Groucho is a transcriptional repressor implicated in Notch signaling and involved in neural development and segmentation in Drosophila. We are investigating the molecular mechanisms underlying the functions of Groucho and its mammalian homologs, the transducin-like Enhancer of split (TLE) proteins. We report that Groucho/TLEs are associated with chromatin in live cells and that they co-purify with isolated histones. Affinity chromatography and far Western blotting studies show further that native Groucho/TLE proteins interact specifically with histone H3 and not with other core histones. This interaction is mediated by the H3 amino-terminal domain previously shown by genetic analysis in yeast to be essential for the role of H3 in transcriptional silencing. We also demonstrate that Groucho/TLEs form oligomeric structures in vivo. These combined findings suggest that transcription complexes containing Groucho/TLEs may associate with chromatin through interactions with the amino terminus of histone H3 and that these interactions may be propagated along the chromosome due to the ability of Groucho/TLEs to participate in higher order structures.

In both invertebrates and vertebrates, signaling mechanisms mediated by the cell-surface receptor Notch control the differentiation of a variety of cell types both by restricting the competence of precursor cells to respond to specific differentiation cues and by providing inductive signals (reviewed in Refs. 1 and 2). Our understanding of the molecular mechanisms underlying the restrictive functions of the Notch pathway points to an important role for proteins involved in transcriptional repression. In particular, molecular genetic analysis of Notch signaling during Drosophila neural development shows that the basic helix-loop-helix (bHLH)3 proteins encoded by the Enhancer of split complex act at the end point of the Notch cascade by repressing the expression of genes that promote the neural fate (proneural genes) (3–8). This transcriptional function involves the activity of the product of another locus, referred to as groucho (3, 9–12). This gene was originally implicated in Notch signaling by genetic analysis showing pheno typic interactions between alleles of groucho and other genes involved in the regulation of proneural gene expression (1–3, 13). Molecular studies subsequently showed that the bHLH proteins encoded by the Enhancer of split complex interact with the Groucho protein (10, 12). Groucho also interacts with other bHLH factors related to Enhancer of split proteins; these include the product of the pair rule gene hairy and will be hereafter collectively referred to as Hairy and Enhancer of split-like (HES) proteins (2, 4–6, 10–12). It is thought that HES factors recruit the broadly expressed Groucho protein, which lacks DNA-binding ability, to specific DNA sites. Once targeted to DNA, Groucho can repress both basal and activated transcription (12). This particular observation and the demonstration that groucho mutations affect the expression of a variety of genes and have pleiotropic consequences in Drosophila (10, 11–16) strongly suggest that Groucho is involved in the regulation of multiple promoters. However, virtually nothing is known about the molecular mechanisms underlying its transcriptional repressor functions.

Recent studies have characterized a number of mammalian groucho homologs, which have been designated as the transducin-like Enhancer of split (TLE) or groucho-related genes 1 through 4 (hereafter referred to as TLE1-4) (17–22). Comparative analysis of the structural and functional properties of Groucho and TLE proteins has revealed that they resemble Tup1p, a general transcriptional repressor in yeast (23–28). Tup1p and Groucho/TLEs share a similar overall domain structure including carboxyl-terminal tandem WD40 repeats (17, 23, 29), an amino-terminal Gln-rich region that mediates protein dimerization (30, 31), and internal Ser/Thr/Pro-rich sequences (17, 23). Like Groucho/TLEs, Tup1p lacks DNA-binding ability and is recruited to specific DNA sites through interactions with DNA-binding proteins, like the homeodomain factor a2 (26). Once targeted to DNA, Tup1p can repress the expression of a variety of genes (23–26).

These observations suggest that similar mechanisms may mediate the transcriptional activities of Tup1p and Groucho/TLE proteins. In this regard, genetic and molecular studies have revealed that the general transcriptional functions of Tup1p involve direct interactions between multimeric Tup1p complexes and nucleosomes (24, 32, 33). These associations are mediated by binding of Tup1p to the amino termini of histones H3 and H4 (24). It is thought that these interactions result in local chromatin remodeling effects that establish repressive structures, thereby preventing access to transcriptional activators (24). Based on these findings, we have investigated the possibility that interactions with nucleosomes play a role in the transcriptional functions of Groucho/TLEs. Our analysis shows
that these proteins co-fractionate with histones in live cells and interact specifically with the amino terminus of histone H3 in vitro. We also show that Groucho/TLEs exist as high Mr oligomeric structures. Taken together, these findings suggest a model in which transcription complexes containing Groucho/TLEs may regulate gene expression by interacting with adjacent nucleosomes through associations with the amino termini of H3 proteins.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Rat monoclonal antibodies against the conserved carboxyl-terminal WD40 domain of Groucho/TLEs (pan-TLE antibodies) (17, 34, 35), mouse monoclonal antibodies against Drosophila Groucho (13, 35), and rabbit polyclonal antibodies against the amino terminus of yeast histone H3 (24) have been described previously.

**Cell Culture and Preparation of Cell and Embryo Lysates**—Human Jurkat and HeLa cells and Drosophila S2 cells were cultured as described previously (35). Staged Drosophila embryos were collected and dechorionated as described previously (13). For the preparation of cell/embryo lysates, samples were collected, washed with ice-cold phosphate-buffered saline, and homogenized in buffer 1 (30 mM HEPES, pH 7.6, 60 mM KCl, 30 mM NaCl, 250 mM sucrose, 1 mM EDTA, 20 mM iodoacetamide, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 mM leupeptin, 2.5 mM/ml aprotinin, 2.5 mM/ml pepstatin A, and 2.5 mM/ml antipain) by trituration through 25-gauge needles, followed by addition of Triton X-100 to a final concentration of 1% and incubation at 4 °C for 20 min. Lysates were centrifuged at 12,000 × g for 15 min, and the supernatant was recovered.

**Isolation of Chromatin**—Nuclei were isolated from actively growing Jurkat cells as described previously (35). Chromatin was obtained by subjecting nuclei to sequential homogenization/sedimentation steps in the presence of decreasing concentrations of Tris/HCl, pH 8.0, as described previously (36), except that all buffers were supplemented with 1 mM phenylmethylsulfonyl fluoride, 2 mM leupeptin, 2.5 mM/ml aprotinin, 2.5 mM/ml pepstatin A, and 2.5 mM/ml antipain. Chromatin was recovered after the last homogenization/sedimentation step in the presence of 1 mM Tris/HCl, pH 8.0. This pellet was resuspended in 10 volumes of Tris/HCl, pH 8.0, and sheared by heating at 95 °C. The solution was then sequentially homogenized through 21- and 25-gauge needles prior to analysis by SDS-PAGE and Western blotting with pan-TLE antibodies (1:20 dilution) (35).

**Isolation of Histones**—Chromatin was resuspended in 10 volumes of buffer 2 (50 mM Tris/HCl, pH 8.0, 0.6 M NaCl) and homogenized through 18-gauge needles. After recovery of the chromatin pellet by sedimentation at 8,000 × g for 10 min, this step was repeated once. This procedure resulted in the removal of the majority of non-histone proteins (37). The obtained pellet was then incubated in the presence of 10 volumes of buffer 3 (50 mM Tris/HCl, pH 8.0, 0.9 M NaCl) and homogenized through 18-gauge needles. The supernatant obtained after centrifugation at 8,000 × g for 10 min was collected and shown by SDS-PAGE to contain predominantly histone proteins (see Fig. 2). Alternatively, histones were isolated from nuclei in the presence of dilute sulfuric acid as described previously (24), except that all buffers contained 1 mM phenylmethylsulfonyl fluoride, 2 mM leupeptin, 2.5 mM/ml aprotinin, 2.5 mM/ml pepstatin A, and 2.5 mM/ml antipain. Purified histone H4 was obtained from Boehringer Mannheim.

**Batch Affinity Chromatography**—A mixture of purified histones H1, H2A, H2B, H3, and H4 from calf thymus, as well as hen egg white lysozyme, were obtained from Boehringer Mannheim. Proteins were coupled to CNBr-activated Sepharose 4B according to the manufacturer’s specifications (Pharmacia Biotech Inc.) by using 8–10 mg of protein per g of dry gel. Affinity chromatography was performed at 4 °C. Beads were equilibrated in “binding buffer” (20 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 mM leupeptin, 2.5 mM/ml aprotinin, 2.5 mM/ml pepstatin A, and 2.5 mM/ml antipain) (24), followed by incubation in the presence of Jurkat cell lysate (roughly 1 mg of total protein) for 3 h. Beads were then collected by centrifugation, and the supernatant was recovered (unbound fraction). After extensive washing with binding buffer, beads were incubated sequentially in Tris-buffered solutions containing 0.4, 0.6, 0.8, and 1.0 mM NaCl, followed by incubation in 2 × SDS-PAGE sample buffer. The recovered fraction (bound), together with unbound and starting fractions, was analyzed by gel electrophoresis, transfer to nitrocellulose, and Western blotting with pan-TLE antibodies.

**Far Western Blotting**—Jurkat cells were collected, washed extensively with phosphate-buffered saline, and then resuspended in phosphate-buffered saline containing 2% SDS, 10% glycerol, and 50 mM dithiothreitol. The suspension was triturated through 18-, 21-, and 25-gauge needles, followed by incubation at 95 °C for 5 min and additional trituration through 25-gauge needles. After centrifugation at 12,000 × g for 5 min, the supernatant was collected and subjected to SDS-PAGE on 20% gels. After transfer to nitrocellulose, the different histones were identified on the replicas with staining with Ponceau S and comparison of the migration of purified histones. Drosophila and human H3 proteins were visualized by cross-reacting antibodies raised against the conserved amino terminus of yeast H3 (24). Replicas were incubated in “incubation buffer” (25 mM Tris/HCl, pH 7.8, 150 mM NaCl, 0.05% Triton X-100, 3% dry milk) for 2–3 h, followed by a 2-h incubation in the presence of Jurkat cell lysate (approximately 60 μg of protein). After extensive washing, replicas were incubated in the presence of pan-TLE monoclonal antibodies, followed by horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies. In competition experiments, the nitrocellulose replicas were first preincubated in the presence of a 1:400 dilution of anti-histone H3 antibodies and then subjected to far Western blotting. When the binding of Groucho to histone H4 was examined, replicas on nitrocellulose were incubated, followed by incubation with anti-Groucho monoclonal antibodies (1:20).

**Fusion Protein Interaction Assay**—Roughly 5 μg of a fusion protein between GST and residues 21–46 of H3 (GST-H321–46) (38) were incubated for 3 h at 4 °C in binding buffer containing Jurkat cell lysate (approximately 60 μg of protein), followed by recovery of the fusion proteins on glutathione-Sepharose beads, and extensive washing with binding buffer. Washed beads were directly resuspended in SDS-PAGE sample buffer, and the bound material was subjected to electrophoresis on a 10% gel together with serial dilutions of the starting lysate.

**Cross-linking Experiments**—Cell lysates were incubated at room temperature for 30 min in the presence of either no cross-linker or increasing concentrations of DTSSP (Pierce) (0.1–0.6 mM). After this time, the cross-linked sample was quenched by incubation in incubation mixtures to contain 40 mM glycine and 50 mM Tris/HCl, pH 8.0. Reactions were then mixed with 2 × SDS-PAGE sample buffer in the absence of any reducing agent and subjected to gel electrophoresis. To reverse the cross-linking reactions, samples were incubated in the presence of 50 mM dithiothreitol and heated at 65 °C prior to electrophoresis.

**Non-denaturing PAGE**—Native Groucho/TLE protein complexes were resolved by non-denaturing PAGE on 4–18% gels according to the protocol of Andersson et al. (39) (see also Ref. 27). Electrophoresis was conducted at 100 V for 24 h.

**RESULTS**

**TLE Proteins Are Associated with Chromatin in Live Cells**—The current understanding of the molecular mechanisms underlying the transcriptional activities of Groucho/TLE proteins is limited. Their relatedness to yeast Tup1p suggests that Groucho/TLEs may share with the latter the ability to interact with chromatin components. To test this hypothesis, chromatin was recovered from isolated Jurkat cell nuclei by serial homogenization/sedimentation steps in decreasing ionic strength conditions (36). Chromatin, together with the other nuclear subfractions, was then analyzed for the presence of TLE proteins by SDS-PAGE and Western blotting with pan-TLE antibodies. These studies showed that a large amount of the TLE immunoreactivity present in intact nuclei was not associated with chromatin and was readily removed by mild washes (Fig. 1, lanes 4–7). This result is in agreement with previous studies showing that although Groucho/TLEs translocate to the nucleolus, the majority of them (>80%) do not become tightly associated with the nuclear compartment and can be eluted off by exposure to low ionic strength conditions (17, 35; see also Fig. 1, lanes 1 and 2). However, a fraction of the nuclear TLE population, exhibiting a slightly slower electrophoretic mobility, was found associated with isolated chromatin (lane 12), suggesting that certain, but not all, Groucho/TLEs become associated with chromatin after translocation to the nuclear compartment. To determine whether this association was mediated by nucleosomal components, a fraction highly enriched...
in histone proteins was recovered from chromatin (Fig. 2, lane 1). Western blotting analysis of this fraction with pan-TLE antibodies revealed that Groucho/TLEs co-isolate with histones (Fig. 2, lane 3). The same result was obtained when histones were prepared by extractions with dilute sulfuric acid (24) (not shown). Taken together, these findings show that Groucho/TLEs can associate with chromatin and strongly suggest that they can do so through interactions with nucleosomal components.

Groucho/TLE Proteins Interact with Histone H3—The co-isolation of histones and Groucho/TLE proteins suggests that these molecules may interact with each other. To test this hypothesis, we investigated the ability of Groucho/TLEs to bind to histone proteins immobilized on a Sepharose matrix. Histone-Sepharose beads were incubated with Jurkat cell extracts; these samples served as source of both native TLE proteins and intrinsic nonspecific competitors. Unbound and bound fractions were then analyzed by Western blotting with pan-TLE antibodies together with serial dilutions of the input lysate. These investigations showed that TLE proteins bound to the histone-Sepharose matrix and that this interaction, once formed, was insensitive to high ionic strength conditions (Fig. 3). Approximately one-sixteenth of the starting TLE immunoreactivity was retained on the beads (cf. lanes 1 and 6), indicating that significant amounts of TLE proteins interacted with histones. Control affinity chromatography experiments with lyszyme-Sepharose beads revealed no interaction between TLEs and lysosome (Fig. 3, lane 5). Since the latter is a small, highly basic protein, these combined results strongly suggest that TLE binding to the histone-Sepharose matrix was specific and not mediated by nonspecific electrostatic interactions with small, positively charged proteins.

The interaction between histones and Groucho/TLEs was confirmed by far Western blotting studies (24). Nitrocellulose replicas containing fractionated histones (Fig. 4a) were incubated in the presence of detergent extracts from either Jurkat cells (Fig. 4b) or Drosophila S2 cells (which express high levels of Groucho (35)) (Fig. 4c). Bound proteins were visualized by

FIG. 1. TLE proteins associate with chromatin. Chromatin was isolated from Jurkat cell nuclei as described under “Experimental Procedures.” Samples were subjected to SDS-PAGE on an 8% gel, followed by transfer to nitrocellulose and Western blotting with pan-TLE monoclonal antibodies. Lanes were loaded as follows. Lane 1, Jurkat cell lysate (lys., ~60 μg of protein/lane). Lane 2, nuclear extract (ne, ~40 μg of protein/lane). Lanes 4 and 5, nuclear wash fractions obtained in the presence of 250 mM sucrose (nis/EDTA, ~50 μg of protein/lane). Lanes 6 and 7, nuclear wash fractions obtained in the presence of saline/EDTA (sal/EDTA, ~50 μg of protein/lane). Lanes 8–11, supernatants obtained after homogenization/sedimentation in the presence of either 30 mM Tris/HCl, pH 8.0 (50 mM Tris, lane 8, ~50 μg of protein/lane), 10 mM Tris/HCl, pH 8.0 (10 mM Tris, lane 9, ~50 μg of protein/lane), 5 mM Tris/HCl, pH 8.0 (5 mM Tris, lane 10, ~40 μg of protein/lane), or 1 mM Tris/HCl, pH 8.0 (1 mM Tris, lane 11, ~40 μg of protein/lane). Lane 12, isolated chromatin (Chrom., ~70 μg of protein/lane). No samples were loaded onto lane 3. The positions of migration of Mr standards are indicated.

FIG. 2. TLE proteins co-isolate with histones. Aliquots of histones obtained by high-salt extraction of isolated chromatin (lane 1, ~15 μg of protein/lane; lane 3, ~25 μg of protein/lane) and histones purified as described (47) (lane 2, ~15 μg of protein/lane) were subjected to SDS-PAGE on a 14–18% gradient gel. Samples in lanes 1 and 2 were stained with Coomassie Blue. Sample in lane 3 was transferred to nitrocellulose, followed by Western blotting with pan-TLE antibodies. The antibodies decorated a roughly 95-kDa band(s). The position of migration of each of the core histones is indicated along the left-hand side of the panel. The more slowly migrating bands of roughly 34–36 kDa correspond to histone H1 proteins. The positions of migration of Mr standards are indicated.

FIG. 3. TLE proteins bind to a histone-Sepharose matrix. Jurkat cell lysate (~1 mg of protein) was subjected to batch affinity chromatography on either a histone-Sepharose 4B matrix (lanes 4 and 6) or a lysozyme-Sepharose 4B matrix (lanes 3 and 5). Aliquots of the starting material (SM, lane 1, ~60 μg of protein/lane and lane 2, ~30 μg of protein/lane), the unbound fraction after chromatography on lysozyme-Sepharose (BLy, lane 3, ~30 μg of protein/lane), the unbound fraction after chromatography on histone-Sepharose (UBHi, lane 4, 30 μg of protein/lane), the entire fraction eluted from lysozyme-Sepharose (BHi, lane 5), and the entire fraction eluted from histone-Sepharose (BHHi, lane 6) were subjected to SDS-PAGE on an 8% gel, followed by transfer to nitrocellulose, and Western blotting with pan-TLE monoclonal antibodies. The positions of migration of Mr standards are indicated.

TLE Proteins with H3
incubation with either pan-TLE monoclonal antibodies or previously characterized (19, 35) anti-Groucho monoclonal antibodies. Groucho/TLEs bound to a component that co-migrated with histone H3 and not to other abundant proteins present on the nitrocellulose replicas, including histones H2A, H2B, and H4 (Fig. 4, b and c; cf. lanes 1 and 3). Importantly, this interaction was significantly reduced by preincubation in the presence of antibodies that recognize the amino-terminal domain of H3, strongly suggesting that Groucho/TLEs interact with the amino-terminal region of this protein (Fig. 4, b and c; cf. lanes 2 and 3). This competition was specific, since the presence of the anti-H3 antibodies had no effect on the binding of Groucho/TLEs to an as yet uncharacterized protein(s) of roughly 36–38 kDa (Fig. 4c; see arrowhead).

The binding of Groucho/TLEs to the amino-terminal region of H3 was confirmed by separate solution interaction assays with previously described (38) fusion proteins containing portions of the amino terminus of H3 fused to GST. Groucho/TLEs bound to fusion proteins containing either residues 1–46 (not shown) or residues 21–46 of H3 (GST-H321–46) (Fig. 5). Approximately one-twelfth of the TLE immunoreactivity present in the input lysate bound to GST-H321–46 (cf. lanes 3 and 4), in good agreement with the results shown in Fig. 4. Taken together with previous studies showing that Groucho/TLEs do not interact with GST under the same conditions (12, 40), these results show that these proteins bind to histone H3 and not to other core histones and that this interaction is mediated by the amino-terminal region of H3.

Groucho/TLE Proteins Are Part of Large Oligomeric Structures—The results described above show that Groucho/TLEs share with Tup1p the ability to interact with nucleosomal components, suggesting that similar mechanisms may mediate the transcriptional functions of these proteins. Analysis of Tup1p properties shows that this protein can multimerize, suggesting that Tup1p complexes can engage multiple nucleosomes (24, 27, 28). To determine whether Groucho/TLEs exhibit a similar ability to form oligomeric structures in vivo, we performed cross-linking studies. Lysates from Drosophila embryos were incubated in the presence of increasing concentrations of the water-soluble, reversible cross-linker DTSSP. Western blotting analysis revealed that under nonreducing conditions Groucho migrated as a roughly 87–90-kDa protein (Fig. 6, lane 1). A minor immunoreactive form of roughly 170,000 was also visible (lane 1). Incubation in the presence of DTSSP resulted in the appearance of a ladder-like immunoreactive profile, suggesting the existence of complexes containing Groucho proteins (lanes 2 and 3). Cross-linked products exhibiting apparent M, of roughly 170,000 and 240,000 (see arrowheads) were observed, suggesting that the roughly 90-kDa Groucho protein may multimerize (the apparent M, standards are indicated).

FIG. 4. Groucho/TLE proteins interact with histone H3. Jurkat cell extract (lanes 1–5) and purified calf thymus histone H4 (lane 6) were fractionated on a 20% SDS-polyacrylamide gel, followed by transfer to nitrocellulose. a, the resolved histones were visualized on the nitrocellulose replica by staining with Ponceau S. The position of migration of each of the core histones is indicated along the left-hand side of the panel. b and c, nitrocellulose replicas were subjected to far Western blotting analysis in the presence of the following reagents. Lane 1, polyclonal anti-H3 antibodies (1:2000), followed by horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies. Lane 2, anti-H3 antibodies (1:400), followed by either Jurkat (b) or S2 (c) cell lysate (100 µg of protein). Either pan-TLE (b) or anti-Groucho (c) antibodies were then added (1:20), followed by horseradish peroxidase-conjugated goat anti-rat IgG (b) or anti-mouse IgG (c) secondary antibodies (1:4000). Lane 3, either Jurkat (b) or S2 (c) cell lysate (100 µg of protein), followed by either pan-TLE (b) or anti-Groucho (c) antibodies (1:20) followed by appropriate secondary antibodies (1:4000). Lane 4, either pan-TLE (b) or anti-Groucho (c) antibodies (1:20) followed by appropriate secondary antibodies (1:4000). Lane 5, horseradish peroxidase-conjugated goat anti-rat (b) or anti-mouse (c) IgG secondary antibodies (1:4000). Lane 6, either Jurkat (b) or S2 (c) cell lysate (100 µg of protein), followed by either pan-TLE (b) or anti-Groucho (c) antibodies (1:20) and appropriate secondary antibodies (1:4000). Bands were visualized using either color development with 3,3′-diaminobenzidine (b) or enhanced chemiluminescence (c). The positions of migration of M, standards are indicated.

FIG. 5. Interaction between the amino terminus of H3 and TLE proteins. Roughly 5 µg of GST-H321–46 fusion protein was incubated in the presence of Jurkat cell lysate (60 µg of protein), followed by recovery of the fusion protein on glutathione-Sepharose beads, and analysis of the bound material by Western blotting with pan-TLE monoclonal antibodies. Lanes 1–3 were loaded with one-third (20 µg of protein), one-sixth (10 µg of protein), and one-twelfth (5 µg of protein), respectively, of the amount of starting lysate used in the binding assay. Lane 4 was loaded with the entire fraction bound to the beads. Approximately one-twelfth of the starting TLE immunoreactivity bound to GST-H321–46. The positions of migration of M, standards are indicated.
The presence of either no DTSSP (Drosophila malian Groucho/TLEs exhibited migrations indicative of a par-
linking pattern of species of slower mobilities was observed after cross-
also visible under nonreducing conditions), a ladder-like pat-
ters decorated a 90–95-kDa multiplet in the absence of DTSSP similar cross-linking pattern. Although the pan-TLE antibod-
7
ries resembling those detected in embryonic extracts, were ob-
phoresis was performed under reducing conditions, indicating
reactions.

FIG. 6. Detection of oligomeric Groucho forms by cross-linking reactions. Lyate from Drosophila embryos (2–10 h) was incubated in
the presence of either no DTSSP (lane 1, ~80 µg of protein/lane), 0.2 mM DTSSP (lane 2, ~80 µg of protein/lane), or 0.4 mM DTSSP (lane 3, ~80 µg of protein/lane). Samples were then subjected to SDS-PAGE on a 4–15% gradient gel, followed by transfer to nitrocellulose, and Western blotting with anti-Groucho monoclonal antibodies. Arrowheads point to the positions of migration of the major cross-linked products. The arrow points to the position of migration of a minor cross-linked product (see text). The positions of migration of Mr standards are indicated.

These findings were confirmed and extended by performing cross-linking studies with both Drosophila and human cell lines. Immunoreactive Groucho species of high apparent Mr, resembling those detected in embryonic extracts, were observed in insect S2 cell lysates after exposure to DTSSP (Fig. 7a, lanes 1–3). These products were not observed when electrophoresis was performed under reducing conditions, indicating that they resulted from the activity of the cross-linking agent (Fig. 7a, lanes 4–6). Studies with HeLa cell extracts showed a similar cross-linking pattern. Although the pan-TLE antibodies decorated a 90–95-kDa multiplet in the absence of DTSSP (Fig. 7b, lane 1) (a slightly slower form of roughly 110 kDa was also visible under nonreducing conditions), a ladder-like pattern of species of slower mobilities was observed after cross-linking (lane 3).

These findings were confirmed by performing nondenaturing gel electrophoresis studies (27, 39). Both Drosophila and mammalian Groucho/TLEs exhibited migrations indicative of a participation in high Mr complexes. For instance, Western blotting analysis of resolved native TLE proteins revealed a heterogeneous immunoreactive profile comprising species of very high apparent Mr (Fig. 8). These results, also confirmed by gel filtration chromatography studies (not shown), indicate that Groucho/TLEs are part of large protein complexes that exist at physiologically significant levels in a variety of tissues and cell lines.

FIG. 7. Detection of oligomeric forms of Groucho/TLEs by cross-linking reactions. a, Drosophila S2 cell lysate was incubated in
in the presence of either no DTSSP (lanes 3 and 6, ~50 µg of protein/lane), 0.2 mM DTSSP (lanes 2 and 5, ~50 µg of protein/lane), or 0.4 mM DTSSP (lanes 1 and 4, ~40 µg of protein/lane). Samples were then subjected to SDS-PAGE on a 4–15% gradient gel in the absence (lanes 1–3) or presence (lanes 4–6) of dithiothreitol, followed by transfer to nitro-
cellulose, and Western blotting with anti-Groucho monoclonal antibod-
arrowheads point to the positions of migration of the major cross-linked products. The positions of migration of Mr standards are indicated. b, HeLa cell lysate was incubated in the presence of either no DTSSP (lane 1, ~60 µg of protein/lane), 0.2 mM DTSSP (lane 2, ~60 µg of protein/lane), or 0.4 mM DTSSP (lane 3, ~60 µg of protein/lane). Samples were then subjected to SDS-PAGE on a 4–15% gradient gel, followed by transfer to nitrocellulose, and Western blotting with pan-
TLE antibodies. Arrowheads point to the positions of migration of the major cross-linked products. The positions of migration of Mr standards are indicated.

tiple promoters. However, virtually nothing is known about the molecular mechanisms that underlie their transcriptional functions. In this paper, we have investigated the possibility that interactions with chromatin components may play a role in the activities of Groucho/TLEs.

Several findings show that histone H3 interacts with native Groucho/TLE protein complexes in a specific fashion and that this interaction is physiologically significant. Groucho/TLEs are present in the chromatin fraction isolated from live mammalian cells. Furthermore, isolation of histone proteins from chromatin results in the co-isolation of TLEs. In agreement with these findings, Groucho/TLEs bind to a histone-Sepharose matrix. This binding does not result from nonspecific electrostatic interactions with highly basic proteins, since it persists even in the presence of very high ionic strength, and Groucho/ TLEs do not interact with an affinity matrix containing lysisozyme, which is also a small, highly basic protein. Additional studies with both native histones and fusion proteins contain-

DISCUSSION

Previous analysis of the properties of Groucho/TLE proteins in both invertebrates and mammals has shown that these molecules are transcriptional repressors that regulate gene expression together with bHLH proteins of the HES family (1, 2, 10, 13, 14). The broad tissue distribution of Groucho/TLEs (13, 34), their ability to repress both basal and activated transcription (12), and the pleiotropic effects of groucho mutations (10, 11, 15) strongly suggest that Groucho/TLE proteins inter-

FIG. 6
To Groucho/TLEs is also suggested by the observation that only a fraction of the native Groucho/TLE proteins used in our binding assays interacted with H3. This situation is analogous to the previous demonstration that only a fraction of total H3 and H4 bound to Tup1p in in vitro binding assays (24). It is also possible that only subpopulations of the native Groucho/TLE proteins present in the cell lysates that we utilized can interact with H3. Specific post-translational modifications, e.g. phosphorylation (35), and/or restricted associations with other proteins may increase the affinity of Groucho/TLEs for H3. This possibility would be consistent with previous results showing that Groucho/TLEs translocate to the nucleus, but only a fraction of them becomes strongly associated with the nuclear compartment, likely the result of association with chromatin (35).

Our studies have also shown that Groucho/TLEs form large multiprotein complexes that can be readily detected under native conditions. Although the composition of these complexes remains to be elucidated, it seems likely that they contain multimeric forms of Groucho/TLEs. This possibility is suggested by the recent demonstration that these proteins can homo- and heterodimerize in vitro through their conserved amino-terminal Q domain (30). Moreover, the electrophoretic mobilities of the major cross-linked products are in agreement with the apparent molecular weights expected for Groucho/TLE multimers. It is also possible that members of the HES family may be part of these molecular complexes. A number of TLE and HES family members are co-expressed in a variety of tissues and cell lines (18, 34, 42, 43), and Groucho/TLEs bind to HES proteins in both Drosophila and mammals (10, 12, 40), suggesting that they may be part of the high M, complexes that we have detected. Finally, it is also possible that these complexes contain other molecules, in addition to Groucho/TLEs and HES proteins. For instance, genetic studies in Drosophila suggest that the product of the runt gene may interact with Hairy and Groucho proteins (11, 44). Interestingly, the Runt protein and its mammalian homologs contain a carboxyl-terminal WRPY motif that closely resembles the WRPW motif that mediates binding of Groucho/TLEs to HES proteins (10, 12, 40, 44), suggesting that Groucho-like and Runt-like proteins may interact in invertebrates and vertebrates.

The demonstration that Groucho/TLE proteins can form oligomeric structures and interact with H3 is consistent with a number of recent studies showing that the recruitment of chromatin components by multimeric transcription factors plays an important role in the regulation of gene expression (24, 38, 41). Studies in yeast have shown that the amino termini of H3 and H4 participate in the transcriptional repression of a number of genes, including the silent mating loci (38), and a cell-specific genes in haploid a and diploid a/α cells (24, 32). H3 and H4 mediate repression at these loci by directly interacting with the Sir3p and Sir4p silent information regulatory proteins and Tup1p, respectively (24, 38). It is thought that the recruitment of oligomeric forms of these proteins to DNA results in the formation of heterochromatic complexes that spread along the chromosome through interactions with the amino termini of H3 and H4. The consequent “coating” of the nucleosomes likely leads to repression by restricting the access of transcription factors to their DNA targets. The present findings suggest that protein complexes containing Groucho/TLEs may regulate transcription through similar mechanisms involving interactions with nucleosomes (although their apparent inability to interact with H4 suggests that Groucho/TLEs may utilize dis-

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2 A. Baratz and S. Stifani, unpublished data.

3 R. Grbavec, R. Lo., Y. Liu, and S. Stifani, manuscript submitted for publication.
tinct mechanisms that do not require interaction with this histone). By virtue of these associations, complexes containing Groucho/TLEs may promote formation of repressive chromatin configurations in the vicinity of their binding sites. In this scenario, they might be able to both establish and maintain transcriptional repression.

Finally, our findings provide additional support to the recent suggestion that activation of the Notch signaling pathway may ultimately result in transcriptional regulation through mechanisms involving alterations of chromatin structure. This possibility was first raised by the demonstration of a physical association between the intracellular domain of the Notch receptor and the EMB-5 protein (45). C. elegans EMB-5 resembles the yeast protein Spt6p, which was implicated in controlling chromatin structure by directly interacting with H3 (46). Genetic analysis suggests that the emb-5 gene functions in the same pathways as lin-12 and glp-1 and that the EMB-5 protein acts downstream of these receptors (45). It is possible that, by activating the expression of genes through the action of fairly general mechanisms involving chromatin reorganization.

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