Mammalian Scratch: A neural-specific Snail family transcriptional repressor

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Members of the Snail family of zinc finger transcription factors are known to play critical roles in neurogenesis in invertebrates, but none of these factors has been linked to vertebrate neuronal differentiation. We report the isolation of a gene encoding a mammalian Snail family member that is restricted to the nervous system. Human and murine Scratch (Scrt) share 81% and 69% identity to *Drosophila* Scrt and the *Caenorhabditis elegans* neuronal antiapoptotic protein, CES-1, respectively, across the five zinc finger domain. Expression of mammalian Scrt is predominantly confined to the brain and spinal cord, appearing in newly differentiating, postmitotic neurons and persisting into postnatal life. Additional expression is seen in the retina and, significantly, in neuroendocrine (NE) cells of the lung. In a parallel fashion, we detect hScrt expression in lung cancers with NE features, especially small cell lung cancer. hScrt shares the capacity of other Snail family members to bind to E-box enhancer motifs, which are targets of basic helix–loop–helix (bHLH) transcription factors. We show that hScrt directly antagonizes the function of heterodimers of the proneural bHLH protein achaete-scute homolog-1 and E12, leading to active transcriptional repression at E-box motifs. Thus, Scrt has the potential to function in newly differentiating, postmitotic neurons and in cancers with NE features by modulating the action of bHLH transcription factors critical for neuronal differentiation.

Small cell lung cancer (SCLC), the most virulent form of human lung cancer, is distinguished from typical non-small cell lung cancer (NSCLC) by the presence of neuroendocrine (NE) characteristics. Understanding the molecular mechanisms underlying NE differentiation may provide insight into the generation and progression of SCLC. Previously, we have shown conservation of a critical neural developmental pathway in SCLC (1, 2). Basic helix–loop–helix (bHLH) transcription factors homologous to the *Drosophila* achaete-scute complex are essential for neuronal commitment and differentiation in a variety of organisms (3). We have found that human achaete-scute homolog-1 (hASH1) is constitutively expressed in SCLC and is required for maintenance of the NE phenotype (1). In addition, transcriptional repression of hASH1 by hairy-enhancer-of-split-1 in SCLC resembles the lateral inhibition mechanism best characterized in *Drosophila* neurogenesis (2). Moreover, expression of a hASH1 transgene contributes to the formation of lung cancers with NE features in mice (4). Using *Drosophila* neural development as a paradigm, we sought to identify genes that may function in SCLC NE differentiation and, by extension, mammalian neural development.

In *Drosophila*, members of the Snail family of transcription factors play critical roles in neural development. Combined deletion of *Snail*, *Escargot*, and *Worniu* results in abnormal central nervous system (CNS) formation (5). The CNS defects can be rescued to varying degrees by transgenic expression of each Snail family member, suggesting redundancy in function. *Drosophila Scrt* (*dScrt*) is expressed in all neuronal precursors; loss of *dScrt* mutants are weak and uncoordinated and exhibit a rough eye phenotype (6). Ectopic expression of *dScrt* results in extra neuron formation. A *C. elegans* gene, *ces-1*, has been identified that encodes a protein most similar to dScrt (7). *ces-1* gain-of-function mutations prevent the apoptosis of specific neuronal populations: the sister cells of serotoninergic neurosecretory motor neurons and I2 sisters.

Of the known vertebrate Snail family members, none exhibits significant nervous system expression. Mammalian Snail inhibits E-cadherin expression, promoting epithelial–mesenchymal transitions (8, 9). Vertebrate Slug appears to regulate cell migration (10, 11) and exhibits antiapoptotic activity in pro-B cells (12). Mouse *Smuc* is expressed in muscle and thymus and may influence muscle differentiation (13). All vertebrate Snail family members are C_2H_2-type zinc finger transcription factors that possess an N-terminal SNAG domain that is not present in invertebrates. In the Gfi1 proto-oncoprotein, the SNAG domain appears to confer repressor and nuclear localization activities (14). Snail family proteins bind an E-box (CAGGTG) motif that is also recognized by bHLH transcription factors.

We now report the isolation of a mammalian gene most similar to *dScrt* and *ces-1*. Mammalian *Scrt* retains the neural-specific expression pattern of *dScrt*. We found that *mScrt* is expressed in newly differentiating neurons of the CNS and in lung neuroepithelial bodies (NEBs), composed of NE cells that closely resemble SCLC. In addition, *hScrt* is specifically expressed in SCLC and other lung cancers with NE characteristics. Furthermore, we provide functional evidence that hScrt may modulate the transcriptional effects of the proneural bHLH protein hASH1 through a common DNA binding motif.

Materials and Methods

Cell Culture. Origins and culture conditions of lung cancer cell lines have been described (1). CV-1 cells were purchased from American Type Culture Collection.

Cloning of Mammalian *Scratch* cDNA. cDNA for reverse transcription (RT)–PCR was made by using RNA from DMS53 cells and amplified by using degenerate primers based on the third (LFSRPWL) and fifth (FALKSYL) zinc fingers of dScrt. Touchdown PCR was performed and the product was used to screen a human fetal brain cDNA library (Stratagene). A partial cDNA clone was identified, termed hScrt8, and used to screen an adult brain cDNA library (Stratagene) to obtain full-length cDNA. To isolate *mScrt*, degenerate primers corresponding to the first and fifth zinc finger of hScrt were used to amplify template cDNA synthesized from mouse brain RNA (Ambion, Austin, TX). The

Abbreviations: bHLH, basic helix–loop–helix; hASH1, human achaete-scute homolog-1; NE, neuroendocrine; NEB, neuroepithelial body; Scrt, Scratch; SCLC, small cell lung cancer; NSCLC, non-SCLC; VZ, ventricular zone; CNS, central nervous system; RT-PCR, reverse transcription–PCR; IVT, *in vitro* transcription/translation.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AY014996 (human Scrt) and AY014997 (mouse Scrt)].

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361-bp product was used to isolate full-length cDNA from a mouse brain cDNA library (Stratagene). All clones were sequenced on both strands with an Applied Biosystems sequencer.

RNA Analysis. A human normal tissue Northern blot (CLONTECH) was probed with a 296-bp *Pst*I fragment of clone hScrt8. First strand cDNA synthesis for RT-PCR was made from poly(A)$^+$ RNA and amplified for 30 cycles. Sequences of primers used for RT-PCR:

S1, 5′-TTCGGCTGCGCGCACTGCGGC
S2, 5′-CTCTTCTTGCAGCGCTTGCAC
H1, 5′-GGGCTCGCCGGTCTCATCCT
H2, 5′-CTCCCCCTCCCAACGCCACT
G1, 5′-CGGAGTCAACGGATTTGGTCGTAT
G2, 5′-AGCCTTCTCCATGGTGGTGAAGAC

Ribonuclease protection assay was performed by using the RPA III kit (Ambion) with 2 μg of poly(A)$^+$ RNA. An hScrt probe yielded a 359-bp protected fragment (nucleotides 924-1282; GenBank accession no. AY014996). A GAPDH probe produced a 180-bp protected fragment (1).

***In Situ* Hybridization and Immunohistochemistry.** C57BL/6 and BALB/c embryos were fixed in 4% paraformaldehyde, infused with 20% sucrose, and cryosectioned at 7 μm. Sections were postfixed in 4% paraformaldehyde, treated with proteinase K 1 μg/ml in TE for 20 min, acetylated with acetic anhydride/triethanolamine, and blocked in ISH solution (Dako). Hybridization was performed with an mScrt digoxigenin-labeled RNA probe (nucleotides 1757–2115; GenBank accession no. AY014997) in ISH solution (Dako) at 55°C followed by washes and detection with a Genpoint kit (Dako) and NBT/BCIP (Pierce). For BrdUrd staining, pregnant mice were injected as described (15). Immunostaining for BrdUrd, calcitonin gene-related peptide (CGRP), and Tuj1 was done as described (4, 15), using diaminobenzidine (BrdUrd, CGRP) and Fast Red (Tuj1) as chromogens.

Expression Constructs. hASH1 and HA-tagged hScrt were generated by PCR and ligated into pcDNA3.1 (Invitrogen) or pBind (Promega). A four-E-box luciferase reporter (E_4-TK-LUC) was made by ligating the herpes simplex virus thymidine kinase (HSV-TK) promoter from pRL-TK (Promega) into pGL3-Basic (Promega) and inserting this oligonucleotide annealed to its complement:

E1, 5′-CCAGGACAGGTGCTGAACAGGTGCTGAAACAGGTGCTGAACAGGTGCTGCAAG

pGL3-Control Vector containing the SV40 promoter was obtained from Promega. Human E12, a gift from G. Kato (Johns Hopkins University School of Medicine, Baltimore; ref. 16), was subcloned into pcDNA3.1. GAL4-MeCP2 has been described (17). G_5TKLUC was kindly provided by C. Dang (Johns Hopkins University School of Medicine, Baltimore; ref. 18). All constructs were sequenced.

Electrophoretic Mobility Shift Assay. The oligonucleotides CCAGGCG*ACAGGTG*CTGCAAG (wild type) and CCAGGCG*TTAGGTA*CTGCAAG (mutant) were end-labeled with [γ-^{32}P]ATP and incubated in 10 μl, containing 4 μl *in vitro* transcription/translation (IVT) protein (TNT; Promega), 100,000 cpm probe, and 1 μg poly(dI-dC) in binding buffer consisting of 5 mM Tris·HCl (pH 7.5), 50 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, and 5% glycerol. Reactions performed on ice for 20 min were analyzed on a 6% nondenaturing polyacrylamide gel. For competitions, up to 2,000-fold excess unlabeled probes were added to binding reactions. Similar amounts of hScrt, hASH1, and E12 IVT protein production were confirmed by incorporation of [^{35}S]methionine.

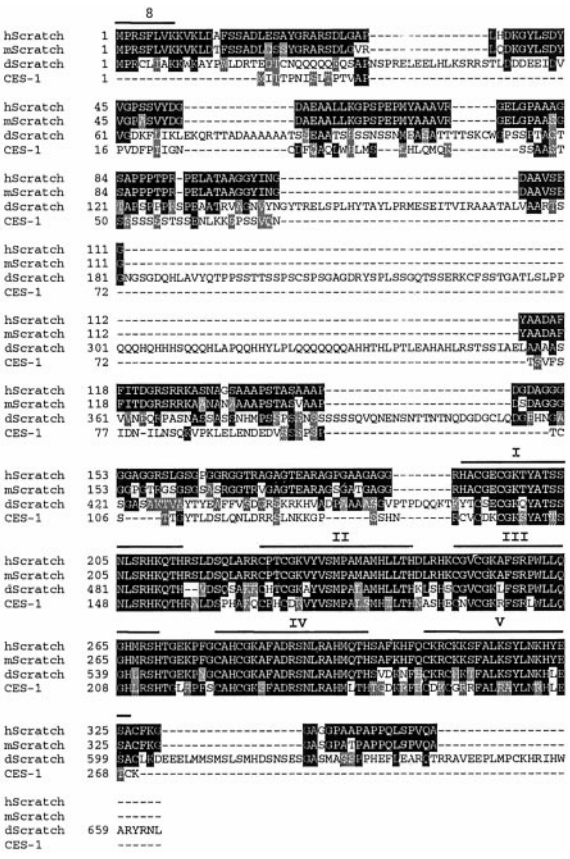

Fig. 1. Comparison of Scratch proteins. Deduced amino acids of hScrt and mScrt were aligned with dScrt (GenBank accession no. AAA91035) and CES-1 (GenBank accession no. AAF01678). The conserved eight amino acids present in vertebrate Snail members is indicated by the number 8 above the black bar. Positions of zinc fingers are indicated by Roman numerals above the black bars. Multiple sequence alignments were performed by using CLUSTALW 1.8. Identical residues are blackened. Similar residues are shaded gray.

Reporter Assays. NCI-H1299 cells (10^5) were seeded in 6-well plates 2 days before transfection. Expression plasmids were cotransfected with luciferase reporter (0.5 μg) and CMV-β-galactosidase (0.5 μg) by using Lipofectamine Plus (Life Technologies, Rockville, MD). Total DNA was kept at 5 μg with pcDNA3.1. After 48 h, luciferase activity was normalized to β-galactosidase activity (Promega). CV-1 cells were seeded at 2×10^5 cells per well 1 day before transfection. GAL4-fusion constructs (1 μg) were cotransfected with G_5TKLUC (0.5 μg) and CMV-β-galactosidase (0.5 μg) and analyzed after 24 h. Experiments were performed in duplicate or triplicate two times.

Results

Identification of Mammalian Scratch. Using a degenerate RT-PCR strategy, we identified a PCR product from the SCLC cell line DMS53 most similar to *dScrt*. We ultimately isolated a clone designated *hScrt* with an ORF encoding a predicted peptide of 348 amino acids. The first methionine in *hScrt* was preceded by an upstream termination codon, and the most N-terminal eight amino acids were identical to other vertebrate Snail family proteins. We also obtained a single *mScrt* clone, encoding a predicted protein with 92% identity to hScrt overall.

Across the five zinc fingers, hScrt shares 81% identity with dScrt and 69% identity with CES-1 (Fig. 1). However, in this region, hScrt is less similar to human Slug (59% identity) and Snail (62% identity), the latter having only four zinc fingers. An N-terminal region of conserved amino acids resembling the

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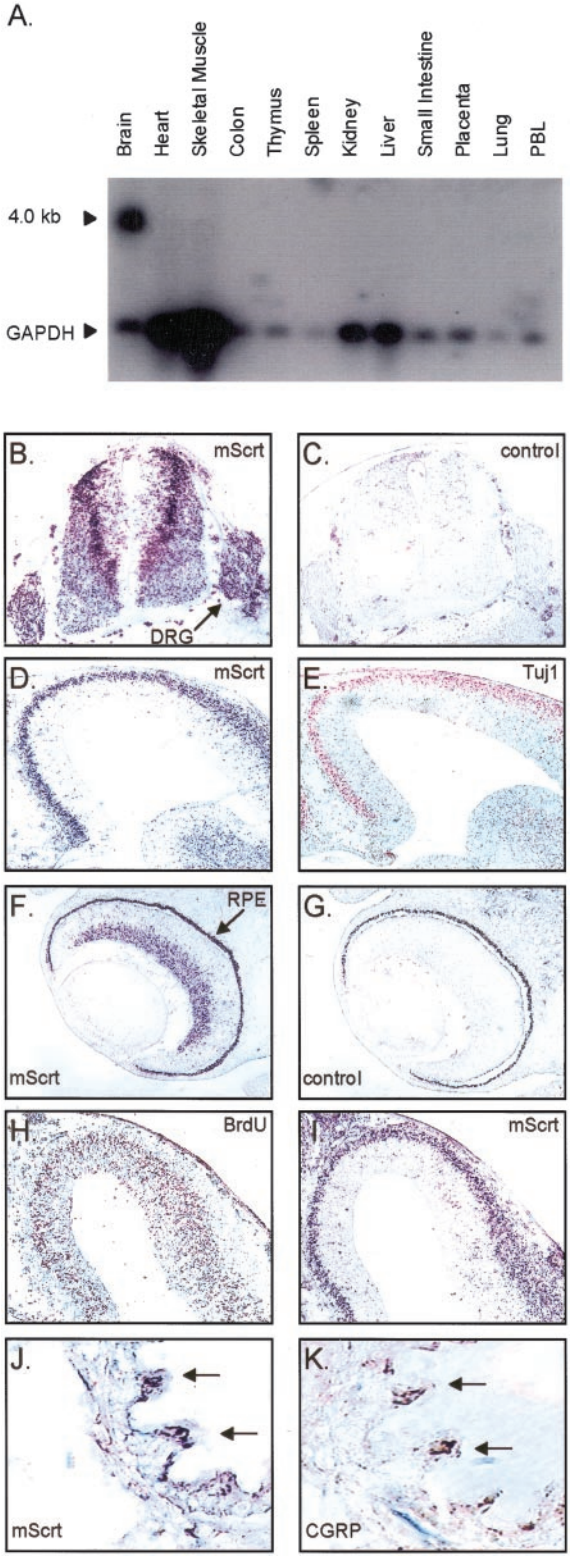

Fig. 2. Neural-specific expression of mammalian *Scrt*. (*A*) Northern blot of hScrt in adult human tissues. (*B–K*) Shown are paired adjacent transverse sections of embryonic mouse tissues.(*B*) In the cervical spinal cord at E11.5, *mScrt* expression detected by *in situ* hybridization was seen throughout the cord, excluding the VZ, and in the dorsal root ganglia (DRG). (*C*) No signal was detected with the sense control probe. (*D*) At E14.5, *mScrt* labeling was present adjacent to the VZ in the telencephalon. (*E*) The adjacent section shows positive staining for Tuj1. (*F*) mScrt expression was seen in the inner nuclear layer of the retina at E13.5. RPE, retinal pigment epithelium. (*G*) Sense control probe showed no labeling. (*H*) Proliferating cells were labeled with BrdUrd and detected in the VZ and SVZ of the telencephalon. (*I*) Mutual exclusivity between *mScrt* expression and BrdUrd staining is shown. (*J*) Clusters of cells expressing *mScrt* (arrows) were seen protruding into lung airway lumen at E17.5. (*K*) Positive immunohistochemical staining for CGRP (arrows) was found in an adjacent section.

SNAG domain is present in hScrt, but absent in dScrt and CES-1 (14). Although hScrt shares the eight amino terminal amino acids with other SNAG-domain-containing proteins, the proximal twenty amino acids are otherwise highly divergent.

A BLAST search using the *hScrt* sequence revealed matching genomic sequence from chromosome 8q24.3 (GenBank accession no. AC006263). A second clone designated *hScrtP* was isolated that matched genomic DNA on chromosome 20 (GenBank accession no. AL121758).

Mammalian *Scratch* Expression Is Restricted to Neural Tissues. By Northern blot, a 4.0-kb hScrt transcript was detected exclusively in adult human brain but not other tissues (Fig. 2*A*). To further characterize *Scrt* expression, *in situ* hybridization was performed on mouse embryos. When using an mScrt-specific probe, labeling was first detected in the spinal cord and dorsal root ganglia at E11.5 (Fig. 2*B*). mScrt was detected throughout the spinal cord except in the ventricular zone (VZ) surrounding the central canal, which contains proliferating neurons. At E12.5, mScrt was seen in the telencephalon, and by E14.5 a distinct laminar pattern of expression was seen in regions adjacent to the VZ (Fig. 2*D*). Diffuse expression of *mScrt* was present in the fore-, mid-, and hindbrain at E13.5, with absence from periventricular regions (data not shown). In the developing eye, *mScrt* expression was detected in the inner nuclear layer of the retina beginning at E13.5 (Fig. 2 *F* and *G*). This laminar domain of *mScrt* expression was absent from the VZ of the retina, which resides next to the retinal pigment epithelium and is comprised mostly of mitotically active cells (19). Throughout the CNS, *mScrt* expression persisted into postnatal life.

In the telencephalon, proliferating neural precursors reside within the VZ and subventricular zone (SVZ). With the onset of differentiation, these cells cease dividing and migrate outward. On serial sections, a laminar domain of *mScrt* expression coincided with that of Tuj1, a marker of early neuronal differentiation (15, 20), adjacent to the VZ (Fig. 2*E*). To confirm the absence of *mScrt* expression in proliferating neural precursors, pregnant mice were injected with BrdUrd before embryo harvest. Proliferating cells detected by BrdUrd uptake were limited to the VZ and SVZ of the telencephalon (Fig. 2*H*). Conversely, a domain of *mScrt* expression was complementary to that of BrdUrd (Fig. 2*I*). These data are consistent with *mScrt* expression in early differentiating neural precursors.

Because a small population of NE cells within the lung, the NEBs, may stem from cellular precursors of SCLC (21) and shares many NE features with this cancer (1), we examined embryonic lung for *mScrt* expression. At E17.5, distinct clusters of mScrt-reactive cells were detected in the epithelium of medium-sized airways, especially at airway branch points, a distribution characteristic for NEBs (Fig. 2*J*). Adjacent sections immunostained for calcitonin gene-related peptide (CGRP), a marker for NEBs, revealed coinciding expression of *mScrt* (Fig. 2*K*). We conclude that *mScrt* is expressed in lung NEBs, a pattern similar to hASH1 expression in fetal lung (1).

Specific Expression of *hScratch* in Lung Cancers with NE Features. Given the expression of *mScrt* in lung NEBs with their potential precursor relationship to SCLC, we were interested in whether hScrt correlated with the NE phenotype in lung cancers. By RT-PCR, hScrt transcripts were detected in cell lines of classic SCLC, bronchopulmonary carcinoid, and non-small cell lung

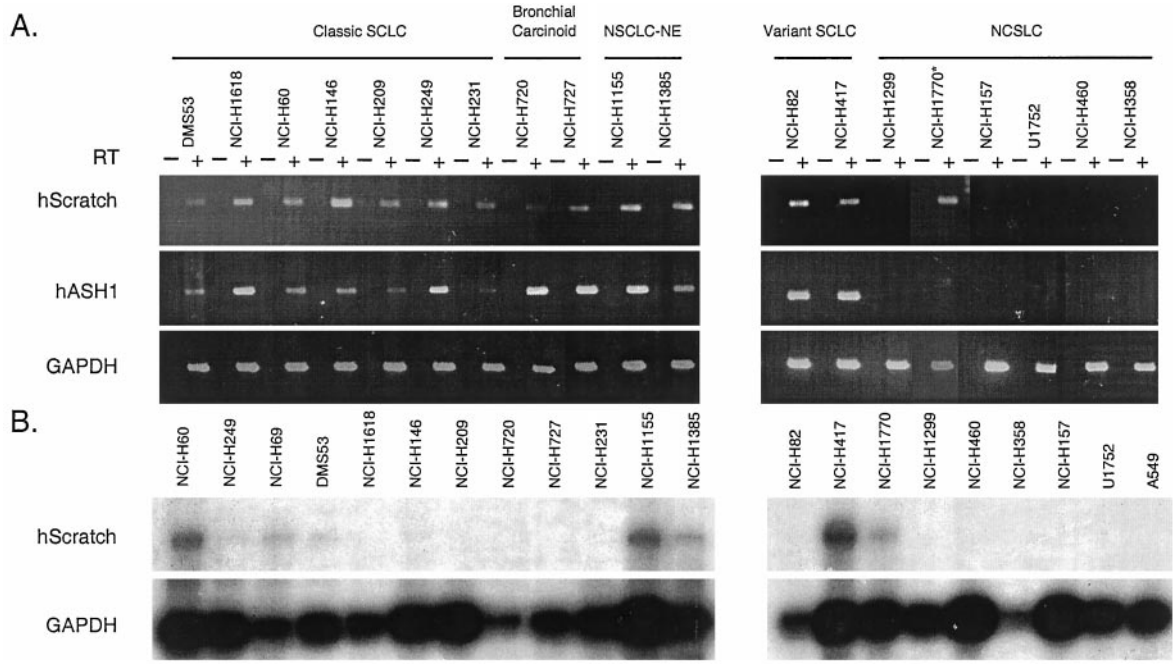

Fig. 3. Specific expression of *hScrt* in lung cancers with NE features. (*A*) RT-PCR analysis of *hScrt* and *hASH1* expression in lung cancer cell lines. First strand cDNA was prepared with (+) or without (−) reverse transcriptase. RNA quality was assessed by amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). *, NCI-H1770 is a NSCLC cell line obtained from a tumor originally described to exhibit NE histology. (*B*) RPA of *hScrt* expression in lung cancer cell lines. A GAPDH probe was used to assess RNA quality and quantity.

cancer with NE features (NSCLC-NE), all of which exhibit a characteristic NE phenotype (Fig. 3*A*). In addition, *hScrt* was expressed in variant SCLC lines, which have lost some NE characteristics, and a NSCLC cell line, NCI-H1770. NCI-H1770 was established from a tumor with NE histology, but lacks NE features in cell culture (http://www.atcc.org). Conversely, hScrt transcripts were not detected in NSCLC cell lines that lack NE characteristics. In 19 of 20 lung cancer cell lines, *hScrt* expression was concordant with that of *hASH1*, which appears to be essential for the NE phenotype in lung cancers (1).

Using RPA, we found significant expression limited to a subset of lung cancers with NE features (Fig. 3*B*). Low levels of *hScrt* expression were generally detected in cell lines that are known to express *hASH1* highly, such as classic SCLC and bronchopulmonary carcinoid cell lines (1); two cell lines in which hASH1 mRNA cannot be detected by Northern blot, NCI-H417 and NCI-H1770, express high levels of *hScrt*. As in normal tissues, *hScrt* expression is limited to lung cancers with neural characteristics. However, the relative abundance of hScrt mRNA does not seem to correlate with that of hASH1 in lung cancer cell lines.

hScratch Binds to a Consensus E-Box Motif. Because Snail family proteins share DNA binding specificity to the hepatanucleotide sequence ACAGGTG (7), we investigated whether hScrt exhibits similar binding properties. hScrt protein was produced by IVT. In an electrophoretic mobility gel shift assay, hScrt bound to an oligonucleotide containing the wild-type Snail family consensus binding site (Fig. 4). Specificity of binding was supported by absence of a shift when a mutant probe containing three base changes was used. Unlabeled wild-type oligonucleotide competed with labeled probe for binding in a dose-dependent manner. This competition was specific because unlabeled mutant oligonucleotide did not compete for binding, even at 2,000-fold molar excess to labeled probe.

Because the Snail consensus binding sequence contains the E-box motif CAGGTG recognized by bHLH transcription factors (22) and functional interactions between Snail and bHLH proteins have been reported (13, 23), we tested the ability of hASH1 to bind to the same sequence. When either hASH1 or E12, a heterodimerizing partner for bHLH proteins, was used alone, no or only a modest shift of the wild-type Snail probe was seen, respectively (data not shown). However, a mixture containing hASH1 and E12 produced a distinct shifted band of the wild-type probe (Fig. 4). No shift was detected with the mutant probe. Competition for binding of the wild-type probe was observed with an excess of unlabeled wild-type, but not mutant, oligonucleotide in a dose-dependent manner. Therefore, hASH1-E12 heterodimers can bind specifically to similar sequences as hScrt.

hScratch Represses hASH1-E12 Transactivation of a Heterologous Reporter. Because hScrt shares DNA binding specificity with hASH1-E12 and both hScrt and hASH1 are expressed in similar normal tissues and lung cancer cell lines, we studied whether they could interact functionally. Before testing the actions of various hScrt constructs in this context, we detected their subcellular expression by confocal microscopy. Wild-type hScrt is expressed in the nucleus (e.g., see Fig. 7, which is published as supplemental data on the PNAS web site, www.pnas.org). An hScrt mutant (Δ Zinc Fingers, amino acids 1–190) containing the N-terminal half but lacking all five zinc fingers was expressed diffusely throughout the nucleus and cytoplasm. However, a mutant containing only the zinc finger domain of hScrt (Δ N terminus, amino acids 173–348) localized to the nucleus indistinguishably from the wild-type protein. Therefore, a critical nuclear localization signal is present within the zinc finger region of hScrt, and the N-terminal SNAG-like domain is dispensable for nuclear localization.

Using a luciferase reporter that contains four E-box motifs, we found that hASH1-E12 could potently activate the reporter in a NSCLC cell line, NCI-H1299, which does not express hASH1 or hScrt (Fig. 3*A*). Wild-type hScrt inhibited hASH1-E12 transactivation of the reporter in transient transfection assays to 32% of the value attained by empty vector (Fig. 5). In the absence of

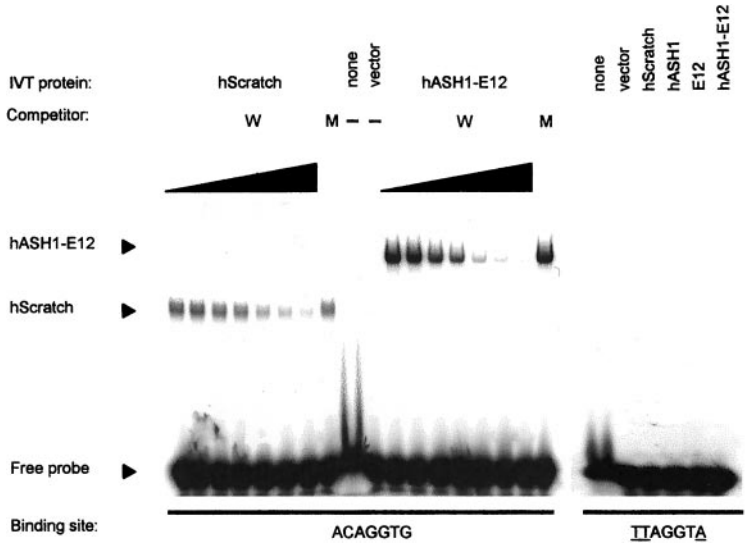

Fig. 4. hScrt and hASH1-E12 bind specifically to a conserved E-box sequence. hScrt and hASH1-E12 protein produced by IVT were used in an EMSA. The wild type probe contains an optimal Snail binding site (ACAGGTG), whereas the mutant probe contains three nucleotide changes (*TT*AGGT*A*). Excess of unlabeled wild type probe (W), amounts indicated by the inclining wedge (0-, 10-, 50-, 100-, 500-, 1,000-, or 2,000-fold), or 2,000-fold excess mutant probe (M) was used as a competitor.

hASH1-E12, the TK reporter had minimal activity. Repression required both the zinc finger domain and the N-terminal non-zinc finger region of hScrt because constructs containing either region alone were ineffective. Specificity of hScrt repression was demonstrated by the minimal effect on a reporter containing the SV40 promoter. We conclude that hScrt may potentially modulate the actions of hASH1-E12 on common target genes by binding to E-box motifs in promoter regions.

An N-terminal Repressor Domain in hScrt. To determine whether hScrt exhibits active repressor activity, various hScrt domains were fused in-frame with the GAL4 DNA binding domain and targeted to a reporter containing five GAL4 binding sites upstream of the TK promoter (Fig. 6*A*). Expression of all constructs was verified by Western blot (data not shown). GAL4-fusion constructs containing full-length hScrt (GAL4-

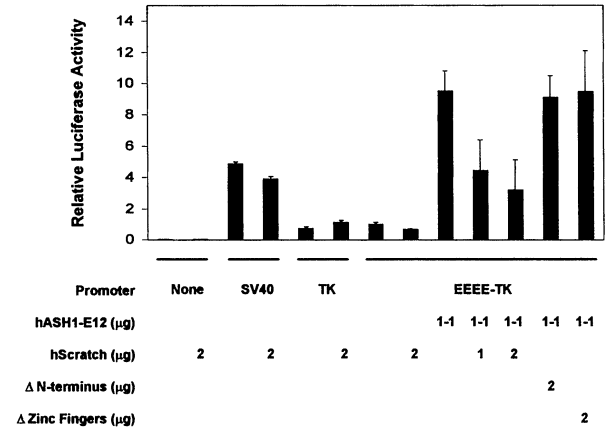

Fig. 5. hScratch is a nuclear protein that can repress bHLH function. In NCI-H1299 cells, cotransfection of hASH1 and E12 expression vectors activated a luciferase reporter downstream of four E-box motifs. Increasing amounts of full-length hScrt inhibited hASH1-E12 transactivation of the reporter to 32% of control levels ($P < 0.001$). No effect on reporter activity was seen with the mutant construct lacking residues N-terminal to the five zinc fingers (Δ N terminus) or a mutant hScrt lacking the five zinc fingers (Δ Zinc Fingers) ($P > 0.05$). Luciferase activity of cells cotransfected without hASH1-E12 was set at 1.0. The data represent averages and standard errors of values obtained from two different experiments performed in duplicate or triplicate. Statistical analysis of a representative experiment was performed by one-way ANOVA followed by the Bonferroni test.

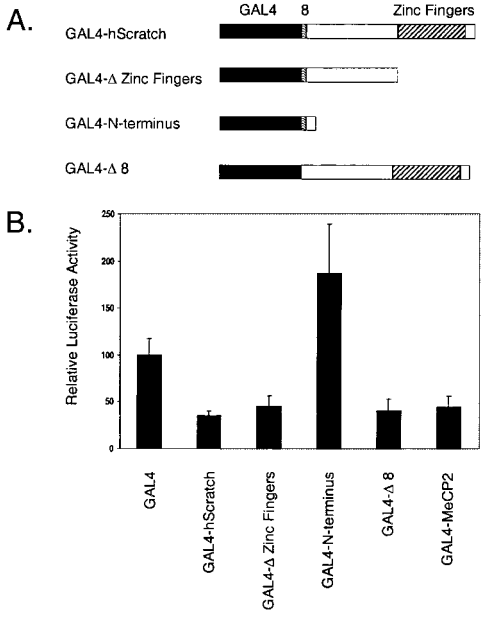

Fig. 6. Repressor domain of hScrt resides in the non-zinc finger region. Various hScrt constructs were fused in-frame to the GAL4 DNA-binding domain (DBD) and targeted to a luciferase reporter containing five GAL4 binding sites upstream of the thymidine kinase promoter in CV-1 cells. (*A*) Diagram of the constructs used. GAL4 DBD is blackened; number 8 represents the first eight amino acid residues conserved among vertebrate Snail members; zinc finger domain is hatched. (*B*) Full-length hScrt (GAL4-hScratch) repressed reporter activity to 35% of control levels, which is comparable to MeCP2, a positive control for repression. hScrt mutants lacking the five zinc fingers (GAL4-Δ Zinc Fingers) or N-terminal eight amino acids (GAL4-Δ 8) also inhibited reporter activity. No repression was seen by the N-terminal 40 amino acids of hScrt (GAL4-N-terminus). Luciferase activity from cells cotransfected with only GAL4 DBD was set at 100%. The data represent averages and standard errors of values obtained from two different experiments performed in triplicate.

hScratch), hScrt lacking all five zinc fingers (GAL4-Δ Zinc Fingers), and hScrt minus the first eight amino acids of the SNAG domain (GAL4-Δ 8) all repressed reporter activity as effectively as a construct containing the known repressor MeCP2 (Fig. 6*B*). In contrast, when the first 40 amino acids of hScrt were expressed as a GAL4-fusion protein (GAL4-N terminus), no repression was seen. Therefore, hScrt repressor activity resides in the non-zinc finger region and is not dependent on the conserved N-terminal eight amino acids of the SNAG domain, which has been reported to be important for repressor activity in Snail family and other zinc finger transcription factors (8, 14, 24).

Discussion

Drosophila neurogenesis has served as an invaluable model for the discovery of genes that regulate vertebrate neural development and NE differentiation in human cancers, including SCLC. In this study, we have identified a mammalian gene that encodes a Snail family zinc finger transcription factor most similar to *Drosophila* Scrt and *C. elegans* CES-1. Mammalian *Scrt* is the first vertebrate Snail family member known to be expressed highly and specifically in neural tissues. Outside the nervous system and lung NE cells, we did not detect significant levels of *Scrt* expression. In flies, *dScrt* is expressed in dividing neuronal precursors and persists in postmitotic neurons (6). In contrast, *mScrt* transcripts were not detected in regions known to contain proliferating neurons—the VZ of the telencephalon, spinal cord, and retina. Rather, prominent domains of *mScrt* expression were seen adjacent to the VZ in these tissues. Specifically, we detected mScrt expression in a distribution similar to BrdUrd−/Tuj1+ neurons in the telencephalon. Thus, *mScrt* expression in the CNS appears to be confined to newly differentiating, postmitotic neurons, suggesting a potential role in neuronal differentiation.

NEBs are most prominent in late fetal lung development and share a common NE phenotype to SCLC, potentially reflecting a common precursor cell population (21). We have found that *mScrt* is expressed in NEBs, whereas *hScrt* is expressed specifically in SCLC and other lung cancer cell lines with NE features. hASH1 exhibits a similar expression pattern and is essential for NE differentiation in the lung and SCLC (1). In a mouse model, hASH1 cooperates with SV40 large T antigen to promote highly aggressive NE lung tumors (4). Thus, dysregulation of critical neural developmental programs in lung cancer cells may contribute to tumorigenesis.

Like other Snail family members, hScrt is a nuclear protein that functions as a transcriptional repressor. Repressor activity resides within the N-terminal non-zinc finger region. However, the conserved N-terminal eight amino acids that hScrt shares with other SNAG domain containing proteins are not required for repressor function. This observation is in contrast to other reports ascribing important repressor function to the N-terminal twenty amino acids of the SNAG domain of vertebrate Snail, Slug, Smuc, and Gfi1 proteins (8, 13, 14, 24). Though modest nuclear targeting activity has been described for this N-terminal region of Gfi1, effective nuclear localization depends on the full-length protein, including the zinc finger domain (14). We have found that the N-terminal non-zinc finger region of hScrt is not sufficient for proper expression in the nucleus; conversely, information within the zinc finger domain is necessary and sufficient to effect nuclear localization.

During vertebrate development, *Mash1* and *Neurogenin1* and *-2* are transiently expressed in proliferating neurons of the nervous system and exhibit determination and differentiation functions (15, 25–27). We have shown that *mScrt* is expressed in an adjacent layer characteristic of newly differentiating neurons and that hScrt can repress hASH1-E12-mediated reporter transactivation. Taken together, Scrt may modulate the effects of ASH1-E12 on common target genes, thereby potentially affecting neuronal determination and differentiation. Because Scrt expression is more widespread than Mash1, it is possible that Scrt may interact functionally with other bHLH transcription factors, such as the Neurogenins. Others have reported functional interactions between Escargot and Scute-Daughterless (23), as well as Smuc and MyoD-E12 *in vitro* (13). Thus, interactions between Snail family and bHLH factors may be a common theme in development. Further studies of Scrt should provide insight into programs of neural differentiation that appear conserved in normal and neoplastic tissues.

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