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Conservation of the *Drosophila* lateral inhibition pathway in human lung cancer: A hairy-related protein (HES-1) directly represses achaete-scute homolog-1 expression

(neuroendocrine/hairy-enhancer-of-split-1/transcriptional regulation/small cell lung cancer/basic helix–loop–helix)

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ABSTRACT **The achaete-scute genes encode essential transcription factors in normal *Drosophila* and vertebrate nervous system development. Human achaete-scute homolog-1 (hASH1) is constitutively expressed in a human lung cancer with neuroendocrine (NE) features, small cell lung cancer (SCLC), and is essential for development of the normal pulmonary NE cells that most resemble this neoplasm. Mechanisms regulating achaete-scute homolog expression outside of *Drosophila* are presently unclear, either in the context of the developing nervous system or in normal or neoplastic cells with NE features. We now provide evidence that the protein hairy-enhancer-of-split-1 (HES-1) acts in a similar manner as its *Drosophila* homolog, hairy, to transcriptionally repress achaete-scute expression. HES-1 protein is detected at abundant levels in most non-NE human lung cancer cell lines which lack hASH1 but is virtually absent in hASH1-expressing lung cancer cells. Moreover, induction of HES-1 in a SCLC cell line down-regulates endogenous hASH1 gene expression. The repressive effect of HES-1 is directly mediated by binding of the protein to a class C site in the hASH1 promoter. Thus, a key part of the process that determines neural fate in *Drosophila* is conserved in human lung cancer cells. Furthermore, modulation of this pathway may underlie the constitutive hASH1 expression seen in NE tumors such as SCLC, the most virulent human lung cancer.**

Basic helix–loop–helix transcription factors homologous to the *Drosophila* achaete-scute complex (AS-C) are critical to nervous system development in multiple organisms (1–9). Specifically, mouse transgenic knockout studies indicate that transient expression of achaete-scute homolog-1 (termed MASH1) in neural precursor cells is necessary for establishment of a subset of autonomic, olfactory, and enteric neurons, and adrenal chromaffin cells (1, 6). Recently, we have shown that human achaete-scute homolog-1 (hASH1) is constitutively expressed in an important tumor, small cell lung cancer (SCLC) (10), which accounts for 25% of over 150,000 new cases of lung cancer each year. In this extremely virulent and metastatic cancer where the 5-year survival is less than 5%, hASH1 expression appears tightly linked to the neuroendocrine (NE) properties that characterize SCLC (11–13). The possibility that this transcription factor could be integral to the process of NE differentiation is underscored by our recent finding that pulmonary NE cells, the normal bronchial cells that most resemble the SCLC phenotype, fail to develop in transgenic mice homozygous for MASH1 deletion (13). Furthermore, depletion of hASH1 in classic SCLC lines results in a significant reduction of NE marker expression (13). These data indicate that delineating the molecular events which lead to constitutive hASH1 expression may prove essential for understanding the establishment of the NE phenotype in SCLC.

To date, there have been no studies that detail the regulation of achaete-scute homolog-1 expression. However, critical pathways controlling *Drosophila* AS-C genes provide important clues to potential regulatory mechanisms in mammalian homologs (14, 15). During development of peripheral sensory organs in *Drosophila*, neural fate is specified by focal restriction of AS-C gene expression as a consequence of direct transcriptional repression by hairy (16, 17) and other enhancer-of-split family members (18). In turn, these genes respond to signals transmitted by other factors in conjunction with regulation of Notch (19–23), in a process termed lateral inhibition. Hairy and a mammalian homolog, hairy-enhancer-of-split-1 (HES-1) (24, 25), have been previously shown to act as transcriptional repressors by binding to a class C site (CACGCG) in the *Drosophila* achaete promoter (16, 17) or similar sequences termed N-boxes (CACNAG) in the rodent HES-1 promoter (26, 27), respectively. Although *Drosophila* hairy has been shown to directly interact with the achaete promoter to inhibit transcription (16, 17), corresponding interactions in mammals have not yet been explored.

In the present study, we investigated the possibility that alterations in HES-1 activity could be implicated in the constitutive hASH1 expression seen in human SCLC. We show that human HES-1 is found at abundant levels in several non-SCLC (NSCLC) lines which do not express hASH1 but is virtually absent in hASH1-expressing SCLC lines. Introduction of HES-1 into SCLC cells results in a marked reduction of hASH1 mRNA through a process that requires direct binding of HES-1 to a class C site in the hASH1 promoter.

MATERIALS AND METHODS

Cell Culture. Origins and culture conditions of the NSCLC cell lines (NCI-H157, NCI-H358, U1752, NCI-H1299, NCI-H1385, and NCI-H1770) and the SCLC cell lines (DMS53, NCI-H209, NCI-H249, and NCI-H1618) (28–32), as well as the colon cancer cell line RKO (33), have been described.

Western Blot Analysis. Western blot analysis of HES-1 was performed as described (34), using total protein from 1×10^6 cells, with affinity-purified polyclonal rabbit antisera raised against a C-terminal peptide common to human and rat HES-1 (amino acid sequence, SGTSVGPNAVSPSSGSSLTADSMWRPWRNC). Equivalent protein loading was verified by staining with Fast Green. A rat HES-1 cDNA construct,

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Abbreviations: AS-C, achaete-scute complex; MASH1, mammalian achaete-scute homolog-1; hASH1, human achaete-scute homolog-1; SCLC, small cell lung cancer; NSCLC, non-SCLC; NE, neuroendocrine; HES-1, hairy-enhancer-of-split-1; 2NB, sequence containing the two N-box HES-1 binding sites; WT, wild type.

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pHES-1 (24), was used for *in vitro* translation using a transcription and translation (TNT) kit (Promega).

Reporter Plasmid Construction. We have cloned 16 kb of 5′ flanking hASH1 genomic DNA and localized the transcription start site by primer extension, RNase protection, and reverse transcription–PCR (33). Fragments of this region (−7,900/+37, −3,600/+37, −2,500/+37, −611/+37, −308/+37, and −234/+37) were cloned into the luciferase reporter gene in pGL2 (Promega), and sequences within the constructs have been numbered in relation to the major transcription initiation site (33). A hASH1 promoter construct, −282/+37, containing a wild-type (WT) class C site was made using a PCR approach. A similar construct containing a mutated class C site at position −258, −282/+37M (CACGCA changed to AGTCAA), was also constructed by PCR. All plasmids were verified by DNA sequencing.

Transient Transfections. DMS53 and NCI-H157 cells were seeded at 0.2–0.3 × 10^6 cells per well 3 days before transfection. hASH1 promoter constructs (1 μg) were cotransfected into cells with CMV–β-galactosidase (0.5 μg), and 2 μg of either pHES-1 or pRc/CMv using Lipofectamine (GIBCO/BRL). After 48 hr, cells were assayed for luciferase activity using a Monolight 2010 Luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI). Luciferase activity was expressed relative to β-galactosidase activity (Promega) in a minimum of three experiments performed in duplicate. Percent activity was reported relative to the construct −234/+37.

Gel Mobility-Shift Assays. Nuclear extracts were prepared according to Dignam *et al.* (35). Extracts were dialyzed into 1 × HAC (25 mM Hepes, pH 7.5/50 mM KCl/0.1 mM EDTA/20% glycerol/0.5 mM DTT/50 μg/ml phenylmethylsulfonyl fluoride). For nuclear extracts from DMS53 cells transfected with a tetracycline-inducible HES-1 or reverse-HES-1 vector (see next paragraph), cells were treated for 96 hr in the presence or absence of 1 μg/ml doxycycline before harvesting. Annealed oligonucleotides were filled in with Klenow DNA polymerase and [α-^{32}P]dCTP. Plus-strand oligonucleotide sequences are shown in Fig. 5*A*. Labeled probe (1 ng) was bound to 4 μg of nuclear extract at 22°C in 1 × HAC and 1.5 μg of poly(dI–dC) for 15 min, with competitor oligonucleotides added just before the labeled probe. For mobility-shift reactions in the presence of antibody, nuclear extracts were incubated with HES-1 antibody, polyclonal rabbit antisera raised against glutathione *S*-transferase (GST)–hASH1, or rabbit pre-immune sera for 30 min at 4°C; the labeled probe was added; and the samples were incubated another 15 min at 22°C. Samples were electrophoresed on 5% nondenaturing polyacrylamide gels at 4°C for 2 hr.

Tetracycline-Inducible HES-1 Constructs. A 1.7-kb *Hin*dIII/*Apa*I fragment from pHES-1 (24) containing the coding region of rat HES-1 was subcloned into pBluescript SK(+/−). A 1.7-kb *Xba*I fragment was then excised and subcloned into pUHC10–3-hygromycin in the sense (pUH-HES-1) and antisense (pUH-rev-HES-1) directions. All constructs were confirmed by DNA sequencing. DMS53 cells were transfected with pUHD172–1 (36) using Lipofectamine and selected in 0.4 μg/ml G418. Fifteen G418-resistant DMS53/172–1 clones were screened for tetracycline-dependent inducibility of reporter genes by the ability to transactivate the luciferase reporter in pUHC13–3 (36). DMS53/172–1 clone #7, which reproducibly induced the luciferase gene by 100-fold, was transfected with pUH-HES-1, pUH-rev-HES-1, or pUHC10–3-hygromycin using Lipofectamine. Transfected cells were selected in 0.4 μg/ml hygromycin. Resistant DMS53-HES-1, DMS53-rev-HES-1, and DMS53-vector clones were treated with 1 μg/ml doxycycline for 8, 48, and 146 hr, and screened for HES-1 protein by Western blot analysis. Total RNA (20 μg) was subjected to Northern blot analysis using riboprobes for hASH1 (base pairs 1350–1635) and GAPDH as described (37). Hybridization signals were quantified by use of a PhosphorImager (Molecular Dynamics).

RESULTS

HES-1 Expression in Lung Cancer Lines. During neural development in *Drosophila*, hairy plays a critical role in restricting the domains of AS-C gene transcription (16, 17). To explore the possibility that a hairy homolog could play an analogous role in mammalian cells, we examined HES-1 protein expression patterns in a panel of lung cancer cell lines previously characterized for hASH1 expression. We used affinity-purified antisera for HES-1 that recognized a 32-kDa protein from a HES-1 *in vitro* translation product (Fig. 1*B*). A similar 32-kDa protein (Fig. 1*A*)

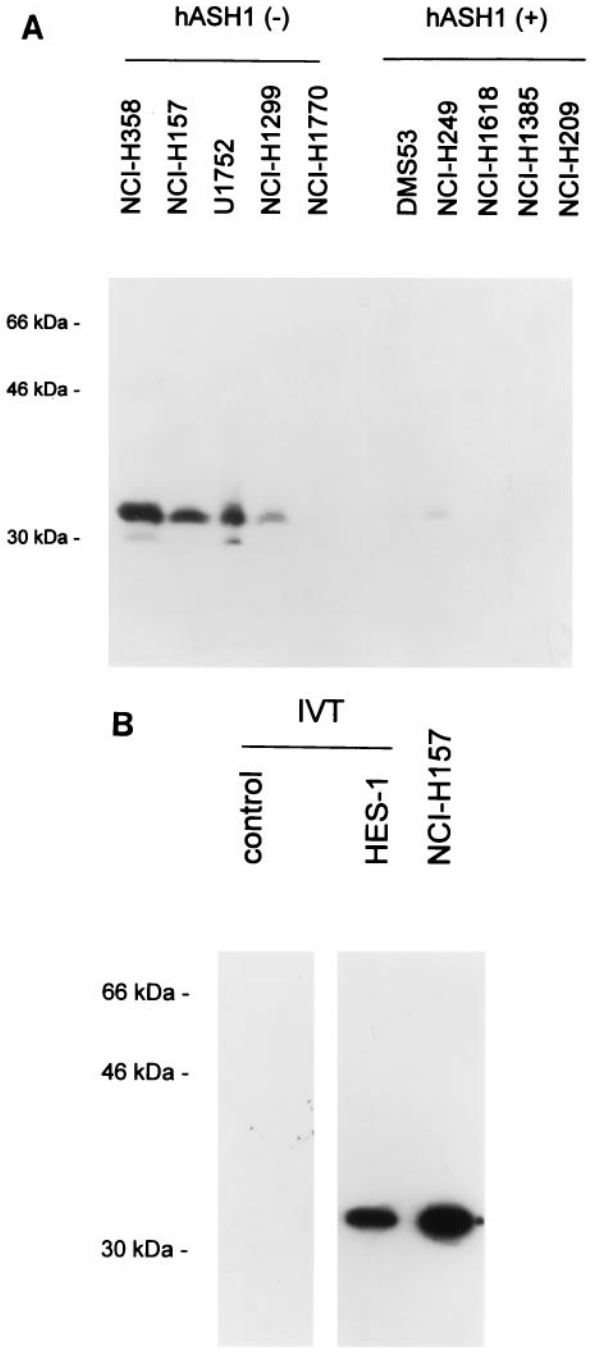

FIG. 1. (*A*) Western blot analysis of HES-1 protein in lung cancer cell lines, grouped by hASH1 mRNA expression status. Extracts from 1 × 10^6 cells separated by SDS/PAGE were analyzed using polyclonal rabbit antisera raised against a C-terminal peptide common to rat and human HES-1. (*B*) Lysates (5 μl of 50 μl) from *in vitro* translation reactions containing no plasmid (control) or a HES-1 expression construct, as well as an extract from NCI-H157 cells, were analyzed for HES-1 protein by Western blotting as above.

was readily detected in four NSCLC cell lines (NCI-H358, NCI-H157, U1752, and NCI-H1299) that do not express hASH1 mRNA, whereas a fifth NSCLC line was negative (NCI-H1770). In contrast, five of five hASH1-expressing cell lines (SCLC cell lines DMS53, NCI-H249, NCI-H1618, and NCI-H209, and a large cell NE line, NCI-H1385) exhibited absent or trace HES-1 protein. Based on the apparent inverse association of HES-1 and hASH1 expression in lung cancer cell lines, we explored potential interactions of HES-1 with the native hASH1 promoter.

Induction of HES-1 Down-Regulates Transcription of the Native hASH1 Gene. We next tested whether induction of HES-1 in SCLC cells with a NE phenotype could down-regulate the transcription of the constitutively expressed, endogenous hASH1 gene. For this study, we constructed a tetracycline-inducible DMS53-HES-1 cell model. No HES-1 protein was seen in untreated or treated DMS53 cells with an inducible antisense HES-1 construct (DMS53-rev-HES-1) (Fig. 2*A*, lanes 7 and 8) or cells with vector alone (DMS53-vector) (lanes 5 and 6) at 48 hr, or in untreated DMS53 cells containing the inducible sense construct (DMS53-HES-1) at 48 and 146 hr (lanes 1 and 3). However, after doxycycline treatment, the 32-kDa HES-1 protein was markedly induced in the DMS53 cells with the inducible HES-1 gene (DMS53-HES-1) at 48 hr (lane 2) and 146 hr (lane 4). This induction of HES-1 resulted in a progressive reduction of hASH1 mRNA which was first detected at 8 hr, reached 60% at 48 hr, and exceeded 75% by 146 hr (Fig. 2*B*). This reduction in hASH1 was not seen in DMS53-vector or DMS53-rev-HES-1 cells treated with doxycycline. Therefore, insertion of HES-1 into DMS53 cells can repress expression of the endogenous hASH1 gene.

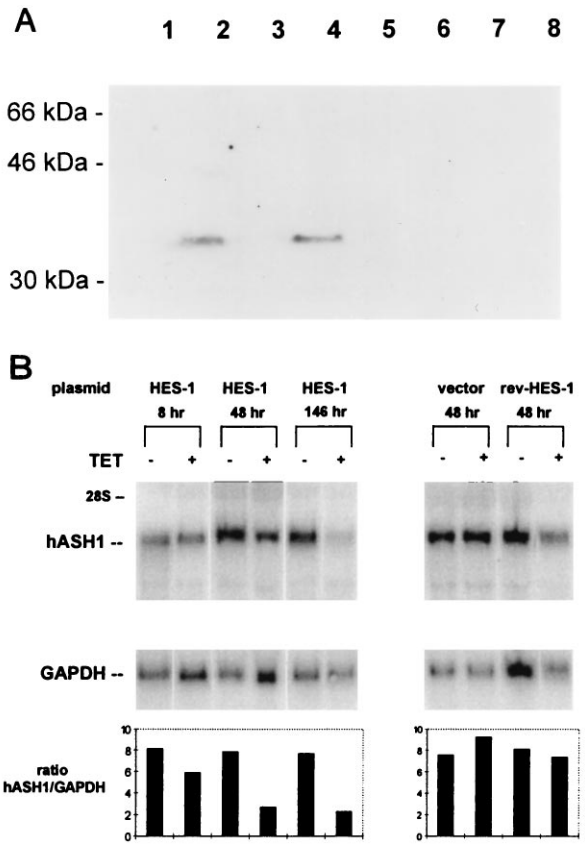

FIG. 2. Induction of HES-1 in SCLC cells. (*A*) DMS53 cells containing a tetracycline-inducible transcriptional activator were stably transfected with HES-1 plasmids driven by a tetracycline response element, treated with carrier or doxycycline (1 μg/ml) for various time periods, and analyzed for HES-1 protein expression by Western blotting as described in Fig. 1. Lanes: 1, carrier-treated DMS53-HES-1 (sense orientation HES-1 construct) cells, 48 hr; 2, doxycycline-induced DMS53-HES-1 cells, 48 hr; 3, carrier-treated DMS53-HES-1 cells, 146 hr; 4, doxycycline-treated DMS53-HES-1 cells, 146 hr; 5, carrier-treated DMS53-vector cells, 48 hr; 6, doxycycline-treated DMS53-vector cells, 48 hr; 7, carrier-treated DMS53-rev-HES-1 (antisense orientation HES-1 construct) cells, 48 hr; 8, doxycycline-treated DMS53-rev-HES-1 cells, 48 hr. (*B*) Effect of HES-1 induction on hASH1 mRNA levels. Total RNA (20 μg) from DMS53 cells transfected with tetracycline-inducible constructs, and treated with carrier (TET−) or 1 μg/ml doxycycline (TET+) for 8, 48, and 146 hr, was analyzed for hASH1 and GAPDH mRNA as described in *Materials and Methods*. In the bottom panels, the ratios for the relative intensities of the hASH1 and GAPDH hybridization signals were calculated from PhosphorImager values.

HES-1 Represses hASH1 Transcription Through a Class C Site in the hASH1 Promoter. We next explored whether the ability of HES-1 to repress hASH1 gene expression might be directly mediated through interaction of this protein with the hASH1 promoter. We have recently cloned and characterized over 16 kb of the hASH1 gene 5′ flanking region and found that tissue-specific expression of hASH1 in NE tumors is controlled by a proximal generalized, basal enhancer (bp −234 to −46) and two tissue-specific repressor regions: a distal segment (base pairs −15,900 to −13,500) and a proximal site (base pairs −308 to −234) (33). Inspection of the proximal hASH1 repressor region revealed a class C hairy binding site (CACGCA) at bp −258 (Fig. 3*A*) similar in sequence and location to the one in the *Drosophila* achaete gene (17).

To determine if HES-1 could repress hASH1 transcription at this class C site, we employed transient transfection assays. Fragments of hASH1 5′ flanking genomic DNA were inserted into a luciferase reporter vector and cotransfected with HES-1 (24) or control vectors. In DMS53 cells [hASH1(+), HES-1(−)], reporter activity from hASH1 promoter constructs containing the class C site (−7,900/+37, −3,600/+37, −2,500/+37, −308/+37, and −282/+37) was inhibited 60–80% by cotransfection with HES-1 (Fig. 3*B*). In sharp contrast, HES-1 had no effect when cotransfected with a construct with a mutated class C site (−282/+37M). A 5′ end deletion of the class C site (plasmid −234/+37) similarly resulted in no difference between the hASH1 promoter activity after cotransfection with HES-1 or vector controls. These data indicate that overexpression of HES-1 can lead to repression of transcription from hASH1 promoter fragments containing the class C site.

As noted previously, NCI-H157 NSCLC cells, unlike DMS53 cells, contain abundant HES-1 and no detectable hASH1. Cotransfections of HES-1 and the hASH1 promoter fragments into these cells revealed a pattern distinctly different (Fig. 3*C*) from the DMS53 cells studied above. hASH1 promoter activity was not affected by cotransfection of HES-1 regardless of the status of the class C site, suggesting that maximal concentrations of endogenous HES-1 were already present in these cells. Indeed, basal hASH1 promoter activity in these cells compared with the DMS53 cells was relatively lower in constructs possessing the class C site (−7,900/+37, −3,600/+37, −611/+37, −308/+37, and −282/+37) versus those lacking it (−282/+37M and −234/+37). Direct comparison of plasmids −282/+37 and −282/+37M indicates an approximately 6-fold repression associated with the class C site in NCI-H157 cells. Together, these data are consistent with a hypothesis that the endogenous HES-1 is capable of modulating transcription through the class C site.

We consistently observed an approximately 30% decrease in reporter activity of plasmid −282/+37M compared with −234/+37, suggesting a weak repressor element in addition to the class C site in both cell lines. A decrease in reporter activity after deletion from base pairs −308 to −282 may also suggest the presence of weak enhancer activity in NCI-H157 cells that was not apparent in DMS53 cells. In summary, these results indicate that endogenous or transfected HES-1 can act through the class C site in the hASH1 gene promoter to inhibit the transcription of this gene.

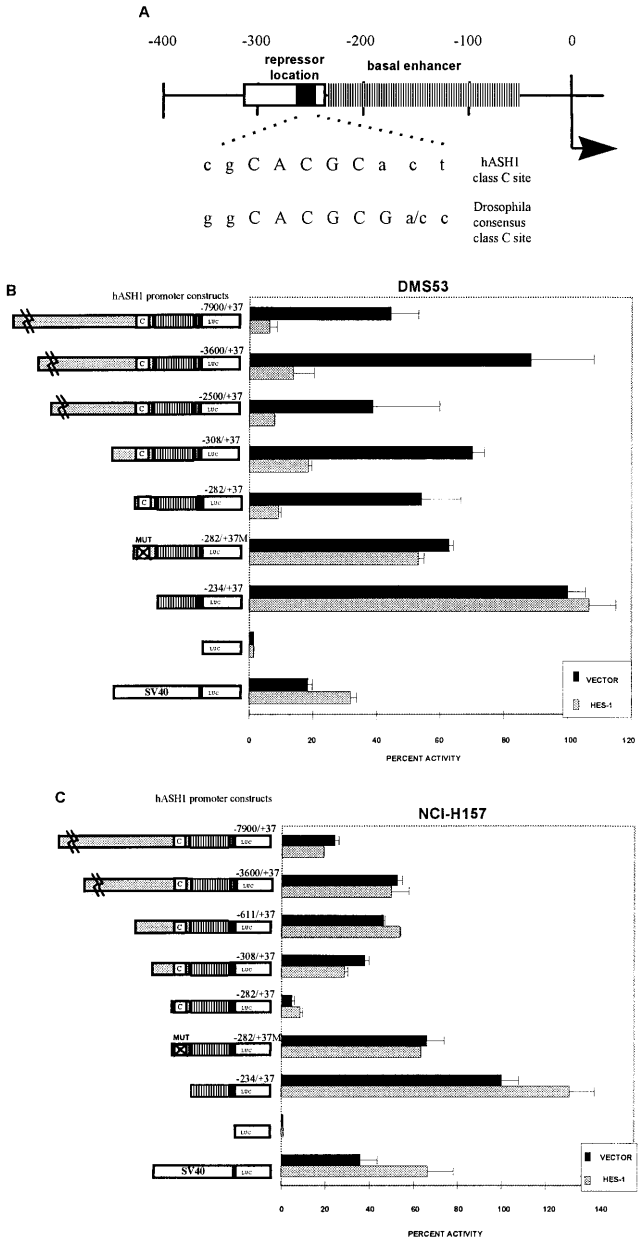

FIG. 3. (*A*) Schematic representation of the class C site (solid box, bp −258) in the proximal hASH1 repressor region (open box). The striped box indicates the basal hASH1 enhancer (base pairs −234 to −46). (*B* and *C*) Transient transfection assays of hASH1 promoter constructs. Fragments of the hASH1 promoter region containing 5′ flanking sequence (shaded box), the candidate class C site (open box labeled with the letter C), the generalized enhancer region (striped box), the transcription initiation site (at position +1), and a short 5′ untranslated region were subcloned into the luciferase reporter vector pGL2 and then cotransfected with CMV–β-galactosidase and either HES-1 (shaded bars) or control (solid bars) vectors into DMS53 cells [HES-1(−), hASH1(+)] (*B*) and NCI-H157 cells [HES-1(+), hASH1(−)] (*C*). Luciferase activity is normalized to CMV–β-galactosidase and expressed in relation to the most active construct (plasmid −234/+37). The letter X over the class C site represents mutation of this motif.

HES-1 Protein Binds Directly to the hASH1 Class C Site. Gel mobility-shift assays were performed to determine whether the endogenous HES-1 protein binds to the hASH1 class C site. Nuclear extracts from NCI-H157 NSCLC cells, which have high levels of HES-1 protein and do not express hASH1, yielded a broad DNA–protein band with a probe containing the WT hASH1 class C site (Fig. 4*A*, lane 2). In contrast, nuclear extracts

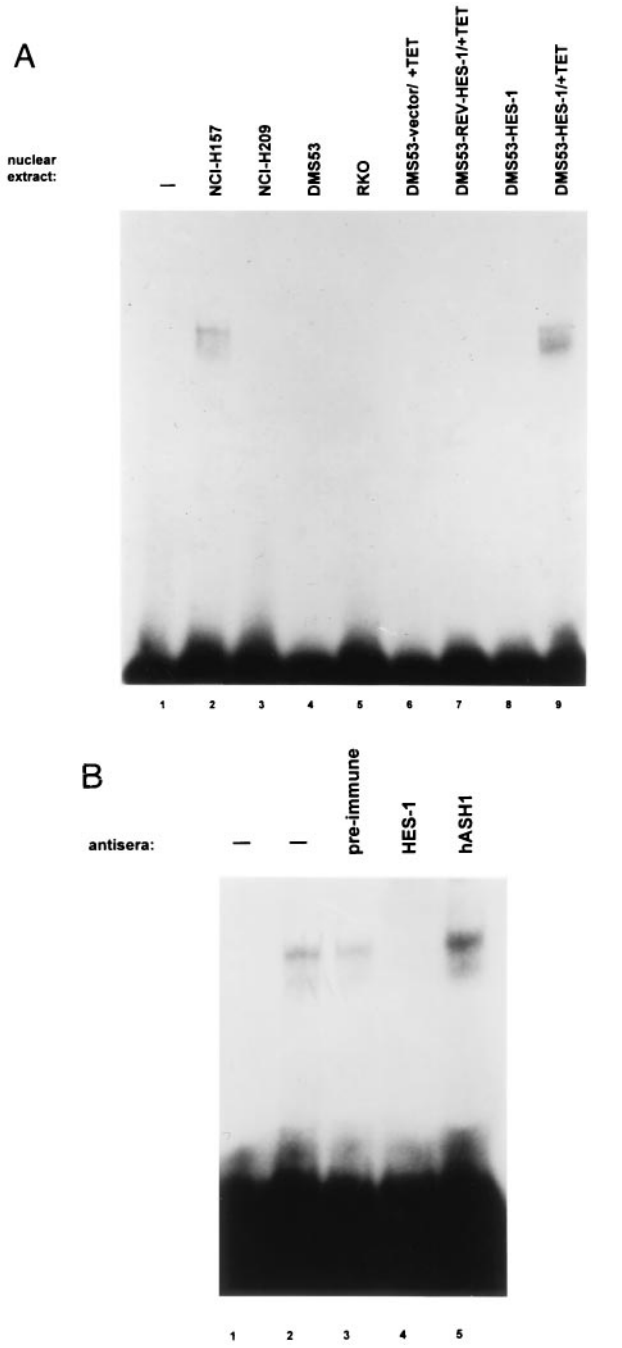

FIG. 4. Gel mobility-shift analysis of the hASH1 promoter class C site. (*A*) Cell-type-specific shift patterns. Nuclear protein extracts (4 μg) from native cells (lanes 2–5) or from DMS53 cells expressing HES-1 (lane 9) or control constructs (lanes 6–8) were incubated with a WT probe, containing the class C site from the hASH1 promoter. (*B*) Effect of HES-1 antisera on complexes with the hASH1 promoter class C site. NCI-H157 nuclear extracts (4 μg) and various antisera were preincubated for 30 min, then incubated with WT probe. Lanes: 1, free probe; 2, extract with buffer alone, no antisera; 3, preimmune sera; 4, HES-1 antisera; 5, control hASH1 antisera.

from NCI-H209 SCLC, DMS53 SCLC, and RKO colon cancer cells, none of which express HES-1, failed to produce any significant DNA–protein complexes. We next tested whether insertion and induction of exogenous HES-1 into the DMS53 cell line would result in the same DNA–protein complexes detected with the NCI-H157 nuclear extracts. With the same tetracycline-inducible HES-1 system described above in DMS53 cells, we found that nuclear extracts prepared after 96 hr of doxycycline

treatment resulted in DNA–protein complexes similar to those seen with NCI-H157 extracts (Fig. 4*A*, lane 9), whereas vector (lane 6), antisense (lane 7), and noninduced DMS53-HES-1 control (lane 8) nuclear extracts produced no such pattern. Therefore, these DNA–protein complexes appear to result from HES-1 induction in SCLC cells that do not express the endogenous HES-1 gene.

To demonstrate that the above DNA–protein complexes contain HES-1, we performed gel mobility-shift assays in the presence of HES-1 antisera (Fig. 4*B*). Nuclear extracts from NCI-H157 cells were pre-incubated with buffer (Fig. 4*B*, lane 2), preimmune sera (lane 3), HES-1 antibody (lane 4), and hASH1 antibody (lane 5), and then incubated with labeled WT hASH1 class C site probe. A total loss of the complexes was seen only after preincubation with the HES-1 antisera, whereas preimmune sera and the unrelated hASH1 antibody had little or no effect on the DNA–protein complex pattern. These results show that specific HES-1 antisera can inhibit formation of protein–DNA complexes, indicating that native HES-1 is a component of the binding complexes.

To determine if the HES-1–DNA complexes represented specific interactions with the class C site, competition studies were performed with NCI-H157 nuclear extracts using the WT probe together with competitor oligonucleotides (Fig. 5*A*). Addition of WT competitor oligonucleotide effectively competed away the entire broad band (Fig. 5*B*, lanes 3 and 4). Furthermore, competition using the 2NB oligonucleotide, containing two HES-1 binding sites from the rat HES-1 promoter, (Fig. 5*B*, lanes 9 and 10) competed to a greater degree than even WT, which contains only one binding site. In contrast to the above results, a competitor mutated at the class C site had little effect (Fig. 5*B*, lanes 5 and 6), indicating that these HES-1–DNA complexes represented direct binding to the class C site. This interpretation is further supported by competition with unrelated oligonucleotide (CT7–8) which also had no effect (Fig. 5*B*, lanes 7 and 8). In summary, these gel mobility-shift studies show that HES-1 has a high-affinity, direct interaction with the class C site in the hASH1 promoter.

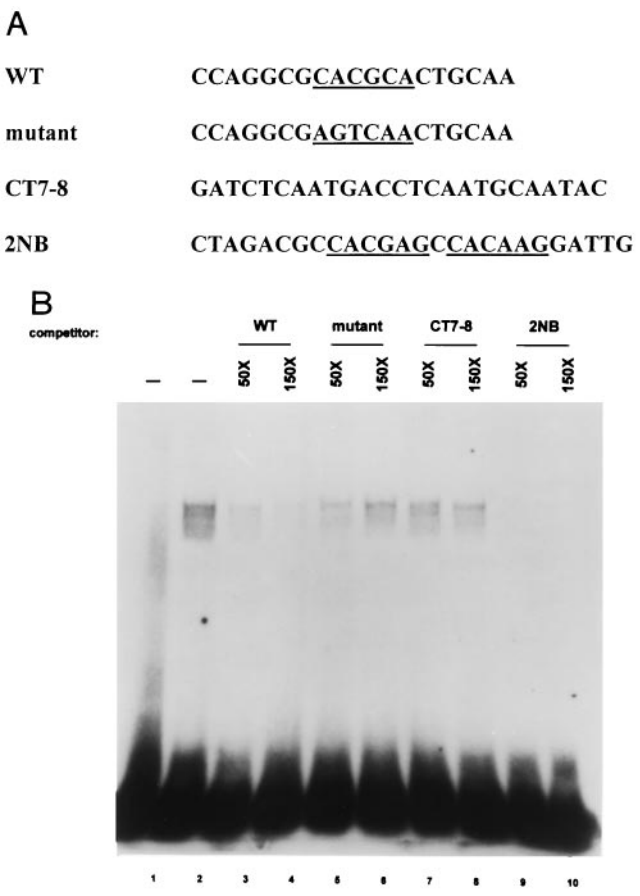

FIG. 5. (*A*) Oligonucleotide sequences used for gel mobility-shift analysis: WT, derived from the hASH1 promoter (base pairs −265 to −247) containing the class C site (underlined); mutant, identical to WT except for five point mutations in the class C site (underlined); CT7–8, an irrelevant probe derived from the calcitonin gene promoter (38); and 2NB, containing the two N-box HES-1 binding sites (underlined) in the rat HES-1 promoter (27). (*B*) Competitor studies of the hASH1 promoter class C site using NCI-H157 nuclear protein extracts (4 μg), WT probe, and competitors shown in *A*. Lanes: 1, free probe; 2, no competitor; 3 and 4, 50- and 150-fold excess WT; 5 and 6, mutant; 7 and 8, CT7–8; 9 and 10, 2NB.

DISCUSSION

In this study, we have shown that components of the *Drosophila* lateral inhibition pathway appear to be conserved in human lung cancer such that the protein HES-1 can regulate the constitutive hASH1 expression seen in lung neoplasms with a NE phenotype. HES-1, a mammalian homolog of the *Drosophila* hairy-enhancer-of-split complex, was found at abundant levels in the majority of NSCLC cell lines with no NE characteristics but was virtually absent in hASH1-expressing SCLC cell lines with NE features. Insertion of HES-1 into SCLC cells resulted in a marked reduction of native hASH1 gene expression. As in the lateral inhibition of achaete gene expression in *Drosophila*, HES-1 can repress hASH1 through direct binding to a class C site in the promoter of the target gene. In addition, the class C site in the proximal hASH1 promoter is similar in orientation to the analogous site in the *Drosophila* achaete promoter.

Recent data from our group and others (6, 33, 39) illustrate that the organization of human and rodent achaete-scute homolog-1 genes is highly conserved. Although rodent MASH1 promoters and their direct interaction with HES-1 have not been previously characterized, transgenic studies clearly suggest that HES-1 influences MASH1 gene expression. HES-1 knockout mice have severe defects in neurulation, resulting in exencephaly and anencephaly, and die late in gestation or shortly after birth (40, 41). Strikingly, these mice have enlarged fields of MASH1 expression that persist abnormally during development (40), indicating that HES-1 is responsible for modulating MASH1 levels in the mammalian nervous system.

As in the hASH1 promoter, a single class C element within the *Drosophila* achaete promoter appears to be the site of interaction with hairy (17). In transfection assays using the achaete promoter, hairy inhibits achaete transcription activity to a similar degree to that which we now observe for the hASH1 promoter (16). These parallel observations indicate a remarkable degree of functional conservation between the *Drosophila* and mammalian homologs. The importance of the HES-1 binding site in the human gene is further emphasized in *Drosophila* genetic studies, in which mutation of the class C site in the achaete promoter results in a phenotype that is similar to flies carrying a mutation in the hairy gene itself (17). The *Drosophila* hairy class C binding site (CACGCG) differs only slightly from the previously reported HES-1 binding sites in its own promoter (CACNAG), which have been termed N-boxes. The possibility of high affinity interactions of HES-1 with class C sites is further supported by reports that HES-1 is capable of binding to such a site *in vitro* to repress the achaete promoter (42) and that the *Drosophila* hairy protein can bind to N-box sequences present in the HES-1 promoter, but at a lower affinity (17).

In our nuclear binding assays, the broad nature of the HES-1 complex makes it unclear whether the protein binds as a monomer, homomultimer, or heteromultimer with other factor(s). Both Drosophila hairy (43–45) and mammalian hairy-related proteins (17, 27), have been shown to bind DNA as homomultimers or heterodimers. However, two findings indicate that the components of the shifted complex are specific for HES-1. First, effective competition with the 2NB oligonu-

cleotide suggests that proteins in all of the complexes share binding affinity to N-boxes. Second, incubation with HES-1 antisera blocks formation of all of the complexes.

Our recent studies indicate that achaete-scute homolog-1 is essential for the development of the normal pulmonary NE cells, which have a NE phenotype similar to SCLC and may share a common differentiation pathway with this cancer (13). The regulation of hASH1 expression in NE tumors such as SCLC appears to be a complex process including a proximal generalized promoter and two tissue-specific repressor regions (33). It is likely that the activity of the proximal and distal repressor regions regulates hASH1 transcription in a tissue-specific fashion. For example, one NSCLC line in the present series (NCI-H1770) lacks HES-1 protein but does not express hASH1, similar to the pattern seen in several unrelated cancer lines, including RKO colon cancer cells. It is possible that the class C site modulates hASH1 expression in a graded fashion, whereas the distal repressor blocks expression outside of a neural or NE context. Therefore, a defect in either of these candidate repression pathways, such as the lack of HES-1, could contribute to constitutive hASH1 expression. In addition, mammalian homologs of Notch, which appear to play a predominant role in HES-1 induction (41), have been implicated in other malignancies as regulators of tissue differentiation (46–49). This growing body of data, including our present observations, underscores the potentially important role of hASH1 and its regulatory factors in human neoplasia. Our findings emphasize the importance of future studies to explore the interplay of lateral inhibitory pathway components in the development of the mammalian nervous system and, especially, in events underlying the evolution of the different forms of human lung cancer.

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