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TLS (FUS) binds RNA in vivo and engages in nucleo-cytoplasmic shuttling

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SUMMARY

TLS, the product of a gene commonly translocated in liposarcomas *(TLS)*, is prototypical of a newly identified class of nuclear proteins that contain a C-terminal domain with a distinct RNA recognition motif (RRM) surrounded by Arg-Gly-Gly (RGG) repeats. Its unique N terminus serves as an essential transforming domain for a number of fusion oncoproteins in human sarcomas and leukemias. In this study we use an in vivo UV crosslinking procedure to probe the interactions of TLS with RNA. TLS is found to bind RNA in vivo and the association of TLS with RNA is rapidly diminished by treating cells with transcriptional inhibitors. This suggests that the species bound by TLS turns over rapidly. Surprisingly, the RRM was found to be dispensable for RNA binding by TLS in vivo, suggesting that at any one time most of the interactions between TLS and RNA in the cell are not sequence specific. Analysis of inter specific heterokaryons formed between human and mouse or *Xenopus* cells revealed that TLS engages in rapid nucleocytoplasmic shuttling, a finding confirmed by the ability of anti-TLS antibodies to trap TLS when injected into the cytoplasm of HeLa cells. Cellular fractionation experiments suggest that TLS binds to RNA in both the nucleus and cytoplasm and support the hypothesis that TLS functions as a heterogeneous ribonuclear protein (hnRNP)-like chaperone of RNA. These findings are discussed in the context of the role altered forms of TLS play in cellular transformation.

Key words: Transcription, Nuclear localization, Monoclonal antibody, Sarcoma

INTRODUCTION

Many human sarcomas are associated with chromosomal translocations that lead to the expression of fusion oncoproteins whose N-termini are comprised of peptide sequence from one of two highly related proteins: TLS (also known as FUS) and EWS (for review see Rabbitts, 1994; Ron, 1996). In the oncogenic fusion proteins, the conserved C terminus of TLS or EWS is replaced by a DNA-binding domain from one of several unrelated transcription factors. The N-termini of TLS and EWS are essential to the transforming capability of the fusion oncoproteins (May et al., 1993a,b; Zinszner et al., 1994). The N-terminal domain of TLS or EWS serves as a potent transcriptional activator when fused to a heterologous DNA-binding protein (May et al., 1993a,b; Prasad et al., 1994; Sanchez-Garciá and Rabbitts, 1994; Zinszner et al., 1994). It also has a topogenic role in specifying association of both the normal TLS and its oncogenic counterpart with a novel nuclear structure (Zinszner et al., 1997). However, despite these recent advances in the characterization of the activities associated with the 'oncogenic' N terminus of TLS and EWS, we know little about the functional properties of the normal proteins.

In their C-termini, both TLS and EWS contain peptide sequences that are often found in RNA-binding proteins. These consist of a conserved approx. 100 amino acid domain known as the RNA recognition motif (RRM) flanked on both sides by multiple repeats of the tripeptide sequence RGG (Delattre et al., 1992; Crozat et al., 1993). Both these structural motifs are believed to participate in RNA binding (Kenan et al., 1991; Haynes, 1992; Burd and Dreyfuss, 1994). Several observations are consistent with the idea that TLS and EWS are indeed RNA-binding proteins. First, the C-termini of TLS or EWS, when expressed in bacteria will bind RNA in vitro. However, the potential significance of this finding is diminished by lack of evidence that binding is sequence specific (Crozat et al., 1993; Ohno et al., 1994). Secondly, TLS is an abundant nuclear protein with many features of a heterogeneous ribonuclear protein (hnRNP): it is found in association with other hnRNPs in a stable complex (Zinszner et al., 1994; Calvio and Lamond, 1995; Zinszner et al., 1997) and it can be purified from nuclear extracts by single-stranded DNA affinity chromatography (Calvio and Lamond, 1995). The integrity of the TLS-hnRNP complex is furthermore dependent on the integrity of its constituent RNA (Zinszner et al., 1994). This latter observation suggests that at least some component(s) of the complex has RNA-binding activity, but does not directly implicate TLS in that role. Thirdly, a *Drosophila* homologue of TLS and EWS, CAZ (Stolow and Haynes, 1995), also named SARFH (Immanuel et al., 1995), is localized to areas of active transcription in the polytene chromosomes of salivary glands (Immanuel et al., 1995). CAZ thus resembles other *Drosophila* hnRNP proteins that are known to bind RNA (Matunis et al., 1992). However, our attempts to determine if localization of CAZ to areas of active transcription is dependent on an RNA component have been frustrated by the fact that RNase treatment of the polytene squashes leads to their disintegration.

Finally, when transcription is inhibited TLS undergoes striking re-localization, from a diffuse nucleoplasmic distribution to accumulation in the cytoplasm and association with a discrete subnuclear structure (Zinszner et al., 1994, 1997). Cytoplasmic accumulation under conditions in which transcription is inhibited is a feature of certain RNA-binding proteins, particularly hnRNPs that shuttle from the nucleus to the cytoplasm (Pinol-Roma and Dreyfuss, 1992).

Despite much suggestive evidence, proof that TLS binds RNA in vivo has so far not been obtained. Similarly, though accumulation of TLS in the cytoplasm when transcription is inhibited is suggestive of nuclear-cytoplasmic shuttling (Pinol-Roma and Dreyfuss, 1992), direct evidence that this is the case has also not been obtained so far. In this manuscript we describe the adaptation of an in vivo UV cross-linking procedure to study the binding of TLS to RNA and use of this procedure to obtain evidence that the RNA-species bound by TLS is likely to be an mRNA or pre-mRNA. In addition two independent techniques are used to document nucleocytoplasmic shuttling by TLS. In light of these findings we discuss possible roles for an oncogenic N-terminal transcriptional activation domain in the context of a shuttling RNA-binding protein.

MATERIALS AND METHODS

Cell culture and UV crosslinking of TLS to RNA in vivo

HeLa and COS1 cells were grown in 10 cm plates to approx. 80% confluence in DMEM with 10% fetal calf serum. To inhibit RNA polymerase activity, cells were treated where indicated with either 5 μg/ml of actinomycin D (Boehringer Mannheim) or 50 μg/ml of α-amanitin (Sigma). The plates were removed from the incubator and placed on a flat bed of crushed ice. Cells were rapidly washed with ice-cold phosphate-buffered saline (PBS) and the cellular monolayer left in a thin layer of PBS (2 ml/plate). The plates were placed on ice in a UV Stratalinker 2400 oven (Stratagene) and irradiated with 900 mJ/cm^2 total energy (i.e. max energy output for about 4 minutes). Immediately thereafter the cells were scraped on ice into 1 ml of PBS with 2 mM EDTA, pelleted by slow-speed spin and the cellular pellet was re-suspended in 300 μl of TNE-450 lysis buffer (20 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 1 mM $CaCl_2$, 450 mM NaCl, 0.6% Triton X-100 and the protease inhibitors: 2.2 μg/ml aprotinin, 0.25 mM PMSF, 1 μg/ml leupeptin and 1 μg/ml pepstatin. The cellular pellet was vortexed gently and left on ice for 20 minutes. Following this lysis and extraction step that strips TLS from the ribonuclear particles, the salt concentration of the buffer was adjusted to 150 mM with 2 volumes of the same buffer lacking NaCl. RNase treatment of the lysate was carried at 20°C for 30 minutes using either 100 units of RNase T1 or 30 μg of RNase A (Boehringer Mannheim). SDS and deoxycholate were added to the lysate to a final concentration of 0.1% and 0.5% w/v, respectively, to further enhance the solubility of TLS and increase the stringency of the subsequent immunoprecipitation step. The extracts were clarified by spinning for 30 minutes at 90,000 rpm (TLA-100-2 rotor) in a Beckman tabletop ultracentrifuge (Optima TL). The supernatant (600 μl) was further clarified by incubation in a rotator for 15 minutes at 4°C with 10 μl of Sepharose 4B beads (Pharmacia). The supernatant was transferred to a new tube containing Protein A/G agarose beads (Santa Cruz Biotech) that had been pre-incubated with monoclonal antibodies to TLS. Immunoprecipitation was performed for 1 hour at 4°C on a rotator. The beads were washed three times in 1 ml RIPA buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1% SDS, 0.5% deoxycholate, 1% NP-40), once in RIPA buffer containing 500 mM NaCl, and three times in 1 ml kinase buffer (50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM DTT containing the aforementioned protease inhibitors). Following this last wash the immune-complex containing beads were re-suspended in 30 μl of kinase buffer, then 100 μCi of [γ-^{32}P]ATP (7,000 Ci/mmole) and 5 units of T4 kinase (NEB) were added and the sample was placed for 30 minutes at 37°C, with periodic shaking. Following completion of the kinase reaction the beads were washed three times with 1 ml RIPA (to remove the unincorporated [γ-^{32}P]ATP), re-suspended in 20 μl of SDS-loading buffer, boiled for 3 minutes and resolved on an 8% SDS-PAGE gel. The gel was autoradiographed or in certain cases (where indicated) the proteins in the gel were transferred to a nitrocellulose filter for autoradiography and western blotting.

To recover the labeled RNA molecules cross-linked to TLS, the unfixed gel was dried in a gel dryer and the portion of the gel corresponding to the labeled band was excised and washed two times in 10% methanol for 30 minutes at room temperature. After lyophilization to dryness, the acrylamide was rehydrated for 30 minutes in 300 μl of proteinase K buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.1% SDS, 10 mM EDTA) containing 200 μg of proteinase K (Boehringer Mannheim) and 20 μg of yeast tRNA to serve as a carrier. The volume was then adjusted to 1 ml with proteinase K buffer and the reaction continued for 16 hours at 37°C. The supernatant was recovered, the labeled RNA extracted with phenol and chloroform, ethanol precipitated and resolved on a 12% acrylamide gel containing 8 M urea.

Heterokaryons and cytoplasmic injection of antibodies

Heterokaryons were produced exactly as described (Borer et al., 1989). Briefly, the two cells types to be fused were co-cultured on glass coverslips. The coverslip was incubated in 50% PEG (M_r 1,500, Boehringer Mannheim) adjusted to 150 mM NaCl for 2 minutes at 37°C, and placed back in culture after several rinses in serum containing medium. Cells were pre-treated with 20 μg/ml cyclohexamide for 20 minutes before fusion and maintained in medium containing the inhibitor until fixation, four hours later. For immunostaining, cells were fixed in 4% methanol-free ultra pure formaldehyde (Polysciences Inc.) for 15 minutes and permeabilized with 0.2% Triton X-100 in PBS for 5 minutes. Immunochemical detection of TLS, CHOP and the hnRNPs was carried out as described previously (Zinszner et al., 1997).

Cellular micro injection was performed as previously described using a Zeiss automated AIS system (Barone et al., 1994; Immanuel et al., 1995). Monoclonal antibodies to TLS (4H11) and the CHOP control (9C8) were produced as ascites in Balb/c mice. IgG was purified from the ascites by chromatography on Protein A Sepharose. Following gel filtration on a NAP25 desalting column (Pharmacia) equilibrated with phosphate buffered saline, the IgG was concentrated on a Microcon 100 (Amicon) to a final concentration of 5 mg/ml. The IgG was injected into the cytoplasm of HeLa cells growing on glass coverslips that had been pre-treated for 30 minutes before injection with 20 μg/ml cyclohexamide. Cells were fixed with 4% neutral buffered formalin and stained with rabbit polyclonal serum to TLS (Crozat et al., 1993) and a fluoroscein-conjugated donkey anti-rabbit IgG and a Texas Red-conjugated donkey anti-mouse IgG were used to reveal the injected antibody.

Expression plasmids

The full length 9E10 MYC epitope tagged TLS as well as the C-TLS and C-NLS-TLS deletion mutants have all been described elsewhere (Zinszner et al., 1997). TLS and C-TLS lacking the RRM motif were obtained by digestion of the corresponding human cDNAs with *Bsp*HI and *Eco*RI restriction enzymes, followed by Klenow repair and re ligation. This results in an in-frame deletion that encompasses the RRM. Integrity of the protein was ascertained by western blot analysis of transfected cells.

Fractionation of lysates by sucrose gradients

Cytoplasmic extracts of HeLa cells were prepared in lysis buffer (10 mM Tris, 100 mM NaCl, 3 mM $MgCl_2$, 0.6% Triton X-100 and protease inhibitors) and clarified by a short spin in a tabletop micro centrifuge at maximal speed. Nuclear extracts were obtained by sonication of the nuclear pellet prepared by the procedure of Schreiber et al. (1989) into a Triton-free version of the lysis buffer. Extracts from the equivalent of two 10 cm plates were loaded on 10-40% sucrose gradients in 13 ml tubes. Fractionation was carried out at 4°C by spinning at 24,000 rpm for 15 hours in a Beckman SW41 rotor. Fractions (1 ml) were recovered from the gradient and proteins precipitated by addition of 1/4 volume of 100% TCA solution containing 4 mg/ml DOC. Precipitation was carried at 4°C for 2 hours and the precipitates were washed two times with 100% ice-cold ethanol. After air-drying, pellets were directly re-suspended into SDS loading buffer, boiled and resolved by 10% SDS-PAGE. Western blotting with anti-TLS antibodies was performed as previously described (Zinszner et al., 1994, 1997).

RESULTS

TLS binds RNA in vivo

UV light efficiently cross-links nucleic acids to proteins in vivo (Wagenmakers et al., 1980). To determine if TLS is bound to a polynucleotide in vivo, HeLa cells were irradiated with UV light, whole cell extracts prepared and TLS was immune precipitated from the soluble phase using specific antibodies. The TLS-polynucleotide cross-linked adduct in the immune-precipitates was revealed by labeling the free 5′ OH end of the polynucleotide with ^{32}P using T4 polynucleotide kinase (van Eekelen et al., 1982). The sample was boiled in denaturing buffer to dissociate the antibody and any non-covalently bound labeled species from TLS and the adduct was resolved by denaturing SDS-PAGE (Fig. 1A). The presence of the adduct was revealed by autoradiography of the gel in which the labeled species appears as a discrete band migrating at approximately the size of TLS (Fig. 1B). To interpret the in vivo UV cross-linking assay, several technical features must be considered: because of the large amount of RNA in the cellular lysates, even the most stringent immunoprecipitation procedure results in a pellet that is heavily contaminated by non-cross-linked RNA. When radio labeled with T4 kinase, this gives rise to a very high non-specific background. To reduce this background we found it necessary to clear the lysate of non-cross-linked RNA by ultra centrifugation prior to immunoprecipitation of TLS. However, ultra centrifugation was also found to result in significant selective loss of the covalent RNA-TLS adduct from the soluble phase; non-cross-linked TLS remained soluble. Therefore, following UV cross-linking (but before the ultra centrifugation step), the lysates were treated with RNase (RNase T1 or RNase A). This led to recovery, in the soluble phase, of a substantial amount of TLS cross-linked to a remnant of RNA. Both RNases leave a free 5′OH group allowing efficient labeling by polynucleotide kinase, and, because the bound RNA remnant is relatively small (see below), the resulting RNA-TLS adduct is similar in size to the native TLS molecule.

Fig. 1B shows that the labeled species is immunoprecipitated specifically by anti-TLS antibodies and is present only when the cells have been irradiated with UV. A smaller amount of radio labeling was reproducibly found even when TLS was immunoprecipitated from cells that had *not* been irradiated by UV (such low level labeling is not observed at the exposure shown here). Furthermore, this low-level radio labeling was *not* dependent on the addition of T4 kinase and is due, most probably, to an endogenous kinase that co-immunoprecipitates with TLS and phosphorylates it upon addition of Mg^{2+} and ATP. This latter finding is consistent with the observation that TLS contains phosphoserine in vivo (J. Sok, unpublished observations).

We next sought to determine the molecular nature of the radiolabeled species in this immunoprecipitation-polynucleotide kinase assay. The labeled band was eluted from the SDS-PAGE gel, the protein component subjected to proteolytic degradation and, following phenol extraction, the remaining soluble macromolecules were precipitated by ethanol and resolved by PAGE in the presence of 8 M urea. A labeled smear with a size of 12-20 nucleotides was revealed by autoradiography (Fig. 1C). The size of the labeled species in this experiment provides a rough estimate of the size of the RNA fragment protected from RNase by being bound to TLS (the TLS ‘footprint’ on the RNA). The average size was larger when the cellular lysates had been treated with RNase T1 than when they had been treated with RNase A. This is consistent with the fact that the former nuclease cleaves RNA less frequently than the latter. After digestion of the proteinaceous component, the purified radiolabeled species is rendered completely degradable by RNase, identifying it as an RNA (Fig. 1D).

The RNA-binding domain of TLS has been localized by in vitro assays to the C-terminal portion of the molecule (Crozat et al., 1993). This region contains the RRM and is flanked on both sides by RGG repeats. Both types of motifs have been associated with RNA-binding activity in various proteins, although only in the case of the RRM motif has sequence-specific binding been demonstrated (reviewed by Burd and Dreyfuss, 1994). To determine if RNA-binding by TLS in vivo is also mediated by the C-terminal half of the molecule and if this is dependent on the integrity of the RRM, we prepared expression plasmids encoding epitope-tagged versions of full-length TLS and mutant forms of the protein in which the N terminus or the RRM had been deleted (see Fig. 2A for a schema of the deletion mutants used). COS1 cells transfected with these plasmids were subjected to UV cross-linking and lysates prepared for immunoprecipitation-polynucleotide kinase assay using a tag-specific monoclonal antibody. Both full-length tagged TLS (FL-TLS) and an N-terminal deleted derivative (C-TLS) are efficiently cross-linked to RNA (Fig. 2B). This is consistent with the in vitro data and suggests that the C terminus of TLS contains peptide sequences sufficient for RNA binding in vivo. Interestingly, immunoprecipitates from cells expressing the C terminus of TLS consistently had an additional labeled band migrating with the 68 kDa marker (marked by the asterisks in lanes 4 and 6, Fig. 2B). The molecular nature of this species has not been determined.

Removal of the N terminus of TLS results in a protein that is localized primarily to the cytoplasm (Fig. 2C). Therefore, we also studied the RNA-binding properties of a C-terminal derivative of TLS that has been rendered constitutively nuclear (by fusion to a heterologous nuclear localization sequence).

This derivative, C-NLS-TLS, also binds RNA. The diminished ^{32}P signal of C-NLS-TLS when compared with C-TLS (Fig. 2B, lanes 6 and 7) is due to a lower level of recovery of this peptide in the immunoprecipitates (data not shown). The large amount of C-TLS cross linked to RNA (Fig. 2B, lane 6) and the predominantly cytoplasmic localization of the protein in vivo (Fig. 2C) suggests that TLS may be capable of binding RNA not only in the nucleus but also in the cytoplasm. However, it is also possible that only a small nuclear fraction of C-TLS is contributing all of the in vivo RNA binding activity detected by this assay. Our attempts to isolate C-TLS-RNA adducts from cytoplasmic extracts prepared from cells that had been irradiated with UV were frustrated by the fact that even though the vast majority of the C-TLS is cytoplasmic, after UV cross-linking virtually all the RNA-bound form is associated with the post-lysis low speed pellet (the 'nuclear fraction', Fig. 2D, compare lanes 1 and 2). This may reflect the fact that the RNA-bound C-TLS is indeed predominantly nuclear, but it is also consistent with the possibility that UV irradiation causes cross-linking of RNPs that contain TLS-RNA adducts to cytoskeletal elements and these are then lost from the soluble cytoplasmic fraction when the nuclei, membranes and cytoskeletal elements are pelleted by low speed spin. To explore this issue further we UV irradiated in vitro rapidly-prepared cytoplasmic and nuclear fractions from cells expressing C-TLS. The in vitro irradiated fractions were extracted with high salt, treated with RNase and an immunoprecipitation-kinase assay was performed on the solubilized proteins using a monoclonal antibody to the C terminus of TLS. The endogenous TLS is found cross-linked to RNA in both the nucleus and to a lesser degreee in the cytoplasm (Fig. 2D, lower panel, lanes 3 and 4). C-TLS is predominantly cross-linked to RNA in the cytoplasmic fractions (Fig. 2D, upper panel, lanes 3 and 4). We interpet these results to be consistent with TLS binding RNA in both the nucleus and cytoplasm.

To examine the role of the RRM in RNA binding, this domain was deleted in the context of either the full-length tagged TLS (FL-TLSΔRRM) and in the context of the C-terminal derivative of TLS (C-TLSΔRRM). Both ΔRRM versions of TLS associated with RNA in proportion to their level of recovery in the immunoprecipitation-polynucleotide kinase assay (Fig. 2E, compare lanes 1 and 2 and lanes 3 and 4). This result indicates that at any given moment in time (at least as captured by the 'snap-shot' of the UV cross-linking procedure) the bulk of the association of TLS with RNA is *not* dependent on the RRM.

Inhibition of RNA synthesis blocks the formation of the TLS-RNA adduct

In *Drosophila* larval salivary glands the TLS homologue, CAZ,

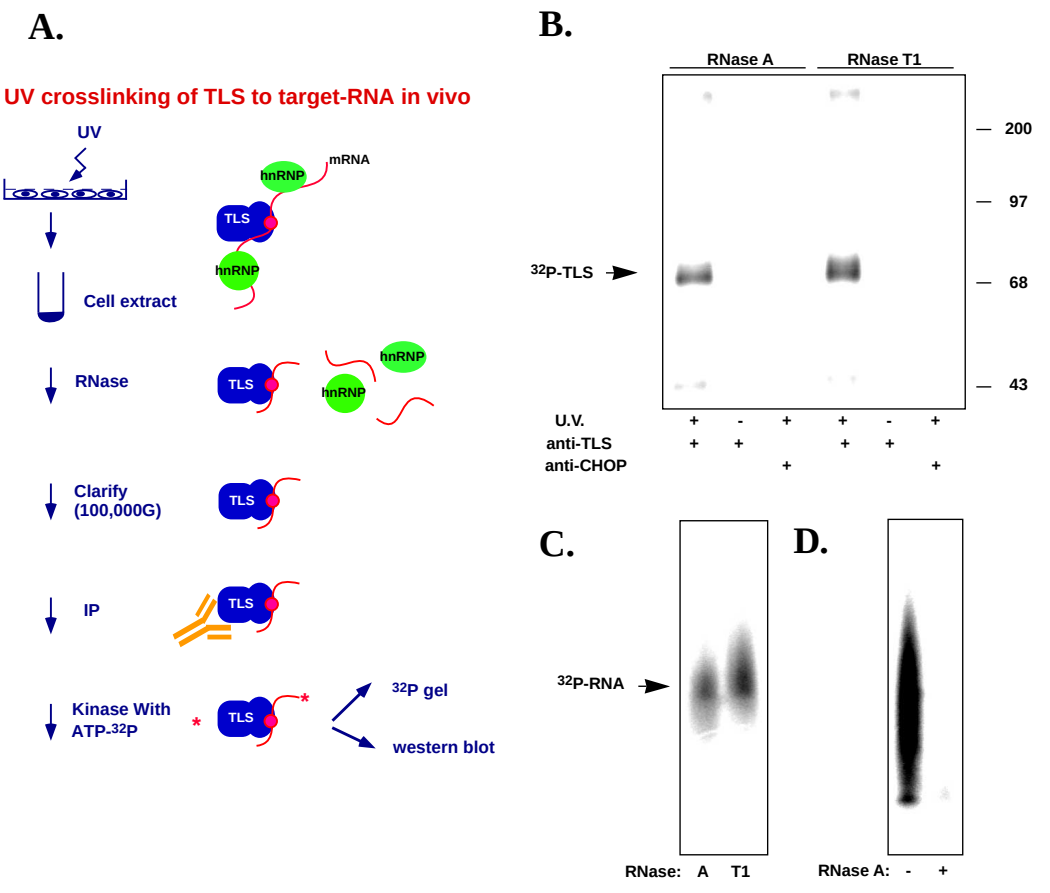

Fig. 1. Cross-linking of TLS to RNA in vivo. (A) The procedure used to detect TLS-RNA complexes in schematic form. (B) Autoradiogram of an 8% SDS-PAGE of proteins immunoprecipitated with anti-TLS antibodies and anti-CHOP antibodies (as a control) from UV crosslinked cell extracts that had been treated with either RNase A or RNase T1 and then labeled in vitro with [γ-^{32}P]ATP using polynucleotide kinase. A band of labeled material, migrating at the position of the 68 kDa marker (the size of TLS) is observed only in lysates from UV cross-linked cells and only in the TLS immunoprecipitated lanes. (C) Autoradiogram of the deproteinated radiolabeled polynucleotide that had been cross-linked to TLS in vivo. The labeled material in B was excised from the gel, digested with proteinase K and resolved by 12% PAGE in 8 M urea. Note that the labeled material recovered from the immunoprecipitate of the RNase A treated lysates was smaller in size than that of the RNase T1 lysates. (D) Autoradiogram of the radiolabeled deproteinated material recovered in C and subsequently re digested with RNase A and resolved by 12% PAGE in 8 M urea. Note that the deproteinated labeled polynucleotide that had been cross-linked to TLS is sensitive to degradation by RNase A.

is rapidly localized to areas of ongoing transcription (Immanuel et al., 1995). If this recruitment reflects an association with newly synthesized RNA, then treatment of cells with inhibitors of transcription should lead to a decreased association of TLS with RNA. HeLa cells were treated with actinomycin D and the amount of RNA associated with TLS in vivo was estimated by the immunoprecipitation-polynucleotide kinase assay. Within 30 minutes of actinomycin D treatment, the amount of ^{32}P signal associated with TLS decreased more than 4-fold (Fig. 3A). A western blot shows that this decrease is *not* due to a change in the amount of TLS immunoprecipitated (its level does not change even after 4 hours of treatment with the inhibitor, Fig. 3A, lower panel) but rather reflects a decrease in the amount of cross-linked RNA. Actinomycin D at this dose inhibits most RNA polymerase activity in the cell. The same experiment was therefore performed using α-amanitin, an inhibitor that preferentially affects RNA polymerase II. The latter inhibitor had a similar effect on the association of TLS with RNA (Fig. 3B). It is, however, important to point out that we have not excluded a possible effect of α-amanitin at this dose on transcription by other polymerases. These results, when viewed in conjunction with the observation that the TLS *Drosophila* homologue is associated with areas actively transcribed by RNA polymerase II (Immanuel et al., 1995), are consistent with the interpretation that TLS associates with a pool of mRNAs or pre-mRNAs that turn-over

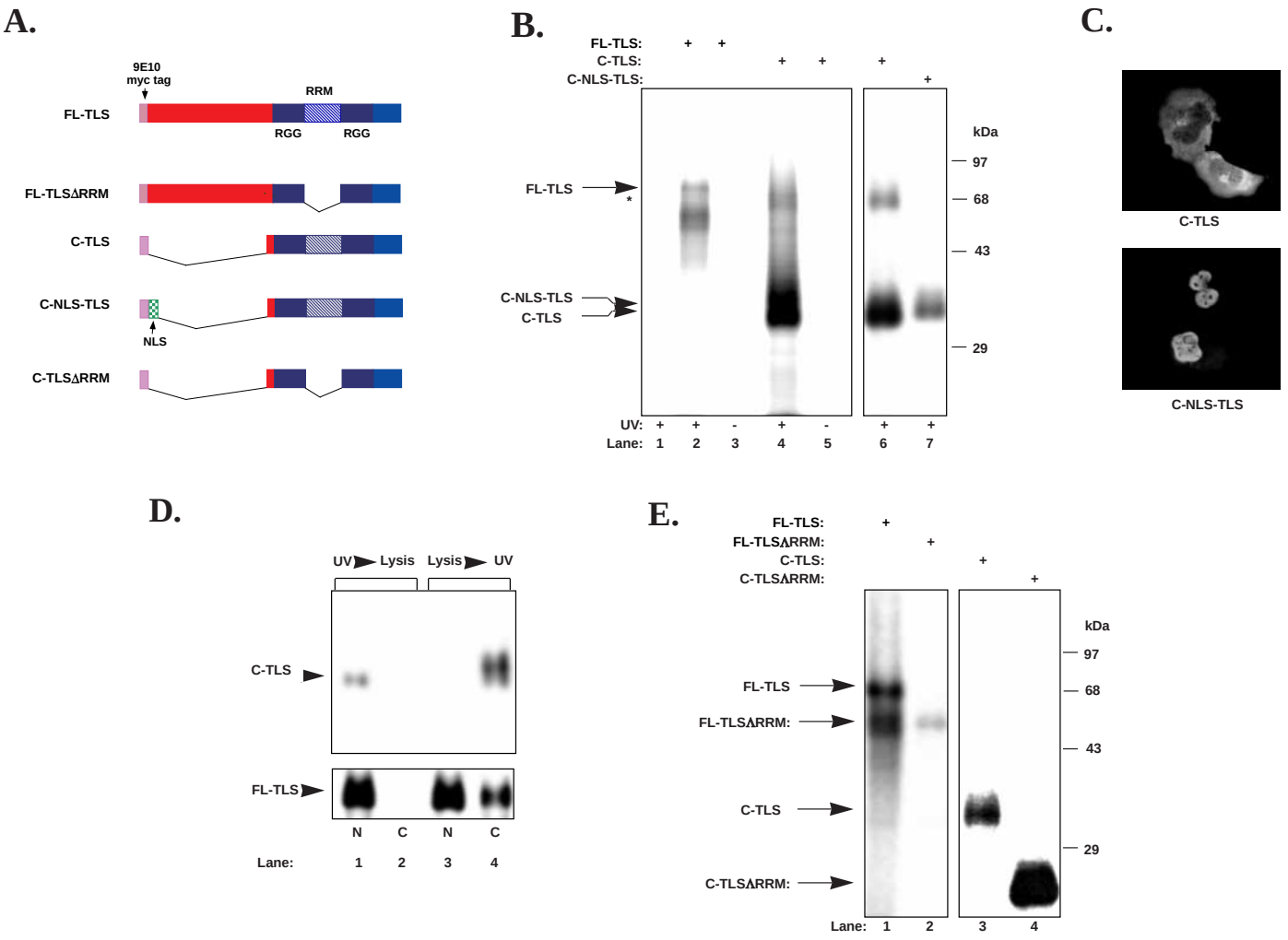

Fig. 2. RNA binding is localized to the C terminus of TLS and does not require the RNA recognition motif (RRM). (A) The linear structure of the TLS derivatives used in this experiment is depicted in schematic form. 9E10: the MYC epitope used to tag the recombinant proteins. RGG: Arg-Gly-Gly tripeptide repeats. NLS: heterologous nuclear localization signal used to direct the deleted form of TLS to the nucleus.
(B and E) Autoradiogram of immunoprecipitated and kinase-labeled TLS derivatives expressed in COS1 cells and UV cross-linked in vivo. The identity of the proteins is indicated above the lanes and the position of the proteins is marked by arrows to the left of the autoradiogram. The slower migrating species present in the C-TLS lanes (*) is a reproducible finding of unknown significance. (C) Immunolocalization of C-TLS and C-NLS TLS in transfected COS1 cells. The recombinant proteins are revealed by staining with an antibody directed against the 9E10 epitope tag. (D) COS1 cells expressing C-TLS were irradiated with UV light in vivo (UV→Lysis). Monoclonal antibodies to the C terminus of TLS were used to immunopurify both endogenous and recombinant C-TLS from nuclear (lane 1) and cytosolic (lane 2) fractions and the immunopurified proteins were subjected to an IP-kinase assay. In lanes 3 and 4 the C-TLS expressing cells were first lysed and then proteins in the nuclear (lane 3) and cytoplasmic (lane 4) fractions were UV crosslinked in vitro and subjected to the IP kinase assay (Lysis→UV). Both upper and lower panels are from the same autoradiogram but the exposure time is approx. 10× longer in the one showing C-TLS.

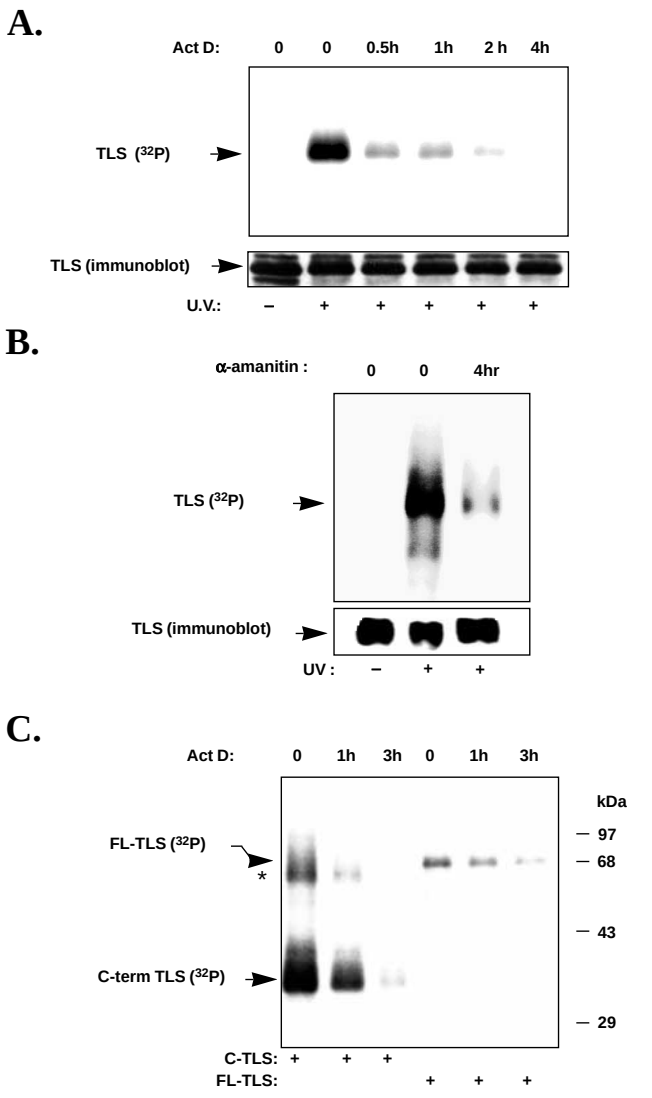

Fig. 3. The association of TLS and RNA is sensitive to transcriptional inhibitors. (A) HeLa cells were treated with actinomycin D (5 μg/ml) for the indicated period of time followed by UV cross-linking and a TLS immunoprecipitation-polynucleotide kinase assay as in Fig. 1. The gel was transferred to a nitrocellulose filter, exposed for autoradiography (upper panel) and a TLS immunoblot was performed (lower panel). Note that actinomycin D treatment leads to rapid decrease in the amount of RNA cross-linked to TLS but has little effect on the amount of TLS recovered in the immunoprecipitation. (B) Identical to A except that α-amanitin (50 μg/ml) was used to inhibit the polymerase. (C) COS1 cells transfected with tagged full-length TLS or C-TLS were treated with actinomycin D as in A. Note that treatment causes a diminution in the amount of RNA cross-linked to both TLS (a predominantly nuclear protein) and to C-TLS (a cytoplasmic protein, see Fig. 2).

rapidly. Interestingly, our results suggest that the association of TLS with these labile mRNA species can occur in either the nucleus or cytoplasm: inhibition of transcription also rapidly blocks the association of the cytoplasmic C-terminal fragment of TLS with RNA (Fig. 3C).

TLS participates in nucleocytoplasmic shuttling

The presence of TLS in a complex with other hnRNPs (Zinszner et al., 1994; Calvio and Lamond, 1995), the redistribution of TLS immunoreactivity when transcription is inhibited (Zinszner et al., 1994) and the observations suggesting that TLS can bind RNA in both the nucleus and cytoplasm (see above) led us to examine the possibility that TLS participates in nucleo-cytoplasmic shuttling. Two different assays were used to address this issue. In the first, a monoclonal antibody that specifically recognizes human TLS but not TLS from other vertebrate species (3D4) was used to examine TLS immunoreactivity in inter-specific heterokaryons (see Borer et al., 1989; Pinol-Roma and Dreyfuss, 1992, for the original description of the assay). In this experiment, the movement of TLS from human nuclei to mouse nuclei can be monitored by the accumulation of 3D4 immunoreactivity in the mouse nuclei of heterokaryons. Cell fusion is performed in the presence of cycloheximide (an inhibitor of new protein synthesis), therefore the human TLS accumulating in the murine nuclei must be pre-formed TLS that had been present in the human nuclei at the time of fusion and had migrated to the mouse nuclei through the common cytoplasm (Fig. 4A). The advantage of using mouse-human heterokaryons lies in the ease with which one can distinguish the two nuclei; the AT rich mouse centromeres have high affinity for the karyophilic dye H33258 and give rise to bright spots in the murine nucleus (Fig. 4A). However, we did not have at our disposal a species specific antibody that would recognize a non-shuttling human protein in such human-mouse heterokaryons, an important negative control. The 4F4 monoclonal antibody reactive against mammalian hnRNP C1/C2 does not recognize the *Xenopus* protein (Pinol-Roma and Dreyfuss, 1992), we therefore prepared heterokaryons between HeLa cells and *Xenopus* A6 cells and stained them with anti-TLS (3D4) and, as a control, anti-hnRNP C1/C2 (4F4). Human TLS immunoreactivity was found in the *Xenopus* nuclei but hnRNP C1/C2 immunoreactivity was not detected in identically-prepared human-*Xenopus* heterokaryons (Fig. 4B). This result indicates that under the conditions we used to prepare heterokaryons a non-shuttling protein such as hnRNPC1/C2 remains in the original nucleus, attesting to the specificity of the shuttling of TLS.

To confirm these results we injected antibodies to TLS into the cytoplasm of cycloheximide treated HeLa cells and examined the effect this would have on the distribution of the protein. Cells injected with a monoclonal antibody to the C terminus of TLS (4H11) were fixed 2 hours later and stained with rabbit anti-N-terminal TLS. The injected cells were revealed by staining for mouse IgG. Injection of TLS antibody (αTLS), but not a control monoclonal antibody (αCHOP) led to profound re-distribution of TLS from the nuclei to the cytoplasm (Fig. 3C). This result is most consistent with trapping of TLS in the cytoplasm by the injected antibody and suggests that TLS is continuously shuttling between the nucleus and the cytoplasm. The fact that such trapping is virtually complete 2 hours after injection, suggests that TLS is shuttling at a relatively rapid rate. An alternative explanation, by which anti-TLS antibodies disrupt nuclear membrane integrity and cause non-specific leakage of proteins into the cytoplasm, is rendered unlikely by the observation that other nuclear proteins such a CHOP and C/EBPα retain their nuclear localization in injected cells (data not shown).

Fractionation of cell lysates on sucrose gradient reveals that TLS and core hnRNPs have different biochemical properties

The features of TLS described above, RNA-binding and nucleocytoplasmic shuttling, are also shared by core hnRNPs such as hnRNP A1. We therefore sought to determine if TLS co-fractionates with core hnRNPs in ribonuclear particles (RNPs). Cell extracts prepared under mild lysis conditions that preserve the integrity of RNPs were fractionated on sucrose gradients and the quantity of TLS in fractions of various densities was estimated by western blotting. TLS immunoreactivity was reproducibly found in two widely separate fractions in both nuclear and cytoplasmic extracts: a very low molecular mass fraction at the top of the gradient and a very heavy fraction at the bottom (in addition, a significant amount of TLS was present in the insoluble pellet). This bimodal distribution of TLS is very different from that of core hnRNPs such as hnRNP A1 or hnRNP C1/C2. The latter are found predominantly in heavier fractions with almost no free material at the top of the gradient (Fig. 5A).

To determine which of the fractions of TLS corresponds to the RNA-bound one, we fractionated lysates from cells that had been irradiated with UV light in vivo and performed TLS immunoprecipitation-polynucleotide kinase assays on the heavy and light peak TLS fractions. RNA was found associated with TLS only in the heavy fraction (Fig. 5B). This experiment also revealed that UV cross-linking shifts a substantial portion of the total TLS immunoreactivity to the heavier fraction (compare the immunoblot in the upper panel of Fig. 5A with the lower panel of Fig. 5B). To the extent that UV cross-linking stabilizes the in vivo protein-RNA complexes, the presence of large amounts of TLS in the lighter fractions of lysates prepared from non-UV treated cells is due, at least in part, to in vitro dissociation of TLS from RNPs. Though strictly-speaking artifactual, this dissociation nonetheless appears to have some biological specificity, in so much as it is not a property of other hnRNPs; these remain in heavier fractions and are presumably bound in RNP particles (RNase treatment of lysates results in a shift of hnRNP A1 to lighter fractions, data not shown). We thus conclude that a substantial portion of the TLS in the cell at any one time is either not contacting RNA or is easily dissociated from the RNP.

DISCUSSION

In this study evidence is presented that TLS bind RNA, most likely a product of RNA polymerase II. In vivo RNA binding by TLS was revealed using an adaptation of an existing UV cross-linking protocol. Most previously described in vivo procedures for detecting proteins cross-linked to RNA have relied on purification of complexes containing poly(A)$^+$ RNA by oligo-dT affinity chromatography and identification of the protein by immunochemical methods. These procedures could only detect abundant proteins that are strongly associated with the RNA (Economidis and Pederson, 1983; Dreyfuss et al., 1984). The procedure used here relies on ex-vivo radio labeling of the immunopurified protein species via the cross-linked RNA remnant and is therefore much more sensitive in detecting RNA-protein interactions. Furthermore, this procedure should work independently of the type of RNA bound (mRNA, rRNA etc). Its relative simplicity allows one to analyze multiple points in a single experiment and has been utilized here to follow the association of TLS with RNA in cells in which transcription has been inhibited. A potential limitation of the technique described here is that proteins that co-immunopurify with a kinase activity are susceptible to direct labeling which may obscure the labeling of any attached nucleic acid species.

One of the most surprising findings to emerge from this study is that the RRM of TLS is dispensable for binding to RNA in vivo. The RRM is the region most highly conserved between TLS, EWS, CAZ and other members of this family. Furthermore, in other proteins this motif has been implicated in sequence-specific RNA binding (Kenan et al., 1991; Burd and Dreyfuss, 1994). Apart from the RRM, the C terminus of TLS contains multiple repeats of the tripeptide R-G-G. Such RGG-rich motifs are present in many RNA-binding proteins and may also participate directly in RNA binding (Burd and Dreyfuss, 1994). However, their limited structural complexity suggests that they do not impart sequence specificity to such binding, serving instead as an auxiliary RNA-protein interaction domain. If this is indeed the case, then the dispensability of the RRM suggests that most of the TLS-RNA interactions fixed by UV cross-linking are *not* sequence specific. Yet the cross-linking is performed in vivo and for relatively short periods of time (association of TLS with RNA is observed after as little as 30 seconds of cross-linking performed on ice), it therefore seems unlikely that the procedure is reporting on the artifactual association of TLS with RNA, such as might occur in cell lysates or in in vitro RNA-binding assays. One attractive hypothesis, consistent with dispensability of the RRM, is that TLS extensively 'scans' the RNA via its RGG repeats and that at any one time most of the TLS molecules are contacting RNA with no sequence specificity. These transient interactions are immortalized by the UV cross-linking procedure. The observation that the C terminus of TLS binds RNA non-selectively in vitro supports this hypothesis (Crozat et al., 1993). The role of sequence-specific RNA binding by TLS remains to be demonstrated.

The quantity of RNA bound to TLS is rapidly diminished in response to treatment of cells with inhibitors of RNA polymerase, including inhibitors with selectivity for Pol II. This suggests that the RNA species contacted by TLS is a product of that polymerase, a conclusion supported by previous experiments in which it had been found that CAZ, a *Drosophila* homologue of TLS, co-localizes with the active form of Pol II on the puffs of polytene chromosomes (Immanuel et al., 1995). The sensitivity of RNA binding by TLS to transcriptional inhibitors is consistent with the possibility that the RNA species bound by TLS turns over rapidly. Alternatively, it is possible that TLS associates with the RNA soon after it is synthesized and remains bound to it only for a portion of the life span of the RNA. Rapid association of TLS with RNA soon after its biogenesis is again suggested by experiments performed on polytene chromosomes of *Drosophila*, in which it was found that CAZ is recruited to the heat shock puffs within minutes of their formation (Immanuel et al., 1995). Transient association of TLS with RNA is also consistent with the demonstration that the protein undergoes rapid nucleocytoplasmic shuttling. The fact that

TLS can be found cross-linked to RNA both in the nucleus and in the cytoplasm suggests that the protein shuttles in a complex that contains RNA, serving in effect as a chaperone for mRNA. The *Chironomus* hnRNP A1 homologue, hrp36, has been directly observed to perform such a task (Visa et al., 1996) and TLS may participate in a similar activity. It remains possible, however, that TLS binds to different RNAs in both compartments of the cell and does not shuttle in a complex with RNA.

As pointed out above, shuttling is a property that TLS shares with other hnRNPs (Pinol-Roma and Dreyfuss, 1993). However, in contrast to hnRNP A1 in which both nuclear import and export are mediated by a small peptide (the M9 sequence; Michael et al., 1995), neither nuclear localization nor export of TLS could be mapped by us to a linear peptide domain. In fact both N-terminal and C-terminal TLS fragments, when expressed in cells are localized predominantly to the cytoplasm (though a small fraction of the N-terminal fragment is also found in the nucleus; Zinszner et al., 1997). When these TLS peptide fragments are directed to the nucleus through artificial fusion with a heterologous nuclear localization signal (C-NLS-TLS, Fig. 2) or as a natural occurrence in the case of the TLS-CHOP oncogene, we found no evidence that they are actively exported to the cytoplasm (unpublished

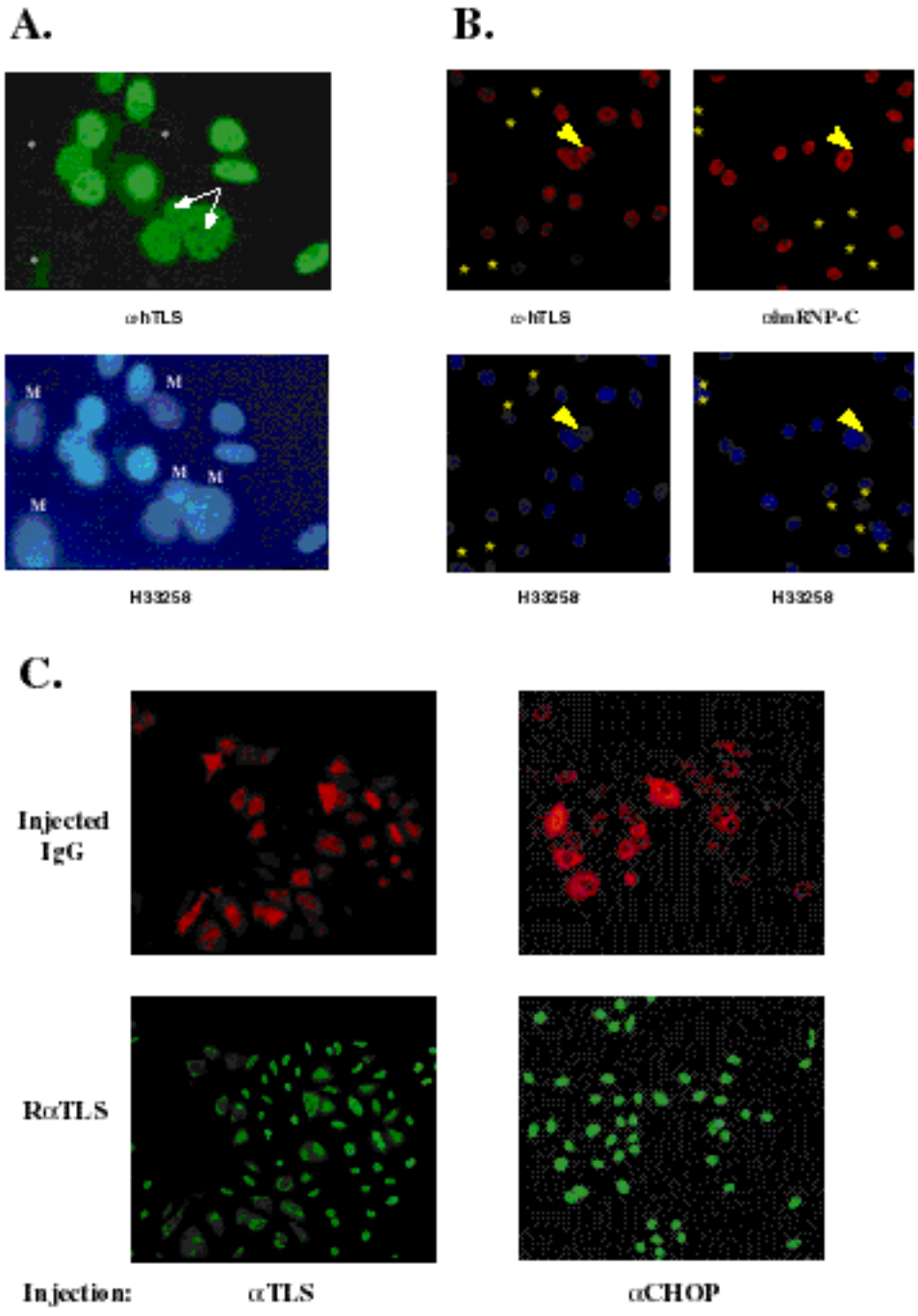

Fig. 4. TLS engages in nucleo-cytoplasmic shuttling. A. Heterokaryons formed between HeLa cells (human) and NIH 3T3 cells (mouse) were stained with the human-specific anti-TLS monoclonal antibody 3D4. In the upper panel (fluorescein) the two murine nuclei that are part of a heterokaryon (arrows) are stained with the TLS antibody whereas the murine nuclei that are not part of heterokaryons are unstained (*). The lower panel is an H33258 stain of the same field in which the murine nuclei (M) are identified by the bright spots corresponding to their AT-rich centromeres (Blau, 1989). (B) Similar heterokaryons were formed between *Xenopus* A6 cells and HeLa cells. These were stained by either the human-specific anti-TLS 3D4 monoclonal antibody (left panel) or with a mammalian-specific antibody to hnRNP C1/C2 (right panel). Note that the TLS antibody stains the *Xenopus* nucleus in the heterokaryon (arrow) but not in the non-fused cells (*), whereas the anti-C1/C2 antibody does not stain the *Xenopus* nuclei in the heterokaryon. (C) Monoclonal antibodies to TLS were micro-injected into the cytoplasm of HeLa cells growing on glass coverslips. After 2 hours the cells were fixed and stained with a Texas Red-conjugated antibody to mouse IgG (to identify the injected cells, red) and with rabbit polyclonal antiserum to TLS (detected with a fluoroscein-conjugated anti-rabbit IgG secondary antibody, green). Note that in cells injected with antibodies to TLS, TLS immunoreactivity is localized predominantly in the cytoplasm (lower left panel). As a control, cells were injected with purified monoclonal antibodies to CHOP; in these cells TLS remains localized in the nucleus (lower right panel).

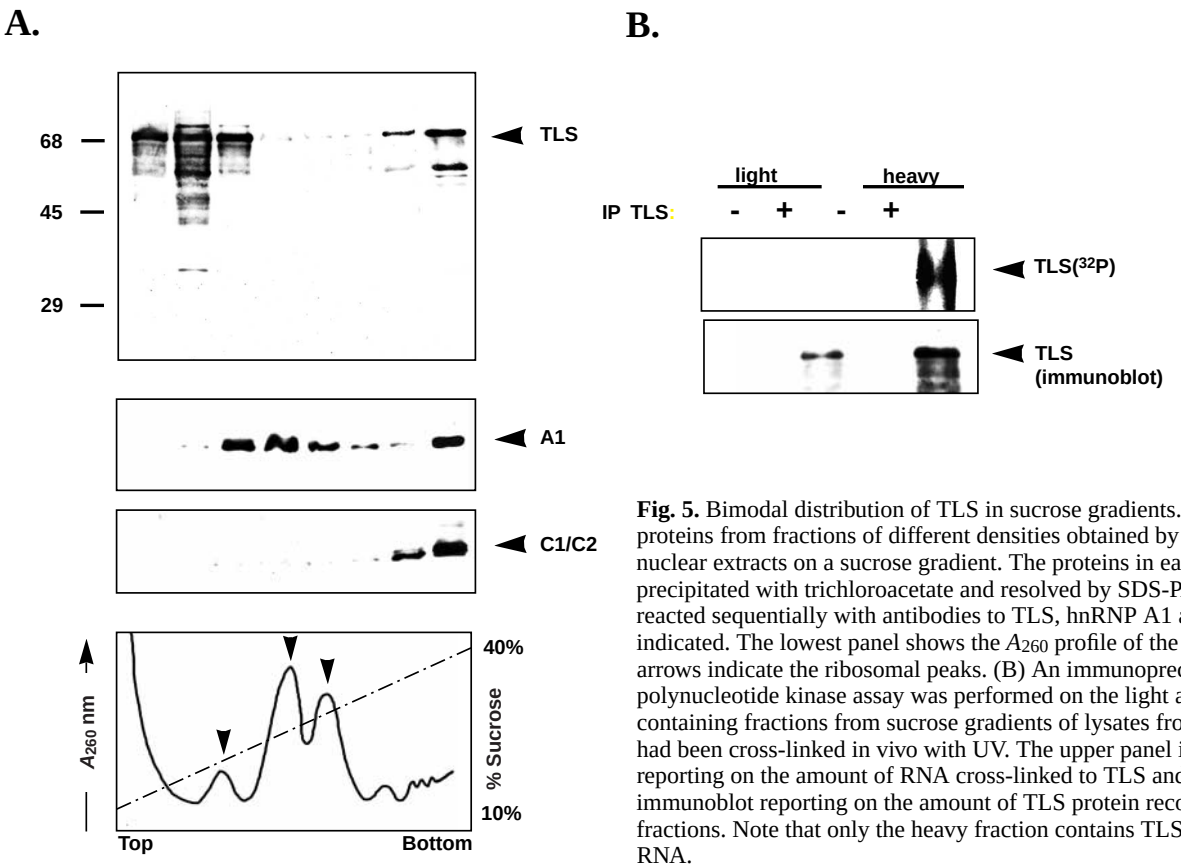

Fig. 5. Bimodal distribution of TLS in sucrose gradients. (A) Immunoblot of proteins from fractions of different densities obtained by separation of HeLa nuclear extracts on a sucrose gradient. The proteins in each fraction were precipitated with trichloroacetate and resolved by SDS-PAGE. The blot was reacted sequentially with antibodies to TLS, hnRNP A1 and hnRNP C1/C2 as indicated. The lowest panel shows the A_{260} profile of the gradient and the arrows indicate the ribosomal peaks. (B) An immunoprecipitation-polynucleotide kinase assay was performed on the light and heavy TLS-containing fractions from sucrose gradients of lysates from HeLa cells that had been cross-linked in vivo with UV. The upper panel is an autoradiogram reporting on the amount of RNA cross-linked to TLS and the lower panel an immunoblot reporting on the amount of TLS protein recovered in the two fractions. Note that only the heavy fraction contains TLS cross-linked to RNA.

data). This suggests that sub-cellular localization of TLS is mediated by multiple domains and is different mechanistically from that of hnRNP A1.

Fractionation of cell lysates according to density has revealed that TLS is present in two distinct fractions: a heavy fraction in which it co-localizes with other core hnRNPs and a lighter fraction that appears to be unique to TLS. The RNA-bound form of TLS is present only in the heavier fraction whereas the lighter one contains free TLS. At this point we do not know what proportion of TLS is free in vivo, since it is clear that at least some of the TLS in the lighter fraction is due to its post-lysis dissociation from the RNP particles. Regardless of mechanism, the presence of TLS but not other hnRNPs in this light fraction suggests that important differences exist between members of the hnRNP class of RNA-binding proteins.

TLS, EWS and the related protein TAFII68 (collectively referred to as TET proteins) have other features that distinguish them from most hnRNPs. Notable are the presence of an N-terminal peptide sequence that can serve as a transcriptional activation domain (May et al., 1993a,b; Zinszner et al., 1994) and the ability to directly interact with components of the transcriptional machinery (Bertolotti et al., 1996). It is tempting to speculate that this unique N terminus serves to link transcription to the process by which the RNA becomes associated with hnRNPs. Similar linkage between transcription and RNA processing has recently been suggested to result from an interaction between cleavage-polyadenylation factors and Pol II (McCracken et al., 1997). In this speculative scheme, once loaded on the RNA the TET proteins assume the role of 'typical' hnRNPs, they bind RNA and they shuttle, as demonstrated here in the case of TLS. In the sarcomas, fusion of the N terminus of the TET proteins to a heterologous DNA-binding domain reverses the direction of the interaction and recruits components of the transcriptional machinery to the target gene contacted by the chimeric oncoprotein, thus unmasking a latent transcriptional activation function.

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