and subnuclear localization of splicing factors. Our initial attempts to uncouple these signals with constructs containing only a stretch of basic residues and RS dipeptides or a nucleoplasmin-like NLS have been unsuccessful (data not shown).

Although the NLS-RS signal is present in many proteins involved in splicing, it varies considerably with regard to copy number and complexity. A likely explanation for this difference is that the SR region functions in both subnuclear localization and splicing. In fact, the SR domains of SF2/ASF (43, 44) and Tra2 (26) have been shown to be essential for their splicing activities, and overexpression of the U1 70K RS region affects both splicing and subnuclear localization (28).

The speckle domains were previously thought to represent sites of pre-mRNA splicing (45–47). However, more recent evidence demonstrates that they are regions of spliceosome storage or assembly, with splicing occurring at the site of transcription (refs. 48 and 49; S. Huang and D. L. Spector, personal communication). The RS region is necessary for the manifestation of both these characteristics since it serves as a multifunctional protein–protein interaction domain that is crucial for the subnuclear localization of SR proteins to nuclear speckles and for the role these factors play in the splicing reaction. In both cases this domain appears to mediate the assembly of multicomponent complexes—in one case on the nuclear matrix and in the other on nascent pre-mRNA.

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