Biochemical and Biophysical Research Communications **274,** 22–31 (2000)
doi:10.1006/bbrc.2000.3090, available online at http://www.idealibrary.com on IDEAL®

HASH-1 and E2-2 Are Expressed in Human Neuroblastoma Cells and Form a Functional Complex

Paula Persson, Annika Jögi, Anna Grynfeld, Sven Påhlman, and Håkan Axelson[1]
Department of Laboratory Medicine, Division of Molecular Medicine, Lund University, University Hospital MAS, S-205 02 Malmö, Sweden

Received June 15, 2000

The basic helix-loop-helix (bHLH) transcription factor *mammalian achaete-scute homolog-1* (*MASH-1* in mouse and *HASH-1* in human) is essential for proper development of olfactory and most peripheral autonomic neurons, and for the formation of distinct neuronal circuits within the central nervous system. We have previously shown that *HASH-1* is expressed in neuroblastoma tumors and cell lines, and in this study we have used the yeast two-hybrid system to isolate HASH-1 interacting proteins from a human neuroblastoma cDNA library. Two of the isolated clones contained cDNA from the *E2-2* gene (also known as *ITF2/SEF2-1*). We show that E2-2 interacts with HASH-1 in both yeast and mammalian cells. The HASH-1/E2-2 complex binds an E-box (CACCTG) *in vitro,* and transactivates an E-box containing reporter construct *in vivo.* Furthermore, E2-2 seems to be one of the major HASH-1 interacting proteins in extracts from neuroblastoma cells. In conclusion, E2-2 forms a functional complex with HASH-1, and might therefore be involved in the development of specific parts of the central and peripheral nervous systems.

Key Words: **bHLH; E2-2; SEF2-1; HASH-1; neuroblastoma; sympathetic nervous system; differentiation; yeast two-hybrid.**

The basic helix-loop-helix (bHLH) family of transcription factors are momentous for the proper development of many tissues (1). These factors have been implicated in the development of malignancies due to improper regulation and/or structural alterations (2, 3). The basic domain of the bHLH proteins binds to a consensus sequence, CANNTG, known as the E-box (4). The helix-loop-helix domain mediates protein-protein interactions and the formed complexes can bind DNA either as homo- or heterodimers (5). The bHLH proteins can show a widespread expression pattern (the class A-type, also called E-proteins) or be more tissue-specifically expressed (the class B-type) (5). In addition, there is a third type of proteins, the Id proteins, which contain the HLH domain but lack the basic domain (6). They exert their function mainly by binding to E-proteins, and thereby sequestering them from interaction with tissue-specific bHLH proteins (7). These different types of HLH proteins provide the framework for an intricate network of transcription factors controlling tissue-specific gene expression, governed by the interaction affinities, expression levels and stabilities of the different protein complexes.

The *mammalian achaete-scute homolog-1* (*MASH-1*) gene was isolated on the basis of sequence homology to the *Drosophila* proneuronal *achaete-scute* genes (8). *MASH-1* is expressed during embryogenesis in specific regions of the developing central nervous system and in sympathetic and enteric, but not in sensory precursor cells (8, 9). The pivotal role for *MASH-1* in the development of the central and peripheral nervous systems has been demonstrated in gene targeting experiments. *MASH-1* null mice die at birth and show loss of most peripheral autonomic neuroblasts and olfactory epithelial cells (10). Recent reports also indicate a role for *MASH-1* in the development of the telencephalon (11, 12).

Neuroblastoma is a childhood tumor derived from the sympathetic nervous system (13, 14). We have previously shown that many neuroblastoma tumors and all neuroblastoma cell lines studied express *HASH-1* (the human homolog of *MASH-1*) (15), demonstrating the embryonal features of this tumor. When cultured neuroblastoma cells are induced to differentiate with several different protocols, there is a rapid decrease in *HASH-1* expression (16).

In vitro studies have shown that MASH-1 can form a functional heterodimeric complex with the ubiquitously expressed *E2a* gene products E12 and E47, and that this complex binds to a consensus E-box DNA binding site (17). The E12 and E47 proteins belong to

[1] To whom reprints requests should be addressed at Department of Laboratory Medicine, Division of Molecular Medicine, Lund University, University Hospital MAS, Entrance 78, S-205 02 Malmö, Sweden. Fax: +46-40337322. E-mail: hakan.axelson@molmed.mas.lu.se.

0006-291X/00 $35.00

the E-protein family, which also includes HEB (also ITF4) and E2-2 (also ITF2 or SEF2-1) (18–20). These proteins show a high degree of homology, especially in the bHLH domain (21). The E-proteins seem to be functionally equivalent to some extent, but gene targeting experiments demonstrate some fundamental differences in their biological functions (22). For example, the E12 and E47 proteins have been specifically implicated in the lymphogenesis, since mice lacking the *E2a* gene show a severe perturbation in B-cell development (23). The HEB protein can functionally replace E12/E47 in B-cell development, exemplifying that the E-proteins to a certain extent are functionally redundant (22).

Here we report the isolation of human *E2-2* when screening a yeast two-hybrid library derived from the human neuroblastoma cell line SH-SY5Y, using HASH-1 as bait. A HASH-1/E2-2 DNA-binding complex was further demonstrated *in vitro* and *in vivo*. We conclude that HASH-1 can form a functional complex with E2-2, and that E2-2 is an important heterodimerization partner of HASH-1 in neuronal cells.

MATERIALS AND METHODS

Cell culture. The neuroblastoma cell lines SH-SY5Y and SK-N-BE(2) (kindly provided by Dr. J. Biedler, Sloan Kettering Institute, New York) were grown at 37°C in 5% CO_2 in Eagle's Minimum Essential Medium (MEM, Life Technologies) supplemented with 10% fetal calf serum (FCS, Life Technologies), penicillin (100 U/ml) and streptomycin (100 μg/ml). The SH-SY5Y cells were differentiated for 0, 2, 4, 8, 24 and 96 h with 16 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in MEM containing 10% FCS. HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) with 10% FCS and the antibiotics described above.

Construction of GAL4-HASH-1 chimeras and the SH-SY5Y two-hybrid cDNA library. The bait plasmids containing the GAL4-DNA binding domain used in the two-hybrid system were based on the pAS2-1 vector (Clontech). Plasmids encoding the full-length HASH-1 protein, pAS-HASH-1 (amino acids 1–238) or partial HASH-1 sequences, pAS-HASHΔC (amino acids 1–180), pAS-HASHΔN (amino acids 120–238) and pAS-HASH-HLH (amino acids 120–180) were constructed by inserting PCR-generated fragments, using cDNA from SH-SY5Y as template, into NdeI/BamHI digested pAS2-1 expression vectors. The constructs were verified by automated DNA sequencing and Western blot analyses of yeast expression of GAL4-HASH-1 fusion proteins were made according to the manufacturer's protocol (Clontech). Immunodetection was performed with an anti-GAL4-DNA binding domain antibody (Santa Cruz) diluted 1:1000, followed by incubation with a sheep anti-mouse polyclonal antiserum coupled to horseradish peroxidase (Amersham Pharmacia Biotech) diluted 1:5000. Immunoreactivity was detected using the enhanced chemiluminescence (ECL) method (Pierce). The yeast two-hybrid cDNA library was made from RNA prepared from SH-SY5Y cells using Stratagene RNA-isolation and poly(A)$^+$ kits according to the manufacturer's instructions. A directional cDNA library was prepared using the Stratagene cDNA synthesis kit starting from 5 μg poly(A)$^+$RNA. The cDNA was ligated into EcoRI/XhoI digested HybriZAP DNA (Stratagene) and packaged using a Gigapack II in vitro packaging extract (Stratagene). The library, which had a complexity of 2.5×10^6 cfu, was amplified and the pAD-GAL4 phagemid vector was excised according to the manufacturer's protocol (Stratagene).

Yeast transformation and two-hybrid library screening. Plasmids were introduced into the yeast strain CG1945 by lithium-acetate transformation, and interactions were tested by growth on the appropriate SD medium. The ability of different GAL4-HASH-1 fusion proteins to transactivate the *lacZ* reporter in CG1945 was tested by filter lift assays (Clontech). Semiquantitative analyses of transactivation/interaction in yeast were performed using chlorophenol red-B-D-galactopyranoside (CPRG, Roche), as described in the manufacturer's protocol (Clontech). To screen for HASH-1 interacting clones the SH-SY5Y cDNA library was cotransformed with pAS-HASHΔC plasmid according to the lithium-acetate protocol. The transformed yeast was plated on SD medium lacking tryptophan, leucine and histidine. The plates were incubated at 30°C for seven days and filter lift assays were performed as described above. Positive clones were isolated, purified, and propagated in *E. coli* for plasmid isolation. The inserts were sequenced by automated DNA sequencing and compared with sequences in genome databases using the NCBI database (NIH, USA). To test for specificity of interactions, positive clones were retransformed with a series of heterologous bait plasmids, including pAS-laminin and the empty pAS2-1 vector as described by the manufacturer (Clontech).

Mammalian two-hybrid analyses. Mammalian two-hybrid analyses were performed using the Checkmate system (Promega). GAL4-DNA binding (pBIND) and VP16 transactivating (pACT) fusion proteins were constructed using PCR-generated fragments cloned into the BamHI/SalI digested vectors. The following fragments were used: HASHΔC, as described above, E47 (amino acids 508–654, which includes the basic helix-loop-helix domain) and E2-2 (amino acids 1–668). The reporter plasmid pG5*luc* contains a GAL4 binding site upstream of the coding sequence of firefly luciferase. The analyses were made in HeLa cells (2.5×10^5) grown in 60 mm dishes. The cells were transfected with appropriate expression plasmids and the reporter plasmid (5 μg of each plasmid per dish) using Lipofectin (Life Technologies). The cells were harvested two days after transfection. The luciferase activity was measured using the Dual Luciferase Assay kit (Promega) according to the manufacturer's instructions. The transfection efficiency was calculated based on the level of Renilla luciferase activity constitutively expressed by the pBIND vector.

GST-HASHΔC pulldown experiments on in vitro translated proteins. A pGEX-HASHΔC plasmid was made by inserting a PCR-generated DNA fragment, using pAS-HASH-1 as template, into an EcoRI/XhoI-digested pGEX-5X-1 plasmid (Amersham Pharmacia Biotech). Glutathione *S*-transferase (GST)-HASHΔC or GST alone were expressed in BL21 (DE3) *E. coli*. The bacteria were induced and lysed according to the manufacturer's protocol (Amersham Pharmacia Biotech). The lysates were incubated with Glutathione Sepharose 4B (Amersham Pharmacia Biotech) for 30 min at room temperature. The beads were washed three times in PBS and thereafter incubated with blocking buffer (20 mM Hepes pH 7.4, 100 mM NaCl, 10% glycerol, 0.1% NP40 and 1% BSA) for 1 h at +4°C. The GST-HASHΔC or GST sepharose beads were thereafter incubated with 8 μl *in vitro* translated, ^{35}S-methionine labeled E2-2 (see below) in binding buffer (20 mM Hepes pH 7.4, 100 mM NaCl, 10% glycerol, 0.1% NP40 and 0.15% BSA) for 1 h at 4°C. The beads were washed four times with binding buffer and once with pulldown buffer (20 mM Hepes pH 7.4, 100 mM NaCl, 10% glycerol, 0.1% NP40). The bound proteins were released from the beads by boiling and were subjected to 10% SDS–PAGE followed by autoradiography.

Electrophoretic mobility shift assay (EMSA). The oligonucleotides were labeled by a kinase reaction using γ^{32}P-ATP (5000 Ci) (Current Protocols). Unincorporated nucleotides were removed using Pharmacia G25 columns (Amersham Pharmacia Biotech). *In vitro* transcription and translation of HASHΔC, E47 and E2-2 were carried out using the TNT coupled reticulocyte lysate kit (Promega), using the plasmids pBIND-HASHΔC, pBIND-E47 (see above) and pcDNA-E2-2. The E2-2 insert was generated by PCR using pAD-E2-2

as a template and cloned into BamHI/XhoI digested pcDNA3.1/HisA (Invitrogen). Three μl of the respective *in vitro* translated products were mixed and incubated at 37°C for 20 min. A DNA-binding mix (20 mM HEPES pH 7.4, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 1 μg of double-stranded poly(dI-dC) (Amersham Pharmacia Biotech)) with labeled wt or E-box mutated oligonucleotides (25 000 cpm/reaction) was added to the *in vitro* translated products. Binding reactions were performed at room temperature for 15 min, and then immediately loaded on a 4% polyacrylamide (30:1 acrylamide:bis-acrylamide) gel in 0.5 × TBE and run for approximately 4 hours. Gels were dried and autoradiographed. Sequences of oligonucleotides used in EMSAs with the E-box underlined; muscle creatine kinase (MCK) enhancer wt top strand: 5′-GATCCC-CCCAACACCTGCTGCCTGA-3′, MCK enhancer mutated top strand: 5′-GATCCCCCCAAATGAGGCTGCCCTGA-3′.

Transient transfections and luciferase assays. Syrian hamster E47 and E12 (24) cloned in frame with the myc tag BamHI site of the MD3 vector were provided by Dr. M. Sigvardsson, Dept. of Cell and Molecular Biology, Lund University, Sweden. The MD3 vector contains six copies of the myc epitope, cloned into a BamHI/EcoRI digested cDNA3 vector (Invitrogen). The His tagged pcDNA-E2-2 plasmid is described above. The pHA-HASH-1 plasmid was made by inserting a PCR-generated DNA fragment, using pAS-HASH-1 as template, into a BglII/XhoI digested pHA vector (Clontech). The E-box reporter plasmid was based on the pGL3-fos vector, which contains a basal fos promoter inserted into the pGL3 luciferase vector (25). The E-box reporter contains two copies of the E1E2 containing region of the MCK enhancer (kindly provided by Dr. M. Sigvardsson, Dept. of Cell and Molecular Biology, Lund University, Sweden). HeLa cells (2×10^5) were seeded in 35 mm dishes and grown over night. The cells were transfected with 300 ng of appropriate expression plasmids and 500 ng of the reporter plasmid using Lipofectin (Life Technologies). 500 ng of a pCMV-gal plasmid (kindly provided by Dr. L.-G. Larsson, Uppsala University, Sweden) was cotransfected as a control of transfection efficiency. The total amount of DNA used in each transfection was adjusted by adding pBluescript II plasmid (Stratagene). The cells were harvested 40 hours after transfection and protein extracts were prepared in lysis buffer (Dual Light, Tropix). To assay cells for luciferase activity, the Dual Light kit (Tropix) was used according to the manufacturer's recommendations. The transfection efficiency was calculated based on the level of β-galactosidase activity expressed by the pCMV-gal plasmid.

RNA preparation and Northern blotting. Twenty million cells were washed in PBS, and then lysed in 0.4 ml lysis buffer (0.15 M NaCl, 10 mM Tris pH 7.4, 1 mM $MgCl_2$, 0.5% NP40). The lysate was centrifuged at 12,500*g* for 5 min and 50 μl 10% SDS was added to the supernatant and the RNA was extracted twice with phenol. After precipitation the RNA was dissolved in water. Fifteen μg total RNA was separated on a formaldehyde agarose gel and blotted onto Hybond-N nylon membrane (Amersham Pharmacia Biotech). The following probes were used: *E2-2;* a 2 kb EcoRI/XhoI fragment from the human E2-2 clone isolated in the yeast two-hybrid screen, *HASH-1;* nucleotides 1350–1635 (16), *E47;* nucleotides 1555–1995 encompassing the bHLH region of *E47*. The *E47* cDNA was generated by RT-PCR using SH-SY5Y cDNA as template. cDNA probes for *growth associated protein 43* (*GAP-43*) and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) were generated as previously described (26).

Western blot analyses. Total cell homogenates from SH-SY5Y or SK-N-BE(2) cells were prepared by lysing cells in NP40 lysis buffer (1% NP40, 10% glycerol, 20 mM TrisHCl pH 8.0, 137 mM NaCl and 4% complete protease inhibitor cocktail mix (Roche)). Twenty μg of protein per lane was subjected to 10% SDS–PAGE, followed by blotting to nitrocellulose filters. The filters were probed with either a monoclonal anti-E2-2 antibody (Pharmingen) or a polyclonal anti-E12/E47 antiserum (Santa Cruz) diluted 1:1000. The blots were then incubated with either a sheep anti-mouse polyclonal antiserum coupled to horseradish peroxidase (Amersham Pharmacia Biotech) or a donkey anti-rabbit polyclonal antiserum coupled to horseradish peroxidase (Amersham Pharmacia Biotech) diluted 1:1000. For immunodetection of HASH-1, the filters were incubated with a monoclonal anti-MASH-1 antibody (kindly provided by Dr. D. J. Anderson, Howard Hughes Medical Institute, CA) (9) diluted 1:100, followed by incubation with a sheep anti-mouse polyclonal antiserum coupled to horseradish peroxidase (Amersham Pharmacia Biotech) diluted 1:2000. Immunoreactivity was detected by using the ECL method.

GST-HASHΔC pulldown experiments on SK-N-BE(2) extracts. SK-N-BE(2) cells (8×10^6) in 100 mm dishes were lysed in NP40 lysis buffer and the lysates were incubated with GST-HASHΔC or GST glutathione-sepharose beads for 1 h at 4°C. The beads were washed four times with lysis buffer and bound proteins were subjected to 7.5% SDS–PAGE and transferred to nitrocellulose membranes. Twenty μg of crude cell extract from SK-N-BE(2) cells was run on the same gel for subsequent Western blot analysis. The blots were incubated with either a monoclonal anti-E2-2 antibody (Pharmingen) diluted 1:1000 or a monoclonal anti-E12/E47 antibody (Pharmingen) diluted 1:250, followed by incubation with a goat anti-mouse polyclonal antiserum (Jackson ImmunoResearch Laboratories Inc.) diluted 1:5000. Immunoreactivity was detected using the ECL method. In ^{35}S-methionine labeling experiments, SK-N-BE(2) cells (2×10^6) were seeded in 100 mm dishes. After 24 h the cells were washed twice with MEM lacking L-methionine (Life Technologies). 8 ml of medium was added to the cells and they were incubated for 30 min. The medium was removed and 2 ml of MEM supplied with L-methionine (0.75 mg/l) and 0.1 mCi/ml ^{35}S-methionine was added to the cells. After a 4 h incubation at 37°C in 5% CO_2 the cells were lysed in NP40 lysis buffer and after spinning down the cell debris, the supernatant was incubated with GST-HASHΔC or GST glutathione-sepharose beads for 1 h at 4°C. The beads were washed four times with binding buffer and once with pulldown buffer. The proteins were subjected to 7.5% SDS–PAGE and transferred to nitrocellulose membranes. Twenty μg of crude cell extract from SK-N-BE(2) cells was separated on the same gel for subsequent Western blot analysis. The filter was exposed to X-ray film overnight. Immunodetection was performed using a monoclonal anti-E2-2 antibody (Pharmingen) diluted 1:1000, followed by incubation with a sheep anti-mouse polyclonal antiserum coupled to horseradish peroxidase (Amersham Pharmacia Biotech) diluted 1:1000. Immunoreactivity was detected using the ECL method.

RESULTS

Transactivation by GAL4-HASH-1 in Yeast

Our aim was to isolate HASH-1 interacting proteins by using the yeast two-hybrid system. Initial data showed that full-length GAL4-HASH-1 had transactivating activity in the reporter yeast strain CG1945. We therefore tested different deletion constructs of HASH-1 (Fig. 1A). In filter lift assays both the full-length HASH-1 and the HASHΔN containing constructs activated the *lacZ* reporter gene, while HASHΔC and HASH-HLH did not. To verify these results a semi-quantitative liquid β-galactosidase assay was performed, showing that the HASHΔN construct was approximately ten times more potent than the full-length construct in transactivating the *lacZ* reporter (Fig. 1A). Thus, the C-terminal domain of HASH-1 (amino acids 180–238) has a transactivating activity in yeast, which might be of importance for the normal function of HASH-1. We used the HASHΔC

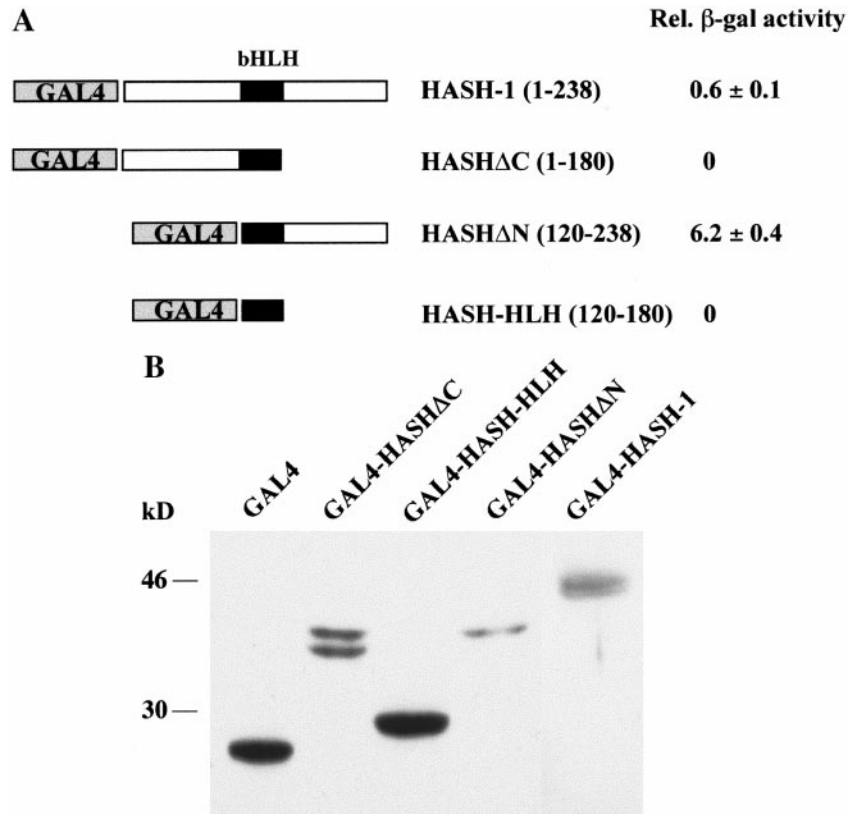

FIG. 1. The C-terminal domain of HASH-1 transactivates the *lacZ* reporter gene in yeast. (A) A schematic representation of the GAL4-HASH-1 fusion proteins. Numbers indicate the amino acids of HASH-1 included in the constructs. The GAL4-DNA binding fusion part is indicated by grey boxes and the basic helix-loop-helix domain of HASH-1 is indicated by black boxes. The transactivating capacity of the GAL4-HASH-1 fusion proteins in yeast was analyzed in a liquid β-galactosidase assay using CPRG as substrate. The assay was performed with three individual transformants, the average values and standard deviations are shown. (B) Western blot analysis of expression of yeast two-hybrid fusion proteins. GAL4-HASH-1 fusion proteins expressed in pAS2-1 were detected with an anti-GAL4-DNA binding domain antibody. The low molecular weight protein generated from the GAL4-HASHΔC construct most likely represents a degradation product. Size markers (in kilodaltons) are shown.

construct as a bait, devoid of the transactivating C-terminal domain, for library screening. Expression of appropriately sized fusion proteins was verified by Western blot analysis, using an antibody directed against the GAL4-DNA binding domain (Fig. 1B).

Isolation of E2-2 from a Neuroblastoma cDNA Library Using HASHΔC as a Bait

pAS-HASHΔC was used as a bait plasmid when screening a yeast two-hybrid cDNA library derived from the SH-SY5Y neuroblastoma cell line. Out of 1.4×10^6 yeast clones screened, 31 scored positive both regarding growth on SD selection media and expression of β-galactosidase. Two of these clones were coding for the E2-2 bHLH transcription factor. None of the other 29 clones coded for the other E-proteins, E12, E47 or HEB. One *E2-2* clone contained the full-length coding sequence and one started in the basic domain at amino acid 567. The *E2-2* containing plasmids were reintroduced into the CG1945 yeast strain together with the pAS-HASHΔC plasmid, a bait plasmid expressing the non-related protein laminin, or empty pAS2-1 vector (results not shown). These reintroduction experiments showed that the interaction between E2-2 and HASH-1 was specific in yeast cells.

GST-HASHΔC Binds in Vitro Translated E2-2

The interaction between HASHΔC and E2-2 was tested in a GST pulldown assay. *In vitro* translated, ^{35}S-labeled E2-2 protein of the expected size was mixed with GST-HASHΔC or GST protein linked to glutathione-sepharose beads. After extensive washing of the beads, bound proteins were released by boiling and subjected to SDS–PAGE. No E2-2 was bound to GST alone, while E2-2 protein was readily detected after incubation with GST-HASHΔC, providing evidence for an *in vitro* interaction between HASH-1 and E2-2 (Fig. 2).

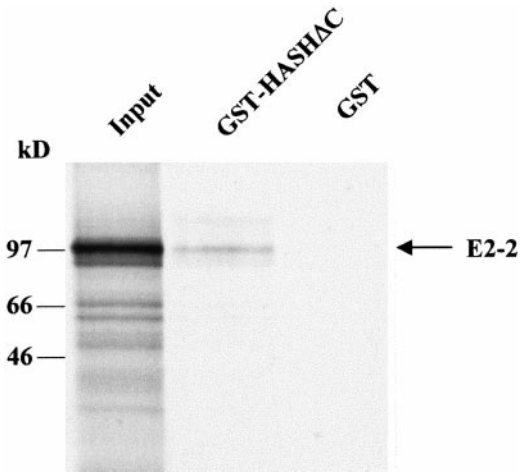

FIG. 2. GST pulldown assays confirming the HASH-1/E2-2 interaction in yeast. *In vitro* translated, ^{35}S-labeled E2-2 protein was incubated with GST or GST-HASHΔC linked to glutathione-sepharose beads. After washing, the bound proteins were released by boiling and subjected to 10% SDS–PAGE. Ten percent of the *in vitro* labeled product used in the pulldown experiments was run in parallel to indicate the amount of protein bound to HASHΔC.

HASHΔC and E2-2 Interact in Mammalian Cells

To verify the interaction between HASH-1 and E2-2 in mammalian cells we used the mammalian two-hybrid system. Transfection with pBIND-HASHΔC alone gave a baseline luciferase activity, while cotransfection with pACT-E2-2 resulted in a moderate transactivation (Fig. 3). In line with previous studies showing that E12 and MASH-1 interact (17), cotransfection of pBIND-HASHΔC with pACT-E47 or pACT-HASHΔC with pBIND-E47 gave an activation of the reporter gene (Fig. 3). When pBIND-E2-2 was introduced alone, some activation of the reporter gene could be seen, but when combined with pACT-HASHΔC a robust interaction was detected (Fig. 3). Furthermore, when pBIND-E2-2 and pACT-E2-2 were combined, a strong interaction could also be demonstrated, in line with previous data showing that E2-2 efficiently forms homodimers (27). HASHΔC showed a limited capacity to form homodimers, implicating that the function of HASH-1 is dependent on heterodimerization with E-proteins. In conclusion, these data show that both E2-2 and E47 can form heterodimeric complexes with HASH-1 *in vivo* in mammalian cells.

HASHΔC Binds to an E-Box in Complex with E2-2

To establish whether HASH-1 can bind to an E-box in complex with E2-2, EMSAs were performed. We chose to use a MCK enhancer derived oligonucleotide, containing a canonical E-box (CACCTG), since it previously was shown that *in vitro* translated MASH-1 and E12 in complex can bind to this sequence (17). When adding *in vitro* translated E2-2 or E47 alone, one major complex in each reaction could be detected (Fig. 4, lane 1 and 6, complex I and III). Most likely, these complexes represent E2-2 or E47 binding the E-box as homodimers. When mixing E2-2 with *in vitro* translated HASHΔC, an additional complex appeared (Fig. 4, lane 2, complex II). Complex I and II were specifically competed by unlabeled wildtype oligonucleotide, while no competition of complexes I and II was detected when unlabeled E-box mutated oligonucleotide was added (Fig. 4, lane 3 and 4). No HASH-1 specific binding complexes were detected when adding HASHΔC alone, in line with our mammalian two-hybrid data showing that HASHΔC do not readily form homodimers (Fig. 3). This shows that a HASHΔC/E2-2 complex can bind an E-box and that HASHΔC is unable to form DNA-binding homodimers.

The HASH-1/E2-2 Complex Has Transactivating Capacity

Having established the physical interaction between HASH-1 and E2-2, and the capacity of this complex to bind an E-box sequence, we next wanted to analyze the transactivating capacity of this complex. E2-2, E12 and E47 were examined for their abilities to transactivate an E-box controlled reporter gene when transfected with or without HASH-1 into HeLa cells. HASH-1 alone transactivated the reporter gene approximately

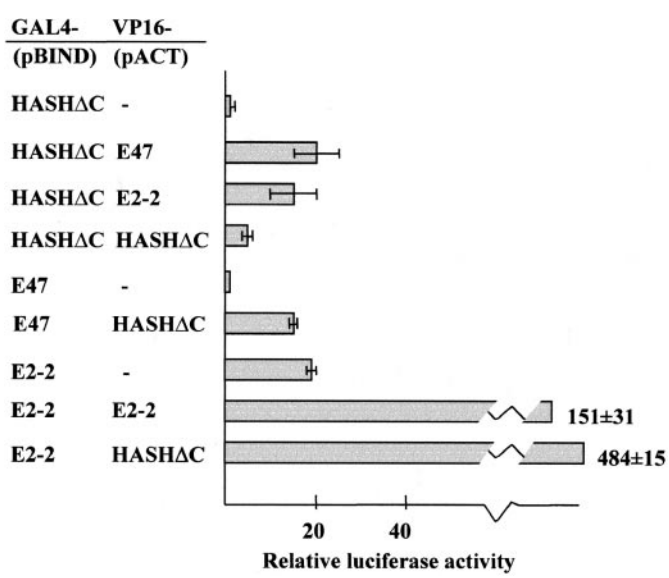

FIG. 3. Mammalian two-hybrid analyses demonstrating the ability of HASHΔC, E2-2 and E47 to form complexes in HeLa cells. The cells were transfected with the indicated GAL4 and/or VP16 expression plasmids together with a firefly luciferase reporter plasmid. Protein-protein interactions were reflected by the firefly luciferase activity relative to the Renilla luciferase activity which serves as a control of transfection efficiency. Results are shown as the mean of at least three independent experiments. Standard deviations are indicated by error bars, and in numbers for the E2-2/E2-2 and E2-2/HASHΔC interactions.

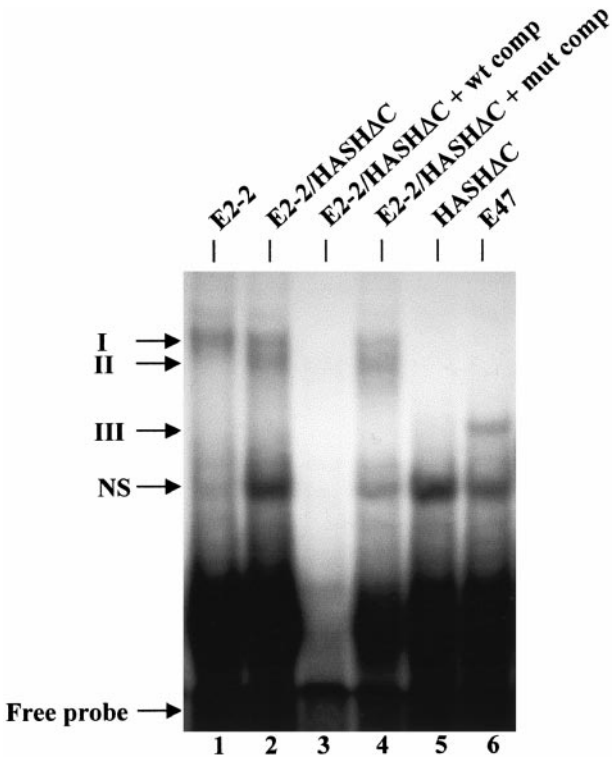

FIG. 4. EMSA of complex formation with a MCK enhancer derived oligonucleotide. A ^{32}P-labeled MCK enhancer derived oligonucleotide containing a CACCTG E-box sequence was incubated with indicated combinations of *in vitro* translated E2-2, HASHΔC and E47. Roman numerals indicate specific complexes formed by the respective proteins. For competition experiments, a 50-fold excess of wt or E-box mutated oligonucleotides were added. NS indicates nonspecific complexes, as shown by competition with an E-box mutated oligonucleotide.

five-fold compared to cells transfected with reporter plasmid (Fig. 5). Since HASH-1 did not form homodimers (Figs. 3 and 4), we assumed that this transactivating capacity was due to interaction between HASH-1 and endogenous E-proteins expressed in HeLa cells (28). A 50-fold induction was seen when combining E12 or E47 with HASH-1, while E2-2 gave rise to a 25-fold induction together with HASH-1 (Fig. 5). As previously reported, we showed that E12/E47 can transactivate the MCK enhancer as homodimers (29). Interestingly, no transactivation was detected with E2-2 (Fig. 5), despite that E2-2 can form homodimers (Figs. 3 and 4). These results show that HASH-1 can bind and transactivate an E-box containing reporter gene together with E12, E47 or E2-2.

E2-2 Expression Is Transiently Induced in Differentiating SH-SY5Y Cells

The expression of *E2-2* and *HASH-1* in differentiating neuroblastoma cells was analyzed by Northern blotting. When the SH-SY5Y cells are treated with TPA, a well documented neuronal differentiation occurs as indicated by the extension of neurites and upregulation of a number of neuronal markers such as *NPY* and *GAP-43* (30). In unstimulated SH-SY5Y cells a moderate *E2-2* expression was detected, while the *E47* expression was hardly detectable. The expression of *E2-2* was transiently upregulated during the first eight hours of TPA treatment, whereafter it declined to an expression level similar to that of untreated cells (Fig. 6). A weak transient increase in *E47* mRNA levels was seen between 2 and 4 hours after TPA treatment. As reported previously, the *HASH-1* expression was rapidly downregulated upon TPA treatment and 2 hours after treatment most *HASH-1* mRNA was eliminated (16). Thus, *HASH-1* and *E2-2* are coexpressed in human SH-SY5Y neuroblastoma cells, but are differently regulated upon induced differentiation.

GST-HASHΔC Binds E2-2 in Extracts from SK-N-BE(2) Cells

In order to establish the expression at the protein level of E2-2, E12/E47 and HASH-1 in neuroblastoma cell lines we performed Western blot analyses on extracts from SH-SY5Y and SK-N-BE(2) neuroblastoma cells. Both E2-2 and E12/E47 were expressed in these cells, together with HASH-1 (Fig. 7A). Since the E12

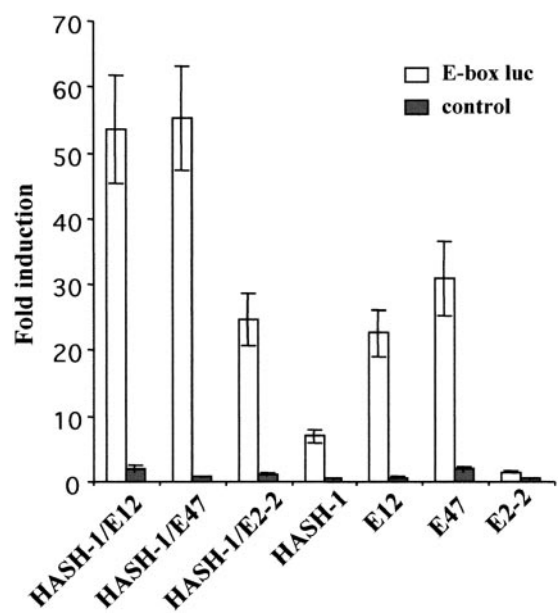

FIG. 5. HASH-1 and E-proteins form complexes that can transactivate an E-box containing reporter. HeLa cells were transfected with an E-box reporter plasmid, with two copies of the E1E2 containing region of the MCK enhancer, or a control luciferase reporter plasmid together with CMV-driven vectors expressing HASH-1, E12, E47 or E2-2. Values are expressed as the fold induction of luciferase activity over the activity of reporter vector cotransfected with pBluescript II. The data shown are based on three independent transfection experiments. Standard deviation is indicated by error bars.

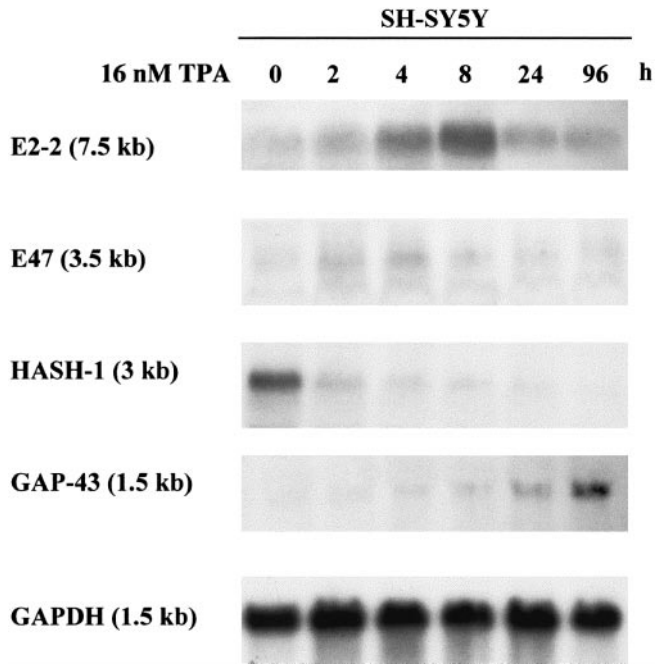

FIG. 6. Expression of *E2-2, E47, HASH-1* and *GAP-43* in SH-SY5Y cells induced to differentiate with TPA. Northern blot analyses of 15 μg total RNA isolated from SH-SY5Y cells treated with 16 nM TPA for the indicated length of time. The *GAPDH* mRNA levels serve as loading controls.

and E47 proteins have a similar molecular weight, only one band could be detected in the cell extracts. The multiple bands in the E2-2 blot can be due to different previously reported splice-variants of E2-2, premature termination of translation or downstream translation initiation codons (27). In order to identify the preferential HASH-1 interacting proteins in neuroblastoma cells co-immunoprecipitation experiments of endogenous proteins were tested. We chose to use extracts from SK-N-BE(2) cells, as these cells express higher levels of HASH-1 than SH-SY5Y cells. These attempts failed and we therefore performed GST pulldown assays instead. SK-N-BE(2) cell extract was incubated with GST-HASHΔC or GST coupled to glutathione-sepharose beads. Bound proteins were subjected to SDS–PAGE and transferred to nitrocellulose filters. Immunodetection of the filters showed that both E2-2 and E12/E47 bound to GST-HASHΔC (Fig. 7B). Furthermore, ^{35}S-labeled SK-N-BE(2) cells were lysed and mixed with GST-HASHΔC or GST protein linked to glutathione-sepharose beads. After washing, the bound proteins were released by boiling, subjected to SDS–PAGE and transferred to a nitrocellulose filter, which was exposed to an X-ray film. The bands appearing on the film represent GST-HASHΔC interacting proteins in SK-N-BE(2) cells. A prominent band of approximately 90 kD, corresponding to the size of E2-2, was detected only in the fraction of eluted proteins from the GST-HASHΔC glutathione-sepharose beads (Fig. 7C). Immunodetection with a monoclonal anti-E2-2 antibody of the same filter revealed an identically sized band in crude cell extract from SK-N-BE(2) cells. Interestingly, only a small amount of proteins within the size-range of E12 or E47 (approximately 75 kD) were bound to GST-HASHΔC (Fig. 7C). These results indicate that E2-2, and not E12 or E47, is the preferential interaction partner of GST-HASHΔC in extracts from human neuroblastoma cells.

DISCUSSION

The importance of bHLH proteins for neuronal determination and differentiation is well established. Studies in *Drosophila* show that proneuronal bHLH genes control many aspects of neurogenesis, and a number of mammalian homologs of these genes have been identified (31). One of these homologs, *MASH-1*, is involved in the development of the neural crest-derived peripheral nervous system. The precursor cells of the childhood tumor neuroblastoma are of neural crest origin, and several studies indicate that the tumor cells arise as a consequence of blocked differentiation of sympathetic neuroblasts (13, 15). We have recently shown that *HASH-1* is expressed in neuroblastoma cell lines, and that this expression is down-regulated during induced differentiation (16). It is therefore of interest to understand the role of HASH-1 in neuroblastoma cells and one strategy to do that would be to identify interaction partners that might influence the transactivating capacity of HASH-1.

In order to form a functional DNA binding complex MASH-1 has to heterodimerize with other bHLH transcription factors. Additional factors, such as the MEF2 proteins, can modulate the transactivating capacity through direct interaction with MASH-1, or by synergistic activation of target genes with neighboring MASH-1 and MEF2 binding sites in their promoter region (32, 33). In an attempt to further explore this network of cross-talk between different types of transcription factors we screened a yeast two-hybrid cDNA library from neuroblastoma cells for proteins interacting with HASH-1. Two of the isolated clones encoded E2-2, a bHLH transcription factor belonging to the E-protein family. The interaction was verified both *in vitro* using GST pulldown assays, and *in vivo* using the mammalian two-hybrid system. We also showed that E2-2 and HASH-1 form a functional E-box binding complex with transactivating activity.

These results raise several questions regarding the formation of dimeric complexes between HASH-1 and E-proteins. Despite a low, but detectable expression of *E47* in SH-SY5Y cells, from which the yeast two-hybrid library was derived, no E12/E47 encoding clones were isolated when screening with HASHΔC as a bait. This could however partly be explained by our finding that a combination of HASH-1 and E47 in the yeast two-

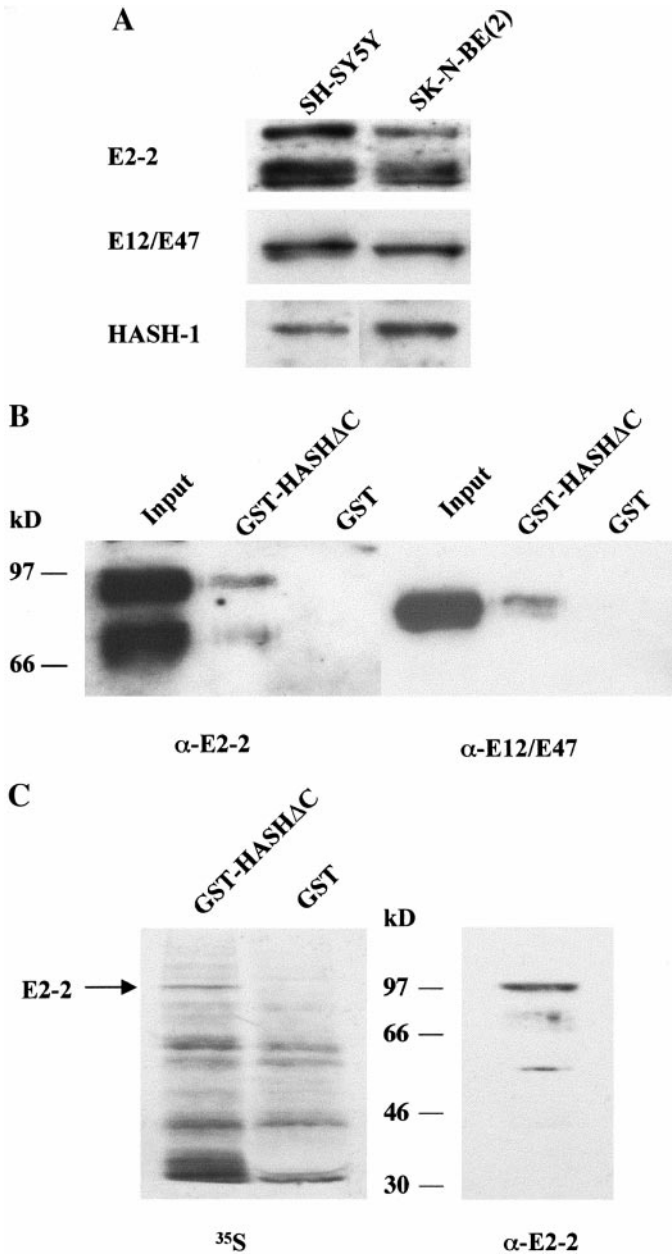

FIG. 7. GST pulldown assays confirming the HASH-1/E2-2 interaction. (A) Western blot analyses of extracts from neuroblastoma cell lines. Extracts were prepared from SH-SY5Y and SK-N-BE(2) neuroblastoma cells. After SDS–PAGE, the proteins were detected using antibodies directed towards E2-2, E12/E47 or MASH-1. (B) SK-N-BE(2) cell extract was incubated with GST or GST-HASHΔC glutathione-sepharose beads and the bound proteins were subjected to 7.5% SDS–PAGE. Crude cell extract from SK-N-BE(2) cells was run in a parallel lane. The proteins were transferred to a nitrocellulose membrane and the immunodetection was performed using a monoclonal anti-E2-2 antibody or a monoclonal anti-E12/E47 antibody. Size markers (in kilodaltons) are shown. (C) ^{35}S-methionine *in vivo* labeled cell extract from SK-N-BE(2) was incubated with GST or GST-HASHΔC coupled to glutathione-sepharose beads. After extensive washing, bound proteins were released by boiling and separated on a 7.5% SDS–PAGE gel. Crude cell extract from unlabeled SK-N-BE(2) cells was run in a parallel lane. The proteins were transferred to a nitrocellulose membrane and subjected to autoradiography (left panel). The size of E2-2 using crude cell extract was determined by immunoblotting using a monoclonal anti-E2-2 antibody (right panel). Size markers (in kilodaltons) are shown.

hybrid system gave rise to a very low reporter gene activation (data not shown). Since interaction between HASH-1 and E47 was readily detected in the mammalian two-hybrid system, in line with previously published data (29), one might speculate that factors present in mammalian cells facilitating HASH-1/E47 complexes are missing in yeast cells. In pulldown experiments using SK-N-BE(2) cell extracts we could show that both E12/E47 and E2-2 bound to GST-HASHΔC. Since these experiments are made with different antibodies, a quantitative estimation of the relative abundance of the different protein complexes is difficult to perform. In order to circumvent these problems we therefore analyzed GST-HASHΔC interacting proteins using ^{35}S-labeled SK-N-BE(2) cell extracts. The most prominent band obtained in these experiments corresponded to the size of E2-2, suggesting that E2-2, and not E12 or E47, is the preferred interaction partner of HASH-1 in neuroblastoma cells (Fig. 7C). More specific biochemical assays are however needed for a quantitative assessment of the binding affinities between HASH-1, E2-2 and the *E2a* gene products.

There are several different splice-variants of *E2-2*, with or without an additional exon of 4 amino acids (RSRS) just upstream of the basic domain (20). These splice-variants differ in their transactivating potential as shown in previous reports (21, 27). The full-length clone described in this paper represents a long splice-variant without RSRS, as described by Corneliussen *et al.* (20), and the E-box reporter data presented here show that this variant forms a transactivating complex together with HASH-1 (Fig. 5). These experiments also demonstrate that a homodimeric complex of E2-2 does not transactivate the E-box reporter construct, in line with previous studies on E2-2 using a B-cell-specific E-box containing reporter plasmid (34). Furthermore, recently published data show that homodimeric E2-2 without RSRS not only lacks transcriptional activity, but also inhibits transcription of MCK enhancer controlled reporter genes (35). It is therefore possible to envisage a situation in neuronal cells where E2-2 lacking RSRS, repress transcription of target genes by forming a homodimeric complex. By increased expression of tissue-specific bHLH proteins, such as HASH-1, a HASH-1/E2-2 complex is formed which is capable of transactivating the same target genes. We are currently investigating which splice-variants of *E2-2* that are predominantly expressed in neuroblastoma cells, and whether they repress or transactivate reporter gene activity.

To elucidate the biological function of E2-2, knockout mice have been produced. These mice are born with

low frequency and die for unknown reasons during their first postnatal week (36). Since our data suggest a role for E2-2 as being a functional partner of HASH-1, we investigated whether the *E2-2* knock-out mice showed any defects in the sympathetic nervous system that could be attributed to a functional interaction with HASH-1. We could however not detect any apparent structural changes or loss of cells in sympathetic ganglia, when serial sections were analyzed after immunohistochemical staining using an anti-tyrosine hydroxylase antibody (data not shown).

Albeit expressed in many tissues, some evidences indicate that E2-2 might have a specific function in neuronal tissues. Firstly, E2-2 is expressed at relatively high levels in the brain compared to the expression in most other tissues (27), and secondly, E2-2 has been shown to bind and regulate some neuron- and neuroendocrine-specific promoters, including the tyrosine hydroxylase enhancer and the somatostatin receptor II promoter (37, 38). In a recent study, Liu *et al.* (1998) reported the binding of E2-2 to the fibroblast growth factor promoter FGF-1.B (27). This promoter is specifically active in defined regions of the brain, and the FGF-1.B transcript has been found in the brain, gliomas and some glioblastoma cell lines (27). Interestingly, when crude extracts from brain were used in EMSAs using an E-box from the FGF-1.B promoter, an E2-2 containing complex was detected, and the authors conclude that E2-2 was associated with an unknown neuron-specific bHLH protein (27). In light of our data regarding the avid interaction between HASH-1 and E2-2 in neuroblastoma cells and the significant expression levels of both genes in the developing brain, we hypothesize that the unknown partner to E2-2 could be HASH-1.

Taken together, our study shows that HASH-1 can form a functional complex together with E2-2 as well as with E12 or E47, but in neuroblastoma cells E2-2 might be the preferential interaction partner. Furthermore, the splice-variant of E2-2 isolated from neuroblastoma cells does not activate an E-box reporter gene as a homodimer, but in complex with HASH-1 a potent transactivating complex is formed. This leads to a model where the regulation of bHLH neuronal target genes can be governed by several parameters, including not only expression levels of tissue-specific bHLH proteins, but also the expression levels and splice-pattern of the E-proteins.

ACKNOWLEDGMENTS

Special thanks are given to Dr. Thomas Grundström and Dr. Dan Holmberg for providing the *E2-2* knock-out mice. We also thank Dr. Mikael Sigvardsson for providing luciferase reporter and expression plasmids. This work was supported by grants from the Swedish Cancer Society, the Children Cancer Foundation of Sweden, Inga and John Hain's, Åke Wiberg's, HKH Kronprinsessan Lovisas förening för barnasjukvård, and Hans von Kantzow's Foundations, the Crafoord Foundation and Malmö University Hospital Research Funds.

REFERENCES

1. Littlewood, T. D., and Evan, G. I. (1995) *Protein Profile* **2,** 621–702.
2. Look, A. T. (1997) *Science* **278,** 1059–1064.
3. Rabbitts, T. H. (1994) *Nature* **372,** 143–149.
4. Ephrussi, A., Church, G. M., Tonegawa, S., and Gilbert, W. (1985) *Science* **227,** 134–140.
5. Murre, C., McCaw, P. S., Vaessin, H., Caudy, M., Jan, L. Y., Jan, Y. N., Cabrera, C. V., Buskin, J. N., Hauschka, S. D., Lassar, A. B., *et al.* (1989) *Cell* **58,** 537–544.
6. Benezra, R., Davis, R. L., Lockshon, D., Turner, D. L., and Weintraub, H. (1990) *Cell* **61,** 49–59.
7. Norton, J. D., Deed, R. W., Craggs, G., and Sablitzky, F. (1998) *Trends Cell Biol.* **8,** 58–65.
8. Johnson, J. E., Birren, S. J., and Anderson, D. J. (1990) *Nature* **346,** 858–861.
9. Lo, L. C., Johnson, J. E., Wuenschell, C. W., Saito, T., and Anderson, D. J. (1991) *Genes Dev.* **5,** 1524–1537.
10. Guillemot, F., Lo, L. C., Johnson, J. E., Auerbach, A., Anderson, D. J., and Joyner, A. L. (1993) *Cell* **75,** 463–476.
11. Tuttle, R., Nakagawa, Y., Johnson, J. E., and O'Leary, D. D. (1999) *Development* **126,** 1903–1916.
12. Casarosa, S., Fode, C., and Guillemot, F. (1999) *Development* **126,** 525–534.
13. Hoehner, J. C., Gestblom, C., Hedborg, F., Sandstedt, B., Olsen, L., and Påhlman, S. (1996) *Lab. Invest.* **75,** 659–675.
14. Brodeur, G. M., Maris, J. M., Yamashiro, D. J., Hogarty, M. D., and White, P. S. (1997) *J. Pediatr. Hematol. Oncol.* **19,** 93–101.
15. Gestblom, C., Grynfeld, A., Öra, I., Örtoft, E., Larsson, C., Axelson, H., Sandstedt, B., Cserjesi, P., Olson, E. N., and Påhlman, S. (1999) *Lab. Invest.* **79,** 67–79.
16. Söderholm, H., Örtoft, E., Johansson, I., Ljungberg, J., Larsson, C., Axelson, H., and Påhlman, S. (1999) *Biochem. Biophys. Res. Commun.* **256,** 557–563.
17. Johnson, J. E., Birren, S. J., Saito, T., and Anderson, D. J. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 3596–3600.
18. Henthorn, P., McCarrick-Walmsley, R., and Kadesch, T. (1990) *Nucleic Acids Res.* **18,** 678.
19. Hu, J. S., Olson, E. N., and Kingston, R. E. (1992) *Mol. Cell. Biol.* **12,** 1031–1042.
20. Corneliussen, B., Thornell, A., Hallberg, B., and Grundström, T. (1991) *J. Virol.* **65,** 6084–6093.
21. Skerjanc, I. S., Truong, J., Filion, P., and McBurney, M. W. (1996) *J. Biol. Chem.* **271,** 3555–3561.
22. Zhuang, Y., Barndt, R. J., Pan, L., Kelley, R., and Dai, M. (1998) *Mol. Cell. Biol.* **18,** 3340–3349.
23. Bain, G., Maandag, E. C., Izon, D. J., Amsen, D., Kruisbeek, A. M., Weintraub, B. C., Krop, I., Schlissel, M. S., Feeney, A. J., van Roon, M., *et al.* (1994) *Cell* **79,** 885–892.
24. German, M. S., Blanar, M. A., Nelson, C., Moss, L. G., and Rutter, W. J. (1991) *Mol. Endocrinol.* **5,** 292–299.
25. Sigvardsson, M., O'Riordan, M., and Grosschedl, R. (1997) *Immunity* **7,** 25–36.
26. Bjelfman, C., Meyerson, G., Cartwright, C. A., Mellström, K., Hammerling, U., and Påhlman, S. (1990) *Mol. Cell. Biol.* **10,** 361–370.
27. Liu, Y., Ray, S. K., Yang, X. Q., Luntz-Leybman, V., and Chiu, I. M. (1998) *J. Biol. Chem.* **273,** 19269–19276.

28. Bain, G., Gruenwald, S., and Murre, C. (1993) *Mol. Cell. Biol.* **13,** 3522–3529.
29. Johnson, J. E., Zimmerman, K., Saito, T., and Anderson, D. J. (1992) *Development* **114,** 75–87.
30. Påhlman, S., Meyerson, G., Lindgren, E., Schalling, M., and Johansson, I. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 9994–9998.
31. Lee, J. E. (1997) *Curr. Opin. Neurobiol.* **7,** 13–20.
32. Mao, Z., and Nadal, G. B. (1996) *J. Biol. Chem.* **271,** 14371–14375.
33. Black, B. L., Ligon, K. L., Zhang, Y., and Olson, E. N. (1996) *J. Biol. Chem.* **271,** 26659–26663.
34. Henthorn, P., Kiledjian, M., and Kadesch, T. (1990) *Science* **247,** 467–470.
35. Chen, B., and Lim, R. W. (1997) *J. Biol. Chem.* **272,** 2459–2463.
36. Zhuang, Y., Cheng, P., and Weintraub, H. (1996) *Mol. Cell. Biol.* **16,** 2898–2905.
37. Pscherer, A., Dorflinger, U., Kirfel, J., Gawlas, K., Ruschoff, J., Buettner, R., and Schule, R. (1996) *EMBO J.* **15,** 6680–6690.
38. Yoon, S. O., and Chikaraishi, D. M. (1994) *J. Biol. Chem.* **269,** 18453–18462.