A novel effector domain from the RNA-binding protein TLS or EWS is required for oncogenic transformation by CHOP

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In human myxoid liposarcoma, a chromosomal rearrangement leads to fusion of the growth-arresting and DNA-damage-inducible transcription factor CHOP (GADD153) to a peptide fragment encoded by the TLS gene. We have found that wild-type TLS and a closely related sarcoma-associated protein, EWS, are both abundant nuclear proteins that associate in vivo with products of RNA polymerase II transcription. This association leads to the formation of a ternary complex with other heterogeneous RNA-binding proteins (hnRNPs), such as A1 and C1/C2. An NIH-3T3-based transformation assay was used to study the oncogenic role of the sarcoma-associated domain of these RNA-binding proteins. Transduction of the TLS–CHOP oncogene into cells by means of a retroviral expression vector leads to loss of contact inhibition, acquisition of the ability to grow as colonies in soft agar, and tumor formation in nude mice. Mutations that interfere with the function of the leucine zipper dimerization domain or the adjacent basic region of CHOP abolish transformation. The essential role of the TLS component was revealed by the inability of truncated forms to fully transform cells. Domain swap between TLS- and EWS-associated oncogenes demonstrated that the component contributed by the RNA-binding proteins are functionally interchangeable, whereas the transcription factor component specifies tumor phenotype. The sarcoma-associated component of TLS and EWS contribute a strong transcriptional activation domain to the fusion proteins; however, transforming activity cannot be fully substituted by fusion of CHOP to other strong *trans*-activators. The juxtaposition of a novel effector domain from sarcoma-associated RNA-binding proteins to the targeting domain of transcription factors such as CHOP leads to the creation of a potent oncogene.

[*Key Words*: Nuclear localization; transcription; leucine zipper; hnRNP; estrogen receptor; nude mice]

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In adipose tissue, CCAAT/enhancer-binding protein (C/EBP) family members participate in the process of terminal differentiation and growth arrest (Cao et al. 1991; Samuelsson et al. 1991; Umek et al. 1991; Williams et al. 1991; Freytag and Geddes 1992; Lin and Lane 1992), they drive the expression of a variety of adipose-specific genes (for review, see McKnight et al. 1989), and may play a role in mediating the response of this tissue to hormonal cues such as inflammatory cytokines (Alam et al. 1992; Ron et al. 1992; Stevens and Pekala 1992). CHOP (C/EBP homologous protein), also known as GADD153, is a member of the C/EBP family (Fornace et al. 1989; Ron and Habener 1992). The CHOP gene is normally expressed at very low levels in most cells, including adipocytes; however, it is markedly activated by perturbations that induce cellular stress (Fornace et al. 1989; Chen et al. 1992; Luethy and Holbrook 1992; Carlson et al. 1993). Microinjection of CHOP protein into NIH-3T3 cells induces G_1/S arrest of the cell cycle (Barone et al. 1994; Zhan et al. 1994). This observation suggests that CHOP may participate in regulating cell growth under certain stressful conditions (Ron 1994).

CHOP is found in cells as a dimer with other members of the C/EBP family, with heterodimerization being strongly preferred to homodimerization (Ron and Habener 1992; Barone et al. 1994) Dimerization is essential for growth arrest induced by CHOP (Barone et al. 1994), yet the biochemical activity of CHOP–C/EBP heterodimers is not completely understood. Such dimers are clearly incapable of binding to certain C/EBP sites such as those present in the promoter of the angiotensinogen gene. Consequently, in such genes, CHOP functions as a dominant-negative inhibitor of activation by C/EBP family members (Ron and Habener 1992). Other aspects of CHOP function may, however, require DNA binding because deletion of the CHOP basic region reduces the ability of CHOP to induce growth arrest (Barone et al. 1994).

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Additional evidence of a role for CHOP in regulation of cell growth comes from the observation that the adipose tissue tumor myxoid liposarcoma is associated with a structural rearrangement of the CHOP gene (Åman et al. 1992). A *t(12;16)* chromosomal translocation, present in the vast majority of these tumors, leads to the fusion of CHOP to the 5′ end of a novel gene named *translocated in liposarcoma* (*TLS*) or *FUS* (Crozat et al. 1993; Rabbitts et al. 1993). The fusion gene encodes a protein that consists of the amino terminus of TLS fused to the full-length CHOP-coding region. This chimeric protein is present at high levels in the nuclei of myxoid liposarcoma cells, where it associates with at least one other C/EBP protein, C/EBPβ (Barone et al. 1994). Unlike wild-type CHOP, microinjection of TLS–CHOP does not lead to growth arrest (Barone et al. 1994). This suggests that the presence of peptide sequence from the amino terminus of TLS modifies the function of CHOP.

Comparison of the peptide sequence of TLS to that of other proteins in the data bank reveals striking similarity to two other proteins. One of these is a potential open reading frame in a *Drosophila melanogaster* anonymous cDNA (Dpen p19; Haynes et al. 1987). TLS is most similar to Dpen p19 in a region that corresponds to a putative RNA recognition motif (RRM) in the 3′ end of TLS. This part of TLS is lost in the t(12;16) gene fusion event. Recently, we have found that Dpen p19 encodes a functional homolog of TLS that is associated in vivo with the majority of RNA polymerase II(Pol II) transcripts (D. Immanuel, H. Zinszner, and D. Ron unpubl.). The other protein in the data bank exhibiting sequence similarity to TLS is EWS (Ewing's sarcoma). Interestingly, EWS is encoded by a gene that is also involved in human tumor-specific chromosomal rearrangements. EWS is >50% identical to TLS throughout its coding region, with extreme similarity in the carboxy-terminal putative RRM (Crozat et al. 1993). The architecture of the EWS-based oncogenes is similar to TLS–CHOP in that the amino terminus of EWS is fused to a series of transcription factors such as FLI1, ERG1, ATF1, and WT1. In all of these cases, the RRM-containing portion of EWS is lost (Delattre et al. 1992; Zucman et al 1993a,b; Ladanyi and Gerald 1994). The amino terminus of EWS and TLS is very rich in the amino acids glutamine and proline. As such, it resembles the activation domain of certain transcription factors such as SP-1 (Courey and Tjian 1988). The amino terminus of EWS is capable of contributing to the *trans*-activation potential of various DNA-binding proteins (May et al. 1993a,b; Bailly et al. 1994), and EWS–FLI1 has been shown to transform NIH-3T3 cells in culture (May et al. 1993a,b). However, the role of transcriptional activation in oncogenic transformation by the chimeric proteins is not known.

Many gene fusion events associated with human tumors occur between regions encoding transcription factors (Rabbitts 1991). Because transcription factors regulate gene expression, it is easy to rationalize their involvement in tumor development—illegitimate juxtaposition of domains from two such proteins or the inappropriate expression of a truncated portion of one of them may cause deregulated expression of downstream "cancer-causing" target genes. The involvement of RNA-binding proteins in a similar process poses a new challenge. In this paper we describe our studies on the normal TLS protein and on the molecular dissection of the TLS–CHOP oncogene. We find that TLS (and EWS) normally associates with products of RNA polymerase II in a complex with other hnRNPs. In the tumor cells the non-RNA-binding portion of these proteins contributes an essential function to a novel set of chimeric oncoproteins. It appears that the process of cellular transformation and the precise phenotype of the transformed cells are critically dependent on the targeting of this effector domain to heterologous sites. This occurs because the normal carboxy-terminal RNA-binding portion is substituted by a DNA-binding and dimerization component in the oncogenic chimera.

Results

TLS is an RNA-binding protein found in a ternary complex with hnRNPs

The carboxyl terminus of TLS is capable of binding mRNA in vitro (Crozat et al. 1993). To explore the relevance of this finding to interactions of TLS in vivo, we relied on the observation that many RNA-binding proteins exhibit altered subcellular localization in response to treatment of cells with inhibitors of RNA polymerase (e.g., see Carmeo-Fonseca et al. 1992; Piñol-Roma and Dreyfuss 1992). Because TLS exhibits preferential binding to mRNA in vitro, we reasoned that its intracellular target may be a product of RNA Pol II. Therefore, we treated HeLa cells with various inhibitors of Pol II and studied the consequences of this treatment on the localization of TLS. In untreated cells the polyclonal anti-TLS serum gives rise predominantly to a nuclear pattern of staining (Fig. 1A, left column). Following treatment of the cells with 50 μg/ml of α-amanitin, a significant portion of the TLS immune reactivity was observed to redistribute from the nucleus to the cytosol and much of the residual nuclear reactivity became associated with several clearly defined irregularly shaped nuclear bodies (Fig. 1A, middle column). When the α-amanitin-treated cells were costained with an antibody to the heterogeneous nuclear RNA-binding protein (hnRNP) A1, its distribution was noted to parallel that of TLS (Fig. 1A, middle column), consistent with the findings of Piñol-Roma and Dreyfuss (1992). An indication for the specificity of the phenomenon is provided by the fact that redistribution in response to inhibition of Pol II transcription is not shared by all nuclear RNA-binding proteins. The related hnRNPs C1/C2 do not redistribute to the cytosol, nor do they accumulate in the characteristic nuclear bodies (Fig. 1A, right column; Piñol-Roma and Dreyfuss 1992). In these experiments we made use of a rabbit polyclonal antiserum to TLS that happens to also be reactive with EWS (see Fig. 1D). However, an identical pattern of staining was obtained with a murine monoclonal antibody to TLS that is not reactive with EWS (data not shown).

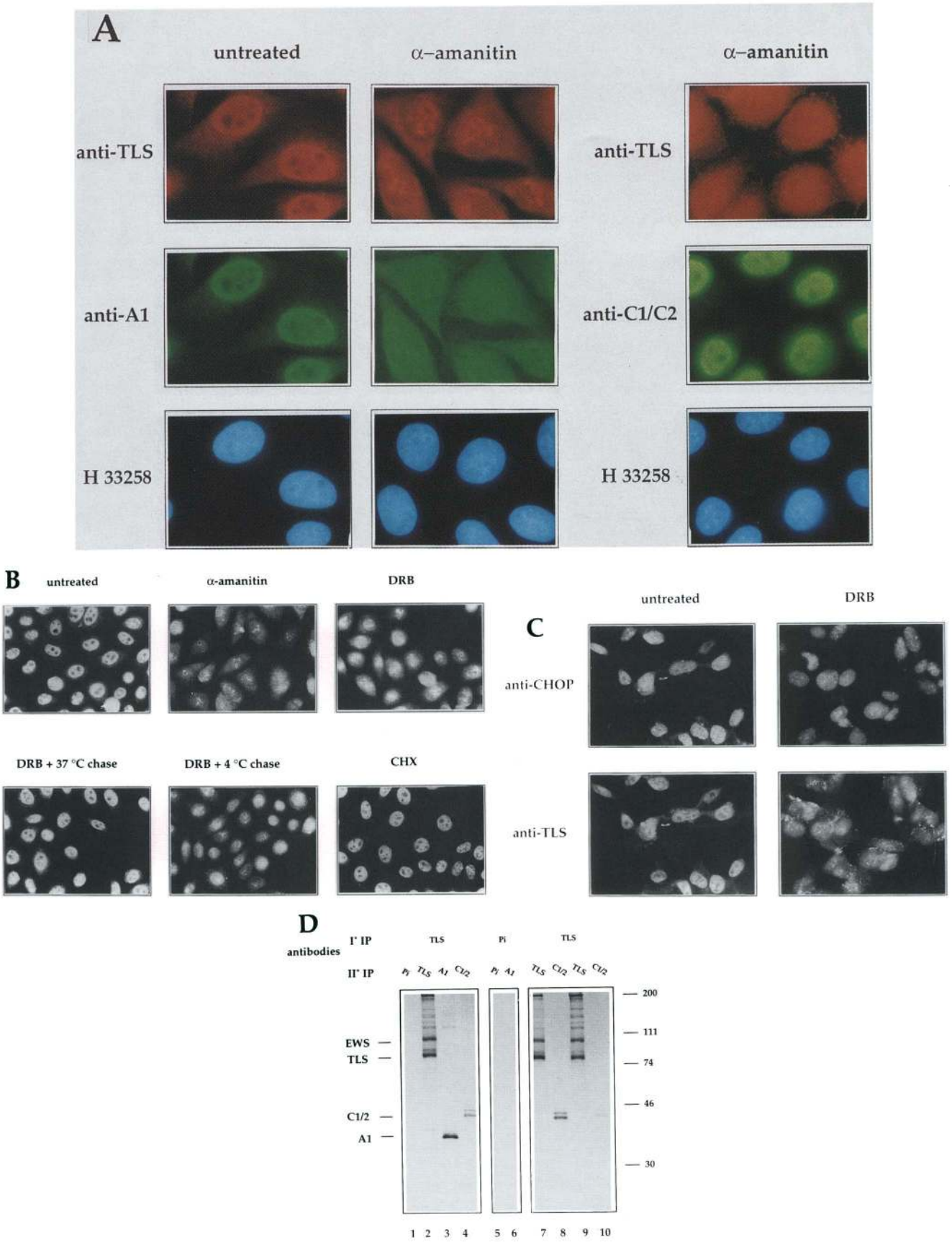

Figure 1. (*See following page for legend.*)

Redistribution of proteins from the nucleus to the cytoplasm in response to inhibition of Pol II transcription has been interpreted as the result of their inability to reaccumulate in the nucleus after loss of an essential nuclear tether (Piñol-Roma and Dreyfuss 1992; Schmidt-Zachmann et al. 1993). We sought to determine whether the integrity of this tether depends on the presence of Pol II products in the case of TLS. To this end, a reversible inhibitor of Pol II processivity, 5,6-dichlororibofuranosyl benzimidazole (DRB) was used. The staining pattern obtained with DRB-treated cells was indistinguishable from that of cells treated with the irreversible inhibitor α-amanitin (Fig. 1B). However, when DRB was washed away, TLS staining reverted to its normal pattern. This occurred only if the cells were incubated at 37°C following the wash but not if they were kept at 4°C (Fig. 1B). The temperature dependence of reaccumulation of TLS in the nucleus indicates that a newly synthesized RNA component is involved. However, these results are also consistent with the possibility that a labile protein, whose level is indirectly reduced by inhibition of mRNA synthesis, provides the tether function. Therefore, we studied the localization of TLS in cells treated with cycloheximide, an inhibitor of protein synthesis. As can be seen in Figure 1B, cycloheximide does not alter the pattern of TLS staining. These experiments are consistent with TLS associating, either directly or indirectly, with an RNA target in vivo.

Having identified a response of TLS to inhibition of Pol II transcription, we studied the extent to which this phenomenon occurs in myxoid liposarcoma cells. These cells contain one normal TLS gene and one altered form, TLS–CHOP. We reasoned that if the TLS–CHOP protein affected the function of the normal TLS protein, this might be reflected in altered localization of TLS either under normal conditions or following inhibition of Pol II transcription. A precedent for such a phenomenon is present in the case of the promyelocyte–retinoic acid receptor (PML–RAR) fusion protein that appears to perturb the localization of the normal PML in acute myeloid leukemia cells (Dyck et al. 1994; Weis et al. 1994). Staining of 1955/91 myxoid liposarcoma cells with antibodies to both TLS and CHOP revealed that whereas the CHOP immune reactivity (reflecting predominantly TLS–CHOP in these cells; Fig. 1B in Crozat et al. 1993) remained nuclear after treatment of the cells with DRB, a fraction of the TLS immune reactivity "leaked" into the cytosol (Fig. 1C). From this experiment we conclude that the presence of TLS–CHOP does not lead to detectable alteration in the localization of TLS and that the redistribution phenomenon is dependent on the RNA-binding carboxy-terminal portion of TLS that is absent in TLS–CHOP.

Many RNA-binding proteins are associated in multimeric complexes consisting of hnRNA and proteins (Dreyfuss et al. 1993). Because TLS colocalizes with hnRNP A1, we sought to determine whether they are part of a complex. A coimmune precipitation assay was utilized in which ^{35}S-labeled nuclear proteins were immune-precipitated first with an antibody to TLS (cross-reactive with EWS but not with the hnRNPs) and then, following disruption of the primary immune complex and destruction of the primary antibodies, with a second antibody against the hnRNPs. Both hnRNP A1 and C1/C2 are present in the TLS immune precipitate (Fig. 1D, lanes 3,4). They are absent from the immune precipitate formed with an unrelated antiserum (lane 6). Furthermore, the association between TLS and the hnRNPs is dependent on the integrity of the RNA in the primary immune precipitate, as treatment of the nuclear extracts with RNase prior to immune precipitation markedly attenuates the TLS-associated hnRNP A1 signal (cf. lanes 8 and 10). These experimental results provide additional evidence that TLS/EWS functions as an RNA-binding protein in vivo.

Figure 1. Inhibition of RNA pol II transcription leads to redistribution of the TLS protein in cells. (*A*) Detection of TLS and hnRNP immune reactivity in cells before and after treatment with α-amanitin. HeLa cells were stained with rabbit anti-TLS serum (red signal) and mouse anti-hnRNP antibodies (green signal). Cells were treated with α-amanitin (50 μg/ml) or left untreated. Costaining was with either anti-hnRNP A1 (*left* and *middle* columns) or anti-hnRNP C1/C2 (*right* column). Note the redistribution of the TLS and hnRNP A1 stain from a diffuse nucleoplasmic pattern in the untreated cells to the cytosol and discrete punctate nuclear structures in the treated cells. To reveal the absence of hnRNP C1/C2 in the cytosol, the photographic exposure *right* was longer by one F-stop than the other two. This tends to obscure the nuclear landmarks in the TLS photomicrograph. (*B*) Reversibility and specificity of the redistribution phenomenon. HeLa cells were treated with the reversible Pol II inhibitor DRB (100 μM) for 3 hr at 37°C and then incubated without the inhibitor for the same period of time. The DRB-free chase was carried out either at 37°C or at 4°C, as indicated. Cells were fixed and stained with antiserum to TLS. Treatment with cycloheximide (50 μg/ml) controlled for the effect reduced protein synthesis might have on TLS distribution, and α-amanitin served as a positive control for the redistribution phenomenon. (*C*) Redistribution of TLS requires the RNA recognition motif and is unaffected by the presence of TLS–CHOP in the cells. Myxoid liposarcoma cells that contain the TLS–CHOP fusion protein were treated with DRB and costained with a rabbit antiserum to CHOP and a mouse antiserum to TLS. Note the redistribution of the TLS signal in response to DRB treatment but not that of TLS–CHOP (detected by the CHOP antiserum). (*D*) TLS is present in a complex with other hnRNPs. Coimmune precipitation of ^{35}S-labeled TLS and hnRNPs from HeLa cell nuclear extracts. Extracts were immune precipitated in the first step in a mild nondenaturing buffer (I° IP) with a TLS antiserum or a preimmune control (lanes *5,6*) and then, following disruption of the immune complex and degradation of the I° antibodies a second immune precipitation was carried out under dissociating conditions (II° IP) with either antiserum to TLS (lanes *2,7,9*), hnRNP A1 (lanes *3,6*), and hnRNP C1/C2 (lanes *4,8,10*) or preimmune serum (lanes *1,5*). Treatment of the sample with RNase prior to the I° immune precipitation (lanes *9,10*) leads to a significant reduction of the intensity of the coprecipitating hnRNP C1/C2 signal.

Transformation requires fusion of the amino terminus of TLS to an intact CHOP protein

To study the role of TLS–CHOP in oncogenesis, we used a cell culture-based transformation assay. Infection of NIH-3T3 cells with a high-titer recombinant ecotrophic retrovirus encoding the TLS–CHOP protein led to a profound growth disturbance. The transformed cells exhibited markedly attenuated contact inhibition and attained higher densities at saturation than the parental cells or cells infected with a control "empty" retrovirus (Table 1). TLS–CHOP-expressing cells also gave rise to many large colonies when plated in soft agar (Fig. 2A). In addition, cells expressing TLS–CHOP rapidly produced tumors when injected into nude mice (Table 1). Oncogenic transformation by TLS–CHOP is restricted to NIH-3T3 cells; despite efficient retrovirus-based gene transfer and high-level recombinant protein expression, we did not observe transformation of related fibroblastic cells such as BALB/c-3T3, adipogenic 3T3-L1, or Rat-1 cells (data not shown).

Truncation of the carboxy-terminal leucine zipper dimerization domain (LZ^-) led to complete loss of the transforming potential of TLS–CHOP (Fig. 2A). This implies an essential role for dimerization in the transformation process. In-frame internal deletion of the CHOP basic region (BR^-) also led to loss of transforming activity (Fig. 2A). This result is explained most easily by a requirement for binding to DNA. However, the level of expression of the BR^- mutant was lower than that of the wild-type protein (Fig. 2B), making it difficult to distinguish between the role for the basic region in protein stability versus its potential role in DNA binding. This issue is addressed by other CHOP derivatives discussed below.

To define the role of the TLS amino-terminal sequence in transformation, we studied the activity of wild-type CHOP, which has no TLS sequence. Infection of NIH-3T3 cells with a CHOP-expressing retrovirus gave rise to colonies that expressed the protein at a high level (Fig. 2B). This is a surprising finding in light of previous work in which CHOP has been found to arrest cell growth (see Discussion; Barone et al. 1994; Zhan et al. 1994). Furthermore, the CHOP-expressing cells exhibited higher saturation densities than parental cells or cells infected with "empty" retrovirus (Table 1). Despite this accelerated growth phenotype, the CHOP-infected cells did not grow efficiently as colonies in soft agar (Fig. 2A). Furthermore, injection of CHOP-expressing cells into nude mice produced only small tumors with much longer latency than that of TLS–CHOP-expressing cells. We conclude that the presence of amino-terminal sequence from TLS in the chimeric protein is necessary for realizing its full oncogenic potential.

The amino terminus of EWS can substitute for TLS in a CHOP fusion oncoprotein

One hypothetical mechanism by which the TLS amino-terminal domain may contribute to transformation is by providing a transcriptional activation function that is absent in wild-type CHOP. We constructed a chimeric protein consisting of the amino terminus of TLS fused to the heterologous DNA-binding domain of the yeast Gal4 protein and studied its ability to activate a reporter gene driven by Gal4 sites. Expression of the TLS amino terminus–Gal4 fusion led to potent *trans*-activation of the reporter gene in Rat-1 and HepG2 cells. Its activity is comparable to that displayed by the activation domains of established transcription factors such as VP16 and C/EBPα and is similar in magnitude to that of the amino terminus of EWS (Fig. 3A). This *trans*-activation potential was also retained when the full-length TLS–CHOP was fused to Gal4 (data not shown).

The observation that the amino terminus of TLS contains a potent *trans*-activation domain led us to address the role of *trans*-activation in the function of the myxoid liposarcoma oncogene. We constructed retroviral expression vectors for chimeric proteins in which CHOP is fused to the *trans*-activation domains of C/EBPα (Friedman and McKnight 1990; Pei and Shih 1991) or VP16 (Sadowski et al. 1988). Despite the fact that both proteins were expressed at high levels in the infected cells and localized appropriately to the nucleus (Fig. 3B,C), these cells did not exhibit a strong transformed phenotype. VP16–CHOP cells produced few colonies in soft agar and were tumorigenic in nude mice, however, only with long

Table 1. *Transformation of NIH-3T3 cells requires the fusion of an intact CHOP to the amino terminus of TLS or EWS*

	"Empty"	TLS–CHOP	TLS–CHOPLZ^-	TLS–CHOPBR^-	CHOP	EWS–CHOP	C/EBPα–CHOP	VP16–CHOP
Density at confluence[a]	1.11 ± 0.11	2.65 ± 0.65	0.91 ± 0.13	1.35 ± 0.23	2.38 ± 0.1	2.4 ± 0.55	2.65 ± 1.08	2.5 ± 0.24
Colonies in soft agar[b]	0	16.7 ± 3.2	0	0	1.5 ± 1	14.2 ± 3.2	2.33 ± 2.1	2.6 ± 2.1
Tumorigenicity in nude mice[c]	−	++	ND	ND	±	++	−	+

[a]Density at confluence was measured by counting the number of cells ($\times 10^6$) on a 60-mm plate at confluence.
[b]Colonies in soft agar were counted in 10 low power fields (50×) per plate; results are expressed as mean and range of experiments performed in duplicate and reproduced at least twice.
[c]Tumorigenicity in nude mice was scored 3 weeks after implantation of 5×10^5 transformed cells: (++) Tumor >10 mm; (+) tumor between 5 and 10 mm; (−) no tumor visible; (ND) not done.

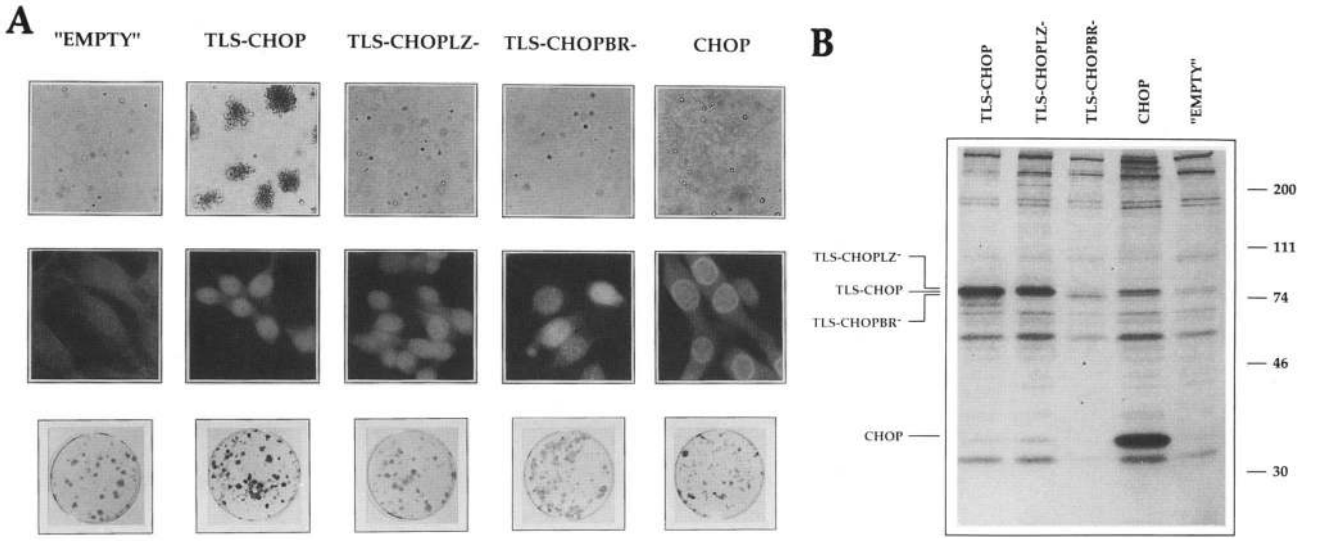

Figure 2. Oncogenic transformation by TLS–CHOP. (*A*) Colony formation in soft agar by cells infected with recombinant retroviruses. Cells were infected with virus encoding wild-type TLS–CHOP, mutant forms of the protein that lack the leucine zipper (LZ^-), basic region (BR^-), or lack the TLS portion (CHOP). The parental virus ("empty") is a control. Cells were plated in soft agar and photographed 2 weeks later by bright-field microscopy (32×, *top*). Infected cells grown on coverslips were stained with a CHOP antibody and a fluorescent secondary antibody and photographed at 400×. The positive nuclei appear bright against a dark background (*middle*). Infected cells were plated at low density and selected for transduction of viral DNA by selection with Geneticin. Ten days later, cells were fixed and stained in situ with crystal violet. The titer of the virus is reflected by the number of resistant colonies forming (*bottom panel*). (*B*) Expression of the recombinant proteins in the infected cells. ^{35}S-Labeled proteins from cells infected with the recombinant retroviruses were immune precipitated with a CHOP antibody, resolved by SDS-PAGE, and visualized by autoradiography. The anomalously slow migration of the leucine zipper minus deletion mutant is a reproducible finding that had been noted previously in the case of murine CHOP–LZ^- (Ron and Habener 1992).

latency. C/EBPα–CHOP produced only background level of colonies in soft agar and was nontumorigenic. On the other hand, a chimera between the amino terminus of EWS and CHOP possesses strong transformation activity, giving rise to as many colonies in soft agar as TLS–CHOP and induces rapidly growing tumors in nude mice (Fig. 3B; Table 1). We conclude that *trans*-activation, as reflected in the Gal4 fusion protein assay, is not sufficient for transformation by CHOP derivatives. Instead, the strong transforming activity of EWS–CHOP suggests that a specific function other than *trans*-activation is conserved between TLS and EWS.

The role of CHOP in oncogenesis in adipose tissue is easy to rationalize in terms of the importance of C/EBP family members in the development of that tissue type. In light of the experiments described above, which indicate interchangeability of the TLS and EWS amino-terminal sequence, we tried to understand the bases for the clinically observed specificity of the association between TLS (but not EWS) and oncogenesis in adipose tissue. We therefore studied the expression of the TLS and EWS genes in various rat tissues by Northern blot analysis. Both mRNAs are abundantly present in many different tissues. However, when the ratio of TLS to actin is used to quantify relative mRNA levels, adipose tissue is found to be particularly rich in TLS and to express EWS very poorly (Fig. 4). This result suggests that the specific association of TLS with myxoid liposarcoma may be simply a consequence of the high level of expression of the gene in the relevant cell type.

The phenotype of NIH-3T3 cells transformed by the sarcoma-related fusion oncogenes is determined by the transcription factor component and not by the contribution from the RNA-binding protein

The participation of TLS and EWS in many different sarcomas raises the possibility that they contribute the only component directly involved in the transformation process, with the transcription factor portion of the fusion protein providing merely a redundant function such as nuclear localization or protein stabilization. If this were the case, the diversity in tumor morphology observed in the different sarcomas should be accounted for by the specific RNA-binding protein involved or by the properties of the cell type in which the oncogene happens to be expressed. To address this issue, we compared the histological appearance of the tumors arising in nude mice injected with NIH-3T3 cells expressing TLS–CHOP, the myxoid liposarcoma oncogene, or EWS–FLI1, the EWS oncogene. TLS–CHOP tumors are composed of slender elongated cells arranged in whorls and bundles, giving rise to a typical "spindle cell" sarcoma. The EWS–FLI1 cells, on the other hand, give rise to tumors composed of sheets of polygonal cells lacking distinct morphological characteristics (Fig. 5). This difference in cell morphol-

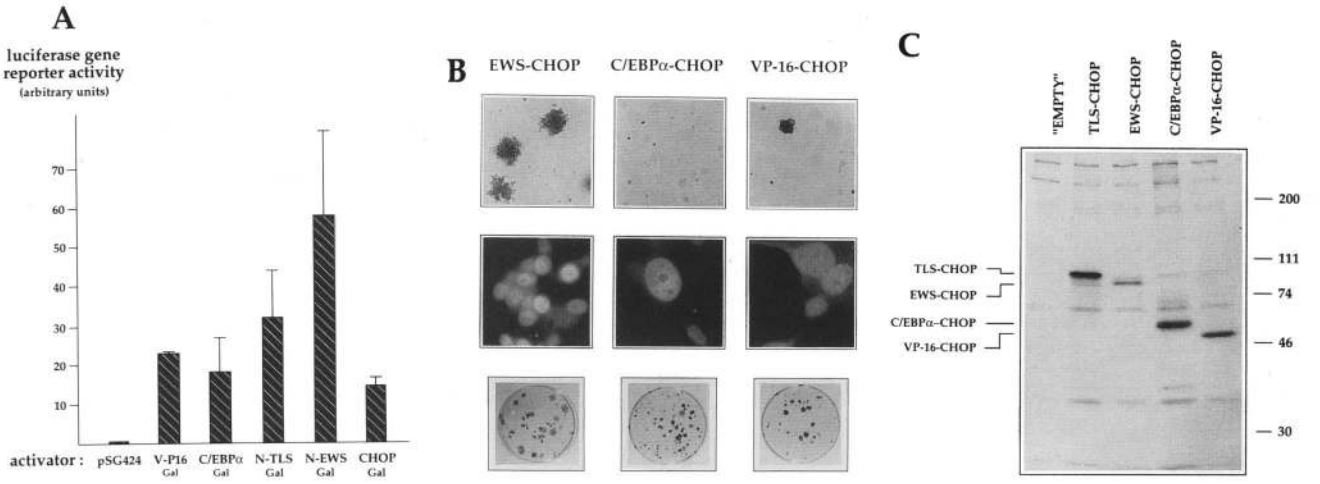

Figure 3. Analysis of amino-terminal peptide sequences that promote transformation when fused to CHOP. (*A*) The amino terminus of TLS possesses strong transcriptional activation properties. Activation of a Gal4-binding- site-driven reporter gene by fusion proteins between the amino terminus of TLS or EWS and the DNA-binding domain of Gal4 is compared with that of other well-characterized transcriptional activators: herpes virus VP16 protein carboxy-terminal *trans*-activator (VP16–Gal4; Sadowski et al. 1988) and the *trans*-activation domain of C/EBPα (C/EBPα–Gal4; Pei and Shih 1991). Results are presented as the mean, and range of a typical experiment is performed in duplicate and reproduced at least three times in two different cell lines, Rat-1 and HepG2. Luciferase reporter gene activity is expressed in arbitrary units, with the activity of the reporter plasmid alone defined as 1 unit. Reporter gene activity was normalized to the activity of the cotransfected internal control CMV β-gal plasmid. (*B*) Colony formation in soft agar by cells infected with recombinant retroviruses. Cells were infected with virus-encoding fusion proteins between the amino terminus of EWS (EWS–CHOP) or the transcriptional activation domains of C/EBPα (C/EBPα–CHOP) or VP16 (VP-16–CHOP). Cells were placed in soft agar and photographed 2 weeks later by bright-field microscopy (50×, *top*). Infected cells grown on coverslips were stained with a CHOP antibody and a fluorescent secondary antibody and photographed at 208×. The positive nuclei appear bright against a dark background (*middle*). Infected cells were plated at low density and selected for transduction of viral DNA by treatment with Geneticin. Ten days later, cells were fixed and stained in situ with crystal violet. The titer of the virus is reflected by the number of resistant colonies forming (*bottom*). (*C*) Expression of the recombinant proteins in the infected cells. ^{35}S-Labeled proteins from cells infected with the recombinant retroviruses were immune precipitated with a CHOP antibody, resolved by SDS-PAGE, and visualized by autoradiography. The positions of the individual proteins are indicated at *left*.

ogy is also reflected in the appearance of the cells in tissue culture plates (data not shown).

Though clonal in origin, NIH-3T3 cells may become heterogeneous in culture. This raised the possibility that the differences in morphology between TLS–CHOP- and EWS–FLI1-induced tumors were attributable to the fact that the two oncogenes transform different target populations in such a heterogeneous pool of cells. To address this possibility experimentally, single subclones of NIH-3T3 cells were isolated by clonal dilution of the parental line and transformed with the two oncogenes. Several subclones were studied, and all were transformable by both TLS–CHOP and EWS–FLI1. The morphology of tumors induced by TLS–CHOP and EWS–FLI1 was analyzed in one defined subclone that exhibits low background oncogenic transformation and is very transformable by both TLS–CHOP and EWS–FLI1 (clone, *III/9*). Tumors developing in nude mice injected with *III/9* cells expressing TLS–CHOP and EWS–FLI1 were identical in appearance to those observed in the parental NIH-3T3 cells, effectively excluding clonal selection as the basis for the morphological differences between tumors induced by the two types of oncogenes.

To determine whether the morphological differences between tumors induced by TLS–CHOP and EWS–FLI1

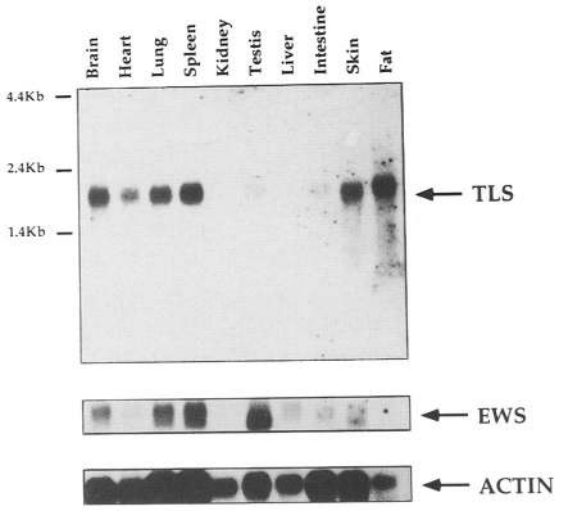

Figure 4. TLS mRNA is abundant in adipose tissue. A Northern blot of poly (A)$^+$ RNA (5 μg/lane) from various adult male rat tissues was hybridized to the labeled human TLS cDNA (*top*), subsequently stripped and hybridized to the human EWS cDNA (*middle*) and mouse actin cDNA (*bottom*). Note the high TLS/actin ratio and low EWS/actin ratio in fat.

Figure 5. CHOP-based oncogenes and FLI1-based oncogenes induce tumors with different morphology. NIH-3T3 cells were infected with retroviruses encoding the indicated oncoproteins. The infected cells were injected subcutaneously into nude mice, and the resultant tumors were fixed, sectioned, and stained by hematoxylin and eosin. Shown are photomicrographs of the sections at a magnification of 118×. Note the elongated spindle cell morphology of the TLS–CHOP- and EWS–CHOP- transduced cells and the polygonal appearance of the TLS–FLI1- and EWS–FLI1-transduced cells. The results shown were reproduced in all three (separate) experiments performed.

are a consequence of the amino-terminal contribution of the RNA-binding protein or the carboxy-terminal contribution of the transcription factor, the morphology of tumors derived from cells expressing chimeras between TLS–FLI1 and EWS–CHOP was studied. In all four cases, the morphological appearance of the tumor was determined by the transcription factor component of the chimera (Fig. 5). We conclude that the carboxy-terminal portion of these chimeric oncogenes specifies the biologically relevant targeting of a functionally interchangeable amino-terminal effector domain. The differences in tumor morphology are presumably a consequence of the fact that FLI1 and CHOP interact with different nuclear targets.

Transformation by TLS–CHOP is associated with an additional activity distinct from binding to C/EBP partners

Having defined an essential role for the TLS (and EWS) amino terminus in transformation by CHOP fusion proteins, it became important to distinguish between two hypothetical modes of action of the oncogene. In the first mode, TLS–CHOP functions as a "superactive" CHOP, dimerizing with C/EBP target proteins and inhibiting their DNA-binding activity. In this case, the function ascribed to the oncogene is that of a stoichiometric inhibitor, and the contribution of the TLS portion is relatively nonspecific, for example, increased protein stability. In the second mode of action, TLS–CHOP plays an active role in promoting transformation by the targeting of the putative TLS effector domain to new cellular macromolecules via its fusion to CHOP. A conditional mutant form of TLS–CHOP would help address this issue. If the transforming activity of TLS–CHOP amounts to no more than stoichiometric inhibition of C/EBP proteins, then activation of the conditional mutant should be associated with increased dimerization with target proteins; lack of such an increase would provide evidence in favor of an active role for TLS–CHOP.

A ligand-dependent form of TLS–CHOP was therefore created by fusing the estrogen receptor ligand-binding domain to the carboxyl terminus of TLS–CHOP. Chimeric transcription factors constructed in this manner often exhibit ligand-dependent activity (Eilers et al. 1989; Umek et al. 1991). Cells infected with a retrovirus encoding the TLS–CHOP–estrogen receptor fusion protein (TLS–CHOP–ER) displayed a ligand-dependent increase in the number of foci present in confluent monolayers (Fig. 6A). The effect of ligand was most pronounced when added to cells that had already attained confluence (Fig. 6A, cf. columns 3 and 4 with 1 and 2), indicating that ongoing activity of the oncogene is necessary for the transformed phenotype. The level of dimerization partners bound to TLS–CHOP–ER in vivo was compared in the absence or presence of estradiol. Proteins bound to TLS–CHOP in nuclear extracts were coimmunoprecipitated with a TLS antibody, resolved on denaturing SDS-PAGE, and revealed by an in vitro dimerization assay on the gel blot using radiolabeled CHOP as a ligand probe ("zipper blot"). The predominant dimerization partner of TLS–CHOP–ER (and TLS–

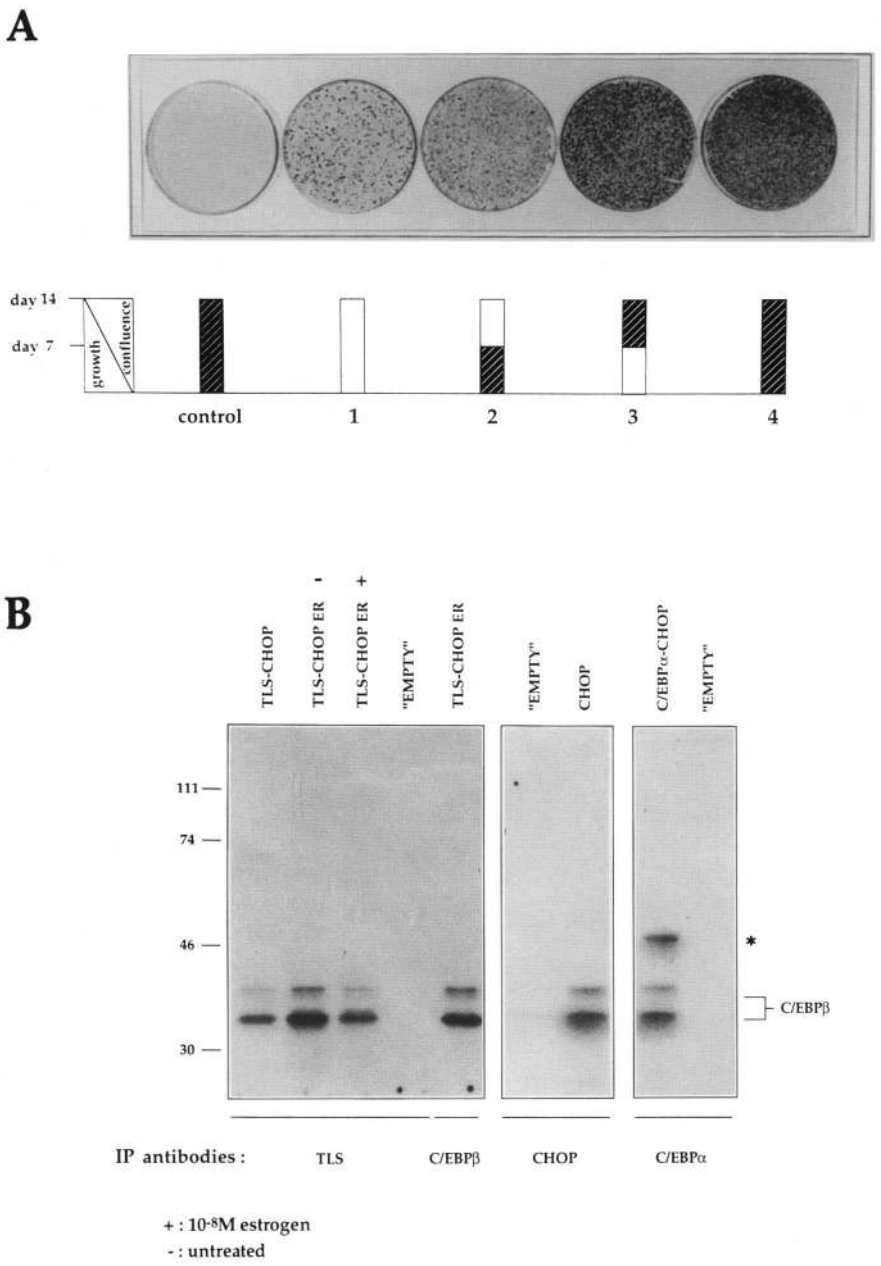

Figure 6. Transforming potential of CHOP derivatives does not correlate with the degree of dimerization with C/EBP targets. (*A*) Ligand-dependent increase in the transforming activity of TLS–CHOP–ER. NIH-3T3 cells were infected with a retrovirus encoding a fusion between TLS–CHOP and the ligand-binding domain of the estrogen receptor (columns 1–4). A plate of uninfected cells ("control") was used to evaluate the nonspecific effects of estrogen. Cells were either left untreated (1), treated only during the first (2) or only during the second week of the 2-week culture period (3), or treated continuously throughout the culture period (4). Following the 2-week culture period, the cells were fixed and stained with crystal violet to reveal the presence of foci. (Hatched bars) 10^{-} M estrogen; (open bars) untreated. (*B*) Identification of in vivo dimerization partners of transforming and nontransforming CHOP derivatives by immune precipitation and zipper blotting. Proteins extracted from nuclei of NIH-3T3 cells expressing TLS–CHOP, TLS–CHOP–ER (+/− treatment with estrogen), CHOP, and C/EBPα–CHOP were immune precipitated under nondisrupting conditions with the indicated antisera; the precipitated proteins were resolved by SDS–PAGE, blotted onto a nitrocellulose filter, and reacted with a ^{32}P-labeled CHOP dimerization probe in a zipper blot. The predominant dimerization partner precipitated with the TLS antiserum is identical with that precipitated by the C/EBPβ antiserum. Note that similar amounts of C/EBPβ associate with the transforming TLS–CHOP, the nontransforming C/EBPα–CHOP, and with TLS–CHOP–ER regardless of the presence of ligand. The asterisk (*) indicates the position of C/EBPα–CHOP that reproducibly reacts with the CHOP zipper probe. The other CHOP derivatives that are also present on this blot do not react with the probe.

CHOP) in NIH-3T3 cells is C/EBPβ (Fig. 6B, cf. lanes 3–5 with 6) The amount of C/EBPβ coprecipitated with TLS–CHOP–ER is not increased by ligand treatment of the cells (cf. lanes 2 and 3). Furthermore, both CHOP and C/EBPα–CHOP are as competent to dimerize with C/EBPβ as TLS–CHOP (lanes 7,8), yet they are not fully transforming. From this line of investigation, we conclude that ligand activation of TLS–CHOP–ER-transforming activity is more consistent with an active role for the oncogene than with a dominant-negative one.

Discussion

The association between *t(12;16)* and myxoid liposarcoma is very strong, being present in >90% of this histological subtype of liposarcoma, and is absent in all other tumors tested to date (Sreekantiaiah et al. 1992). Our finding that TLS–CHOP promotes oncogenic transformation of NIH-3T3 cells in culture supports the presence of causal link between these associated events. The importance of the CHOP component of the fusion oncogene is demonstrated by the fact that deletions of the LZ^{-} or $LZ^{-}BR^{-}$ abolish the oncogenic activity of the protein. In addition, the CHOP portion of the oncogene plays an important role in specifying the tumor phenotype as reflected in the morphology of the tumors induced in nude mice. Deletion of the TLS portion of TLS–CHOP also incapacitates the oncogene, leading to significant attenuation of its ability to promote anchorage-independent growth in soft agar and tumor formation in

nude mice. However, cells overexpressing CHOP or any derivative of the wild-type CHOP protein (C/EBPα–CHOP, VP16–CHOP, or TLS–CHOP–ER in the absence of ligand) exhibit a defect in contact inhibition—they reproducibly gave rise to foci when grown to confluence as monolayers and had higher saturation densities than the parental cells.

The CHOP-induced growth disturbance was unexpected: First, wild-type CHOP, when overexpressed in cells by protein or DNA microinjection, causes growth arrest (Barone et al. 1994). Second, previous attempts to produce cell lines that overexpress CHOP have been routinely unsuccessful (Zhan et al. 1994; H. Zinszner, R. Albalat, and D. Ron, unpubl.). The major difference between the experiments performed here and the ones reported previously lies in the method of gene transfer. The biological response to retroviral transduction may be different from that observed with DNA microinjection or transfection either because of gene dose effects or because the high efficiency of gene transfer by retroviral vectors permits one to see effects on small subpopulations of cells that may respond with growth stimulation instead of growth arrest. Cell line-based heterogeneity of biological responses to C/EBP family members has been noted before. For example, C/EBPα that is associated with the induction of terminal growth arrest in 3T3-L1 preadipocytes (Umek et al. 1991; Freytag and Geddes 1992) is perfectly compatible with cell growth in highly related murine fibroblastic cell lines such as NIH-3T3 (Freytag et al. 1994). C/EBPα is also associated with rapid proliferation in myelomonocytic marrow cells (Scott et al. 1992). An additional example for a situation in which overexpression of a protein by microinjection leads to growth arrest, whereas overexpression of the same protein by different means induces cell proliferation, is presented by the case of D1 cyclin (Pegano et al. 1994). Regardless of the mechanism, overexpression of CHOP gives rise to the clonal outgrowth of cells that exhibit a mild growth disturbance. Appending the TLS amino terminus to CHOP converts it to a fully competent oncogene.

One aspect of CHOP activity that lends itself to a direct comparison between transforming and nontransforming derivatives is dimerization with other proteins. In this respect, the various forms studied, TLS–CHOP, C/EBPα–CHOP, and CHOP, all dimerize avidly with C/EBPβ in vivo. In the case of the conditionally active oncogene TLS–CHOP–ER, ligand-dependent increase in transformation is not associated with enhanced dimerization with C/EBP partners. It therefore appears that although a dominant-negative mechanism of action may account for the enhanced growth common to all CHOP derivatives, full transformation must require an additional activity. It is important to point out that although the assay for in vivo dimerization of CHOP derivatives is unbiased toward any specific partner, it is impossible to exclude the existence of a biologically relevant minority partner that is not detected by the assay. Such a hypothetical partner might be subject to ligand-dependent regulation of its dimerization activity with TLS–CHOP–ER and may exhibit amino-terminal fusion partner-specific differences in interaction with the CHOP moiety. In regard to this caveat, it is worth noting the potential for amino-terminal sequences to influence dimerization partner selection as demonstrated by the ability of C/EBPα–CHOP to dimerize in vitro with the CHOP zipper probe (Fig. 6B, note band marked by asterisks); TLS–CHOP and CHOP that are also present on the same blot in comparable quantity do not react with the probe.

Collectively, these experiments are consistent with an active role for TLS–CHOP in promoting transformation, rather than a role only as a superactive C/EBP antagonist. We propose that transformation by TLS–CHOP is dependent on two activities. The first is a "CHOP" activity that accounts for the mild growth disturbance induced by all CHOP derivatives and may be the result of dominant-negative antagonism of C/EBP proteins. The second is dependent on fusion with a TLS/EWS amino-terminal domain and is separable from dominant-negative effects on the C/EBP family. It is not known whether binding to specific target genes plays a role in the transformation process by TLS–CHOP. The fact that the basic region of CHOP is indispensable for the transforming activity is consistent with a role for DNA-binding; however, other possibilities also exist. The CHOP basic region may be important for non-sequence-specific interactions with chromatin that would serve to anchor CHOP–C/EBP dimers and stabilize them; in this case, one may speculate that a TLS effector domain alters the activity of CHOP with respect to its effect on the C/EBP complex in the cell without the need to evoke the presence of specific target genes.

What does the amino terminus of TLS contribute to the activity of the TLS–CHOP oncogene? This portion of TLS clearly possesses strong transcriptional activation potential. The homologous region of EWS also functions as a strong activator (May et al. 1993a,b; Bailly et al. 1994) and can substitute for TLS in transforming cells when fused to CHOP. Nonetheless, the role of these two homologous domains in the transformation process cannot be reduced to provision of a strong "generic" activation domain, as the fusion of CHOP to other activation domains such as that of VP16 or C/EBPα does not promote a high level of transformation. Additional evidence that transformation by CHOP derivatives is not strictly dependent on providing an activation domain comes from experiments with the *Drosophila* homolog of TLS and EWS known as SARFH (D. Immanuel, H. Zinszner, and D. Ron, unpubl.). The amino terminus of SARFH does not *trans*-activate in the Gal4 system; yet, when fused to CHOP, it promotes high-level transformation. Because different activation domains may contact different cellular targets, it remains possible that transcriptional activation by TLS/EWS is relevant to the transformation process. However, our results argue that the role of this domain in transformation cannot be reduced to the generic transcriptional activation that is as assayed in the Gal4-binding-site system.

What might be the role of the TLS/EWS effector domain in the context of the intact RNA-binding protein?

TLS and EWS are associated with RNA products of Pol II transcription in an in vivo complex with other hnRNPs. Our studies on the *Drosophila* homolog of these proteins, SARFH, suggest that they are associated with most actively transcribed genes (D. Immanuel, H. Zinszner, and D. Ron unpubl.). A dual role for RNA-binding proteins in regulating aspects of both transcript handling and polymerase function has been documented previously. Some, like *Xenopus* TFIIIA and hnRNP K bind both RNA and DNA (Theunissen et al. 1992; Takimoto et al. 1993), whereas others like La or the HIV TAT protein function as RNA-binding proteins to regulate dissociation of the Pol III transcript from the polymerase complex and initiation at the HIV LTR, respectively (Berkhout et al. 1989; Selby and Peterlin 1990; Maraia et al. 1994).

We speculate that TLS and EWS normally play a role in some aspect of the cross talk between transcript and transcription apparatus. This function depends on the amino-terminal effector domain being targeted to RNA molecules. We propose that in the transformed cell, fusion to a transcription factor presents this effector domain to a heterologous target not usually accessible when the protein is bound to RNA. Alternatively, some subtle aspect of the spatial or temporal profile by which the transcriptional regulatory apparatus is normally exposed to this potent domain may be altered in the fusion protein. Progress in understanding the relationship between these events and transformation will depend on identifying downstream targets of the fusion oncogenes.

Materials and methods

Pol II inhibition in HeLa and myxoid liposarcoma cells, immune cytochemistry coimmune precipitation of TLS and hnRNPs, and detection of TLS–CHOP dimerization partners

HeLa cells or MLPS 1955/91 myxoid liposarcoma cells (a kind gift of Nils Mandahl and Felix Mitelman, University of Lund, Sweden) growing on coverslips were treated with α-amanitin (50 μg/ml, 4hr), DRB (100 μM, 3 hr), or cycloheximide (25 μg/ml, 4 hr) and processed for immune cytochemistry as described (Carmeo-Fonseca et al. 1992). The rabbit antiserum to TLS is directed against the amino terminus of the protein and was used at a 1:1000 dilution. The murine monoclonal antibodies to hnRNP A1 (mAb 4F4) and hnRNP C1/C2 (mAb 4B10) were a gift of Gideon Dreyfuss (University of Pennsylvania, Philadelphia). They were used at a dilution of 1:1000. Experiments designed to test the reversibility of the effect of Pol II inhibition on the distribution of TLS immune reactivity were performed exactly as described previously (Piñol-Roma and Dreyfuss 1992). Briefly, cells treated with DRB for 3 hr were washed free of the inhibitor and placed in DRB-free media to recover for three hours after which they were fixed and processed for immune cytochemistry. The DRB-free "chase" was carried out either at 37°C or at 4°C.

Coimmune precipitation of TLS with hnRNPs was performed exactly as described previously (Piñol-Roma and Dreyfuss 1992). HeLa cells were labeled overnight with [^{35}S] methionine–cysteine, after which nuclei were isolated and soluble proteins extracted. A primary immune precipitation was performed using the rabbit TLS antiserum or a preimmune serum. The immune complex was disrupted by 15 min of incubation in 10 mM DTT at 65°C, after which the sample was diluted to 1 mM DTT in RIPA buffer. A second immune precipitation was performed using preimmune serum for control, anti-TLS serum, or the anti-hnRNP monoclonal antibodies described above. Where indicated, the nuclear extract was treated with RNase A (100 μg/ml) and RNase T1 (5 U/ml) for 15 min at 30°C prior to the first immune precipitation. The immune-precipitated proteins were resolved on a 12% SDS–polyacrylamide gel and visualized by autoradiography.

Dimerization partners of TLS–CHOP and its derivatives were analyzed in nuclear extracts of NIH-3T3 cells infected with the oncogene-encoding retroviruses. Immune-precipitated dimerization partners were detected by zipper blot assay, using ^{32}P-labeled bacterially expressed CHOP as a probe (Barone et al. 1994). Briefly, proteins were immune precipitated from nuclear extracts prepared in buffer C [20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 0.5% aprotinin, 1 μg/ml of leupeptin, and 0.25 mM DTT (pH 7.6)]. This was diluted to an NaCl concentration of 150 mM, and the immunoprecipitations were carried out with the antiserum to TLS, C/EBPβ, C/EBPα, or CHOP. Immune- precipitated proteins were resolved by 12% SDS-PAGE, transferred to a nitrocellulose filter, and probed with a ^{32}P-labeled CHOP zipper probe (Ron and Dressler 1992).

Retroviral expression vectors

Recombinant replication-defective ecotrophic retroviruses encoding TLS–CHOP and derivatives thereof were constructed by introducing the encoding cDNAs into the pSRα plasmid (Muller et al. 1991; a gift of Charles Sawyers and Owen Witte, University of California, Los Angeles). This plasmid encodes a replication-defective pro-retrovirus in which the target gene is under the control of a murine sarcoma virus long terminal repeat (MSV–LTR) and an internal transcription unit expresses the *neo* gene under the control of the herpes simplex virus thymidine kinase (HSV–TK) promoter. Proviral plasmids were transfected, along with a plasmid encoding helper functions into COS1 cells, and viral particles were harvested from the culture supernatant. The full-length TLS–CHOP cDNA was excised from the TLS–CHOPpcDNA1 plasmid (Crozat et al. 1993) and ligated into pSRα. To create the leucine zipper deleted form of TLS–CHOP (TLS–CHOP–LZ$^-$), a *Bam*HI–*Nhe*I fragment of TLS–CHOP was ligated into pSRα. This encodes a protein truncated at the 34 carboxy-terminal amino acids encoding the leucine zipper of CHOP. To create the basic region mutant form of TLS–CHOP (TLS–CHOP–BR$^-$), the TLS sequence was fused to the previously described CHOPmutCb that has an internal in-frame deletion of the region encoding the carboxy-terminal portion of the CHOP basic region (murine CHOP residues 101–122; Barone et al. 1994). As is the case for CHOPmutCb, this TLS–CHOP intermediate did not accumulate in the nucleus (Barone et al.1994; data not shown). Therefore, a heterologous nuclear localization signal was introduced near the junction of TLS and CHOP. This was achieved by inserting an oligonucleotide (GGCAAGAAAAAGCGTAAGGTTGCA) into the unique *Pst1* site of the cDNA, giving rise to a BR$^-$ version that is localized to the nucleus. Expression of CHOP was achieved by ligating the murine CHOP cDNA into the pSRα plasmid. To be sure that the exogenous CHOP could be distinguished from any endogenous protein, virus-encoded CHOP was tagged with an amino-terminal human Myc epitope tag (Eilers et al. 1989). The tagged CHOP migrates more slowly on a SDS–Polyacrylamide gel than the native protein (32 vs. 29 kD). To construct the TLS–CHOP–ER fusion protein, the stop codon of the murine CHOP cDNA was altered to a leucine by PCR-based mutagenesis. This created a *Bgl*II site into which the ligand-binding do-

main of the human estrogen receptor was ligated in-frame (Umek et al. 1991). The 5′ *Bam*HI–*Nhe*I portion of this CHOP–ER derivative was then replaced by a *Bam*HI–*Nhe*I fragment excised from the TLS–CHOP sequence to create the TLS–CHOP–ER fusion. In the C/EBPα–CHOP construct, the amino terminus of C/EBPα encoding the transcriptional activation domain (*Bam*HI–*Sma*I fragment of the rat C/EBPα cDNA) was introduced in-frame upstream of the full-length murine CHOP-coding region. To create the VP16–CHOP chimera, the stop codon at the 3′ end of the activation domain of the HSV-1 VP16 gene (78 carboxy-terminal amino acids) was altered to a serine by PCR-based mutagenesis. An initiator methionine in an appropriate Kozak context was introduced at the 5′ end, and the fragment was ligated in-frame 5′ to the full-length human CHOP cDNA into the pSRα plasmid. The EWS–FLI1 retrovirus was a gift from Chris Denny (University of California, Los Angeles). To create the EWS–CHOP construct, a *Bam*HI fragment encoding the amino-terminal domain of EWS was ligated in-frame with the full-length human CHOP coding region. To create the TLS–FLI1 construct, the EWS sequence of EWS–FLI1 was excised by *Eco*RI–*Hin*cII digestion of the EWS–FLI cDNA and was replaced by a *Eco*RI–*Pvu*II fragment encoding the TLS amino terminus.

Oncogenic transformation of NIH-3T3 cells by virus-encoded fusion proteins, immune precipitation, and cellular localization of the expressed proteins

NIH-3T3 cells were infected with high-titer culture supernatants ($0.2–2\times10^5$CFU/ml) from virus-producing COS1 cells. Following the infection, cells were cultured for 10 days under selection with G418 (450 mg/l), at which point they were trypsinized and plated into soft agar (2×10^5 cells/60-mm plate; Lange 1989). Two weeks after plating into soft agar, the number of colonies were counted and the plates were photographed at a magnification of 50× under bright-field illumination. To control for differences in the titer of the different retroviral stocks, each stock was titered by counting the number of G418-resistant colonies produced 10 days after infection of cells with 10 μl of virus. Plates were fixed with 4% formaldehyde and stained in a 0.05% crystal violet solution to visualize colonies.

Expression of virus-encoded proteins in the infected cells was monitored by immune precipitation after [^{35}S]methionine–cysteine labeling. Cells were incubated for 3 hr with 500 μCi of ^{35}S and lysed in RIPA buffer. Immune precipitation was performed using 5μl of rabbit polyclonal antiserum to CHOP (Ron and Habener 1992). Immune precipitated proteins were resolved on 8% SDS–polyacrylamide gels and visualized by autoradiography.

The subcellular localization of the expressed protein in the infected NIH-3T3 cells was studied by immune cytochemistry. Cells growing on coverslips were fixed and labeled using the rabbit CHOP antiserum at a dilution of 1:1000. The secondary antibody was a rhodamine-conjugated affinity-purified donkey antirabbit IgG (Chemicon, Temucula, CA) and was used at a 1:100 dilution. Slides were viewed under fluorescent illumination using a Zeiss Axioplan microscope.

Ligand-dependent transformation by TLS–CHOP–ER was studied by culturing NIH-3T3 cells infected with a retrovirus encoding the protein in media containing 15% fetal calf serum in the absence or presence of 10^{-8} M estradiol. Two weeks after infection, cells were fixed in situ in 4% formaldehyde and stained with crystal violet. Dense foci of cells that had lost contact inhibition appear as darkly staining spots above the background of the normal contact-inhibited cells.

Tumor formation in nude mice

Infected NIH-3T3 cells were cultured for 10 days under selection with G418 (450 mg/liter), at which point they were trypsinized, washed with PBS, and injected subcutaneously (5×10^5 cells/mouse) into 6-week-old female Swiss nude mice. Tumor formation in the animals was monitored weekly starting 1 week after injection and continued for 4 weeks when the animals were sacrificed and the tumor mass was fixed in formaldehyde, sectioned, stained with hematoxylin–eosin, and studied by light microscopy.

Trans-*activation of a reporter gene by Gal4 chimeras*

The DNA fragment encoding the amino terminus of TLS was excised by *Pst*I digestion of TLS–CHOPpBS (Crozat et al. 1993), followed by end repair with T4 DNA polymerase. The fragment was ligated in-frame with a Gal4 DNA-binding domain in the pSG424 mammalian expression plasmid (Sadowski et al. 1988). The plasmid encoding the fusion of the amino terminus of EWS and Gal4 DBD was a gift of Chris Denny and William May (May et al. 1993a). C/EBPα–Gal4 (Pei and Shih 1991) and VP16–Gal4 plasmids were kindly provided by Duanquing Pei and Chiaho Shih (University of Pennsylvania, Philadelphia) and Ian Sadowski (Harvard University, Cambridge, MA), respectively. Rat-1a and HepG2 cells plated in 60-mm plates were transfected with 5 μg of the luciferase reporter gene UASp59RLG (Ron and Habener 1992) that contains two Gal4-binding sites upstream of the rat angiotensinogen gene minimal promoter along with 0.1 μg of *trans*-activator plasmid and 1 μg of a cytomegalovirus (CMV) β-galactosidase expressing internal control plasmid. Luciferase activity and β-galactosidase activity were measured 48 hr after transfection.

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