MOLECULAR AND CELLULAR BIOLOGY, Aug. 1995, p. 4562–4571 Vol. 15, No. 8
0270-7306/95/$04.00+0

Association of SARFH (Sarcoma-Associated RNA-Binding Fly Homolog) with Regions of Chromatin Transcribed by RNA Polymerase II

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Received 22 February 1995/Returned for modification 11 April 1995/Accepted 15 May 1995

Many oncogenes associated with human sarcomas are composed of a fusion between transcription factors and the N-terminal portions of two similar RNA-binding proteins, TLS and EWS. Though the oncogenic fusion proteins lack the RNA-binding domain and do not bind RNA, the contribution from the N-terminal portion of the RNA-binding protein is essential for their transforming activity. TLS and EWS associate in vivo with RNA polymerase II (Pol II) transcripts. To learn more about the target gene specificity of this interaction, the localization of a *Drosophila melanogaster* protein that has extensive sequence identity to the C-terminal RNA-binding portions of TLS and EWS was studied in preparations of *Drosophila* polytene nuclei. cDNA clones encoding the full-length *Drosophila* TLS-EWS homolog, SARFH (stands for sarcoma-associated RNA-binding fly homolog), were isolated. Functional similarity to TLS and EWS was revealed by the association of SARFH with Pol II transcripts in mammalian cells and by the ability of SARFH to elicit homologous down-regulation of the levels of the mammalian proteins. The SARFH gene is expressed in the developing *Drosophila* embryo from the earliest stages of cellularization and is subsequently found in many cell types. In preparations of polytene chromosomes from salivary gland nuclei, SARFH antibodies recognize their target associated with the majority of active transcription units, revealed by colocalization with the phosphorylated form of RNA Pol II. We conclude that SARFH and, by homology, EWS and TLS participate in a function common to the expression of most genes transcribed by RNA Pol II.

Recent work on the molecular basis of human sarcomas has revealed a clinically heterogeneous group of tumors that are associated with chromosomal translocations. These gene rearrangements lead to the fusion of the N-terminal portions of two highly similar proteins, TLS and EWS, to the DNA-binding domain of one of several transcription factors: FLI1, ERG, ATF1, and CHOP (6, 7, 23, 31, 32). The EWS and TLS genes are expressed at high levels in many different tissues. They encode proteins that have in their C termini a highly characteristic structural motif, the RNA recognition motif (RRM), found in many RNA-binding proteins (3, 10). In sarcomas, the translocation-derived fusion protein is missing the RRM. Instead, it has been replaced by a portion of the transcription factor that mediates DNA binding (in the case of FLI1 and ERG) and dimerization (in the case of CHOP and ATF1). In two tumor types studied in detail, Ewing's sarcoma containing EWS-FLI1 and myxoid liposarcoma containing TLS-CHOP, the fusion protein is expressed at high levels, whereas the germ line-encoded transcription factor is not (2, 6, 16). However, overexpressing FLI1 or CHOP does not by itself transform cells, whereas expression of EWS-FLI1 or TLS-CHOP does (15, 16, 30). These experiments establish an important role for the N-terminal portions of TLS and EWS in transformation by these fusion oncogenes.

TLS, expressed in bacteria, is capable of binding to mRNA in vitro, and binding is mediated by the C-terminal portion of the molecule (6). In vivo, association of TLS with nuclear structures is dependent on ongoing transcription by RNA polymerase II (Pol II), as it is sensitive to disruption by inhibitors such as α-amanitin (30). Immune precipitation studies show that TLS and EWS are associated in a complex with heterogeneous nuclear RNA-binding proteins (30). These observations suggest that the germ line forms of the sarcoma-associated proteins function intracellularly as RNA-binding proteins and associate with Pol II transcripts. It seems reasonable to speculate that association with the transcript, mediated via the RNA-binding domain, serves to localize the N-terminal oncogene-associated effector domain to the vicinity of its normal cellular contingent molecules. Identification of target transcripts to which TLS and EWS bind in vivo is an important first step in understanding their role in normal cellular physiology as well as the nature of their contribution to oncogenesis.

A database search for proteins with regions of homology to the highly characteristic RRM of EWS and TLS revealed a previously identified open reading frame (ORF) in a *Drosophila* cDNA (Dpen p19) that had been isolated by virtue of the fact that it hybridized to a probe containing 5′-G-G-N-3′ trinucleotide repeats (9). Comparison of the available sequence for this ORF reveals over 50% identity and 67% similarity to the C-terminal, RNA-binding two thirds of TLS and EWS (6). This suggests that the ORF of Dpen p19 encodes a *Drosophila* protein likely to share the transcript-specific localization of TLS and EWS. Availability of such a *Drosophila* homolog permits the exploitation of the phenomenon of polyteny in larval salivary glands to study the association of the *Drosophila* protein with specific transcribed regions of DNA (for a review, see reference 5). To the extent that the *Drosophila* protein is functionally-related to the mammalian ones in terms of its RNA-binding properties, this line of investigation should provide insight into the restricted or general nature of the interaction of TLS and EWS with transcribed genes.

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Here, we report on the isolation of a full-length cDNA encoding this *Drosophila* protein, which we call SARFH (stands for sarcoma-associated RNA-binding fly homolog). An analysis of its functional similarity to TLS and EWS is presented, and its intranuclear localization with respect to transcribed areas of chromatin is explored.

MATERIALS AND METHODS

Isolation and characterization of the SARFH cDNA clones and chromosomal localization of the gene. A UNI-ZAP cDNA library made from *Drosophila melanogaster* embryos (Canton S strain, 2 to 14 h old; Stratagene) was screened with an internal 634-bp *Bam*HI fragment of the Dpen p19 cDNA clone (9). Sixty-three positive plaques were identified after screening of 4×10^5 plaques; of these, 18 were further characterized by restriction mapping. The two largest clones were sequenced by the dideoxy chain termination method with a Sequenase 2.0 kit (U.S. Biochemical Corporation). Sequence entry and comparisons were done on a VAX computer, using the Genetics Computer Group Program Manual for the version 7 package (April 1991) and Intelligenetics GeneWorks software.

Genomic clones of SARFH were isolated from a Lambda FIX II library prepared from partially *Sau*3A-digested *Drosophila melanogaster* genomic DNA (Canton S strain; Stratagene), using the full-length SARFH cDNA as a probe. Four distinct, overlapping genomic clones that form a single contig were isolated. An internal 4.3-kb *Xba*I fragment from one of the clones was used in a Southern blot on *Drosophila* genomic DNA, and it gave a pattern of shared bands with the cDNA probe, indicating that it maps to the SARFH locus. This fragment was subcloned into pBluescribe, and the digoxigenin-labeled plasmid DNA was used as a probe to map the gene by in situ hybridization to fixed squashes of polytene chromosomes from larval salivary glands (14).

Antisense RNA in situ histohybridization of whole-mount embryos. *Drosophila* embryos (0 to 24 h) were prepared for antisense RNA in situ histohybridization as previously described (12). The templates for generating the antisense RNA probe and sense RNA control were constructed from a 229-bp *Bam*HI-*Hae*III fragment corresponding to the RRM of SARFH. RNA labeling with digoxigenin-UTP was performed with T7 (for sense RNA) and T3 (for antisense RNA) RNA polymerases according to the manufacturer's instructions (Boehringer Mannheim Biochemicals). Antisense RNA was used at a dilution of 1:10, and sense RNA was used undiluted.

Preparation of antibodies, Western immunoblots, and immune cytochemistry. Mice and rabbits were immunized with a purified bacterially expressed glutathione *S*-transferase fusion protein that contained the N terminus of SARFH (amino acids [aa] 1 to 125 of the type 2 molecule). Polyclonal rabbit antiserum to SARFH was affinity purified on a matrix consisting of a bacterially expressed peptide of SARFH (aa 1 to 125) covalently linked to Sepharose 4B beads. Monoclonal antibodies to SARFH were prepared by fusing spleen cells from a single immunized BALB/c female mouse to P3X63-AG8.653 myeloma cells obtained from the American Type Culture Collection. The hybridoma referred to as 3F4 recognizes an epitope common to the type 1 and type 2 proteins at the C terminus of the aa 1 to 125 peptide used as an antigen. The hybridoma referred to as 1G5 recognizes an epitope that is present only in the type 2 protein.

To construct eukaryotic expression plasmids for the type 1 and type 2 SARFH proteins, the corresponding full-length cDNAs were cloned into the *Eco*RI-*Xho*I sites of the pcDNA1 plasmid (Invitrogen). COS1 cells were transfected by the DEAE-dextran method, and whole-cell lysates were prepared for Western immunoblotting on sodium dodecyl sulfate (SDS)–12% polyacrylamide gel electrophoresis gels as previously described (24). Western immunoblotting and immune cytochemistry were performed as previously described (24), with the modification that hybridoma supernatants were used at a dilution of 1:50 for Western immunoblotting and at 1:10 for immunocytochemistry.

Transformation of NIH-3T3 cells by SARFH-CHOP fusion proteins. Retroviral expression vectors for SARFH-CHOP were created by fusing the amino-terminal portion of the type 1 protein (aa 1 to 81) or type 2 protein (aa 1 to 125) to the full-length human CHOP coding region at the residue preceding the initiator methionine of human CHOP. The fusion cDNA was introduced into the plasmid pSRα that encodes a replication-defective retrovirus in which the target gene is under the control of the viral long terminal repeat and an internal transcription unit expresses the bacterial *neo* gene. Packaging was performed by transfection of the pSRα plasmid into COS1 cells alongside a helper plasmid (18). TLS-CHOP-expressing virus and CHOP-expressing virus, respectively, were used as positive and negative controls for oncogenic transformation. Virus-containing culture supernatants of the transfected COS1 cells were used to infect NIH-3T3 cells. Following infection, the cells were cultured for 10 days under selection with G418 (450 mg/l), trypsinized, and plated into soft agar (13). Two weeks after the cells were plated into soft agar, the number of colonies was counted and the plates were photographed at ×50 magnification under bright-field illumination. To control for differences in the titers of the different retroviral stocks, the titer of each stock was determined by counting the number of G418-resistant colonies produced 10 days after infection of cells in 60-mm-diameter plates with 10 μl of retroviral stock. The levels of expression of the virus-encoded proteins in the infected cells were monitored by Western blotting (immunoblotting). Intracellular localization of the proteins in COS1 cells transfected with the pSRα constructs was evaluated by immunocytochemistry. Both methods were performed with a rabbit anti-CHOP antiserum (24).

Microinjection of HeLa cells. HeLa cells growing on glass coverslips were microinjected with pCDNA1-based plasmids expressing the full-length SARFH protein, a fusion protein consisting of the 125 N-terminal aa of SARFH linked to the DNA-binding domain of the yeast protein Gal4, the SARFH type 2-CHOP fusion protein described above, or CHOP-Gal4 (32). Microinjection was performed with the Zeiss AIS system as previously described (28). Experiments designed to monitor the intracellular fate of SARFH upon inhibition of RNA Pol II transcription were carried out exactly as previously described (22). Simultaneous detection of TLS or heterogeneous nuclear ribonucleoprotein (hnRNP) C1/C2 and SARFH in the microinjected cells was carried out 48 h after injection. The rabbit anti-TLS serum is directed against the N terminus of the molecule and has been previously described (6); it was used at a dilution of 1:1,000. The murine monoclonal antibody to hnRNP C1/C2 (4F4) was a gift of Gideon Dreyfuss and was used at a dilution of 1:1,000. The secondary antibodies, rhodamine-conjugated affinity-purified donkey anti-rabbit immunoglobulin G (IgG) and fluorescein-conjugated affinity-purified donkey anti-murine IgG, had been cross-absorbed by the manufacturer against the heterologous IgG (Chemicon International, Temecula, Calif.). Both were used at a dilution of 1:100.

Localization of the SARFH protein in larval salivary gland nuclei and on polytene chromosomes. Larvae from the Oregon S strain were grown at room temperature on the Sigma Fruitfly Diet until the third instar stage of development. Where indicated, heat shock preparations were performed by submersion of a 1.5-ml Eppendorf tube containing third-instar larvae into a 37°C water bath for 15 min followed by quick dissection of larval salivary glands as described below. Salivary glands from third-instar larvae were dissected in phosphate-buffered saline (PBS)–0.5% Triton X-100 and rinsed in PBS–0.5% Triton X-100 as described by Weeks et al. (29). Briefly, the glands were fixed in PBS–0.5% Triton X-100–3.7% formaldehyde for 30 s, rinsed in PBS–0.5% Triton X-100, transferred into Eppendorf tubes with PBS–0.1% Tween 20, and washed three times in PBS–0.1% Tween 20. Chromosomal spreads were made by squashing the fixed glands between a slide and a coverslip.

The primary antibodies used were either a 1:10 dilution of pooled monoclonal mouse anti-SARFH antibodies which recognizes both type 1 and type 2 SARFH and a 1:20 dilution of affinity-purified rabbit IgG directed against the hyperphosphorylated C-terminal domain of *Drosophila* RNA Pol II (a gift of John Weeks and Arno Greenleaf) or, in the case of whole-gland staining, a 1:100 dilution of affinity-purified rabbit polyclonal anti-SARFH antiserum that had been preabsorbed against *Drosophila* embryos. Incubation with antibodies was done at room temperature for 60 min in the case of the chromosomal squashes or at 4°C for 14 h on a rotating platform in the case of the whole-gland staining. After primary antibody incubation, the preparations were washed three times in PBS–0.1% Tween 20 for 5 min with gentle agitation. The secondary antibodies used were 1:50 dilutions (in PBS–0.1% Tween 20) of either fluorescein-conjugated donkey anti-mouse or rhodamine-conjugated donkey anti-rabbit antibodies (Chemicon International). The slides were then rinsed in 1 ml of PBS–0.1% Tween 20 with H33258 (1 ng/ml) for 1 min and washed three times in PBS–0.1% Tween 20 for 5 min with gentle agitation. Coverslips were mounted, and immunofluorescence microscopy of chromosome preparations was done with a Zeiss Axiophot microscope through a Plan-neofluar 40× or Plan-Achromat 63× objective.

Nucleotide sequence accession number. The sequence of SARFH has been submitted to GenBank under the accession number U13178.

RESULTS

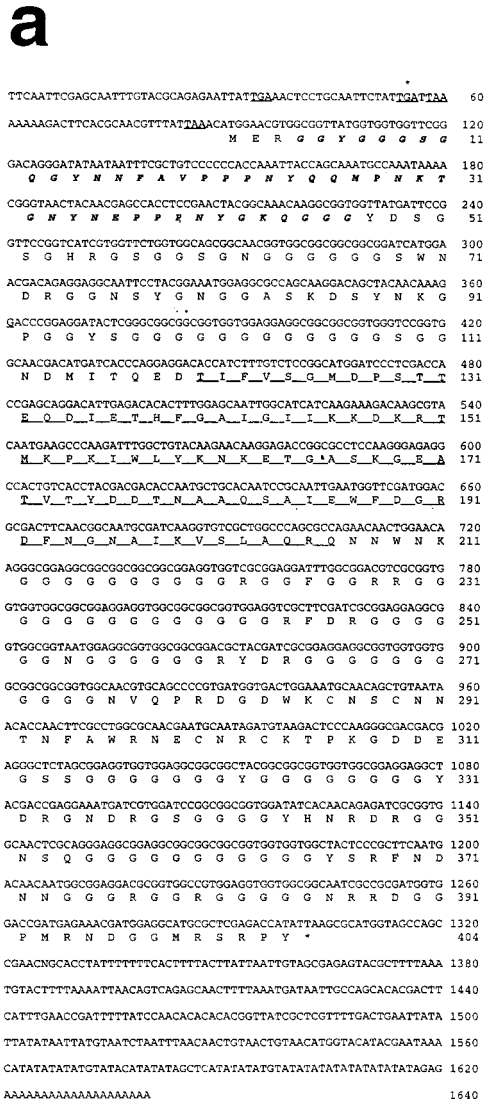
SARFH cDNA encodes a protein with C-terminal sequence identity to EWS and TLS. The Dpen p19 cDNA originally isolated from a pupal library was a partial cDNA (9). An ORF present at one end of the clone encodes a protein with considerable (>50%) identity to the C-terminal RRM-containing portion of TLS and EWS (6, 7). The EWS-TLS homology region of the Dpen p19 cDNA (a kind gift of Susan Haynes) was used as a probe to isolate cDNA clones containing the full-length coding region. In screening 4×10^5 recombinant plaques from a *Drosophila* embryonic (2 to 14 h) cDNA library, we obtained 63 positive clones, of which 18 were characterized further. The largest cDNA was 1,620 bp in length, which is in the size range of the mRNA as estimated by Northern (RNA) blot analysis of embryonic RNA (data not shown). Restriction mapping and sequence analysis of the clones revealed two forms of the cDNA that differ from each other by the presence or absence of a 132-bp insert that encodes a 44-aa stretch in the N terminus of the protein. The ORF of the two transcripts (the type 1 shorter version lacking the 44-aa insertion and the type

2 longer version containing the insertion) initiates at the same methionine and is preceded by an in-frame stop codon. The type 1 version encodes a protein of 360 aa, and the type 2 version encodes a protein of 404 aa. We named the protein SARFH (sarcoma-associated RNA-binding fly homolog) (Fig. 1a).

Comparison of SARFH with TLS and EWS revealed that the previously noted homology between the RRMs extends to other parts of the *Drosophila* protein, including the Arg-Gly-Gly repeats that flank the RRM predominantly on the C-terminal side (such repeats have recently been shown to participate directly in RNA binding [11]) and the extreme C termini of the proteins (Fig. 1b and c). In the N terminus, the aforementioned 44-aa insertion present in the type 2 protein contains blocks of residues with resemblance to the Gln-, Gly-, Tyr-, and Pro-rich domain of the human proteins. Two forms of SARFH are easily detected by PCR analysis of cDNA made from *Drosophila* embryos and likely represent splicing variants of the SARFH gene (data not shown). SARFH type 2 shows 69% similarity and 53% identity to TLS and 60% similarity and 43% identity to EWS (Fig. 1c).

The SARFH cDNA probe was hybridized at high stringency to a *Drosophila* genomic DNA Southern blot digested with *Eco*RI or *Bam*HI, and a simple pattern of one and three bands, respectively, was obtained, which is consistent with the existence of a single-copy SARFH gene (data not shown). We used the SARFH cDNA as a probe to isolate genomic clones, and we used a 4.3-kb *Xba*I fragment from one of these to map the SARFH gene to band 14B on the X chromosome (Fig. 1d). This chromosomal localization has been independently confirmed by Susan R. Haynes and colleagues (8).

To determine if the cDNA clones isolated encode the full-length protein, we raised antisera to the bacterially expressed glutathione *S*-transferase–SARFH fusion protein. Western immunoblots of lysates from COS1 cells transfected with expression plasmids for the type 1 or type 2 form of SARFH were compared with nuclear extracts from *Drosophila* embryos. Two different monoclonal antibodies to SARFH were used. The 3F4 antibody recognizes an epitope common to both the type



1 and type 2 proteins. This antibody recognizes three bands in nuclear extracts from *Drosophila* embryos, a doublet at 49 kDa and a single band at 45 kDa (Fig. 2, lane 1). The doublet corresponds to the type 2 form of SARFH, as it is present only in COS1 cells transfected with type 2 cDNA (Fig. 2, lane 2). The 45-kDa band corresponds to the type 1 form, as it is present only in COS1 cells transfected with type 1 cDNA (Fig. 2, lane 3). The 1G5 antibody reacts with an epitope that is present only on the type 2 protein and consequently detects only the 49-kDa doublet in the embryo extract (Fig. 2, lanes 4 to 6). The absence of shared bands between COS1 cells transfected with type 1 cDNA and those transfected with type 2 cDNA (Fig. 2 [compare lanes 2 and 3 and lanes 5 and 6]) as well as the lack of reactivity of extracts from nontransfected

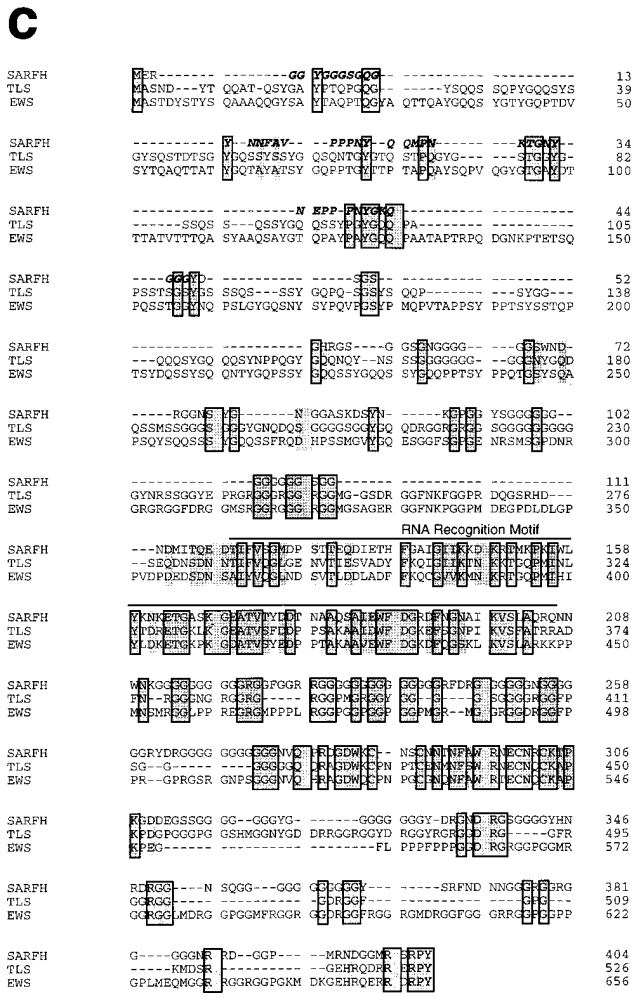


FIG. 1. SARFH sequence and gene localization. (a) SARFH cDNA nucleotide and predicted amino acid sequence. Four upstream stop codons, one of which is in frame (marked by an asterisk) are underlined. The 44-aa sequence present only in SARFH type 2 is indicated in italicized, boldface print. The RRM is indicated by the broken line. (b) Comparison of the structures of SARFH type 1, SARFH type 2, TLS, and EWS. The four sequences were aligned by their RRMs, the most conserved domain among these proteins. Each Arg-Gly-Gly repeat is indicated by dashes, and the conserved portion at the extreme C terminus is shaded dark gray. The 44-aa insertion of SARFH type 2 is shaded light gray. (c) Amino acid alignment between SARFH, TLS, and EWS obtained by using the Intelligenetics GeneWorks software package. Similar residues are shaded, and identical residues are shaded and boxed. The RRM is indicated. The 44-aa insertion of SARFH type 2 is indicated in italicized boldface print. (d) Mapping of the SARFH gene on salivary gland polytene chromosomes by in situ histohybridization. The arrowhead points to the SARFH signal in band 14B on the X chromosome.

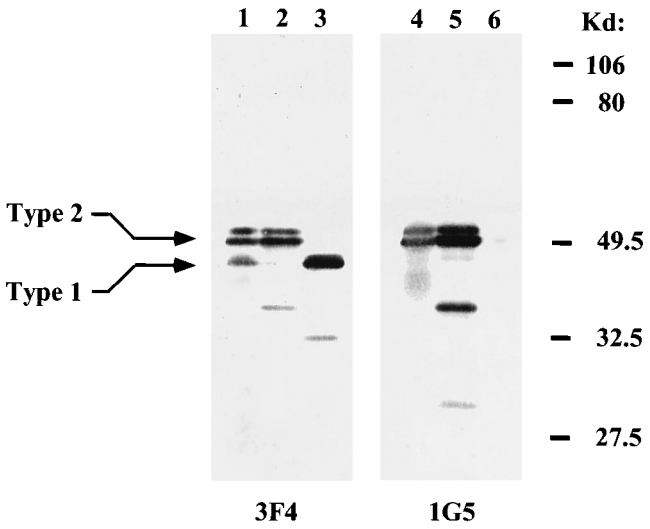


FIG. 2. SARFH cDNA type 1 and type 2 clones encode full-length proteins expressed in *Drosophila* embryos. Lysates of COS1 cells transfected with expression plasmids for SARFH type 2 (lanes 2 and 5) or type 1 (lanes 3 and 6) were compared with nuclear extracts from *Drosophila* embryos (lanes 1 and 4) in a Western immunoblot. The monoclonal antibodies to SARFH, 3F4 and 1G5, were used on identical blots, as indicated. 3F4 reacts with an epitope common to both types of SARFH proteins, whereas 1G5 reacts with an epitope unique to the type 2 protein.

cells (data not shown) indicates that the antibodies do not react significantly with mammalian cellular proteins in this assay. The migration of the type 2 form of SARFH as a doublet may indicate posttranslational modifications of the protein or the existence of multiple conformations that are not fully denatured by SDS and heating. The nature of additional smaller, SARFH-specific bands detected in the COS1 cells is not known. The fact that they are absent from the *Drosophila* embryo nuclear extract is consistent with their being an in vivo artifact of the massive overexpression of the proteins in the transfected cells. From these experiments we conclude that the two forms of SARFH isolated correspond to the two forms of the protein found in *Drosophila* embryos.

The SARFH gene is widely expressed in the developing *Drosophila* embryo. In mammals, TLS and EWS are expressed in many organs (30). To determine if the *Drosophila* homolog SARFH is similarly expressed in a broad range of cell lineages, we performed in situ histohybridization on whole mounts of fly embryos ranging in age from 0 to 24 h postfertilization. The digoxigenin-labeled antisense SARFH riboprobe detected a signal that was present from the earliest stages of blastoderm formation, suggesting that SARFH is expressed from the time of activation of zygotic gene transcription (Fig. 3A and B). No maternal loading of mRNA or protein was observed in the eggs. Expression persists in all regions of the developing embryo, and the intensity of the signal roughly correlates with the degree of cell density in the different regions. For example, heavy staining is noted in the anterior region of later-stage embryos that have already undergone development of central nervous system structures but not prior to that (Fig. 3, compare panels B and C). Staining with the sense probe gave only a background signal, attesting to the specificity of the pattern identified (Fig. 3D). Using immune cytochemistry, we detected high levels of the antigen in virtually all nuclei of the developing embryo as well as in larval salivary glands and ducts (see Fig. 6A). We conclude that SARFH is expressed in many *Drosophila* tissues and cell types.

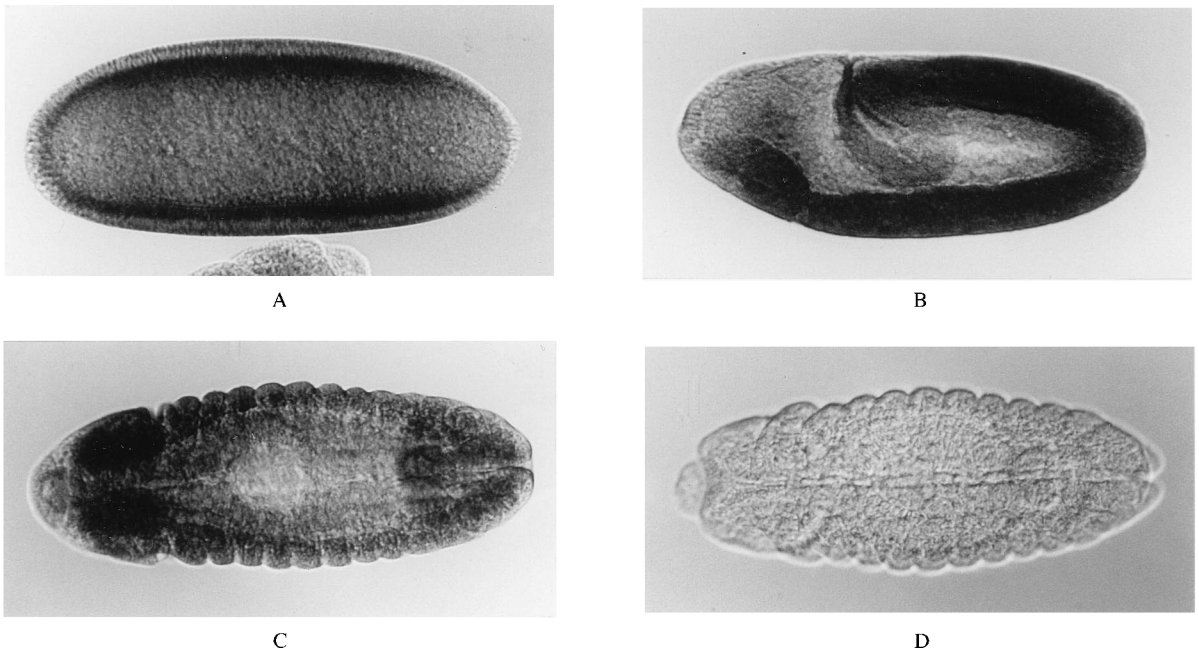


FIG. 3. The SARFH gene is expressed from the earliest stages of development. Digoxigenin-labeled antisense SARFH riboprobe (A through C) and control, sense riboprobe (D) were hybridized in situ to whole-mount *Drosophila* embryos. A signal is present from the earliest stages of blastoderm formation (A [stage 5]) that is maintained throughout development (B [extended germ band] and C [stages 12 to 14]). (D) The negative-control digoxigenin-labeled sense SARFH riboprobe detected only a background signal, confirming the specificity of the pattern identified by the antisense probe.

Evidence for functional homology between SARFH, TLS, and EWS. The similarity in the C-terminal putative RNA-binding portions of SARFH, TLS, and EWS as well as the fact that all three proteins appear to be expressed in many cell types led us to examine the degree to which SARFH exhibits functional similarity to the mammalian proteins. To address this issue experimentally, SARFH was expressed in mammalian cells by microinjecting the cDNA into HeLa cells and staining them for the SARFH antigen. Under normal conditions, SARFH is localized to the nuclei of the injected HeLa cells, giving rise to a diffuse nucleoplasmic staining (Fig. 4A, leftmost panels [upper and lower]). If, however, the injected cells are treated with α-amanitin or actinomycin D, inhibitors of RNA Pol II, SARFH accumulates in the cytosol (Fig. 4A, second panels from the left [upper and lower]; the H33258 DNA stain in the lower panel serves to confirm that redistribution of SARFH is not due to mitosis). Relocation to the cytosol in response to inhibition of RNA Pol II transcription is a property that SARFH shares with TLS, EWS and certain other RNA-binding proteins (22, 30) but not DNA-binding proteins such as CHOP or TLS-CHOP (30).

To determine if cytoplasmic localization in response to inhibition of Pol II is dependent on the C-terminal portion of SARFH, we studied the cellular localization of derivative SARFH proteins in which the C-terminal RRM-containing portion had been replaced by the DNA-binding domain of the yeast Gal4 protein or the CHOP coding region. The fusion proteins, which are nuclear under normal conditions (see Fig. 5C), do not relocalize into the cytosol in response to α-amanitin treatment (Fig. 4A, third and fifth panels [upper and lower]). Similarly, the unrelated CHOP-Gal4 fusion protein remains in the nucleus following inhibition of Pol II (Fig. 4A, fourth panels [upper and lower]). This result is consistent with the hypothesis that the RRM-containing C-terminal portion of SARFH specifies the changes in cellular localization of the protein in response to inhibition of RNA Pol II. It remains possible, however, that retention of the SARFH-Gal4 fusion in the nucleus is due to a dominant effect of Gal4 or CHOP DNA-binding domains rather than reflecting the consequences of the loss of the C-terminal portion of SARFH.

By coimmunostaining microinjected cells with antibodies specific to SARFH and to TLS and EWS, we noted that most but not all cells expressing high levels of type 1 or type 2 SARFH contained lower levels of TLS and EWS immune reactivity (Fig. 4B, left panels). This finding was also observed with COS1 cells transfected with the SARFH expression plasmid (data not shown). To evaluate the degree to which this phenomenon is dependent on the C-terminal TLS-EWS homology region of SARFH, we microinjected the aforementioned SARFH-Gal4 cDNA into HeLa cells. The fusion protein did not induce heterologous down-regulation of TLS or EWS (Fig. 4B, middle panels). This finding indicates that the C-terminal RRM-containing portion of SARFH is essential for heterologous down-regulation to occur. To evaluate the specificity of this phenomenon for TLS and EWS, we costained SARFH-injected cells with an antibody to hnRNP C1/C2. No decrease in the hnRNP signal was observed in the SARFH-expressing cells (Fig. 4B, right panels). The specificity of the heterologous down-regulation is good evidence for a functional similarity between SARFH and both TLS and EWS.

Fusion of the N terminus of TLS or EWS to the DNA-binding domain of CHOP promotes oncogenic transformation of NIH-3T3 cells (30). We tested to see if the corresponding domain of SARFH can likewise serve an oncogenic role. Replication-defective ecotrophic retroviruses that express fusion proteins consisting of either the type 1 or the type 2 form of SARFH and CHOP were produced. SARFH-CHOP-transduced NIH-3T3 cells were compared with TLS-CHOP (a positive control)-and CHOP (a negative control)-transduced cells

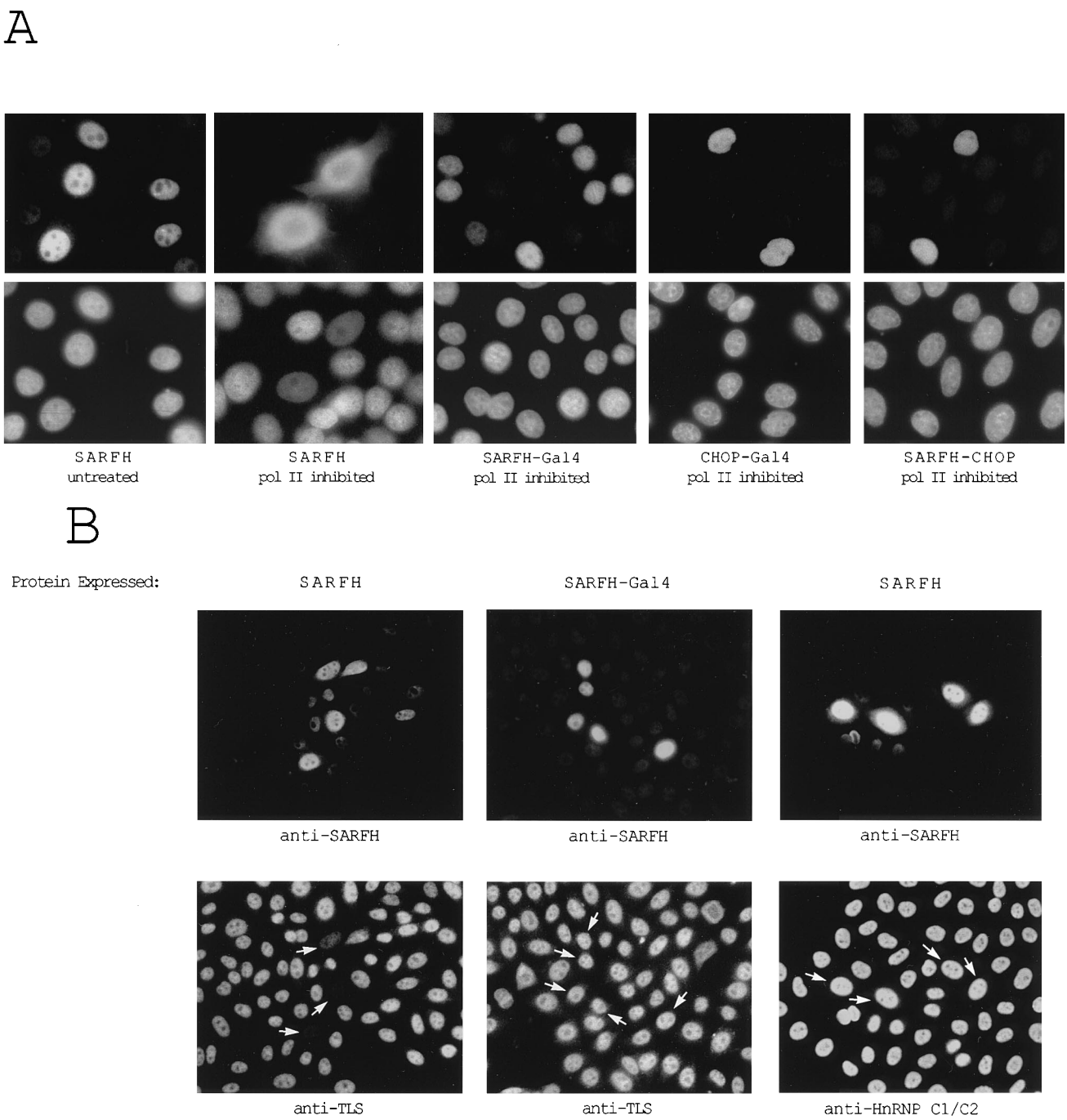


FIG. 4. Tests of functional similarity to mammalian sarcoma-associated proteins. (A) Inhibition of RNA Pol II transcription alters the subcellular localization of SARFH. SARFH localizes to the nucleus under normal conditions, but when cells are treated with α-amanitin, SARFH redistributes to the cytoplasm. HeLa cells grown on coverslips were microinjected with expression plasmids encoding SARFH, a truncated version of SARFH in which the RRM-containing C terminus had been replaced by the DNA-binding domain of Gal4 (SARFH-Gal4), or similar fusions between CHOP and Gal4 or SARFH and CHOP. Forty-eight hours after microinjection, the cells were treated for 5 h with α-amanitin (50 μg/ml) or left untreated as a control. The cells were fixed and then stained with anti-SARFH (first three vertical panels from the left) or anti-CHOP (last two vertical panels from the left) monoclonal antibodies (upper panels) and then with the DNA-binding dye H33258 that preferentially stains nuclei (lower panels). (B) Homologous down-regulation of TLS and EWS levels by SARFH. Microinjected HeLa cells growing on coverslips were costained with a mouse monoclonal antibody to SARFH (left and middle top panels) and a rabbit polyclonal antibody to TLS or EWS (left and middle bottom panels) or a rabbit polyclonal antibody to SARFH (right top panel) and a mouse monoclonal antibody to hnRNP C1/C2 (right bottom panel). The arrows in each lower panel indicate the positions of the microinjected cells as visualized in the corresponding upper panel. Note that cells expressing the highest level of SARFH have markedly decreased TLS immune reactivity but unchanged hnRNP C1/C2 immune reactivity.

for their ability to grow as colonies in soft agar (Fig. 5A). The type 2 SARFH-CHOP protein readily transformed NIH-3T3 cells, giving rise to as many colonies in soft agar as TLS-CHOP. The SARFH-CHOP colonies were, however, smaller than the TLS-CHOP colonies. The type 1 protein was less potent in transforming cells, giving rise to even smaller colonies that numbered less than one third of those obtained with the type 2 protein (4.9 ± 1.9 versus 16.3 ± 4.9 colonies [mean ±

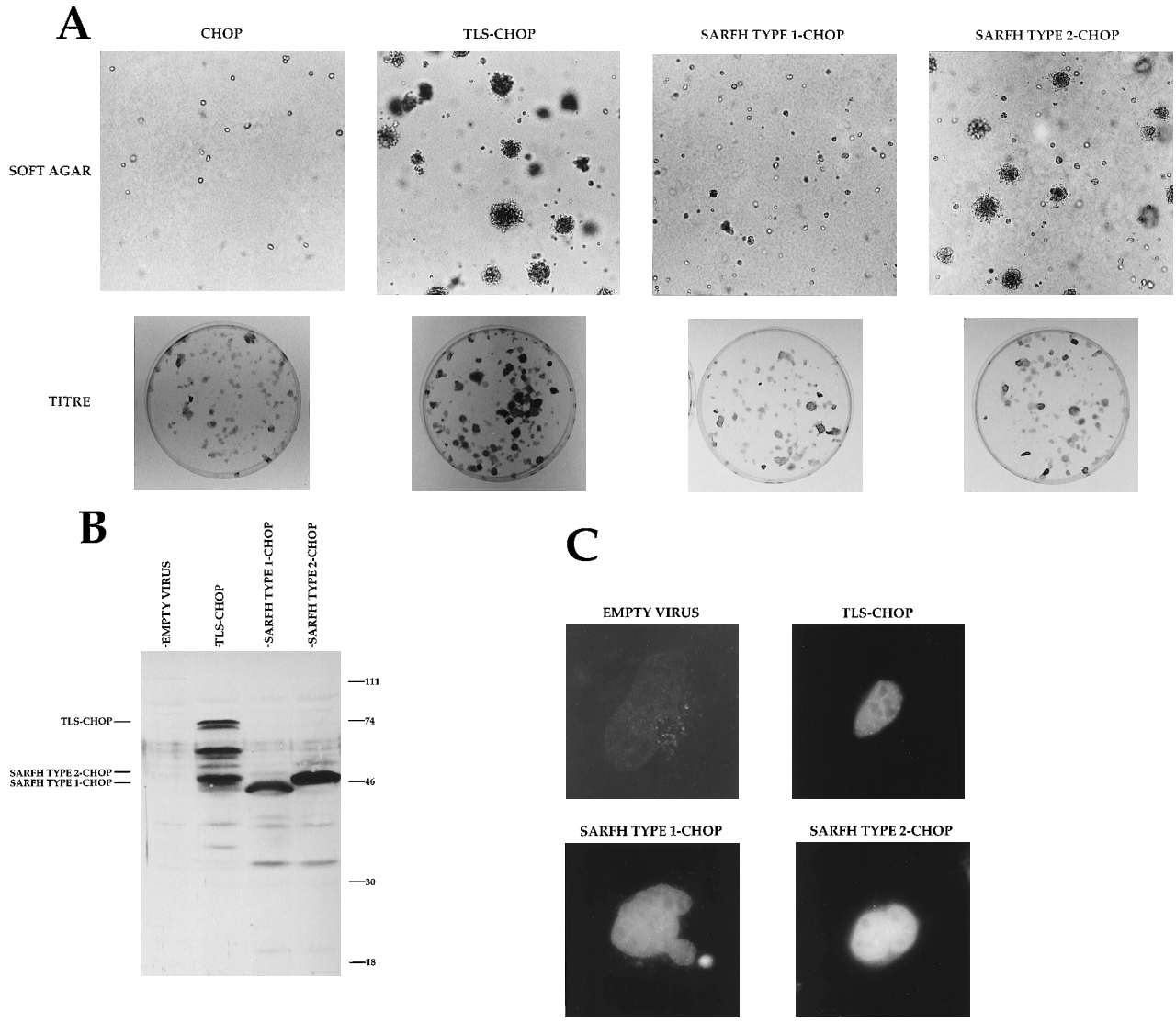


FIG. 5. SARFH-CHOP fusion protein promotes oncogenic transformation of NIH-3T3 cells. (A) Colony formation in soft agar. NIH-3T3 cells were infected with retroviral expression vectors encoding the indicated proteins. Ten days postinfection, the cells were plated in soft agar. Colonies were photographed at 2 weeks postplating at a magnification of ×50 (upper panels). A sister plate was treated with G418 to select for colonies of infected cells. Ten days later, the colonies on the plates were stained with crystal violet; their number reflects the titer of the retroviral stock (lower panels). (B) Expression of the retrovirally encoded proteins in infected cells. Shown is an anti-CHOP Western blot of whole-cell lysates prepared from NIH-3T3 cells infected with the indicated retroviral stocks. The positions of the proteins are indicated to the left of the blot (the multiple bands in the TLS-CHOP-infected cells reflect in vitro degradation of the protein). (C) Nuclear localization of the SARFH-CHOP derivatives. COS1 cells transfected with the proviral plasmids encoding the indicated proteins were stained with CHOP antiserum and a fluorescent secondary antibody. The cells were photographed at ×630 magnification.

standard deviation] per ×50 microscope field). Both SARFH-CHOP proteins were expressed at high levels in the infected cells (Fig. 5B) and localized appropriately to the nucleus (Fig. 5C). This result suggests that the functional similarity between TLS and SARFH extends beyond the C-terminal RNA-binding domain and into the N termini of the proteins.

SARFH protein localizes to the majority of active transcription units in polytene larval salivary gland nuclei. Having established that SARFH satisfies both structural and functional criteria for being a TLS-EWS homolog, we sought to determine the gene-specific spectrum of its association with RNA. First, we determined that SARFH is expressed at high levels in the polytene nuclei of the *Drosophila* third-instar larval salivary glands: the nuclei stain brightly with an antiserum to SARFH (Fig. 6A). We next prepared chromosomal squashes from fixed salivary glands and stained the squashes with the SARFH antibodies. Multiple reproducible bands of SARFH staining were apparent in the chromosomal preparations stained with a pool of monoclonal antibodies to SARFH. Staining was noted in many "puffed" regions of the chromosomes corresponding to developmental stage-specific areas of decondensed chromatin associated with active gene transcription (compare the top left and top right panels in Fig. 6B). The association with actively transcribed genes was further documented by costaining the squashes with antisera that recognize the active (phosphorylated, Pol II°) form of RNA Pol II (Fig. 6B, lower left panel). Precise colocalization of the two signals, reflected in the yellow bands in the lower right panel of Fig. 6B, in which the SARFH and Pol II° images have been superimposed, indicates that SARFH is located at actively transcribed regions of chromatin (29).

In addition to the signal detected on actively transcribed

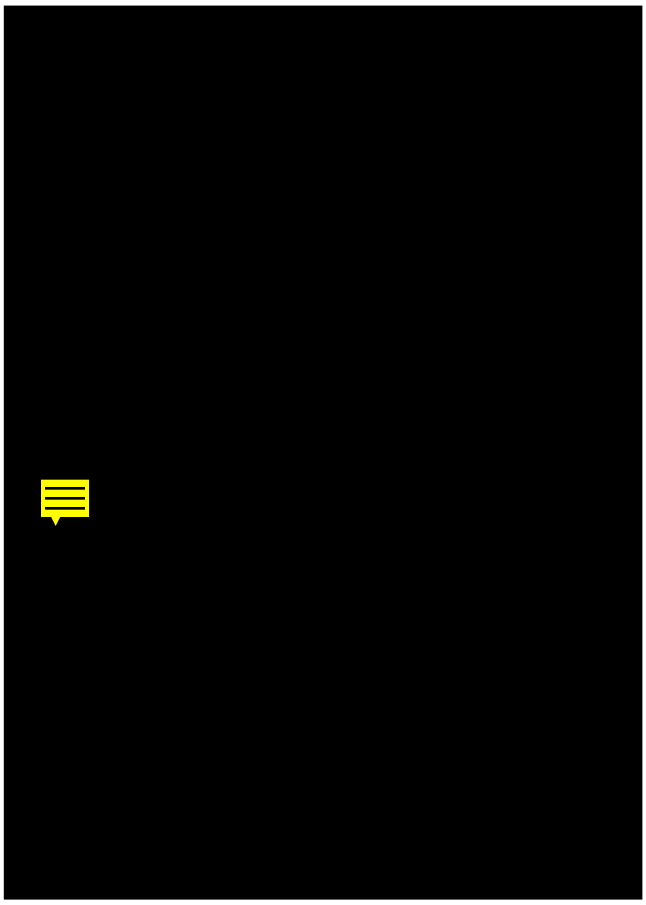


FIG. 6. SARFH binds to most actively transcribed gene loci on polytene chromosomes. (A) Immune staining of SARFH in polytene nuclei of third-instar larvae. Salivary glands were dissected, fixed, and stained with an affinity-purified rabbit polyclonal antiserum to SARFH; this was followed by staining with a rhodamine-conjugated secondary antibody (red). The glands were counterstained with the DNA-binding dye H33258 to delineate the nuclei. (B) SARFH staining colocalizes with the phosphorylated form of RNA Pol II (Pol II°). Fixed chromosomes were stained with an affinity-purified rabbit polyclonal antiserum to *Drosophila* Pol II° (red) and a monoclonal antibody to SARFH (green). In the overlay of both images the regions of costaining are yellow. Arrows denote regions of costaining, with the thick arrows pointing out a prominent developmental puff. (C) SARFH is recruited to heat shock genes upon their transcriptional activation. *Drosophila* larvae were heat shocked for 15 min. Salivary glands were rapidly dissected, fixed, and stained as described above for panel B. Note the colocalization of SARFH and Pol II° to newly formed heat shock puffs at locations 87A and 87C (vertical arrows) and 93D (horizontal arrow).

chromatin, the SARFH antibodies diffusely decorate the chromosomes. This diffuse staining may be due to a nonspecific nucleic acid binding activity commonly associated with RNA-binding proteins. However, careful analysis of the staining pattern demonstrated that the intensity of the SARFH signal did not correlate with that of the DNA-binding dye H33258, indicating that in addition to its background association with chromatin as such, SARFH has a predilection for the most actively transcribing regions. Monoclonal antibodies that recognize an epitope in SARFH common to the type 1 and type 2 forms of the protein (3F4) or an epitope specific to the type 2 form (1G5) gave rise to an indistinguishable staining pattern. This pattern, albeit with a higher background, was also seen with a rabbit polyclonal antiserum to SARFH (data not shown).

In order to determine if the association between SARFH and active transcription is an early event in transcript formation, larvae were heat shocked. Heat shock causes a rapid induction in the transcription of a set of heat shock genes and an equally rapid repression in the transcription of many other non-heat shock genes (1). SARFH staining was absent from the heat shock loci prior to heat shock (data not shown) and became evident at these sites after the larvae were grown for 15 min at 37°C (Fig. 6C, top right panel). SARFH staining colocalized precisely to the intense accumulation of Pol II° at these sites (Fig. 6C, lower left and right panels). We note that SARFH staining of the heat shock puff at location 93D was reproducibly more intense than that of the other heat shock puffs, a feature that SARFH shares with other RNA-binding proteins (20).

DISCUSSION

The structural similarity between SARFH, TLS, and EWS is most apparent in the C-terminal halves of the proteins, with extensive identity revealed at the RRM, the flanking R-G-G repeats, and the extreme C-terminal sequence (Fig. 1b and c). The similarity in the RRMs is particularly striking in view of the fact that all three proteins deviate from the organization of this motif as commonly found in most other RNA-binding proteins. All three proteins contain an acidic residue in the second position and a threonine in the fourth position of the RNP-1 domain of the RRM; most other RNA-binding proteins contain bulky hydrophobic residues in these positions (for reviews, see references 9–11). In addition, all three proteins have an unusually long predicted loop immediately N-terminal to the RNP-1 domain (loop 3 in the solved structure of U1-A [19]). In the case of other RNA-binding proteins, the RNP-1 and loop 3 motifs appear to participate in forming sequence-specific RNA contacts (19, 25). The common special structural features of SARFH and its mammalian homologs suggest that they may bind to RNA in a unique way.

Additional evidence for the similarity between the *Drosophila* protein and the mammalian ones is provided by the fact that SARFH, when expressed in HeLa cells, is targeted to the nucleus and that its cellular localization parallels those of TLS and EWS; inhibition of Pol II transcription leads to an extensive redistribution of both TLS and EWS and of SARFH from the nucleus to the cytoplasm (Fig. 4A) (30). Accumulation in the cytosol in response to inhibition of Pol II transcription is a feature shared by some but not all RNA-binding proteins (17, 22). It may be due to the fact that these proteins shuttle between the nucleus and the cytosol (22). The accumulation in the cytosol following inhibition of polymerase activity implies a dependence of nuclear reimportation on active transcription (21, 22). An alternative, not mutually exclusive, interpretation is that retention of SARFH in the nucleus depends on its continued ability to seek out and bind its RNA target. The role of nuclear retention in determining the intracellular localization of proteins has been demonstrated directly in the case of nucleolin (26).

Evidence for the fact that SARFH is functionally similar to the mammalian sarcoma-associated proteins is provided by the observation that, in most cells, high-level expression of SARFH leads to a reduction in the level of TLS and EWS but has no effect on the related RNA-binding protein hnRNP C1/C2 (Fig. 4B). Because our polyclonal antiserum to TLS may cross-react with EWS in some immunochemical assays and to the extent that both proteins are present in HeLa cells (30), it is impossible to ascribe the phenomena specifically to either one. For SARFH to down-regulate TLS and EWS levels, an intact RNA-binding domain is needed; expression of a SARFH derivative in which the C-terminal RRM-containing region has been replaced by the DNA-binding domain of Gal4 does not

cause a decrease in the TLS or EWS level. The mechanism of this heterologous down-regulation and the reason why it is observed in most but not all cells have not been defined in these studies, but its specificity for TLS or EWS suggests that these sarcoma-associated proteins may regulate their own expression. A precedent for negative autoregulation of the expression of an RNA-binding protein is provided by the case of the small nuclear RNP U1-A protein that binds to its own mRNA and blocks polyadenylation (4). An alternative possibility is that unphysiologically high concentrations of SARFH may compete with TLS or EWS for a common intracellular target, binding to which is necessary for the stability of the mammalian proteins. Regardless of the mechanism, we interpret the ability of SARFH to specifically down-regulate the level of TLS or EWS as evidence of a functional similarity between the proteins.

SARFH is associated with the vast majority of active transcription units in the polytene salivary gland nuclei. Its association with transcribed regions is rapid, as demonstrated by the fact that a brief heat shock of the larvae leads to redistribution of the SARFH signal to the newly formed heat shock puffs. These findings suggest that SARFH associates with the transcript soon upon its formation. In cases of very large genes, this must occur even before transcription of the locus has been completed. The observation that SARFH associates with the heat shock puffs at locations 87A and 87C might suggest that it is not a component of the splicing machinery—these *hsp70* genes do not contain introns (1). However, to the extent that splicing factors may participate in a scanning function that does not distinguish between genes that have introns and those that do not, we cannot effectively rule out a role for SARFH in this aspect of mRNA metabolism. Though in need of direct confirmation by other means, the structural and functional similarities between the putative RNA-binding C-terminal portion of SARFH and those of TLS and EWS suggest that the mammalian proteins may share this promiscuous association with transcribed genes.

The N terminus of SARFH can substitute for that of TLS (and EWS) in transforming NIH-3T3 cells, when fused to CHOP. The functional similarity between SARFH and the mammalian sarcoma-associated proteins thus extends, by this assay, to their N termini. However, the basis for the transforming activity of the N terminus of SARFH is not clear. There is very limited sequence identity between SARFH and the mammalian proteins in this region (Fig. 1c). In addition, we have determined that, in contrast to the N termini of TLS and EWS that possess strong transcriptional activation properties (2, 15, 16, 30), the N terminus of SARFH does not activate transcription in either mammalian or *Drosophila* cells (data not shown). This may indicate that transformation by SARFH-CHOP proceeds by a mechanism that is altogether different from that of TLS-CHOP. On the other hand, transformation by CHOP fusion proteins appears to require something other than mere transactivation (30). And it remains possible that SARFH, in spite of the lack of identity in primary amino acid sequence, retains the relevant TLS-EWS homologous activity. When fused to CHOP, the type 2 form of SARFH transforms much more efficiently than the type 1 form. The compartmentalization of the transforming domain of SARFH to an alternately spliced exon may indicate that this domain plays a distinct regulatory role in the context of the *Drosophila* cell.

SARFH is expressed in many *Drosophila* cell lineages. In situ histohybridization and immunochemistry reveal SARFH mRNA and protein from the earliest stages of development. The mRNA and protein signals roughly parallel the density of cellularization of the embryo, though our data do not rule out the possibility of significant differences in level of expression between various cell types. Embryos contain both the type 1 and type 2 forms of the protein (Fig. 2), whereas later in development (in fly heads and torsos) we detect only the larger, type 2, form of the protein (data not shown). To the extent that the type 2 form contains an effector domain absent from the type 1 form, defined here in terms of its enhanced transforming activity when fused to CHOP, it is tempting to speculate on the possible existence of a developmentally regulated process that specifies the ratio of the two functionally distinct forms of the protein.

A speculative model for the role of SARFH and, by homology, for that of the mammalian sarcoma-associated proteins is that they normally bind the RNA transcript and then contact and regulate the activity of some component of the transcription machinery through the activity of their N-terminal effector domain. The human immunodeficiency virus TAT protein provides a precedent for this speculation, in that it transactivates promoters from its TAR binding sequence on the viral mRNA (27). Perhaps, presentation of a sarcoma-associated effector domain in the context of a DNA-binding protein subtly changes the regulatory properties of the DNA-binding protein and promotes a critical deregulation of the expression of downstream target genes.

ACKNOWLEDGMENTS

We are thankful to Pragna Patel for technical assistance and to Lennart Philipson for comments and suggestions. The following colleagues generously provided assistance, advice, and reagents: Susan Haynes provided the Dpen p19 cDNA; Arthur Kania and Hugo Bellen performed the cytogenetic localization of the SARFH gene; Marcia Simpson-Bose, Anne Goriely, Claude Desplan, and members of his lab assisted with in situ histohybridization; Steve DiNardo and members of his lab advised us on RNA preparation; Leslie Pick provided *Drosophila* embryo nuclear extracts; and John Weeks and Arno Greenleaf provided polyclonal antiserum to the phosphorylated form of *Drosophila* Pol II. Gideon Dreyfuss provided monoclonal antibodies to hnRNP C1/C2, Charles Sawyers and Owen Witte provided the retroviral expression plasmids, and Chris Denny emphasized their utility in oncogenic transformation assays.

This work was supported by a PHS grant to D.R. (CA 60945). Computing was supported by NSF grant DIR-8908095. D.I. is supported by a training grant from NIGMS (5 T32 GM 07308). H.Z. is supported in part by a training grant from INSERM. D.R. acknowledges the Pew Scholars Program in the Biomedical Sciences for support of research in his laboratory.

ADDENDUM IN PROOF

A cDNA virtually identical to SARFH has been isolated and named Cabeza (D. T. Stolow and S. R. Haynes, Nucleic Acids Res. **23:**835–843, 1995).

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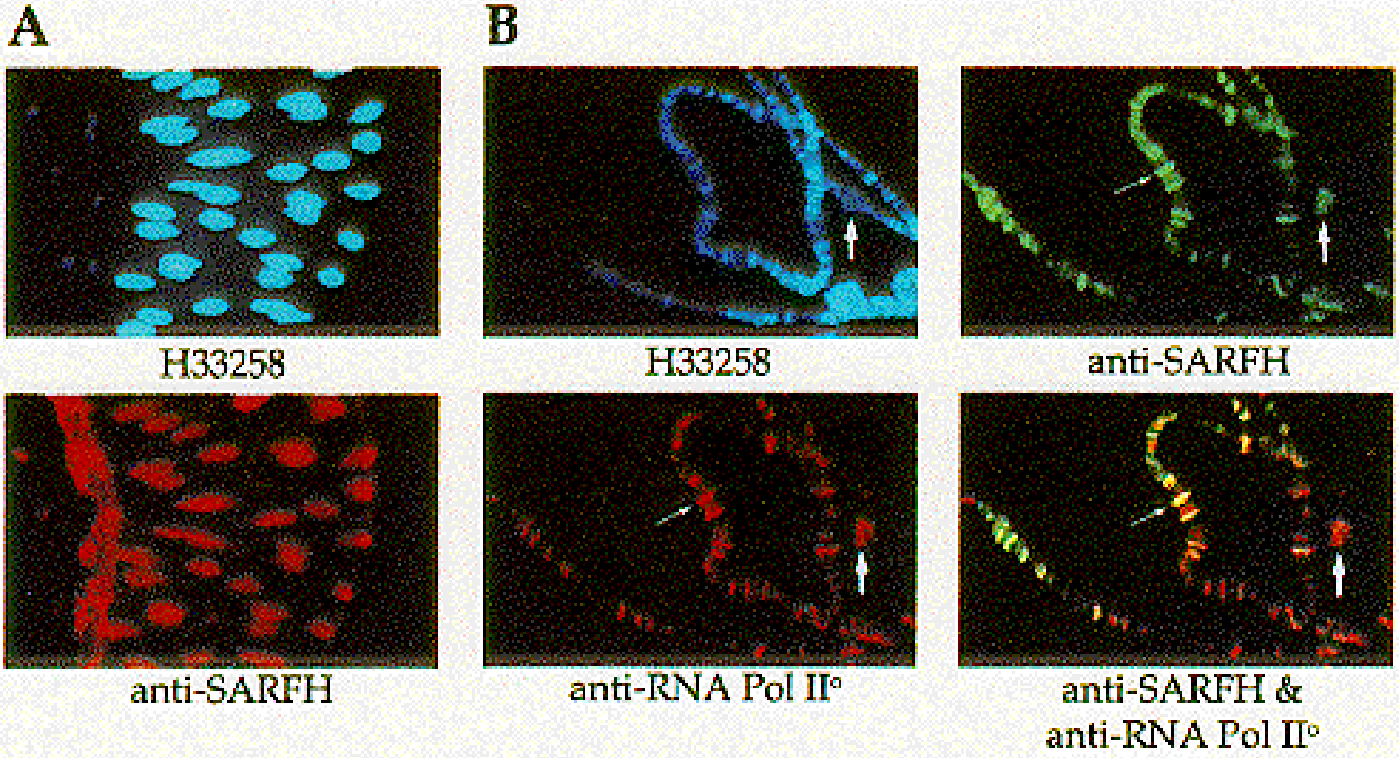
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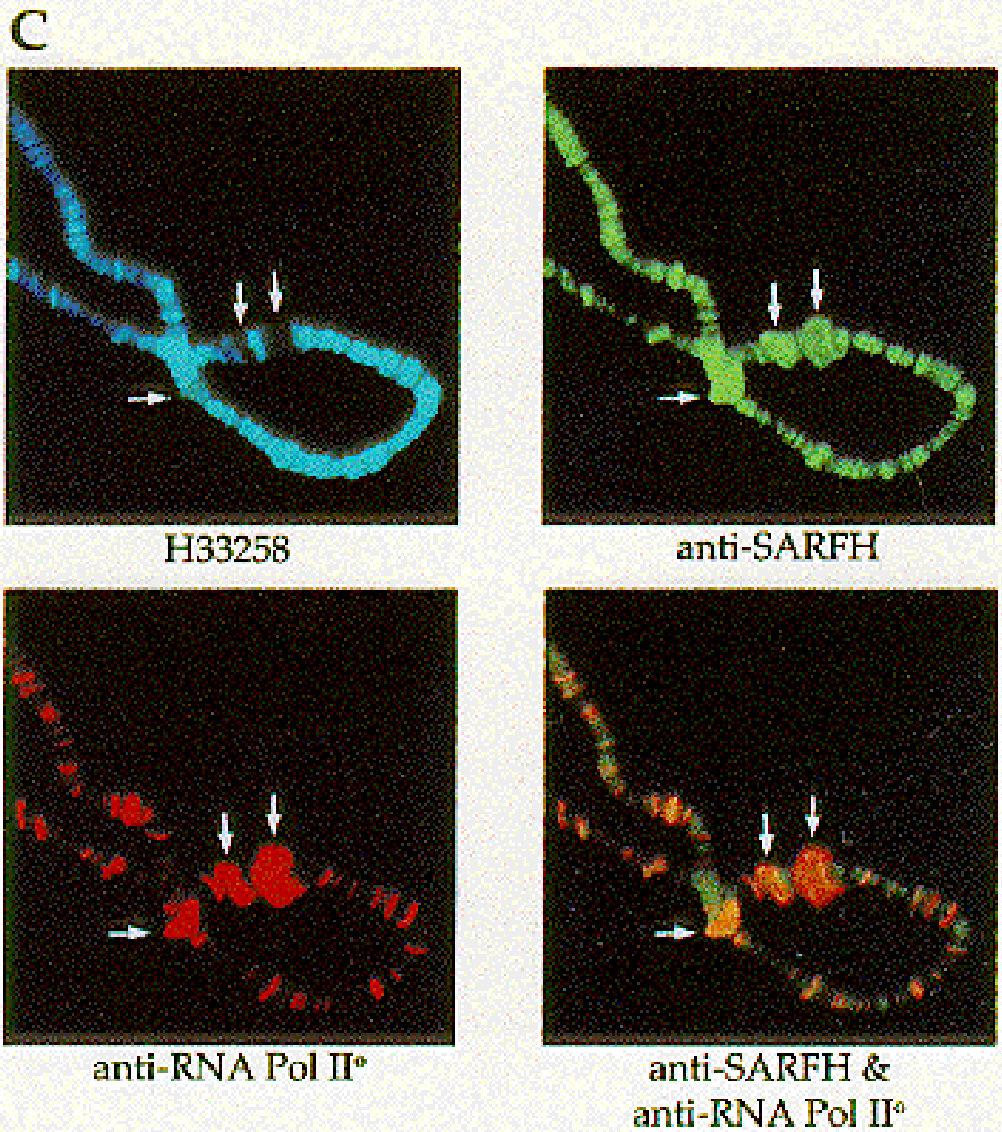
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