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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.

***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-

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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 (Pourquié, 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* (*lfng*), a vertebrate homologue of the *Drosophila* Notch modulator *fringe,* is not only required for somite boundary formation (Evrard *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 *lfng*—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 *lfng* (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 Caudy, 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 dimerisation 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 (CAACTTCCTGTGGGTCCAACC), del.arm-lower (CCAAAGCTGTCACTCTTCCTGCT), and 3′arm-lower (TTAAGCCTCAGTGTAGGGCTG). 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 (AAGGCCGAGATCCTGCARATGACNGT) and cb3 (GAAGGCGCCGACCTCNGTNCCCCA); *c-Hey2,* cbc2 (GCCCACGCCCTGGCCAYNGAYTTYATG) and cbc3r (GCCCCAGGGCCGGTANGGYTTRTT). 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 40 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 I 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 cultured 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 (InsituPro; Abimed) programmed according to the protocol described previously (Leimeister *et al.*, 1998) with the following modifications: instead of using proteinase 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 PMSF. 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 [^{35}S]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 pGBKT7 (GAL4-DBD) and pGADT7 (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 38–175), and the C-terminal half (aa 173–299) of Hey1 were amplified by PCR and cloned into pGBKT7 or pGADT7. Both c-hairy1 isoforms were subcloned from pGEX-chairy1 into pGADT7. The insert was also shuttled into pGBKT7. 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 pGADT7. 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 GBKT7 and GADT7 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/$-2x$) and Ade/His/Leu/Trp (SD/$-4x$). Clones that did not grow on SD/$-4x$ were replica plated from SD/$-2x$ to the high-stringency SD/$-4x$ plates 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 cotransformations 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.

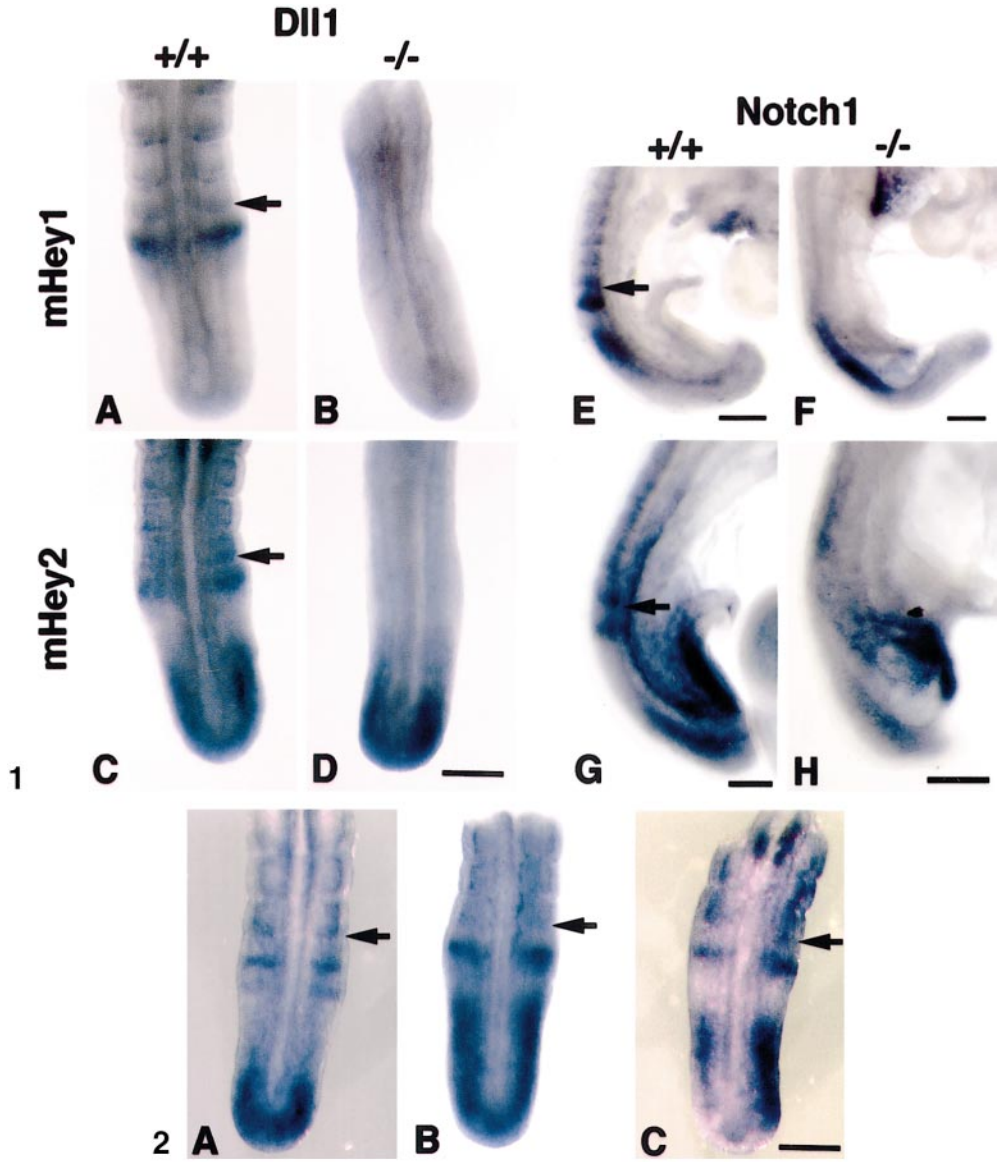


FIG. 1. *mHey2* expression in the caudal PSM is not affected in *Dll1* and *Notch1* knockout mutants. (A–D) Comparison of *mHey1* and *mHey2* expression in wild-type (+/+) and homozygous mutant (−/−) *Dll1* knockout mice at E11.5; dorsal view. (A, B) Expression of *mHey1* at the level of the nascent 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 (I) 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 in Dll1 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* (Maier 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 downstream genes.

Consequently, we analysed *mHey1* and *mHey2* expression in *Dll1* and *Notch1* knockout mice (Hrabe de Angelis *et al.*, 1997; Radtke *et al.*, 1999). In 11.5-day-old *Dll1*−/− embryos *mHey1* and *mHey2* gene expression is absent in the rostral PSM and the somites (Figs. 1A–1D). However, although we never observed staining for either of the *Hey* genes in the disorganized cells of the mutant somites, we cannot rule out the possibility that a weaker and more diffuse expression is still present but falls below the levels of background staining. Interestingly, a strong signal for *mHey2* was still observed in the caudal PSM of *Dll1* mutants, suggesting that *mHey2* expression is partially independent of the Notch signaling pathway (Fig. 1D). In *Notch1* knockout mutants at E9.5 (the last viable stage), we found not only *mHey2* but also *mHey1* expression in the PSM, although this expression no longer showed sharply demarcated boundaries between the region of the PSM and the formed somites (Figs. 1E–1H). The less pronounced perturbation of *Hey* gene expression and somitogenesis in general in *Notch1* knockout mice is very likely due to functional compensation by PSM expression of *Notch2* as has been proposed to be the case for a number of other genes analysed in this same context (Barrantes *et al.*, 1999; Conlon *et al.*, 1995; Swiatek *et al.*, 1994). Thus, the results obtained with the *Dll1* knockout mutants may be more clear cut as to the dependence of *Hey* gene expression on this signaling pathway.

In the PSM, we observed variable expression patterns for the *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/blocks/). 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

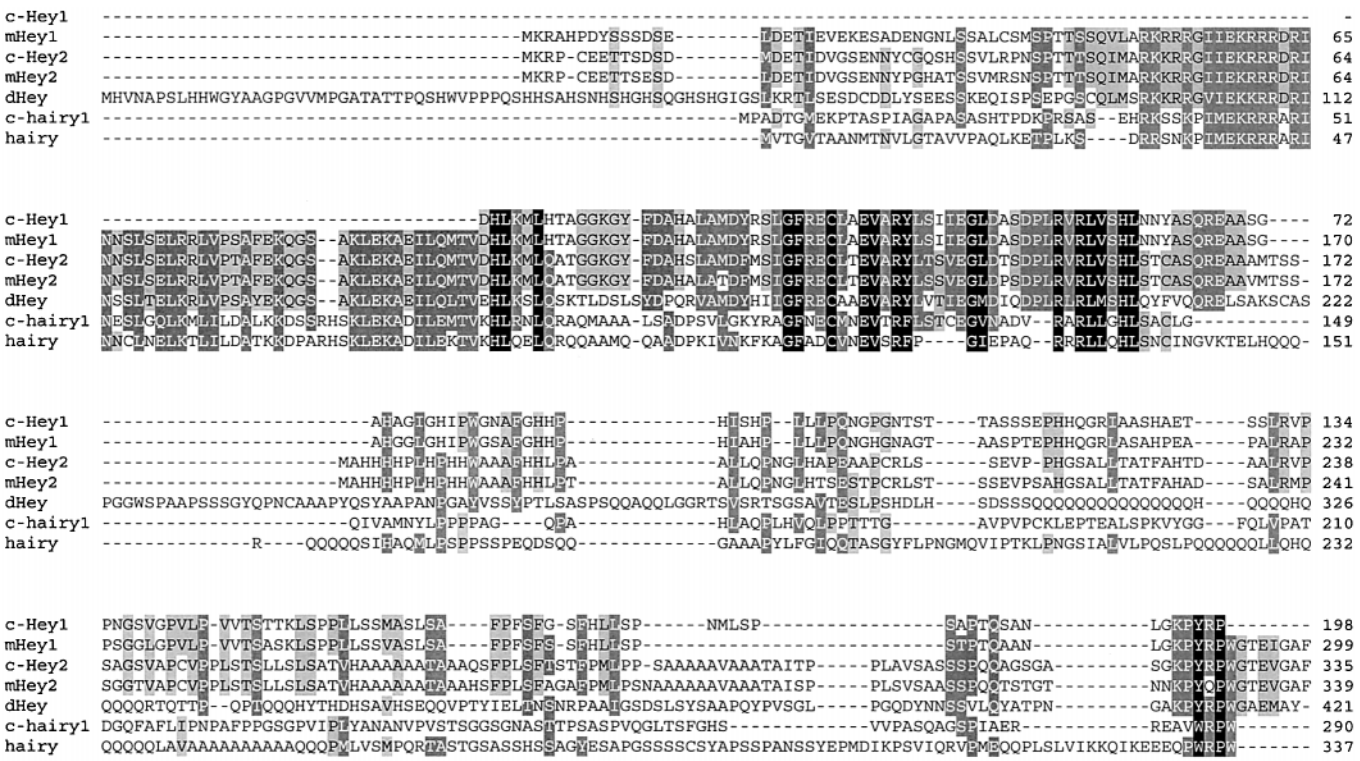


FIG. 3. Sequence alignment of Hey and hairy proteins. Amino acid sequences were aligned using ClustalX and similarities shaded by GeneDoc. For c-Hey2 the coding region has been assembled from cDNA clones and RT-PCR fragments. Only a partial protein sequence is available for c-Hey1. All other sequences were taken from GenBank entries. Basic, HLH, and Orange domains are highlighted by shaded boxes.

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 *lfng* 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 *lfng* 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 *lfng* is expressed in the anterior somite 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 *lfng*, *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 *lfng*, 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 likelyhood 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* 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.

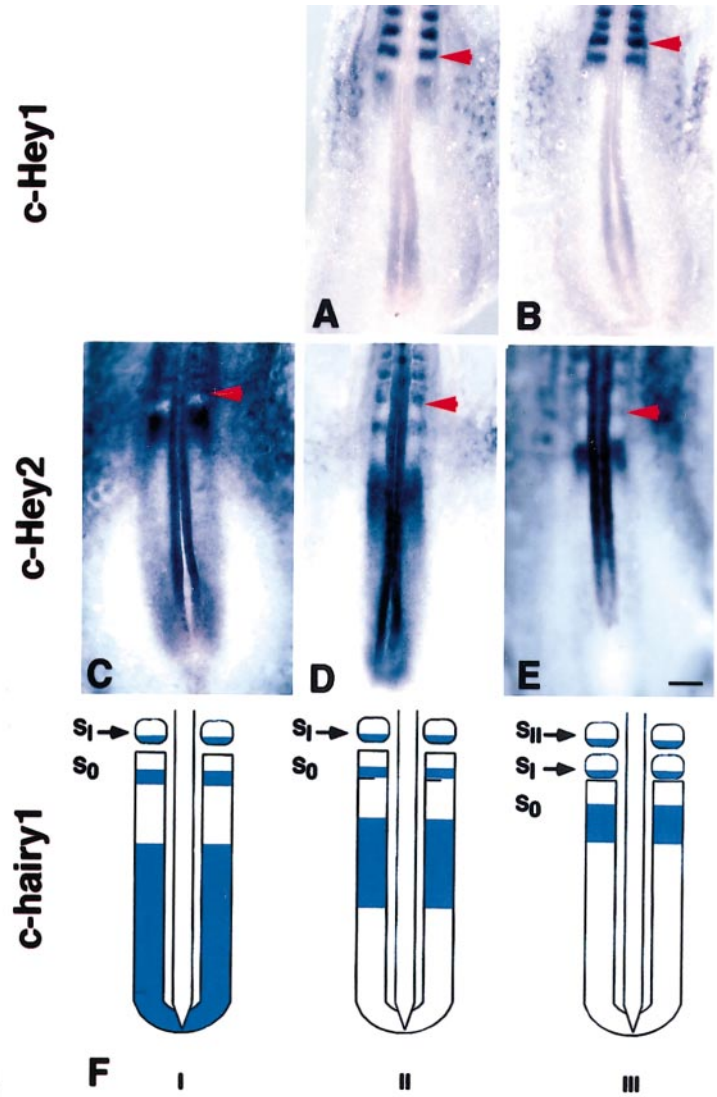
Since it is difficult to quantitate binding strengths in the above assays, we extended the interaction analysis to include the yeast two-hybrid system. Bait and prey vectors contained the same protein domains that were used in the GST pulldown analysis. All constructs were tested for the lack of self-activation 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 tests 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.



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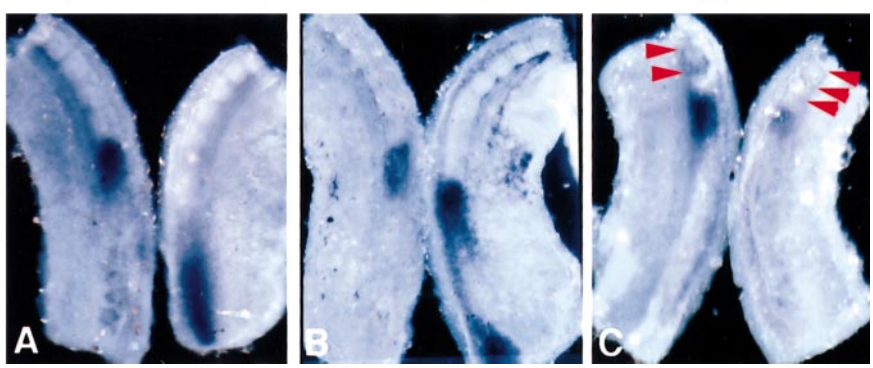

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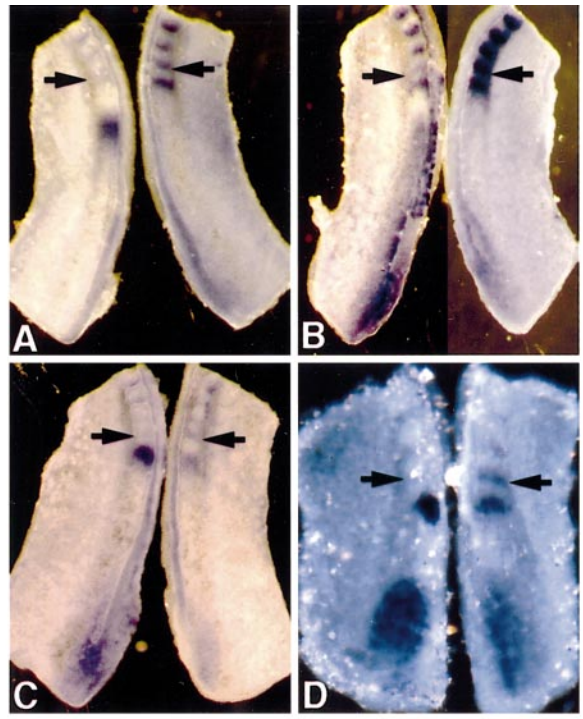

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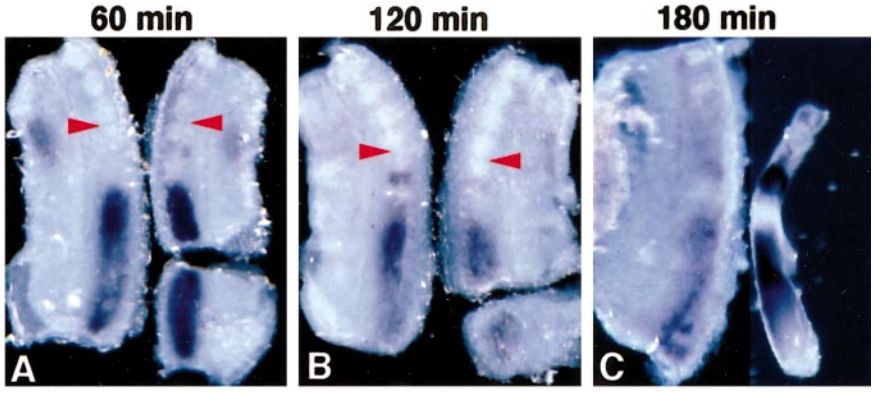

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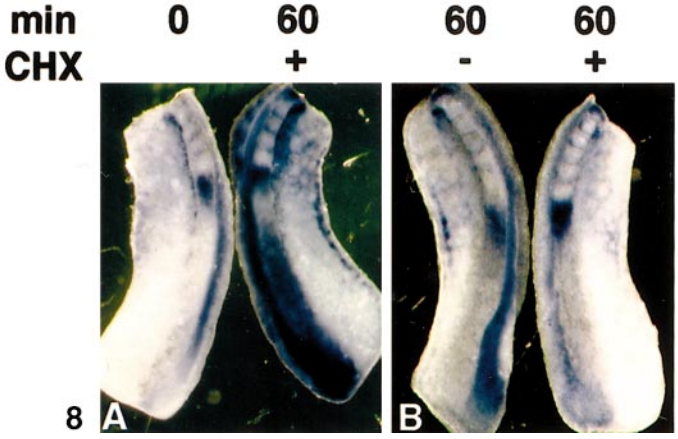

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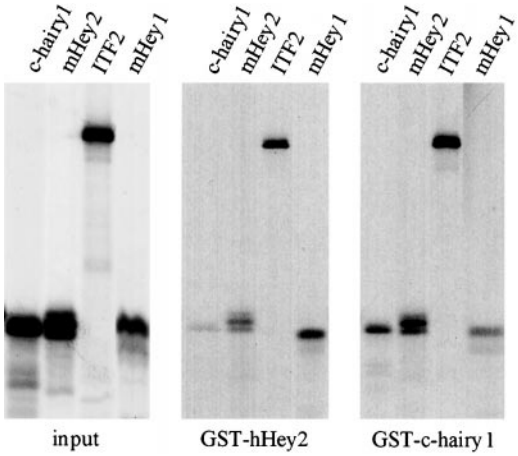

FIG. 9. Interaction of hairy and Hey proteins *in vitro*. Full-length *mHey1, mHey2, c-hairy1,* and *ITF2* cDNAs were transcribed and translated *in vitro* to generate ^{35}S-labeled proteins. Clarified bacterial lysates containing GST–hHey2 and GST–c-hairy1 fusion proteins were used to capture and purify target proteins. A fraction of the labelled input proteins (20%, left) was separated together with bound proteins and visualized by fluorography. Calculated molecular weights are mHey1 (33 kDa), mHey2 (36 kDa), c-hairy1 (30 kDa), and ITF2 (68 kDa).

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.

DISCUSSION

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 YRPW-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 the 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.

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

GAL4 DNA binding domain fusion	GAL4 activating domain fusion								
	Hey1 bHLH	Hey1 bHLH-OR	Hey1 C-terminus	Hey2 bHLH-OR	c-hairy1 bHLH-OR	E12 bHLH	ITF-1 bHLH	ITF-2 bHLH	T antigen
Hey1–bHLH	+	+	−	+	+	−	+	+	−
Hey1–bHLH–OR	+	+	−	+	+++ (7, 80)	−	++ (0, 50)	+	−
Hey1–C-terminus	−	−	−	−	−	−	−	−	−
Hey2–bHLH–OR	+	+	−	+ (0, 61)	+++ (8, 15)	−	++ (2, 75)	+	−
c-hairy1–bHLH–OR	+	+++ (7, 22)	−	+++ (7, 70)	+++ (2, 18)	−	++ (0, 61)	+	−
p53	−	−	−	−	−	−	−	−	+++ (58, 20)

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

Like the *Drosophila* hairy and Enhancer of split (E(spl)) or the mammalian Hes proteins, *Hey* genes could act either independently (*hairy*-like) or as targets (*E*(*spl*)-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*(*spl*)-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 both *Hey* 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 *c-Hey2* 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 *lfng* 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 *lfng* upon inhibition of protein synthesis in chicken explant culture experiments placed *c-hairy1* upstream of or parallel to the *Notch* modulating factor *lfng* (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 *lfng* 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 as 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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