RESEARCH COMMUNICATION

Groucho/transducin-like Enhancer of split (TLE) family members interact with the yeast transcriptional co-repressor SSN6 and mammalian SSN6-related proteins: implications for evolutionary conservation of transcription repression mechanisms

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The yeast proteins TUP1 and SSN6 form a transcription repressor complex that is recruited to different promoters via pathway-specific DNA-binding proteins and regulates the expression of a variety of genes. TUP1 is functionally related to invertebrate and vertebrate transcriptional repressors of the Groucho/transducin-like Enhancer of split (TLE) family. The aim was to examine whether similar mechanisms underlie the transcription repression functions of TUP1 and Groucho/TLEs by determining whether TLE family members can interact with yeast SSN6 and mammalian SSN6-like proteins. It is shown in the present work that SSN6 binds to TLE1 and mediates transcriptional repression when expressed in mammalian cells. Moreover, TLE1 and TLE2 interact with two mammalian proteins related to SSN6, designated as the products of the ubiquitously transcribed tetratricopeptide-repeat genes on the Y (or X) chromosomes (UTY/X). These findings suggest that mammalian TLE and UTY/X proteins may mediate repression mechanisms similar to those performed by TUP1–SSN6 in yeast.

Key words: Notch signalling, TUP1, transcriptional repression, Wnt signalling.

INTRODUCTION

The yeast proteins TUP1 and SSN6 form a transcription repressor complex involved in the regulation of a variety of genes [1,2]. Although neither TUP1 nor SSN6 can bind DNA, they are recruited to selected promoters through interactions with pathway-specific DNA-binding proteins [3,4]. Once targeted to DNA, TUP1 acts as a global transcriptional repressor [1–5]. SSN6 functions as an adaptor between TUP1 and DNA-binding proteins and has no transcriptional repression activity of its own [1,6]. Together, TUP1 and SSN6 form a general repressor of transcription that gains specificity of action by interacting with sequence-specific DNA-binding proteins. TUP1 is functionally related to the *Drosophila* protein Groucho and its mammalian homologues, referred to as transducin-like Enhancer of split (TLE) 1 to 4 [7–10]. Like TUP1, Groucho/TLEs are general transcriptional repressors that lack DNA-binding ability but can be recruited to different promoters through interactions with a variety of sequence-specific DNA-binding proteins [11–16]. Once targeted to DNA, Groucho/TLEs share with TUP1 the ability to interact with chromatin components [2,17]. TUP1 and Groucho/TLEs are phosphorylated proteins of similar size that contain conserved C-terminal domains characterized by tandem copies of a WD40 repeat (WDR), a motif involved in protein–protein interactions [8,13]. In addition, they contain N-terminal domains that, although not highly related from a structural point of view, mediate similar molecular functions, namely transcriptional repression and protein multimerization [12,16,18,19]. Finally, they are both characterized by internal serine/proline-rich sequences that also harbour transcriptional repression functions [8,16,19]. These similar properties suggest that TUP1 and Groucho/TLEs are involved in evolutionarily conserved mechanisms of transcriptional repression.

Recently, two related mammalian genes encoding proteins similar to yeast SSN6 have been identified on either the Y or the X chromosomes of mice and humans [20–23]. The ubiquitously transcribed tetratricopeptide repeat genes on the Y/X chromosomes (UTY/X) encode proteins that exhibit roughly the same size as SSN6 and share with the latter the presence of N-terminal tandem copies of a tetratricopeptide repeat (TPR), a motif involved in protein–protein interactions, as well as internal regions rich in glutamine residues and C-terminal serine/proline-rich sequences [20–26]. Although the mouse *Uty* gene was shown to encode one of the male-specific transplantation antigens (H-Y) responsible for rejection of male tissue grafts by genotypically identical female mice [20], the molecular functions of the UTY and UTX proteins are unknown. However, their structural relatedness to SSN6 and the presence of putative nuclear localization signals within their sequences [20] suggest that they may share functional properties with the latter, including the ability to interact with TUP1-related proteins of the Groucho/TLE family.

In the present work we describe experiments designed to test whether mammalian TLE proteins can interact with yeast SSN6 and mammalian UTY/X. Our results show that SSN6 can mediate transcriptional repression when expressed in mammalian cells and can interact with TLE proteins. Moreover, they demonstrate that UTY and UTX also interact with TLE family

Abbreviations used: GAL4ad, activation domain of GAL4; GAL4bd, DNA-binding domain of GAL4; GST, glutathione S-transferase; TLE, transducin-like Enhancer of split; TPR, tetratricopeptide repeat; UAS, upstream activation sequence; UTY(X), ubiquitously transcribed tetratricopeptide repeat gene on the Y(X) chromosome; WDR, WD40 repeat.

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members, thus providing the first molecular characterization of these proteins. Taken together, these findings suggest that UTY and UTX may be functional counterparts of SSN6 that participate in at least some of the transcriptional roles of the TLE proteins in ways resembling the involvement of SSN6 in the functions of TUP1 in yeast.

MATERIALS AND METHODS

Plasmids

The following is a summary of the names and origins of the constructs used in these studies. Additional information on cloning strategies and oligonucleotide primers used in PCR experiments is available upon request. PCR was used to amplify the following fragments (see [8] for TLE domain organization and nomenclature): (1) TLE1(1–135) (i.e. the Q domain), (2) TLE1(1–435) (i.e. the Q, GP, CcN and SP domains), (3) TLE1 (144–770) (i.e. the GP, CcN, SP and WDR domains) and (4) TLE1(444–770) (i.e. only the WDR domain). Vent DNA polymerase was used and PCR products were routinely sequenced before subcloning into the appropriate vectors. pT7T3D–Pac–hUTY(42–203), containing an alternatively spliced human UTY cDNA encoding the region corresponding to amino acids 42–203 (homologous to residues 40–202 of mouse UTY), was obtained from the I.M.A.G.E. consortium (I.D. number 1062348, GenBank™ accession number AA417679). DNA constructs for yeast two-hybrid assays were obtained as follows. pAS1–SSN6(1–966) [encoding the DNA-binding domain of GAL4 (GAL4bd) fused to full-length SSN6] was provided by Dr S. Roth (MD Anderson Cancer Center, Houston, TX, U.S.A.). pGBT9–mUTY(13–507) (encoding GAL4bd fused to residues 13–507 of mouse UTY) was obtained by subcloning an *Eco*RI fragment from pcDNA3–GAL4bd–mUTY(13–1212) (see below) into the *Eco*RI site of pGBT9. pGBT9–hUTY(42–203) was obtained by digesting pT7T3D–Pac–hUTY(42–203) with *Not*I (followed by filling in with Klenow DNA polymerase) and *Eco*RI and subcloning the ensuing insert into the *Eco*RI and *Bam*HI (filled-in) sites of pGBT9. pGBT9–mUTX(67–524) (encoding GAL4bd fused to amino acids 67–524 of mouse UTX) was obtained by subcloning an *Eco*RI fragment from a pBluescript–mUtx cDNA into the *Eco*RI site of pCITE4-b, followed by digestion with *Eco*RV and *Sal*I and subcloning into the *Sma*I and *Sal*I sites of pGBT9. The PCR products TLE1(1–435) and TLE1(444–770) were subcloned into the *Sma*I site of pGAD424, which encodes the activation domain of GAL4 (GAL4ad). pGAD424–TLE2(31–743) was generated by subcloning an *Eco*RI fragment from a TLE2 cDNA into the *Eco*RI site of pGAD424. DNA constructs used for transcription assays were obtained as follows. pGL2–5XGAL4 [*luciferase* gene under the control of the SV40 promoter linked to five GAL4 upstream activation sequence (UAS) sites] and pcDNA3–GAL4bd have been described previously [12,16]. pcDNA3–GAL4bd–SSN6(1–966) was obtained by subcloning an *Xho*I–*Sal*I fragment from pAS1–SSN6(1–966) into pcDNA3–GAL4bd digested with *Xho*I. pcDNA3–GAL4bd–mUty-(13–1212) was generated by digesting a full-length mouse Uty cDNA with *Avr*II and *Not*I, followed by filling in with Klenow DNA polymerase. This fragment was subcloned into the *Eco*RV site of pcDNA3–GAL4bd. pcDNA3–GAL4bd–hUTY(42–203) was obtained by subcloning an *Eco*RI–*Not*I fragment into pcDNA3–GAL4bd digested with the same enzymes. For expression of glutathione S-transferase (GST) fusion proteins, the PCR products TLE1(1–135), TLE1(1–435) and TLE1(144–770) were subcloned into the *Sma*I site of pGEX2T. pGEX1–TLE1(32–770) was generated by digesting a TLE1 cDNA with *Eco*RI, followed by subcloning into the *Eco*RI site of pGEX1. pcDNA3–TLE1(1–770) was generated by subcloning a *Hin*dIII–*Bam*HI TLE1 fragment into the *Hin*dIII and *Bam*HI sites of pcDNA3.

Transfection/transcription assays

HeLa and 293 cells were transiently transfected with lipofectamine (Gibco–BRL) according to the manufacturer's instructions. The amount of DNA transfected was adjusted with pcDNA3 plasmid so that the total amount of DNA (2.0 μg) used in each transfection was the same. Transcription assays with the 5X UAS–SV40 promoter were performed as described previously [16].

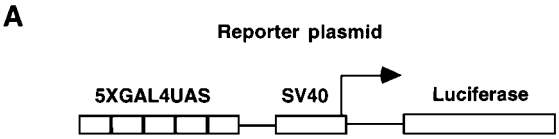
Fusion protein interaction assays

pcDNA3–GAL4bd and pcDNA3–GAL4bd–hUTY(42–203) plasmids were used as templates for *in vitro* translation reactions as described previously [16]. Incubation of proteins translated *in vitro* with GST fusion proteins and analysis of bound material by SDS/PAGE was as described previously [16,27,28].

RESULTS AND DISCUSSION

To examine the possibility that transcriptional mechanisms similar to those mediated by the TUP1–SSN6 complex in yeast may have been conserved during evolution, we first asked whether SSN6 could mediate transcriptional repression when expressed in mammalian cells. Human HeLa cells were transiently transfected with a previously described [12,16] reporter construct containing the *luciferase* gene under the control of the SV40 promoter linked to five GAL4 UAS sites (5XGAL4UAS) (Figure 1A). This modified SV40 promoter is basally active in mammalian cells [12,16]. The reporter construct was transfected alone or in combination with plasmids encoding either GAL4bd or a fusion protein of GAL4bd and full-length SSN6. GAL4bd alone stimulated an approx. two-fold activation of transcription above the basal level. In contrast, GAL4bd–SSN6 had no transactivating function; rather its expression resulted in a partial repression of basal transcription from the UAS–SV40 promoter, showing that SSN6 can act as a repressor of basal transcription when targeted to DNA in transfected HeLa cells (Figure 1B). Further examination of the transcriptional repressor activity of SSN6 revealed that this protein was a better repressor in Hela cells than in 293 cells (Figure 1B). This situation was correlated with the finding that HeLa cells have higher levels of endogenous TLE proteins than 293 cells (Figure 1C), suggesting that TLE proteins may promote the repressive function of SSN6. In possible agreement with this hypothesis, co-transfection of 293 cells with a plasmid driving overexpression of TLE1 potentiated the repressive function of SSN6 (Figure 1B). Taken together, these observations show that SSN6 can mediate transcriptional repression in mammalian cells. Moreover, they suggest that this function is performed in conjunction with TLE proteins, which is consistent with the demonstration that SSN6 requires TUP1 for transcriptional repression in yeast [1,6].

These observations prompted us to determine next whether SSN6 could physically interact with TLE1. Yeast two-hybrid interaction assays were performed using full-length SSN6 and both full-length and truncated forms of TLE1. Co-transformation with plasmids encoding GAL4bd–SSN6 and GAL4ad–TLE1(1–770) (full-length TLE1) resulted in reconstitution of a transcriptionally competent GAL4 complex, indicative of an SSN6–TLE1 interaction (Figure 2A). The N-terminal half of TLE1, TLE1(1–435), which contains each of the TLE structural



B

Cell type	Promoter	Promoter + GAL4bd	Promoter + GAL4bd-SSN6	Promoter + GAL4bd-SSN6 + TLE1	Promoter + TLE1
	Luciferase activity				
HeLa	100	221±27	66±5	N.D.	N.D.
293	100	245±27	93±6	77±10	99±6

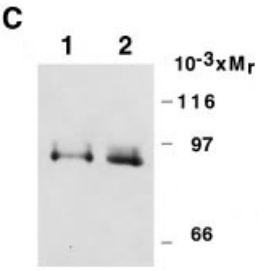


Figure 1 Transcriptional repression by SSN6 in transfected mammalian cells

(**A**) Reporter plasmid used in transient cell transfections. The construct contains the *luciferase* gene driven by the SV40 promoter linked to five GAL4 UAS (5XGAL4UAS). (**B**) Effect of expression of GAL4bd or GAL4bd–SSN6 fusion proteins on transcription of the luciferase reporter gene in HeLa or 293 cells. The reporter plasmid containing the 5XGAL4UAS–SV40 promoter (Promoter; 0.5 μg) was transfected alone or in combination with constructs (0.5 μg) encoding the proteins indicated, followed by determination of luciferase activity. Activity measured in the absence of GAL4bd or GAL4bd–SSN6 (basal activity) was considered as 100%. Values represent the means ± S.D. of at least four independent sets of trials (in duplicate). Expression of TLE1 alone had no effect on the basal activity of the 5XGAL4UAS–SV40 promoter. N.D., not determined. (**C**) Expression of TLE proteins in 293 and HeLa cells. Proteins (~ 50 μg/lane) from either 293 (lane 1) or HeLa (lane 2) cell lysates were subjected to SDS/PAGE (8% gel). Following transfer to nitrocellulose, the replica was stained with Ponceau S to verify that the lanes contained equal amounts of protein and then subjected to Western blotting with panTLE monoclonal antibodies [8,17]. The positions of M_r standards are shown.

domains except for the WDR domain [8], mediated interaction with SSN6 (Figure 2B). In contrast, the C-terminal half of TLE1 containing the WDR domain did not interact with SSN6 under these experimental conditions (Figure 2C), consistent with the results of previous studies in yeast, which showed that SSN6 binds to the N-terminal region of TUP1 and not to the WDR domain of the latter [19]. Similarly to previous results in yeast, which showed that truncated forms of TUP1 lacking the WDR domain interacted with SSN6 better than full-length forms [19], our studies also showed that TLE1(1–435) interacted with SSN6 better than full-length TLE1 (compare Figures 2A and 2B). Taken together with the previous demonstration that SSN6 has no intrinsic transcriptional repressor activity but depends on TUP1 for repression [1,6], these results strongly suggest that SSN6 can interact with TLE family members and form functional transcription repression complexes in transfected mammalian cells. In turn, this situation suggests that mechanisms of transcriptional repression analogous to those mediated by SSN6–TUP1 complexes in yeast may be mediated by TLE and SSN6-related proteins in mammals.

Based on these findings, we asked whether UTY and/or UTX proteins could interact with TLE family members and mediate transcriptional repression. To examine the first possibility, yeast two-hybrid interaction studies were performed. Yeast cells were co-transformed with constructs encoding GAL4ad–TLE1(1–770) and GAL4bd–mUTY(13–507) (a fusion protein containing the N-terminal half of mouse UTY, including the entire TPR domain related to the TUP1-binding TPR domain of SSN6). Transformed cells displayed a specific reconstitution of a transcriptionally competent GAL4 complex, indicative of a UTY/TLE1 interaction (Figure 3A). As observed with SSN6, the N-terminal half of TLE1 was sufficient to mediate UTY binding, and appeared to interact with the latter better than full-length TLE1 (Figure 3B). Interactions were also demonstrated when these assays were performed by fusing mUTY(13–507) to GAL4ad and TLE1 to GAL4bd respectively (results not shown). Similar interaction assays using the TPR domain of mouse UTX,

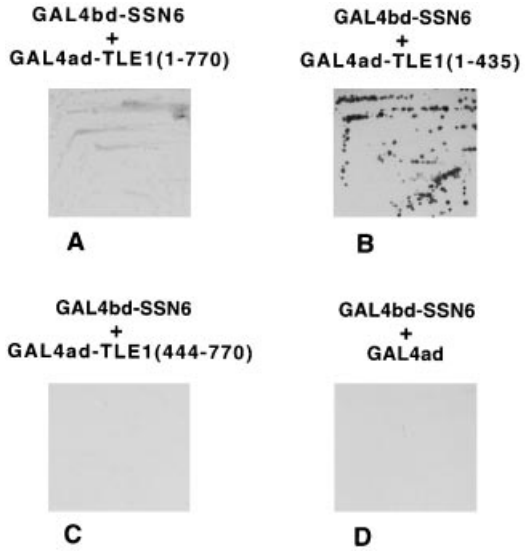


Figure 2 Binding of SSN6 to TLE1 in yeast two-hybrid interaction assays

Yeast cells were co-transformed with plasmids encoding the indicated combinations of proteins, cultured and subjected to a filter assay for β-galactosidase activity as described previously [16,27]. (**A**, **B**) The ability of the transformed cells to turn blue (dark streaks) in the presence of the β-galactosidase substrate, X-gal, indicated that a transcriptionally competent GAL4 complex had been reconstituted due to the interaction of SSN6 and TLE1(1–770) (**A**) or TLE1(1–435) (**B**). No interaction was observed between SSN6 and TLE1(444–770) (**C**) or GAL4ad (**D**).

which is 93% similar to the corresponding region of mouse UTY [20–23], showed that mUTX(67–524) bound to both TLE1(1–770) (Figure 3D) and TLE1(1–435) (Figure 3E). These results suggested that TLE proteins can interact with UTY and UTX and that these interactions are mediated by the TPR domain

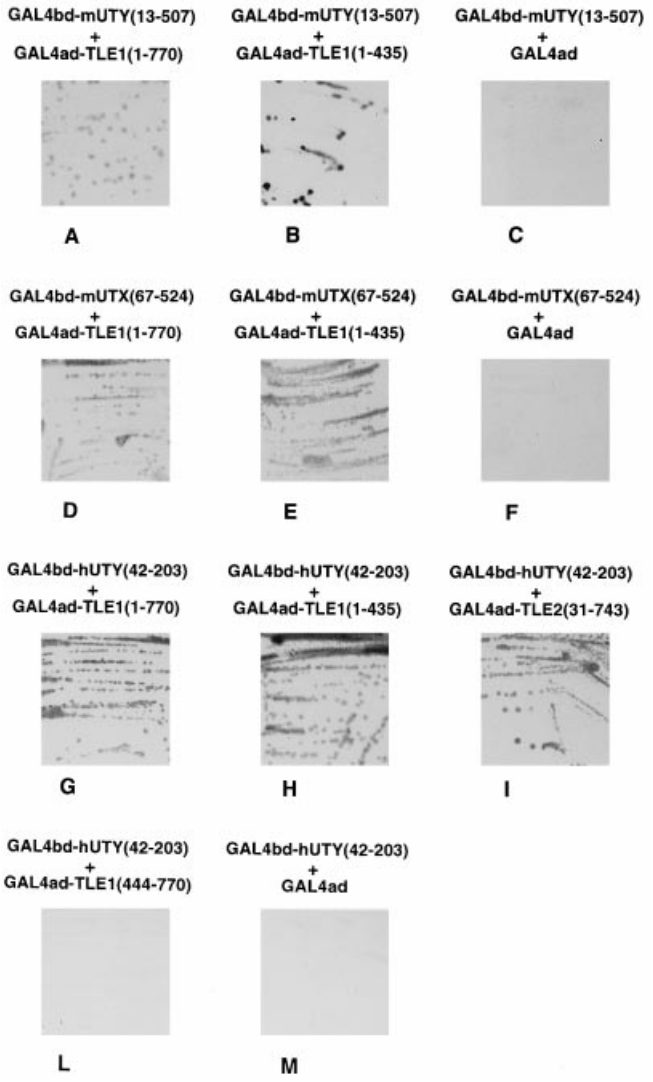


Figure 3 Binding of UTY and UTX to TLE proteins in yeast two-hybrid interaction assays

Yeast cells were co-transformed with plasmids encoding the indicated combinations of proteins, cultured and subjected to a filter assay for β-galactosidase activity as described previously [16,27]. (**A**, **B**, **D**, **E**, **G**–**I**) The ability of the transformed cells to turn blue (dark streaks) in the presence of the β-galactosidase substrate, X-Gal, indicated that a transcriptionally competent GAL4 complex had been reconstituted due to interactions among the indicated pairs of proteins. (**C**, **F**, **L**, **M**) No interactions were observed when cells expressed the indicated combinations of proteins.

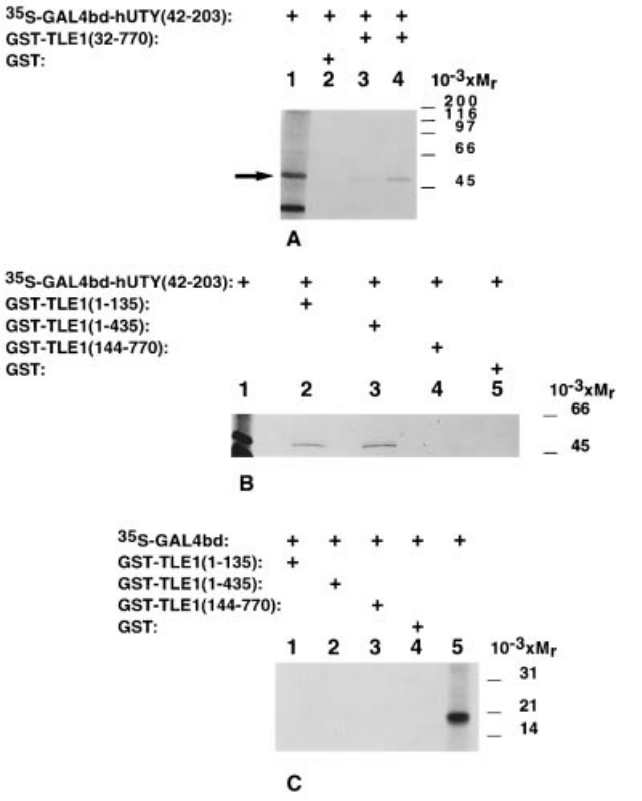


Figure 4 Interaction between the first three TPRs of UTY and the N-terminal domain of TLE1

(**A**) *In vitro* translated ^{35}S-labelled GAL4bd–hUTY(42–203) (indicated with an arrow) (lane 1, one-half of the amount used in each incubation mixture) was incubated in the presence of either GST (lane 2, 2.0 μg of protein) or GST–TLE1(32–770) (lanes 3 and 4, 1.0 and 2.0 μg of protein respectively), followed by addition of glutathione–Sepharose beads as described previously [16,27]. The material still bound to the beads after extensive washing was subjected to SDS/PAGE (10% gel), followed by autoradiography. (**B**, **C**) *In vitro* translated ^{35}S-labelled GAL4bd-hUTY(42–203) (**B**, lane 1; one-half of the amount used in each incubation mixture) or GAL4bd (**C**, lane 5; one-half of the amount used in each incubation mixture) were incubated with the indicated fusion proteins (2.0 μg), then with glutathione–Sepharose beads followed by SDS/PAGE using (**B**) 10% gel or (**C**) 15% gel as described above. GAL4bd–hUTY(42–203) bound to GST–TLE1(1–135) and GST–TLE1(1–435); on occasion, a weak interaction with GST–TLE1(144–770) was observed after prolonged autoradiography (results not shown). The positions of M_r standards are indicated.

of the latter, which is in agreement with the previous demonstration that the first three TPRs of SSN6 mediate interaction with TUP1 in yeast [6]. To examine this possibility further, we performed yeast two-hybrid interaction assays using a portion of the TPR domain of UTY containing only the first three TPRs. Amino acids 42–203 of human UTY, which are 86% similar to residues 40–202 of mouse UTY (including a 92% similarity across TPRs 1–3) [20–23], mediated interaction with TLE1(1–770) (Figure 3G), TLE1(1–435) (Figure 3H), as well as TLE2(31–743), an almost full-length form of TLE2 lacking only the first 30 amino acids (Figure 3I). In contrast, hUTY(42–203) did not interact with the C-terminal half of TLE1, which contains the WDR domain, under these experimental conditions (Figure 3L).

Complementary binding assays with *in vitro* translated UTY and bacterially purified fusion proteins of GST and TLE1 (pull-down assays) were performed to confirm these observations. GAL4bd–hUTY(42–203) interacted with a GST–TLE1(32–770) fusion protein (Figure 4A, lanes 3 and 4), but not with GST (Figure 4A, lane 2). GAL4bd–hUTY(42–203) also bound to GST–TLE(1–135) (containing only the glutamine-rich Q domain of TLE1) (Figure 4B, lane 2) and GST–TLE1(1–435) (Figure 4B, lane 3), but not significantly to GST–TLE1(144–770) (containing all the TLE1 domains except for the Q domain) (Figure 4B, lane 4). GAL4bd did not interact with any GST–TLE1 fusion protein (Figure 4C and [16]). Taken together, these investigations show that UTY and UTX can interact with TLE proteins and strongly suggest that these interactions are mediated, at least in part, by the first three TPRs of UTY. In turn, these repeats can interact with the N-terminal Q domain conserved among Groucho/TLE family members.

Transfection assays with the UAS–SV40–luciferase reporter plasmid and fusion proteins of GAL4bd and mUTY(13–1212)

(which contains the entire mouse UTY sequence except for the first 12 amino acids) were performed next to determine whether UTY could mediate transcriptional repression when targeted to DNA. HeLa cells were transfected with the reporter plasmid alone or in combination with constructs encoding GAL4bd or GAL4bd–mUTY(13–1212). Whereas expression of GAL4bd alone resulted in an approx. 2.0- to 2.5-fold transcriptional activation above basal level, GAL4bd–mUTY(13–1212) had no transactivating function and its expression resulted in a moderate inhibition of basal transcription (results not shown). These findings suggest that, like SSN6, UTY can convert GAL4bd from an activator into a weak transcriptional repressor under these experimental conditions. In contrast to SSN6, however, UTY did not act as a stronger repressor in HeLa cells compared with 293 cells.

In summary, the demonstrated ability of SSN6 to mediate transcriptional repression in cells from species as evolutionarily distant as yeast and humans strongly suggests that this protein is involved in fundamental mechanisms that have been conserved across species boundaries. Its ability to interact with mammalian TLE proteins, which resemble TUP1, the natural functional partner of SSN6 in yeast, suggests further that transcriptional repressive functions analogous to those of SSN6–TUP1 complexes are mediated in mammalian cells by complexes of TLE and SSN6-like proteins. Based on the present studies, we propose that the related proteins UTY/X may be functional counterparts of SSN6 that participate in at least some of the functions of TLE family members. In the future, it will be important to determine the intracellular localization of UTY and UTX, both of which contain potential nuclear targeting sequences and might be able to interact with TLE proteins in the nucleus [20–23]. In addition, it will be important to determine whether UTY/X may act as adaptor proteins that tether TLEs to certain DNA-binding factors that do not have intrinsic TLE-binding properties. Alternatively, UTY/X may strengthen direct interactions between TLEs and other DNA-binding proteins. Both of these possibilities would be consistent with the situation in yeast, where SSN6 has been shown both to mediate indirect interactions between TUP1 and DNA-bound repressors and to interact with transcription factors that also bind directly to TUP1, like the homoeodomain protein $\alpha 2$ [1–6]. These investigations are expected to clarify the roles of the UTY and UTX proteins and facilitate the elucidation of the mechanisms underlying the functions of the Groucho/TLE transcriptional repressors.

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