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Oscillating Expression of c-Hey2 in the Presomitic Mesoderm Suggests That the Segmentation Clock May Use Combinatorial Signaling through Multiple Interacting bHLH Factors

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Vertebrate somitogenesis comprises the generation of a temporal periodicity, the establishment of anteroposterior compartment identity, and the translation of the temporal periodicity into the metameric pattern of somites. Molecular players at each of these steps are beginning to be identified. Especially, members of the Notch signaling cascade appear to be involved in setting up the somitogenesis clock and subsequent events. We had previously demonstrated specific expression of the mHey1 and mHey2 basic helix-loop-helix (bHLH) factors during somitogenesis. Here we show that perturbed Notch signaling in Dll1 and Notch1 knockout mutants affects this expression in the presomitic mesoderm (PSM) and the somites. In the caudal PSM, however, mHey2 expression is maintained and thus is likely to be independent of Notch signaling. Furthermore, we analysed the dynamic expression of the respective chicken c-Hey1 and c-Hey2 genes during somitogenesis. Not only is c-Hey2 rhythmically expressed across the chicken presomitic mesoderm like c-hairy1, but its transcription is similarly independent of de novo protein synthesis. In contrast, the dynamic expression of c-Hey1 is restricted to the anterior segmental plate. Both c-Hey genes are coexpressed with c-hairy1 in the posterior somite half. Further in vitro and in vivo interaction assays demonstrated direct homo- and heterodimerisation between these hairy-related bHLH proteins, suggesting a combinatorial action in both the generation of a temporal periodicity and the anterior-posterior somite compartmentalisation. © 2000 Academic Press

Key Words: Hey1; Hey2; c-hairy1; bHLH; Notch signaling; segmentation clock; somitogenesis; PSM.

INTRODUCTION

Somites establish a metameric body plan along the anterior-posterior axis that remains evident in the segmented pattern of the axial skeleton, skeletal musculature, vasculature, and peripheral nerves. In vertebrate embryos, somites periodically bud off the rostral end of the caudal unsegmented paraxial mesoderm—also known as the presomitic mesoderm (PSM) or the segmental plate—which forms two mesenchymal rods lying either side of the caudal neural tube. Thus, at the rostral developmentally mature end of each PSM rod, groups of cells periodically condense and undergo a mesenchymal-to-epithelial transition concomitant with the addition of new mesenchymal cells at the posterior end or tail bud. These newly formed epithelial somites are already patterned into anterior and posterior segment halves, which differ in their cell-adhesion proper-
ties and gene-expression profiles. The formation of a new somite pair is repeated every 90 min in chicken and mouse embryos to generate a species-specific, constant number of somites. Somitogenesis thus comprises a highly coordinated set of events including generation of periodicity, cell condensation, establishment of anterior–posterior polarity, and epithelialisation (for review see Gossler and Hrabe de Angelis, 1998; Pourquie, 2000). Only recently, gene expression patterns and in vitro experiments in chicken embryos as well as genetic studies in mice have provided insight into some of the molecular mechanisms underlying these processes.

Previously, a number of theoretical models such as the “clock and wavefront” (Cooke and Zeeman, 1976) had proposed the existence of a clock in PSM cells to explain the rhythmic generation of new somites. The first molecular evidence for such an intrinsic clock operating to regulate timing and positioning of somite formation was obtained through the discovery of the chicken hairy-related gene, c-hairy1, which was found to be expressed in cycling waves along the PSM with a temporal periodicity of 90 min (Palmeirim et al., 1997). In explant culture experiments, it was shown that c-hairy1 oscillations are independent of cell movements or of a propagating signal from the tail bud. Instead, the cells within the segmental plate cycle autonomously between c-hairy1 “on” or “off” expression states until they segment and then sustain c-hairy1 expression in the posterior somite half. Recently, a second chicken hairy-related gene, c-hairy2, has been identified and was shown to cycle in synchrony with c-hairy1 across the PSM. In contrast to c-hairy1, c-hairy2 subsequently becomes restricted to the anterior somite compartment (Jouve et al., 2000). A closely related murine gene, Hes1, is also expressed rhythmically within the PSM, suggesting that similar mechanisms operate during somitogenesis in chicken and mice. Interestingly, Hes1 expression in the PSM is absent in Delta-like (Dll1) homozygous null mutants. This indicates that Hes1 transcription is regulated by the Notch signaling pathway which has been demonstrated to play a key role in the establishment of anterior–posterior somite polarity and boundary formation (Pourquie, 1999). These data imply that the cycling expression of hairy-like genes, indicative of segmentation clock activity, requires Notch signaling.

Another component of this pathway, lunatic fringe (Ifng), a vertebrate homologue of the Drosophila Notch modulator fringe, is not only required for somite boundary formation (Evrad et al., 1998; Zhang and Gridley, 1998), but also shows cycling RNA expression in chicken and mouse PSM tissue (Aulehla and Johnson, 1999; Forsberg et al., 1998; McGrew et al., 1998). The finding that oscillating Ifng—but not c-hairy1/2—expression is dependent on de novo protein synthesis places the c-hairy genes as direct outputs of a segmentation clock in which they act upstream of or in parallel to Ifng (McGrew and Pourquie, 1998).

Apart from these expression studies and extrapolations from related murine and Drosophila genes, little is known about c-hairy1/2 function or target genes. Drosophila hairy and the rodent homologues of the Hes gene family encode basic helix-loop-helix (bHLH) proteins that have been shown to function as transcriptional repressors (for review see Fisher and Cadry, 1998; Kageyama and Nakanishi, 1997). They act by forming either homodimers or heterodimers with ubiquitously expressed class A bHLH proteins that directly bind to specific DNA sequences in target promoters to repress transcription (Oellers et al., 1994; Ohsako et al., 1994; Van Doren et al., 1994). There is also evidence supporting another mode of action whereby Hes proteins inhibit the activity of a complexed BHLH partner through formation of a nonfunctional heterodimer (Dawson et al., 1995; Sasai et al., 1992). Therefore, definition of c-hairy1/2 protein dimerization properties and interaction partners will be a crucial step towards our understanding of their role during somitogenesis.

We and others have previously identified a novel subfamily of hairy-related bHLH transcription factors—the Hey genes (also known as HRT or Hesr genes)—that are expressed in the murine PSM and young somites and that are potential targets of the Delta–Notch signaling pathway (Kokubo et al., 1999; Leimeister et al., 1999; Nakagawa et al., 1999). Here we describe the expression of Hey genes in murine Notch pathway mutants. We show that mHey1 and mHey2 expression is affected in Dll1 or Notch1 knockout mice during somitogenesis. However, maintenance of mHey2 expression in the caudal PSM of these mutants suggests that mHey2 transcription is partially independent of Notch signaling. To better address a potential dynamic expression of the Hey genes we cloned the chicken c-Hey1 and c-Hey2 genes. c-Hey2 expression was found to cycle in synchrony with c-hairy1 in the presomitic mesoderm even in isolated PSM explants or after blockade of new protein biosynthesis. Furthermore, we demonstrate a direct interaction between these different bHLH factors that should have functional consequences for either partner.

**MATERIALS AND METHODS**

Genotyping of Mouse Embryos

Dll1−/− embryos were obtained by mating mice heterozygous for the Dll1 targeted mutation (Hrabe de Angelis et al., 1997). Embryos were genotyped by PCR analysis of the yolk sacs as described before. Notch1 homozygous null mutant embryos were generated by intercrossing mice heterozygous for the deletion of part of the putative Notch1 promoter plus the exon containing the signal peptide (Radtke et al., 1999). We observed the same phenotype as described for the knockout mutants made by Swiatek et al. (1994) or Conlon et al. (1995). Primers for genotyping yolk sac DNA were as follows: 5′ arm-up (CAATTCTCGGTGGTGTTCAACC), del.arm-lower (CCAAACGTTCATCTTACCTG), and 3′ arm-lower (TAAAGCTCAGTGGGTGCCT). Separate allele-specific polymerase chain reactions were used to detect wild-type (5′ arm-up/del.arm-lower, 1.8 kb) and mutant (5′ arm-up/3′ arm-lower, 1.3 kb) alleles.
Cloning of c-Hey1 and c-Hey2

Approximately 1 μg of whole chicken embryo total RNA (HH stage 20–22) was employed for RT-PCR using the Titan kit (Roche Biochemicals) as suggested by the manufacturer. Degenerate primers were designed within conserved regions of human and mouse Hey protein sequences based on Blocks and Codehop analysis (Henikoff and Henikoff, 1994; Rose et al., 1998): http://blocks.fhcrc.org/blocks/). Primers used were as follows: c-Hey1, cb2 (AAGGCCGAGACTTCTGCAATGACNGT) and cb3 (GAAG-GCCGCACCTCTNGTCCCCCA); c-Hey2, cb2c (GCCAGGGC-CTGGCCAYNAYTYTATG) and cb3c (GCCCGAGGGCCG-GTANGGYYTRTT). After 40 amplification cycles fragments of the expected size were cut from agarose gels, subcloned into pBluescript or pDK101, and sequenced.

A longer c-Hey2 cDNA clone could be isolated from the RZPD chicken cDNA library No. 573 (HH stage 3–6). Sequences corresponding to exons 2 and 3 are absent from this clone. These sequences were cloned via RT-PCR using primers within flanking exons.

Chick Embryos and Somite Staging

Fertilised chicken eggs (Gallus gallus) purchased from commercial sources (Bayrische Landesanstalt fuer Tierzucht, Kitzingen) were incubated for 48 to 48 h in a humidified atmosphere at 38°C. The embryos were staged by the number of somite pairs. Using the somite staging system developed by Ordahl (1993) the last formed somite was designated somite 1 and the nascent somite somite 0.

Chick Explants

Chick embryos between 10 and 20 somites were isolated and the caudal portion was divided into two halves by cutting along the neural tube. The explants were cultured on polycarbonate filters (0.8 μm; Millipore) floating on top of culture medium composed of Leibowitz L15 (Gibco) supplemented with 5% chicken serum, 10% foetal calf serum, 0.2% sodium bicarbonate, and 50 U/ml penicillin/streptomycin. Five different series of experiments were performed as described by Palmeirim et al. (1997): (A) One half explant was immediately fixed and the other was cultured for 30 to 120 min prior to fixation. (B) One half explant was further subdivided along the anterior–posterior axis into two pieces and both halves were cultured for 1 or 2 h. (C) The PSM of one half was dissected from surrounding tissues and both halves were cultured for the same time period. (D) One half explant was fixed and the other half was cultured for 60 min in medium containing 20 μM cycloheximide (Sigma). (E) One half explant was fixed for 60 min in normal medium and the other half in medium containing 20 μM cycloheximide.

Whole-Mount in Situ Hybridisation

The m-Hey1, m-Hey2, c-hairy1, and lunatic fringe antisense mRNA probes were produced as described (Leimeister et al., 1999; Palmeirim et al., 1997; Sakamoto et al., 1997). c-Hey1 and c-Hey2 cDNAs encompassing most of the coding regions were cloned into pBluescript or pDK101, respectively, and used to generate antisense mRNA transcripts. Mouse embryos were prepared and hybridised as described by Leimeister et al. (1998).

Chicken embryos were fixed overnight in 4% formaldehyde/2 mM EGTA at 4°C, washed two times for 10 min in PBS, dehydrated in a methanol series, and stored in 100% methanol at −20°C. Whole-mount in situ hybridisation of chicken embryos was performed with an automated in situ processor (In situPro; Abimed) programmed according to the protocol described previously (Leimeister et al., 1998) with the following modifications: instead of using protease K the RIPA buffer treatment was extended to three times 20 min. RNase digestion was omitted and the antibody was applied for 4 h at room temperature.

In Vitro Translation and GST Pulldown Assays

GST fusion proteins including the bHLH domains and flanking regions (e.g., Orange domain) were produced for mouse Hey1 (aa 50–200), human Hey2 (aa 31–208), and chicken c-hairy1 (aa 1–163). A GST–E12 clone encompassing the entire coding region of E12 was obtained from Th. Braun (Halle). Induced bacterial cultures (DH5α or BL21-LysS) were sonicated in PBS containing 0.5 mM PMFS. Triton X-100 (1%) was added to the lysates and insoluble material was removed by centrifugation. Cleared lysates containing approximately 1 μg of fusion protein with 100-fold excess of bacterial proteins as nonspecific competitor were incubated for 2 h at 4°C with 4 μl full-length in vitro-translated and [35S]methionine-labelled proteins in bead binding buffer (50 mM phosphate buffer, pH 7.4, 150 mM potassium chloride, 1 mM magnesium chloride, 10% glycerol, 1% Triton X-100). After binding to glutathione–agarose, complexes were washed four times with bead binding buffer. Retained proteins were separated on 10% polyacrylamide gels and visualised by fluorography.

Yeast Two-Hybrid Interaction Assays

All cDNAs were cloned in frame into pGBK7T7 (GAL4-DBD) and pGAD7T7 (GAL4-AD) (Clontech) and confirmed by sequencing. GBK-Hey2 and GAD-Hey2 encode amino acids 1–269 of the murine Hey2 protein. Hey1 cDNA fragments coding for the bHLH (aa 39–121), the bHLH–Orange (aa 39–175), and the C-terminal half (aa 173–299) of Hey1 were amplified by PCR and cloned into pGBK7T7 or pGAD7T7. Both c-hairy1 isoforms were subcloned from pGEX-chairy1 into pGAD7T7. The insert was also shuttled into pGBK7T7. The CDNAs of mouse E12 (aa 48–146, D29919), ITF1 (aa 457–544, X52078), and ITF2 (aa 512–600, X52079) bHLH domains were amplified by PCR and cloned into pGBK7T7. Detailed information about cloning strategies and oligonucleotide primers is available upon request.

Yeast two-hybrid interaction assays were performed according to the manufacturer’s protocol (Clontech; MATCHMAKER Two-Hybrid System 3). Yeast strains AH109 and Y187 were transformed with pGBK7T7 and pGAD7T7 plasmids, respectively, and tested for autonomous activation of the reporter genes HIS3, ADE3, and lacZ. Protein–protein interactions were assayed by cotransformation and mating assays in parallel. Cotransformants and diploids were plated on selection plates lacking Leu/Trp (SD/-Leu/Trp) and Ade/His/Trp (SD/-Ade/His/Trp) and incubated for 6 days at 30°C. To test the activation of the lacZ reporter gene Ade‘His‘ transformants were examined by an X-gal colony-lift filter assay (Breeden and Nasmyth, 1985; Durfee et al., 1993; Staudinger et al., 1993). To quantify the lacZ activity cotransformants were done in strain Y187, and β-galactosidase activity was measured using a liquid assay with ONPG as substrate. Control plasmids (SV40 large T antigen, p53, lamin C) were from Clontech.
mHey2 expression in the caudal PSM is not affected in Dll1 and Notch1 knockout mutants. (A–D) Comparison of mHey1 and mHey2 expression in wild-type (+/+ ) and homozygous mutant (−/−) Dll1 knockout mice at E11.5; dorsal view. (A, B) Expression of mHey1 at the level of the nascent somite seen in wild-type embryos (A) is lost in Dll1−/− mutants (B). (C, D) Although mHey2 expression in the nascent somite (C) is also lost in Dll1−/− mutants (D), the caudal expression domain is apparently normal in extent and intensity. (E–H) Comparison of mHey1 and mHey2 expression in wild-type and homozygous knockout Notch1 embryos at E9.5; lateral view. In wild-type embryos, both mHey1 (E) and mHey2 (G) are expressed in clearly demarcated domains in the forming somite and in the PSM. In mutant embryos, mHey1 (F) and mHey2 (H) staining is diffuse without clearly demarcated expression domains. The caudal margin of the youngest somite is indicated by an arrow. The strong mHey2 signal on the ventral side is derived from the aorta. Rostral is to the top. Scale bar, 200 μm.

FIG. 2. Variable expression patterns for mHey2 are observed in the murine PSM. Dorsal view of the tail region of E10.5 mouse embryos hybridised with mHey2. In each image, the caudal margin of the last formed somite (l) is marked by an arrow. All embryos display a band of mHey2 expression in the rostral PSM at the level of the nascent somite. In the caudal PSM, the mHey2 expression comprises a small (A, one-quarter) or a large (B, three-quarter) domain. The staining at the caudal tip is decreased in a number of embryos, suggestive of cycling expression (C). Rostral is to the top. Scale bar, 200 μm.
RESULTS

Hey2 Expression during Somitogenesis inDll1 and Notch1 Mutant Mice

Hey genes are expressed in the murine PSM and the caudal half of the somites in a way similar to the Notch target Hes1 (Leimeister et al., 1999). Furthermore, all Hey promoters not only contain binding sites for RBPJκ, a transcriptional regulator required for Notch function, but also can be activated in vitro by a constitutively active form of Notch1 (Mair and Gessler, 2000). This strongly suggests that Hey genes may also be direct targets of Notch signaling events. In the mouse, knockout mutants have been instrumental in documenting Notch-dependent expression of Hey genes comparing different mouse embryos of the same developmental stage, such that mHey1 is always restricted to the rostral part of the PSM (Kokubo et al., 1999; Leimeister et al., 1999; Nakagawa et al., 1999), while mHey2 is dynamically expressed throughout this tissue (Fig. 2). These variations in mHey expression patterns are reminiscent of the oscillating expression of the chicken c-hairy1 gene and prompted us to compare their expression domains. Since these genes are derived from different species a direct side-by-side comparison was not possible. We decided to clone the chicken Hey genes because the segmentation clock has been more thoroughly studied in the chick embryo, which is also more amenable to experimental manipulations during embryogenesis.

Cloning of Chicken c-Hey Genes

Amino acid alignments of human, mouse, and Drosophila Hey proteins (Steidl et al., 2000) were used to identify conserved sequence blocks (Blocks server, http://blocks.fhcrc.org/). These were in turn employed to design degenerate primers for RT-PCR based on suggestions from the Codehop web server (Henikoff and Henikoff, 1994; Rose et al., 1998; http://www.blocks.fhcrc.org/codehop.html). Fragments of 595 and 597 bp could be amplified from chicken embryo RNA with primer pairs specific for Hey1 and Hey2, respectively. For Hey2, a longer cDNA clone could be isolated from an E1 chick embryo cDNA library that includes 5′ and 3′ untranslated sequences, but lacks sequences corresponding to exons 2 and 3 of human or mouse Hey2. The latter likely represents a rare splicing artefact since we detected only RNA transcripts including these exons by subsequent RT-PCR of chicken embryo RNA. Conceptual translation and sequence comparison as well as evolutionary analysis using ClustalX clearly identified our chicken c-Hey1 and c-Hey2 clones as the true orthologs of the corresponding human and mouse Hey1 and Hey2 genes (Fig. 3). The chicken and human Hey2 proteins are identical in the basic and HLH domains and the overall similarity is still 92%, which compares favourably with the human–mouse value of 94%. Although we only have limited sequence information for c-Hey1, similarity scores for the Orange domain and the rather divergent C-terminus are nevertheless in the 88% range.

Analysis of c-Hey1 and c-Hey2 Expression during Somitogenesis

To investigate a potential dynamic transcription of the c-Hey1 and c-Hey2 genes in the PSM, we analysed their expression during chick somitogenesis and compared it to that of c-hairy1. By analysing a large number of chicken embryos containing 13–20 somites, we found c-Hey1 to be expressed with some variability, but restricted specifically to the rostralmost PSM just prior to somite condensation (Figs. 4A and 4B). In contrast, c-Hey2 displays the same variety of phases of expression across the entire PSM as previously described for c-hairy1 (Figs. 4C–4E). Thus, c-Hey2 may have the same oscillating expression pattern as c-hairy1, which is outlined schematically in Fig. 4F: a large caudal expression domain is progressively shifted rostrally and finally becomes restricted to a half-somite-wide domain in the rostralmost part of the PSM which is maintained in the formed somite. As soon as the former caudal band becomes reduced to a rostral stripe, a new caudal expression domain appears.

To demonstrate the cyclic nature of the c-Hey2 signal in the PSM and its correlation with somite formation we
performed a series of embryo culture experiments. Caudal chick embryo explants were bisected along the neural tube and one half was immediately fixed while the other half was cultured in vitro for different time intervals. After 60 min of culture the two halves displayed different phases of c-Hey2 expression as shown in Fig. 5: in the immediately fixed half c-Hey2 expression demarcates a broad band in the rostral PSM, while in the cultured half the c-Hey2 signal spans the caudal part (Fig. 5A). This caudal c-Hey2 expression moved farther rostral when the second half was cultured longer (Figs. 5B and 5C). If the culture period is extended to 90 min or longer a new somite pair forms and the expression patterns in the two sides are the same, demonstrating that this expression is cycling and linked to somite formation.

**c-Hey2 Oscillations Display the Same Characteristics as Those of c-hairy1**

For a side-by-side comparison of c-Hey1/2 expression with the c-hairy1 and lfg expression domains in the segmental plate, embryos were cut along the midline and the two halves were processed separately for each probe. In accordance with the expression seen in whole embryos a correlation between c-Hey1 expression with either c-Hey2 or c-hairy1 was not observed in the caudal PSM, but only at the level of the rostral PSM and the nascent somite (Figs. 6A and 6B). In the rostral PSM, all three genes are expressed first in a broader band that seems to condense to the width of the caudal half of the maturing somite I. However, the position of the c-Hey2 signal overlaps precisely with that of c-hairy1 and lfg throughout the PSM (Figs. 6C and 6D). In fully formed somites both c-Hey genes continue to be coexpressed with c-hairy1 in the posterior somitic half, whereas in the chick lfg is expressed in the anterior somitic half.

In a second set of experiments, the caudal part of one half explant was removed and both explants were cultured for the same time. After 1 or 2 h of in vitro development, both halves showed the same c-Hey2 expression pattern and after 2 h an additional somite was formed in both halves (Figs. 7A and 7B). Thus, similar to the observations made with c-hairy1 and lfg, c-Hey2 mRNA cycling does not require a propagating signal from the posterior PSM. To further investigate whether rhythmic c-Hey2 transcription is also independent of surrounding tissues, we analysed c-Hey2 expression in caudal embryo explants in which the PSM of one half was separated from lateral plate, neural
tube, endoderm, and ectoderm. After 3 h in culture, the intact half and the isolated PSM displayed the same expression pattern, indicating that cycling c-Hey2 expression is an autonomous property of this tissue (Fig. 7C).

We then sought to determine whether c-Hey2 mRNA cycling is independent of new protein biosynthesis as demonstrated for c-hairy1. To address this, one embryo half was cultured for 60 min with medium containing cycloheximide, while the other half was immediately fixed. The incubated and the fixed halves showed different expression patterns, indicating that c-Hey2 cycling is not blocked by inhibition of protein synthesis (Fig. 8A). In agreement with this, c-Hey2 expression patterns were identical when one half was cultured with and the other without cycloheximide for the same time period (Fig. 8B). Control experiments demonstrated successful blocking of Ifng, but not c-hairy1 cycling under the same conditions (data not shown). These findings indicate not only that c-Hey2 and c-hairy1 are coexpressed but also that there is a strong likelihood that they are regulated in similar fashions.

**Interaction between Hey and c-hairy1 Proteins**

bHLH proteins reputedly function as homodimers or as heterodimers—frequently with a partner from the more ubiquitously expressed class A bHLH proteins (e.g., E12). Taking into account that c-Hey2 and c-hairy1 mRNAs are coexpressed and potentially coregulated, it is possible that the corresponding bHLH proteins may physically interact in cells of the presomitic mesoderm to form heterodimers. To test this possibility in vitro we performed GST pulldown assays with GST–bHLH or GST–bHLH-Orange domain fusion proteins and full-length target proteins. As there were no full-length c-Hey and c-hairy1 cDNA clones available initially, we relied on their highly similar murine or human counterparts for these experiments. In this assay c-hairy1 and Hey2 proteins not only formed homodimers, but also were able to generate heterodimers with essentially the same efficiency (Fig. 9). Furthermore, both proteins interacted with ITF2, but not with E12 (not shown), two members of the ubiquitously expressed class A bHLH proteins. Interaction was also seen between Hey1 and c-hairy1 proteins, but the expression analysis described above would predict that such heterodimers form only during the last stages of somitogenesis or in newly formed somites.

Since it is difficult to quantitate binding strengths in the above assays, we extended the interaction analysis to include the yeast two-hybrid system. Bait and prey vectors contained the same protein domains that were used in the GST pulldown analysis. All constructs were tested for the lack of self-activation of the reporter genes HIS3, ADE2, and lacZ, either on their own or when cotransformed with the irrelevant partners SV40 large T antigen, p53, and lamin C, respectively.

Interaction between different domains of the Hey1, Hey2, c-hairy1, and class A bHLH proteins was tested by cotransformation of bait and prey vectors as well as by mating assays with identical results. Scoring was performed in a stepwise fashion: robust interactions yielded growth when plated on quadruple selection plates (SD–/–4x; +++ in Table 1). For weaker interactions an intermediate plating step on double-selection plates was necessary before transfer (SD–/–2x; +/++ in Table 1). All positive interactions were further quantitated by β-galactosidase assays via filter lifts or lysate assays of strain Y187. The principally interacting partners detected by the pulldown assay correspond to those observed in vivo, but the strength of the interaction differed considerably.

The Hey1 bHLH–Orange (aa 38–175) domain and Hey2 bHLH–Orange (aa 1–269) domain interacted strongly with the corresponding domain of c-hairy1 (Table 1). This interaction was similar in strength to that seen for c-hairy1 homodimerisation. Hey1 and Hey2 homodimers, on the other hand, generated a much weaker signal in all assays (no direct growth on SD–/–4x plates, very low β-galactosidase activity). Quite interestingly, the shorter Hey1 bHLH protein lacking the Orange domain exhibited a much reduced binding to c-hairy1 compared to the bHLH–Orange version.

**FIG. 4.** Expression of c-Hey1 and c-Hey2 in caudal chicken embryos. (Top and middle) Dorsal view of the caudal region of 15- to 17-somite stage chick embryos hybridised with c-Hey1 (A, B) and c-Hey2 (C–E). (F) Schematic representation of c-hairy1 expression and the correlation with somite formation. (A, B) c-Hey1 is expressed in the rostral part of the PSM with only limited variations. (C–E) c-Hey2 expression in the PSM can be divided into the same three phases (I, II, III) as shown schematically for c-hairy1 (F). Phase I: a large caudal expression domain is observed in the caudal PSM. The rostral staining corresponds to the residual expression domain of the previous wavefront. Phase II: the signal is shifted to a broad intermediate band, while the caudalmost expression disappears. Phase III: in the rostral PSM the next somite is formed. A new caudal expression appears and the former wavefront has condensed in the rostral PSM. The caudal margin of the last formed somite is marked by a red arrowhead. Rostral is to the top. Scale bar, 200 μm.

**FIG. 5.** Cycling expression of c-Hey2 is linked to somite formation. The caudal part of stage 15 to 20 chicken embryos was cut along the midline into two halves. One half (left side) was fixed immediately and the other half (right side) was cultured. Both halves were hybridised with a c-Hey2 probe. (A) The right half was cultured for 60 min. The different expression patterns between the two halves indicate the dynamic nature of the c-Hey2 expression. (B, C) The experimental half (right) was cultured for 90 or 105 min, respectively: the c-Hey2 expression domain in the right half has moved rostrally and after 105 min displays the same pattern as the left half, concomitant with the formation of a new somite (the slight delay in cycling expression may be due to culture conditions). Rostral is to the top. Arrowheads in (C) indicate segmented somites.
The discovery of the Hey genes as a new subfamily of hairy-related genes poses questions concerning functional redundancy or complementary roles for the different members within and between these subfamilies. Shared characteristics of the hairy/E(spl)/Hes proteins are an invariant proline residue in the DNA-binding basic domain, an HLH domain required for protein dimerisation, an Orange domain involved in functional specificity, and a C-terminal WRPW motif that may bind corepressors of the groucho family. Hey proteins differ from these "classical" hairy proteins particularly in the basic domain, which contains a glycine residue in place of the proline, and in the WRPW-containing C-terminus (Kokubo et al., 1999; Leimeister et al., 1999; Nakagawa et al., 1999). The existence of a Drosophila Hey gene/protein provides evidence for a distinct function of Hey proteins that may be independent of that of the classical hairy family (Frise et al., 1997; Leimeister et al., 1999; Steidl et al., 2000). This is supported by the different phenotypes reported for the Drosophila mutants of these two genes: while hairy regulates segmentation and sensory hair development (Ish-Horowicz et al., 1985; Nusslein-Volhard and Wieschaus, 1980; Rushlow et al., 1989), aberrant dHey expression causes disruption of the central nervous system development (Frise et al., 1997). In vertebrates, reports on mutant phenotypes are available only for Hes1 and Hey2. Targeted disruption of Hes1 results in precocious neurogenesis and impaired endodermal endocrine development, whereas no segmentation defect has been observed (Ishibashi et al., 1995; Jensen et al., 2000; Jouve et al., 2000). For Hey2 a specific role in the formation of arterial vessels was recently revealed through the identification of a Hey2 point mutation as the molecular cause of a zebrafish gridlock mutant (Zhong et al., 2000). However, it is likely that in these mutant embryos a lack of a somitogenic phenotype is due to functional redundancy by other bHLH family members.

**DISCUSSION**

This may point to a novel stabilising role of the Orange domain in vivo. The interactions found were specific since all assays with the carboxy-terminal half of Hey1 as either bait or prey essentially generated negative results. Even the rather promiscuous bHLH domain of the ubiquitously expressed E protein E12 did not interact with either Hey or c-hairy1 proteins. However, class A bHLH proteins may still be capable of interacting with these factors since ITF1, in particular, and to some extent ITF2 displayed a weaker, but still robust interaction with all Hey/c-hairy1 proteins.

**FIG. 6.** Comparison of c-Hey1 and c-Hey2 gene expression with that of c-hairy1 and lfng. Caudal regions of chick embryos were divided sagittally and the two halves were hybridised with different probes. (A, B) The left half was hybridised with c-hairy1 and the right half with c-Hey1. While the expression domains of both genes differ in the caudal part of the PSM, they overlap at the nascent somite stage and in the caudal somite half. (C, D) c-Hey2 (right side) is coexpressed with c-hairy1 (C, left side) or lfng (D, left side) in the PSM. Arrows indicate the caudal border of the youngest somite (I). Rostral is to the top.

**FIG. 7.** Rhythmic c-Hey2 expression is independent of adjacent tissues. (A, B) The right side of chicken embryo explants was further divided into two halves. All pieces were cultured for the same time (60 min in A, 120 min in B) and hybridised with c-Hey2. The intact (left) and the subdivided (right) half display the same expression pattern, indicating that c-Hey2 mRNA cycling is independent of a caudal propagating signal. (C) The PSM of the right half was separated from surrounding tissues and cultured together with the left intact half for 180 min. Similar expression patterns observed in both halves show that c-Hey2 cycling is independent of adjacent structures. Arrowheads indicate the caudal border of the youngest somite (I). Rostral is to the top.

**FIG. 8.** c-Hey2 mRNA cycling is independent of de novo protein synthesis. (A) In half-embryo explants in which the left half was immediately fixed and the right half was cultured for 60 min in the presence of 20 μM cycloheximide (CHX) the expression of c-Hey2 differs. (B) The control explant (left) was cultured without and the experimental half (right) was cultured with CHX for 60 min. Here, the expression of c-Hey2 was identical in both halves, indicating that mRNA cycling does not depend on new protein synthesis. Rostral is to the top.
TABLE 1
In Vivo Protein Interaction Analysis Using the Yeast Two-Hybrid System

<table>
<thead>
<tr>
<th>GAL4 DNA binding domain fusion</th>
<th>Hey1 bHLH</th>
<th>Hey1 bHLH-OR</th>
<th>Hey1 C-terminus</th>
<th>Hey2 bHLH-OR</th>
<th>E12 bHLH</th>
<th>ITF-1 bHLH</th>
<th>ITF-2 bHLH</th>
<th>T antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hey1-bHLH</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Hey1-bHLH-OR</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Hey1-C-terminus</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hey2-bHLH-OR</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>c-hairy1-bHLH-OR</td>
<td>+</td>
<td>+++</td>
<td>(7, 22)</td>
<td>(7, 70)</td>
<td>(2, 18)</td>
<td>(0, 61)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>p53</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(58, 20)</td>
<td>+++</td>
</tr>
</tbody>
</table>

Note. Interaction strength is defined as +++ (direct growth on 4x selection plates), ++/+ (intermediate plating on 2x selection plates necessary, but subsequent efficient/retarded growth on 4x plates and positive lacZ filter assay) or – (no growth on 4x selection plates). Numerical values in brackets denote lacZ activity in liquid assays.

Like the Drosophila hairy and Enhancer of split (E(sp1)) or the mammalian Hes proteins, Hey genes could act either independently (hairy-like) or as targets (E(sp1)-like) of the Notch signaling pathway. The striking coexpression of Hey genes with members of the Notch signaling pathway and the Notch targets Hes1 and Hes5 during somitogenesis would suggest a link between Hey gene expression and Notch signaling. However, analyses of mHey1 and mHey2 gene expression in Dll1 and Notch1 knockout mutants have generated conflicting results. On the one hand, an apparently unaltered mHey2 expression within the posterior PSM of Dll1 and Notch1 knockout mice is in line with an independent regulation. Moreover since expression of Hey1, Hes1, and Hes5 is lost in Dll1 mutants, mHey2 is the only hairy/E(sp1)-like gene, thus far, that remains expressed in the PSM of this Notch pathway mutant (Barrantes et al., 1999; Jouve et al., 2000). On the other hand, the expression of both mHey genes is affected at the level of the nascent and fully formed somites, pointing to a Delta-Notch-dependent expression of mHey genes during anteroposterior compartmentalisation and somite formation. This is also in line with analyses of the mHey1/2 promoters that suggest that bothHey genes can be directly regulated by the Notch signaling pathway (Maier and Gessler, 2000). Thus, at least for mHey2 a dual type of regulation with only partial dependence on Notch signaling appears possible.

Hey2 expression in the murine PSM generates patterns similar to the cycling expression of c-hairy1 in chicken PSM tissue. Since the segmentation clock can be more easily studied in the chicken embryo we cloned the chicken c-Hey1 and c-Hey2 genes and employed them for detailed studies and direct comparison with c-hairy1 expression and regulation. Sequence alignment with other hairy-related proteins from chicken or other vertebrate species strongly suggests that c-Hey1/2 represent the true chicken homologues of the previously described human and mouse Hey1 and Hey2 proteins (Leimeister et al., 1999; Sasai et al., 1992).

Like their murine counterparts, c-Hey1 and c-Hey2 are expressed in the posterior compartment of the somites and in the PSM. In the latter tissue c-Hey1 expression is restricted to the anterior PSM while mHey2 mRNA cycles along the entire caudorostral extent of the segmental plate. Furthermore, we demonstrate that the tissue-autonomous oscillating nature of c-Hey2 transcription is not disrupted by blocking protein synthesis. Thus, c-Hey2 mRNA is coexpressed with and presumably regulated in a similar way as the previously described c-hairy1 gene and the related gene c-hairy2. The synchrony observed in the dynamic expression profiles of c-Hey2, c-hairy1, c-hairy2, and Ifng suggests that these genes are all subject to regulation by the same “clock” postulated to generate the temporal periodicity of somitogenesis. The contrasting response of c-hairy1 and Ifng upon inhibition of protein synthesis in chicken explant culture experiments placed c-hairy1 upstream of or parallel to the Notch modulating factor Ifng (McGrew et al., 1998). Likewise, coexpression and presumably coregulation with c-hairy1 implicates c-Hey2 at a position similar to that of c-hairy1 in the signaling hierarchy.

It is interesting to note that c-hairy2, whose expression is similar to c-hairy1 with respect to cycling and independence from new protein biosynthesis, is the putative chicken homologue of the well-established Notch target gene Hes1 (Jouve et al., 2000). With this in mind, transcriptional independence of de novo protein synthesis does not necessarily imply a function of the hairy-like genes upstream of Ifng and the Notch signaling pathway. It would be equally plausible to postulate cycling c-Hey and c-hairy gene expression in the chicken PSM as outputs of a system.
that regulates generation, nuclear access, or transcriptional potency of activated intracellular Notch molecules.

C-Hey1 expression overlaps with that of c-Hey2 or c-hairy1 only at the level of the forming somite and in the caudal somite compartment, implying a role in the establishment of anterior–posterior identity. Thus, c-hairy1 is spatially and temporally coexpressed with c-Hey1 and c-Hey2 during avian somitogenesis. Since we could show that c-hairy1 and Hey proteins efficiently interact to form heterodimers it appears quite likely that they may not act independent of each other. Rather, it could be the combinatorial effect of multiple interacting bHLH factors that ultimately results in the correct regulation of downstream target genes. Nothing is known yet about the specificity of Hey proteins to recruit secondary interacting proteins—e.g., corepressors like groucho-related proteins or other activation factors. Therefore, it remains an open issue to whether this multiplicity of bHLH proteins forms a redundant network or generates diversity of interaction. The complete divergence of more than half of the protein sequence and the presence of a typical C-terminal groucho-binding motif WRPW in c-hairy, but a divergent and embedded YRPW/YQPW sequence in Hey proteins, rather suggest independent or complementary functional properties.

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