

Notch signalling is required for cyclic expression of the hairy-like gene *HES1* in the presomitic mesoderm

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SUMMARY

Somitic segmentation provides the framework on which the segmental pattern of the vertebrae, some muscles and the peripheral nervous system is established. Recent evidence indicates that a molecular oscillator, the 'segmentation clock', operates in the presomitic mesoderm (PSM) to direct periodic expression of *c-hairy1* and *lunatic fringe* (*l-fng*). Here, we report the identification and characterisation of a second avian hairy-related gene, *c-hairy2*, which also cycles in the PSM and whose sequence is closely related to the mammalian *HES1* gene, a downstream target of Notch signalling in vertebrates. We show that *HES1* mRNA is also expressed in a cyclic fashion

in the mouse PSM, similar to that observed for *c-hairy1* and *c-hairy2* in the chick. In *HES1* mutant mouse embryos, the periodic expression of *l-fng* is maintained, suggesting that *HES1* is not a critical component of the oscillator mechanism. In contrast, dynamic *HES1* expression is lost in mice mutant for *Delta1*, which are defective for Notch signalling. These results suggest that Notch signalling is required for hairy-like genes cyclic expression in the PSM.

Key words: Presomitic mesoderm, *HES*, hairy-related genes, *lunatic fringe*, Notch signalling, Chick, Mouse

INTRODUCTION

Segmentation of the vertebrate body is a sequential process which leads to the formation of reiterated mesodermal structures called somites. Somitic segmentation provides the frame on which the segmental pattern of the vertebrae, some muscles and the peripheral nervous system is established (Keynes and Stern, 1988; McGrew and Pourquie, 1998). In amniotes like mouse or chick, a defined number of somites (52 in the chick and approximately 65 in the mouse) are produced in a craniocaudal fashion, one pair every 90 minutes, from an apparently mesenchymal unsegmented tissue called the presomitic mesoderm (PSM). Somites initially appear as epithelial spheres, which subsequently differentiate in response to signals provided by the surrounding structures. They generate the skeletal muscles of the body as well as the axial skeleton and the dermis of the back (Christ and Ordahl, 1995; Gossler and Hrabe de Angelis, 1998). Recently, two distinct but probably interrelated molecular systems have been shown to operate during vertebrate segmentation.

The first one is the Notch signalling pathway for which gain- and loss-of-function studies in mouse and frog have established a role in somitogenesis (McGrew and Pourquie, 1998). The analysis of mice mutant for the Notch1 receptor (Conlon et al., 1995; Swiatek et al., 1994), for its ligands Delta1 (Dll1) (Hrabe de Angelis et al., 1997) and Delta3 (Dll3) (Kusumi et al., 1998) and for RBPJk, a transcription factor mediating its cellular response (Oka et al., 1995), have demonstrated the significance of these proteins for establishing the somitic boundaries as well as in defining anterior and posterior somitic identities. In these mouse mutants, however, the principle segmental arrangement of somitic tissue is retained, indicating that the Notch pathway is unlikely to be responsible for establishing the primary metamer pattern, but rather for the precise positioning of segment borders.

The second system is a molecular oscillator, the 'segmentation clock', which operates autonomously in the PSM and results in the periodic production of mRNAs coding for the transcription factor *c-hairy1* and for the secreted protein *lunatic fringe* (*l-fng*). This oscillator or clock was identified on

the basis of the periodic expression of *c-hairy1* in the PSM (Palmeirim et al., 1997). Expression of *c-hairy1* mRNA appears as a wavefront which sweeps across the whole PSM once during each somite formation. This wave-like appearance of the *c-hairy1* mRNA expression profile does not result from cell movements, nor from signal propagation in the PSM but rather from locally coordinated pulses of mRNA production. These oscillations of *c-hairy1* mRNA have been proposed to identify a molecular clock linked to segmentation (Palmeirim et al., 1997) consistent with previous theoretical models (Cooke, 1998; Meinhardt, 1986; Stern and Vasilaukas, 1998). The existence of such a clock was proposed to generate a temporal periodicity which could then be converted into the spatial periodicity of the somites.

Evidence for the periodic expression pattern of *l-fng* in the chick and mouse PSM has provided a link between the segmentation clock and the Notch signalling pathway (Aulehla and Johnson, 1999; Forsberg et al., 1998; McGrew et al., 1998). *l-fng* is a vertebrate homolog of the fly *fringe* gene which is known to act as a modifier of the Notch response (Irvine, 1999) and which is required for establishment of the dorsoventral boundary of the wing and the eye imaginal disk (Panin et al., 1997; Papayannopoulos et al., 1998). In the mouse, *l-fng* is important for positioning and refinement of somite boundaries and for the normal arrangement of rostral and caudal somitic compartments (Cohen et al., 1997; Evrard et al., 1998; Johnston et al., 1997; Zhang and Gridley, 1998). In mouse and chick, *l-fng* is expressed according to a dynamic pattern similar to that of *c-hairy1* suggesting that it is regulated by the same segmentation clock (Aulehla and Johnson, 1999; Forsberg et al., 1998; McGrew et al., 1998). Therefore one role of the clock might be to periodically modulate the Notch signalling pathway in order to produce somitic boundaries in an iterative fashion (Pourquie, 1999).

Here, we report the identification and characterization of a second avian *hairy*-related gene, *c-hairy2*, which is expressed in a periodic fashion in the PSM. The sequence of this gene is closely related to the mammalian *HES1* gene (Feder et al., 1993, 1994; Sasai et al., 1992), which is thought to act downstream of the Notch signalling pathway (Jarriault et al., 1995, 1998). This prompted us to investigate expression of *HES1* mRNA in the mouse PSM, which proves to be expressed in a cyclical fashion similar to that observed in the chick. In *HES1* mutant embryos (Ishibashi et al., 1995), the periodic expression of *l-fng* is maintained, suggesting that *HES1* is not a critical component of the clock. In contrast *HES1* dynamic expression is lost in the Notch pathway in mice mutant for *Dll1*. These results suggest that Notch signalling is an important component of the segmentation clock mechanism.

MATERIALS AND METHODS

Cloning of *c-hairy2*

c-hairy2 was isolated by screening a chick embryo cDNA library with a *c-hairy1* probe (Palmeirim et al., 1997). A *c-hairy1* fragment of 117 bp was used to screen a random primed cDNA library in λ gt10, prepared from stage 10–14 chick embryos. Three clones were obtained, all of them truncated in the 5' region. A PCR reaction was used to obtain the missing region with an antisense primer: 5'-CATCGGCACCTGGCTTCGAG-3', recognizing a specific sequence

of *c-hairy2* (CKPGAD), and a sense degenerated primer: 5'-ATGCCIGCIGTIATGGA(A/G), corresponding to the most N-terminal sequence, which is highly conserved between HES1 family members (Fig. 1). This produced a 600 bp band, which has been cloned in a pGEMT vector and sequenced. We also detected a sequencing error in the *c-hairy1* sequence published in (Palmeirim et al., 1997). This error introduced a difference of 20 amino acids in the C-terminal part of the protein when compared to the published sequence. The corrected sequence is shown in Fig. 1.

In vitro culture of chick and mouse explants

Fertilised chick (*Gallus gallus*, JA57) eggs, obtained from commercial sources, were incubated for up to 48 hours in a humidified atmosphere at 38°C. The embryos were staged by number of somite pairs formed. The somite staging criteria defined by Ordahl (1993) was used throughout this study. The most recently formed epithelial somite is termed SI, the last but one somite is called SII and so on. We call the forming somite, whose boundaries are not completely formed, somite 0 (S0) and we call blocks of PSM cells of one somite length, located caudal to S0, somite -I, -II and so on.

For the mouse explants, E10.5 embryos were harvested and their caudal part including the PSM with ectoderm, endoderm and the last one or two formed somites were bisected along the midline. Because *HES1* expression in the neural tube obscures the PSM expression pattern, the PSM was dissected out from the surrounding structures for each half embryo. Similar results were obtained with the dissected PSM and with the entire half embryo except that the observation was much easier in the dissected PSM. One half was fixed immediately and the other was cultured for 30 minutes to 2 hours as described for chick explants (Palmeirim et al., 1997) except for the culture medium, which was replaced by HybernateE medium (Gibco BRL).

Whole-mount in situ hybridisation and histology

The *c-hairy2* probe was produced from a 350 bp 3' fragment of the *c-hairy2* cDNA cloned in pGEMT which excludes the highly conserved b-HLH domain. It was linearised with *NcoI* and transcribed with SP6 polymerase. The *c-hairy1* probe is described by Palmeirim et al. (1997). The *HES1* probe was a gift from Dr François Guillemot (IGBMC, Strasbourg). It was produced from a 900 bp fragment cloned in Bluescript, linearised with *PstI*, and transcribed with T7 polymerase. Whole-mount in situ hybridisation was carried out according to the procedure described by Henrique et al. (1995). Stained embryos were processed for sectioning using a Leica vibatome after albumin/gelatin inclusion. 40 μ m sections were collected on microscope slides and mounted in aquatex (Merck).

Genotyping of *HES1* mutant mice

To determine the genotypes of embryos from heterozygote intercrosses, we performed PCR reactions with yolk sac DNA with the following primers. The sense primer, 5'-TGGGATGTGGGG-ACATGCGGG-3', and the antisense primer, 5'-TCACCTCGTTC-ATGCACTCG-3', produced a 850 bp wild-type band. The sense primer, 5'-GCAGCGCATCGCCTTCTATC-3', and the same antisense primer as above, produced a 1500 bp mutant band.

RESULTS

Cloning of *c-hairy2*, a chick gene highly related to chick *c-hairy1* and to mammalian *HES1*

The *c-hairy2* gene is expressed in the PSM in a highly dynamic fashion similar to that reported for *c-hairy1* and *l-fng* (Aulehla and Johnson, 1999; Forsberg et al., 1998; McGrew et al., 1998; Palmeirim et al., 1997). *c-hairy2* was isolated by screening a cDNA library with a *c-hairy1* probe (Palmeirim et al., 1997) (see Materials and Methods).

A

c-hairy2	1	MPADV-MEKSSASPAATPASINATPDKPCTAAEHRKSSKPIMEKRRRRARINESLGQLKT
HER6	1	MPADI-MEKNSSSPVAATPASVNTTPDKPKTASEHRKSSKPIMEKRRRRARINESLGQLKT
mHES1	1	MPADI-MEKNSSSPVAATPASVNTTPDKPKTASEHRKSSKPIMEKRRRRARINESLGQLKT
rHES1	1	MPADI-MEKNSSSPVAATPASVNTTPDKPKTASEHRKSSKPIMEKRRRRARINESLGQLKT
x-hairy1	1	MPADV-MEKNSSSPVAATPASVNTTPDKPKTASEHRKSSKPIMEKRRRRARINESLGQLKT
c-hairy1	1	MPADTGMKPTASPIAGAPASASHTPDKPRSASEHRKSSKPIMEKRRRRARINESLGQLKM
x-hairy2	1	MPADT-MEKPTASPIAGAPASASQTPDKPKSASEHRKSSKPIMEKRRRRARINESLGQLKT
c-hairy2	60	LILDALKKSSSRHSKLEKADILEMTVKHLRSLQRAQMTAALSTDPVTLGKYRAGFSECMN
HER6	60	LILDALKKSSSRHSKLEKADILEMTVKHLRNLQRAQMTAALNTDPTVLGKYRAGFSECMN
mHES1	60	LILDALKKSSSRHSKLEKADILEMTVKHLRNLQRAQMTAALSTDPVTLGKYRAGFSECMN
rHES1	60	LILDALKKSSSRHSKLEKADILEMTVKHLRNLQRAQMTAALSTDPVTLGKYRAGFSECMN
x-hairy1	60	LILDALKKSSSRHSKLEKADILEMTVKHLRNLQRAQMTAALSTDPVTLGKYRAGFSECMN
c-hairy1	61	LILDALKKSSSRHSKLEKADILEMTVKHLRNLQRAQMAAALSDAPSVLTKYRAGFNECMN
x-hairy2	60	LILDALKKSSSRHSKLEKADILEMTVKHLRNLQRAQMAALTSDPSVLTKYRAGFNECMN
c-hairy2	107	EVTRFLSTCEGVNAEVRTRLLGHLASCTQINAINYPV-----PPPLP-----PP
HER6	113	EVTRFLSTCEGVNTEVRTRLLGHLASCTQINAINYPV-----PPPLP-----SV
mHES1	121	EVTRFLSTCEGVNTEVRTRLLGHLASCTQINAINYPV-----PPPLP-----SGPAGPQH
rHES1	121	EVTRFLSTCEGVNTEVRTRLLGHLASCTQINAINYPV-----PPPLP-----SGPAGPQH
x-hairy1	112	EVTRFLSTCEGVNTEVRTRLLGHLASCTQINAINYPV-----PPPLP-----AY
c-hairy1	110	EVTRFLSTCEGVNADVRARLLGHLASCTQINAINYPV-----PPPLP-----LA
x-hairy2	103	EVTRFLSTCEGVNTEVRTRLLGHLSSCTQINAINYPV-----PPSS-----Q-
c-hairy2	157	PAAFGPPLVP---PGGGAGPLPTVPCKPG-----ADAAKVGG-FQLVLASDGGQFAFLI
HER6	169	NPWFKIPSAT---QQANVPLSGVPCKSGSSNLTSDATKVVGG-FQLVLPATDGGQFAFLI
mHES1	177	APFAPPPLVPIPGGAAPPGPSAPCKLGSQA---GEAAKVGG-FQVVPAPDGGQFAFLI
rHES1	176	APFAPPPLVPIPGGAAPPGPSAPCKLGSQA---GEAAKVGG-FQVVPAPDGGQFAFLI
x-hairy1	163	GQPMVQLP----GAAPQSSPAPIACKMGPP---VEAAKVGG-FQLVLPAPDGGQFAFLI
c-hairy1	159	QPLHVQLPP-----TTGAVPVCKLEP---TEALSPKVGG-FQLVLPATDGGQFAFLI
x-hairy2	153	QPLHVQLPS-----STPAPMPISCKVNP---AEAISPKVGG-FQLVLPATDGGQFAFLI
c-hairy2	187	PSAAFAAG-GAVLPIYGGPP-----TAATTASPPGP
HER6	200	PNAAFAAPN-GPVIPYANNSN-----TPVPVAVSPGA
mHES1	210	PNGAFAHS-GPVIPYVTSNSGTS-----VGPNVSPSSG
rHES1	209	PNGAFAHS-GPVIPYVTSNSGTS-----VGPNVSPSSG
x-hairy1	196	TNPAPPHN-GSVIPYVTSNSVGT-----ALPPSVSPSSV
c-hairy1	219	PNPAPPPGSGVPIPLYANANVPVSTSGGSGNASTTPSASPVQGLTSFGHSVVPASQAGSP
x-hairy2	205	PSPAYTSSGPVPIPLYANANVTSPGGRQSQS-----PVQGLTTFGHKMAVSPLGGST
c-hairy2	216	SPGTADS WRPW--
HER6	231	PSVTSDS WRPW--
mHES1	243	SSLTSDS WRPWRN
rHES1	242	SSLTSDS WRPWRN
x-hairy1	229	PSVTIDS WRPW--
c-hairy1	289	IAERREA WRPW--
x-hairy2	267	GADSAES WRPW--

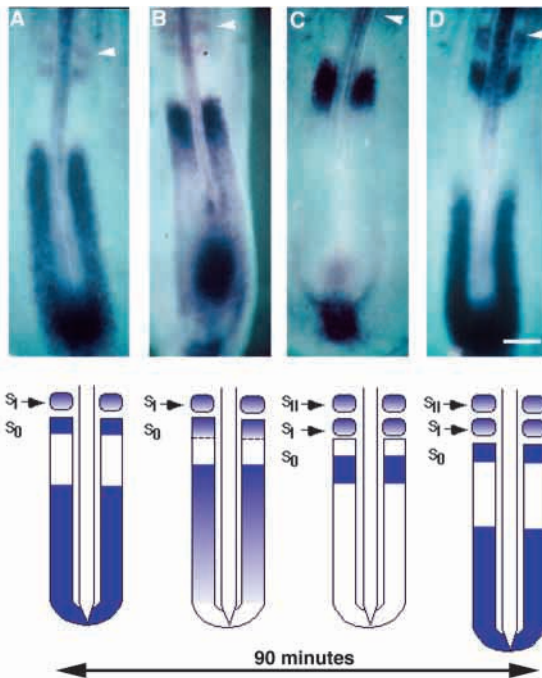
Fig. 1. Sequence comparisons. (A) Comparison of the c-hairy2 protein sequence with that of other vertebrate homologues belonging to the Hairy/Enhancer of split protein (HES) family using the boxshade program. The bHLH domain (between black arrows) and the orange domain (between white arrows) are well conserved between all the HES proteins, as is the tetrapeptide WRPW at the carboxy terminus. The corrected sequence of c-hairy1 is shown in this figure (see methods). c-hairy1 and x-hairy2 are likely orthologues and differ from the HES1 family by an insertion of approximately 20 amino acids in the C-terminal region of the proteins. (B) Percentage identity between sequences of the hairy-like proteins of vertebrates and *Drosophila* hairy and enhancer of split proteins, calculated using the Geneworks program. c-hairy2 shows the highest sequence similarity with mammalian HES1, x-hairy1, zebrafish HER6, whereas c-hairy1 is more closely related to x-hairy2A. This suggests that there are two different classes of hairy-related genes in vertebrates, one including c-hairy2, mammalian HES1, x-hairy1, and Zf HER6, and the second one with c-hairy1 and x-hairy2A.

B

B	c-hairy2 related genes						c-hairy1 related genes		hairy Enhancer of split		
	c-hairy2	mHES1	rHES1	hHES1	x-hairy1	Her6	c-hairy1	x-hairy2A	hairy	Her1	mEspl
c-hairy2		71	72	71	70	73	62	61	30	23	21
mHES1			98	98	72	74	60	62	29	22	22
rHES1				99	72	73	60	62	29	21	22
hHES1					72	74	60	62	29	20	22
x-hairy1						72	63	60	28	20	23
Her6							61	60	26	21	22
c-hairy1								74	29	20	22
x-hairy2A									21	17	17
hairy										21	19
Her1											18
mEspl											

The putative *c-hairy2* protein is 257 amino acids long. It exhibits characteristic features of the *hairy-Enhancer of split* class of bHLH transcription factors such as the characteristic proline in the basic domain and the C-terminal tetrapeptide WRPW involved in the recruitment of the co repressor Groucho (Dawson et al., 1995; Paroush et al., 1994; Fig. 1). *c-hairy2* belongs to the HES1-class of hairy-related proteins

which includes mammalian HES1, *Xenopus* x-hairy1 and zebrafish Her6 (Fig. 1A and B, and Palmeirim et al., 1997). *c-hairy2* is more distantly related to chick c-hairy1 and *Xenopus* x-hairy2A which define a second vertebrate class of hairy-related genes in vertebrates. Therefore, on the basis of sequence similarity *c-hairy2* is likely to correspond to an avian orthologue of the mammalian *HES1* gene.



***c-hairy2* is an output of the segmentation clock and oscillates synchronously with *c-hairy1* in PSM cells**

The expression profile of *c-hairy2* was analyzed by whole-mount in situ hybridization in chick embryos. This analysis revealed very different expression patterns in the PSM of stage-matched embryos (Fig. 2). We were able to establish the expression sequence of *c-hairy2* in the presomitic mesoderm (Fig. 2) from the observation of these expression patterns in relation to somite boundary formation (Fig. 2) and using the

Fig. 2. *c-hairy2* expression appears as a wavefront sweeping across the presomitic mesoderm. (Top) Dorsal view of the caudal region of 17-somite (A-D) chick embryos hybridized with the *c-hairy2* probe. In each panel, the last formed somite (SI) is marked by a white arrow. Expression in the presomitic mesoderm (PSM) appears as a broad caudal domain which progressively moves anteriorly while narrowing as the caudal cleft of somite 0 (S0) forms. *c-hairy2* expression in the rostral PSM becomes restricted to the anterior part of the prospective somite. Rostral to the top. Bar, 110 μ m. (Bottom) Schematic representation of the correlation between *c-hairy2* expression in the PSM with the progression of somite formation. SII, last but one somite.

half embryo technique described by Palmeirim et al. (1997; data not shown). Like *c-hairy1* and *l-fng*, *c-hairy2* is expressed as a wavefront, which sweeps across the PSM once during the formation of each somite (Fig. 2) (Forsberg et al., 1998; McGrew et al., 1998; Palmeirim et al., 1997). In the forming and the newly formed somite (somite 0 and I), *c-hairy2* is strongly expressed anteriorly.

We repeated the *in vitro* tests described in the *c-hairy1* study with the *c-hairy2* probe (Palmeirim et al., 1997), and observed that the propagation of the *c-hairy2* wave-front is regulated exactly like that of *c-hairy1*. It does not rely on cell movement or on a propagatory signal travelling through the paraxial mesoderm (data not shown). This dynamic regulation of *c-hairy2* expression thus constitutes an intrinsic property of the PSM. Furthermore, this expression sequence is not affected by treatment with cycloheximide during a 60 minutes culture period, indicating that like *c-hairy1*, it can proceed without *de novo* protein synthesis (data not shown). Therefore *c-hairy2* is regulated exactly like *c-hairy1* in the PSM by the segmentation clock.

We then compared the expression profiles of *c-hairy2* and *c-*

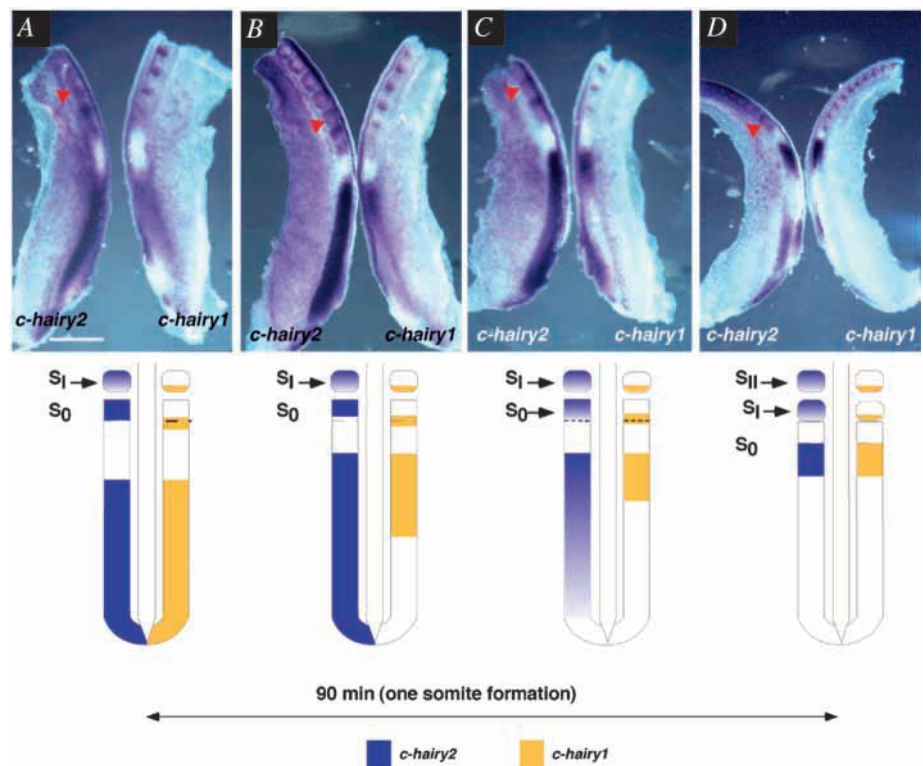


Fig. 3. *c-hairy2* and *c-hairy1* exhibit similar anterior expression borders in the PSM but become expressed in complementary compartments in the rostral-most PSM. (A-D) Caudal portions of embryos were sagittally divided along the midline and the left half was hybridized with *c-hairy2* while the right half was hybridized with *c-hairy1*. A-D show different stages of the progression of the *c-hairy1/c-hairy2* wavefront in the PSM. *c-hairy2* and *c-hairy1* expressions cycle together in the PSM, they share the same expression domain, except that *c-hairy2* extends more caudally in the PSM. At the end of one cycle, the expression domains diverge in the rostral PSM, where *c-hairy2* is located in the anterior part of the prospective somite, whereas *c-hairy1* is found in the posterior part. Red arrowheads indicate SI. Bar, 150 μ m.

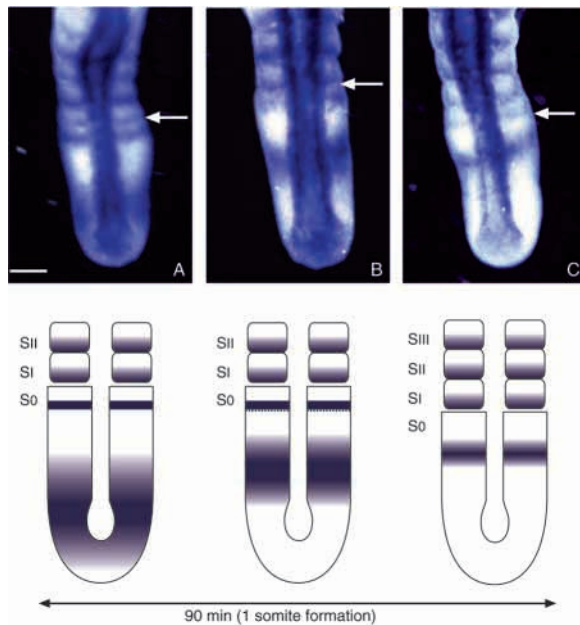


Fig. 4. *HES1* is expressed in a dynamic wavefront in the mouse PSM. (Top) Dorsal view of the tail region of E10.5 mouse embryos hybridized with the *HES1* probe (A-C). In each panel, the caudal boundary of the last formed somite (SI) is marked by a white arrow. Expression in the presomitic mesoderm (PSM) appears as a broad caudal domain (A), which progressively moves anteriorly (B,C), while narrowing as the caudal cleft of somite 0 (S0) forms. *HES1* expression in the rostral PSM becomes restricted to the caudal domain of the prospective somite. Rostral to the top. Bar, 120 μ m. (Bottom) Schematic representation of the correlation between *HES1* expression in the PSM with the progression of somite formation. SII, last but one somite.

hair1 in the two halves of 15- to 20-somite chick embryos (Fig. 3). In 14 out of 27 embryos, the expression pattern of the two genes in the presomitic mesoderm was identical, except in its rostral-most aspect. In the other 13 embryos, *c-hair2* expression differs from *c-hair1* in that it extends more caudally in the PSM. Nevertheless, both genes always exhibit a common anterior expression border in the PSM up to the level of somite -I, indicating that their activation occurs synchronously in this tissue. Therefore, because *c-hair1* and *l-fng* share the same anterior expression border (McGrew et al., 1998), the three genes oscillate in synchrony in most of the PSM. The lag observed caudally in the down-regulation of *c-hair2* compared to *c-hair1* could reflect differences in mRNA stability.

In contrast, at the level of the most anterior part of the presomitic mesoderm (somite 0) and the newly formed somite (somite I), the *c-hair2* and *c-hair1* expression domains are always complementary (Fig. 3). While *c-hair1* is expressed in the most posterior part of the somites 0 and I, *c-hair2* is expressed in the most anterior part of the presomitic mesoderm and in the anterior part of the newly formed somite. Therefore, expression of the two genes becomes stabilized in complementary sets of cells at the time of somite formation.

***HES1* is expressed in a periodic wave-front in the mouse PSM**

Based on sequence comparisons, the closest relative of the *c-*

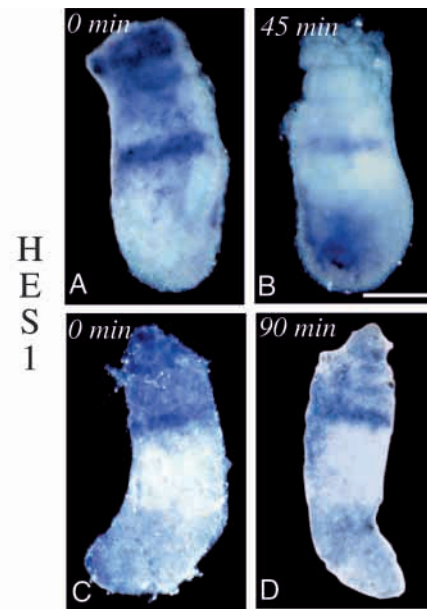


Fig. 5. *HES1* is expressed in a rhythmic fashion correlating with somite formation and is independent of adjacent structures. The two PSMs were dissected from embryo explants and the left PSM was immediately fixed (A,C) while the right PSM was cultured for various time periods (B,D). In all panels, both halves were hybridized with the *HES1* probe. For unknown reasons the quality of the in situ hybridisation is impaired by the culture procedure. (A) Dissected PSM fixed immediately. (B) Contralateral PSM cultured for 45 minutes. A different expression pattern is observed between the two halves. A broad caudal domain has appeared in the cultured PSM. (C) Dissected PSM fixed immediately. (D) Contralateral PSM cultured for 90 minutes. A similar expression pattern is observed in the two PSM. Rostral to the top. Bar, 90 μ m.

hair2 gene is *HES1*, a gene which has been identified in several species ranging from frogs to humans (Dawson et al., 1995; Feder et al., 1993; Feder et al., 1994; Ishibashi et al., 1995; Sasai et al., 1992). This prompted us to explore whether *HES1* might also be expressed in the presomitic mesoderm of mouse embryos in waves correlating with somite formation. Thus far the *HES1* expression pattern has been described in detail only for the nervous system and no dynamic expression has been noted for the PSM (Ishibashi et al., 1995). When mouse embryos, ranging from E9 to E11.5, were hybridized as whole mounts with the *HES1* probe, we observed a series of different expression patterns in the PSM similar to *c-hair2* (Fig. 4). As for *c-hair1* and *c-hair2*, the expression domains of *HES1* appear either as a broad caudal stripe extending into the rostral PSM or as narrower stripes located more rostrally in the PSM. These expression patterns defined a sequence correlating with progression of somitic boundary formation.

***HES1* mRNA expression oscillates in vitro with a 90-minute period correlated with somite formation**

In order to confirm the dynamic nature of *HES1* mRNA expression in the PSM, a culture system of mouse PSM derived from half-embryos similar to that described in the chick was used. In these cultured explants, *HES1* follows a dynamic expression sequence very similar to that observed for *c-hair1* and *c-hair2* in the chick PSM (Fig. 5). Expression first

appears as a broad caudal domain which progressively moves anteriorly and narrows while the somite is forming. This wave-front ends in a domain which corresponds to the prospective caudal part of somite 0, in contrast to chick *c-hairy1* and *c-hairy2* which is eventually restricted to the rostral part of somite 0. This expression sequence is reiterated during every somite formation which in our culture system occurs approximatively every 90 minutes as is seen in the chick embryo (Palmeirim et al., 1997).

***HES1* expression in the mouse is more closely related to *c-hairy1* in the chick**

Strikingly, although on the basis of sequence analysis *HES1* appears to be the closest relative to *c-hairy2*, the two genes are expressed in opposite somitic AP compartments: *HES1* is expressed in the caudal somite half in the mouse, while *c-hairy2* is found in the rostral somite half in the chick. Therefore, based on expression data, *HES1* appears to be more closely related to *c-hairy1*. We have compared the expression of the three genes in other locations such as the neural tube and the limb buds. In the neural tube portion corresponding to the unsegmented region of E2-E3 chick embryos, the two genes are expressed in the whole neural tube. Their expression becomes subsequently regionalized with *c-hairy1* restricted in a ventral domain of the neural tube including the floor plate and in the dorsal third of the neural tube, while *c-hairy2* is found in an intermediate zone (Fig. 6). This pattern appears to be maintained at least up to E5. At the corresponding stages (E9.5 to E12.5), *HES1* is also ubiquitously expressed in the caudal mouse neural tube and becomes expressed in the ventral tube in a domain similar to *c-hairy1* and in a dorsal domain which, however, excludes the roof plate. Therefore, although *HES1* expression more closely resembles the expression of *c-hairy1* than *c-hairy2*, the pattern of the two genes in the neural tube is not exactly similar. Similarly, in the developing limb buds, both *HES1* and *c-hairy1* are expressed in the progress zone and the apical ectodermal ridge (AER) whereas *c-hairy2* is only found in the AER.

The segmentation clock remains functional in the *HES1* null mouse

In order to test whether *HES1* is crucial for the clock function we have analyzed cycling gene expression in the *HES1*^{-/-} mouse (Ishibashi et al., 1995). Homozygous null *HES1* mutants exhibit strong neurogenesis defects but do not show overt segmentation defects (Ishibashi et al., 1995). The only cycling gene described in mouse to date is *l-fng* (Forsberg et al., 1998). We have therefore analyzed the *l-fng* expression profile in a series of *HES1* homozygous mutant embryos ranging from E9.5 to E10.5, and compared this pattern to that of their wild-type and heterozygous littermates (Fig. 7). In all these embryos, very different expression patterns of *l-fng* were observed in the PSM indicating that the dynamic expression of *l-fng* is maintained even in the homozygous mutants.

To further ensure that the cycling behavior of *l-fng* expression was not affected we took advantage of the half-embryo explants culture system to demonstrate that the cycling nature of *l-fng* was maintained. Mutant embryos were bisected along the sagittal axis and one half was fixed immediately

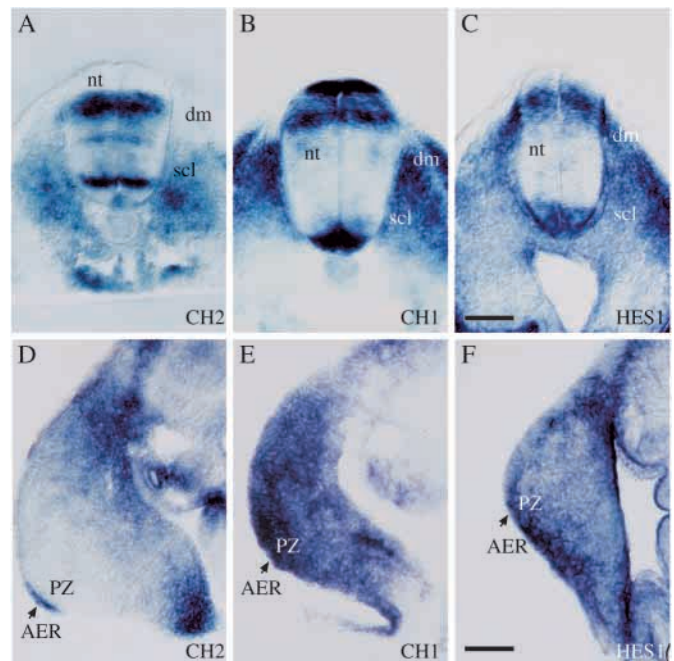


Fig. 6. Expression of *c-hairy1* and *c-hairy2* compared to *HES1* in the neural tube and limb bud. Expression of *c-hairy2* (CH2), *c-hairy1* (CH1) and *HES1* are regionalized in the neural tube (nt). *c-hairy2* is found in an intermediate region of the neural tube (A). *c-hairy1* is expressed in the floor plate and the dorsal-most third of the neural tube, including the roof plate (B). *HES1* is expressed in the floor plate and the most dorsal third of the neural tube, except the roof plate (C). In the limb bud, *c-hairy1* and *HES1* are expressed in the progress zone (PZ) and the AER (E,F), while *c-hairy2* is found in the AER (D). dm, dermomyotome; scl, sclerotome. Bars, 85 µm (for A-C); 65 µm (for D-F).

while the other one was cultured for 45 minutes. When the two halves were hybridized with the *l-fng* probe, a completely different expression pattern was observed indicating that the segmentation clock is still functional in the *HES1* mutant embryos (Fig. 7).

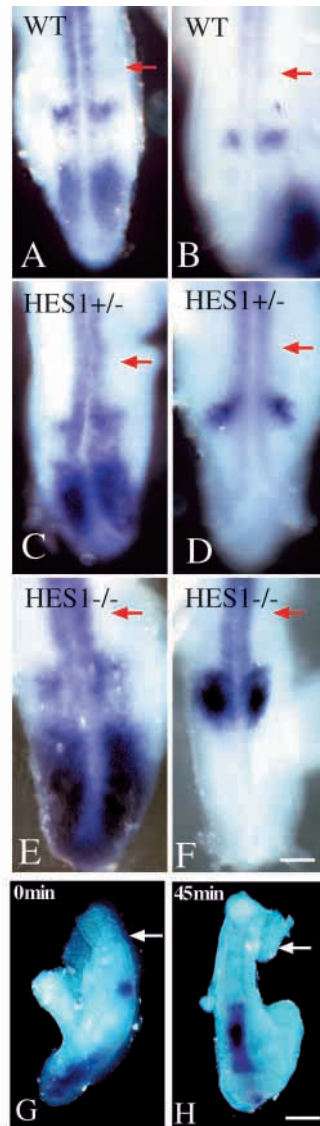
Loss of *HES1* expression in the *Dll1* null mutant mice

In order to investigate the relationship between the dynamic *HES1* expression in the PSM and the Notch signalling pathway, we re-examined *HES1* expression in the *Dll1* homozygous mutant mice in which Notch activation is impaired in the PSM. Homozygous null mutant for the *Dll1* gene exhibit strong segmentation defects and a severe down-regulation of *l-fng* expression (del Barco Barrantes et al., 1999; Hrabe de Angelis et al., 1997). We have compared the expression of *HES1* in wild type, heterozygous and homozygous null mutants at E10.5 by in situ hybridization (Fig. 8). The dynamic expression of *HES1* in the PSM is maintained in *Dll1*^{+/-} embryos as shown by the different expression patterns observed in the PSM. In contrast, all *Dll1*^{-/-} embryos as shown the same global downregulation of *HES1* expression in the PSM (*n*=7). This observation suggests that *HES1* expression in the PSM is dependent on the Notch signalling pathway.

Fig. 7. Lunatic fringe dynamic expression is not affected in *HES1*^{-/-} mutants. (A-F) Expression of *l-fng* in E10.5 mouse wild type (WT), *HES1*^{+/-} (C,D) and *HES1*^{-/-} (E,F) mutant embryos. Different expression patterns of *l-fng* are observed even in the -/- embryos indicating that the dynamic expression of *l-fng* is not disrupted. Red arrow marks the level of somite I. Bar, 110 μ m. (G-H) Expression of *l-fng* in mutant half embryos. The caudal part of an E10.5 *HES1*^{-/-} mouse embryo was separated in two halves and one half was fixed immediately while the other was cultured for 45 minutes. Both halves exhibit different patterns of *l-fng* expression indicating that the *HES1* null mutation does not block the dynamic expression pattern of *l-fng*. Bar, 150 μ m.

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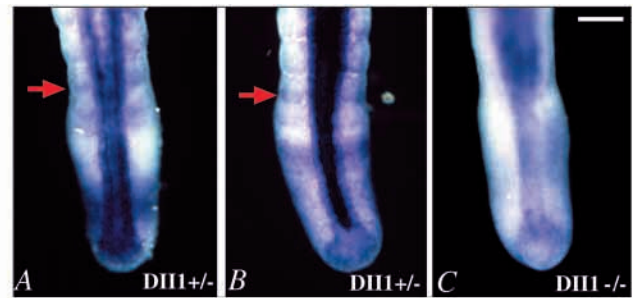


Fig. 8. Loss of *Dll1* disrupts dynamic *HES1* expression. Expression of *HES1* mRNA in the tails of two heterozygous *Dll1* mutants (A,B) showing different patterns of expression of *HES1*, suggesting that the clock is still functional in these embryos. In contrast, in *Dll1*^{-/-} mutants (C), no somites are seen and *HES1* is downregulated indicating that its expression in the PSM requires Notch signalling (labelling seen in the right PSM is only due to background). Red arrows mark the level of somite I. Bar, 120 μ m.

in the PSM. This indicates that Notch signalling is required for the cyclical expression of *HES1* mRNA in the PSM.

Cycling Hairy-like genes in the mouse

Our data indicate that in addition to being structurally closely related to *c-hairy2*, the mouse *HES1* gene is expressed in the PSM in a highly dynamic fashion like the chick *c-hairy1* and *c-hairy2* genes. This identifies *HES1* as the first mammalian hairy homologue expressed in a rhythmic fashion in the mouse PSM. Although, on the basis of sequence similarity, *HES1* appears to be more closely related to *c-hairy2*, on the basis of its expression, *HES1* more closely resembles *c-hairy1*. Therefore, it remains unclear whether this gene is the mouse orthologue of *c-hairy1* or of *c-hairy2*. No mouse homologue of *c-hairy1* has been identified thus far despite extensive efforts (unpublished observations). Thus, it cannot be ruled out that only one cycling hairy-like gene exists in the mouse. However, comparing the sequences of genes related to *c-hairy1* and *c-hairy2* in different vertebrates species suggests the existence of two different classes which arose early in evolution since they are also found in *Xenopus* (Fig. 1).

Is Notch signalling part of the core mechanism of the segmentation clock?

Short term experiments involving blocking protein synthesis do not disrupt the dynamics of *c-hairy1* or *c-hairy2* expression pattern (this study; Palmeirim et al., 1997), indicating that hairy-like genes are not likely to be part of the clock mechanism, but rather represent a read-out of the clock. Our results showing that the segmentation clock remains functional in the *HES1* null mutant are in agreement with this conclusion, although we cannot rule out the possibility that an as yet unidentified second mouse hairy-like homologue is able to compensate for the loss of *HES1* and rescue the clock function.

HES1 and *l-fng* dynamic expression are lost in the PSM of *Dll1* mutants, in which Notch signalling is disrupted (this study; del Barco Barrantes et al., 1999; Hrabe de Angelis et al., 1997). This result is in contrast to previous observations reporting that *HES1* expression was not affected in Notch pathway mutants (del Barco Barrantes et al., 1999). However, in these studies the dynamic expression pattern of *HES1* in the

DISCUSSION

HES1 is a well described downstream target of the Notch pathway, which has been implicated in many developmental and pathological processes (Chen et al., 1997; Kageyama and Ohtsuka, 1999; Tomita et al., 1999). Here we report the identification of *c-hairy2*, a chick gene related to the mammalian *HES1* gene, whose mRNA expression oscillates in synchrony with *c-hairy1* and *l-fng* in the chick PSM. This prompted us to reinvestigate *HES1* expression during somitogenesis in the mouse. We observed that *HES1*, like *c-hairy1* and *c-hairy2* in the chick, is expressed in a dynamic wave, which sweeps across the PSM once during the formation of each somite. These results indicate that *HES1* like the chick hairy-like genes is regulated by the clock linked to segmentation. We observed that expression of another cycling gene, *l-fng*, is not affected in the *HES1* null mouse mutants indicating that the segmentation clock remains functional in these mutants. In contrast, *HES1* expression is severely downregulated in the *Dll1* mutants which disrupts Notch signalling

PSM had not been observed. Various clock outputs appear, therefore, to be severely downregulated when Notch signalling is disrupted. These observations raise the possibility that in addition to being an output of the segmentation clock as previously proposed (Aulehla and Johnson, 1999; Forsberg et al., 1998; McGrew et al., 1998), the Notch signalling pathway might also be an important component of the oscillator.

Notch activation upon ligand binding involves a proteolytic cleavage liberating the intracytoplasmic domain (NICD), which translocates into the nucleus where together with Su(H)/RBPjk it activates the transcription of genes such as *HES1* in vertebrates (Artavanis-Tsakonas et al., 1999; Jarriault et al., 1995, 1998). Our observations in the *Dll1*^{-/-} mice indicate that *HES1* is downstream of the Notch pathway in the PSM. Since *c-hairy1* and *c-hairy2* share a high similarity in their sequence and in their expression patterns to *HES1*, they are also likely targets of Notch signalling in the chick PSM. A direct regulation of *c-hairy1* and *c-hairy2* expression by oscillating Notch activation would explain why *c-hairy1* expression is insensitive to cycloheximide, since protein synthesis is not required for transduction of the Notch signal.

To achieve oscillations of Notch signalling, the activity of the pathway would need to be modulated by a feedback mechanism. However, known output events resulting from Notch signalling are transcriptional regulation of target genes and the clock is partly independent of protein synthesis. *Notch1* and *Delta1* are present along the whole presomitic mesoderm and could generate constitutive activation of the pathway in the tissue (Palmeirim et al., 1998). The rhythmic modification of this activation could, in principle, be achieved by the periodic expression of *l-fng*.

We have shown that cycloheximide treatment does not disrupt *c-hairy1* and *c-hairy2* dynamic expression whereas it blocks that of *l-fng* (this report and McGrew et al., 1998; Palmeirim et al., 1997). This led us to propose that *l-fng* was acting downstream of the *c-hairy* genes (McGrew et al., 1998). The observation that in the mouse *HES1* null mutant, *l-fng* cycling expression is maintained suggests that *HES1* is not upstream of *l-fng*, although we cannot rule out the possibility that a second as yet unidentified mouse *hairy*-like homologue compensates this defect. Such a regulatory network involving *hairy* and *l-fng* genes regulated by periodic Notch signalling activation in the PSM cells could in principle constitute the basis of a molecular oscillator.

Such a hypothesis implies that disrupting Notch signalling will disrupt the segmentation clock. Therefore, given the phenotype of the mouse Notch pathway mutants, which retain a metamerical organization of somitic derivatives, the clock would be unlikely to play a role in segment generation but rather in the coordination or refinement of the process. Alternatively, it cannot be ruled out that the clock is a biochemical oscillator of unknown nature, which interacts with Notch signalling, for instance, by preventing access of NICD to the nucleus in a periodic fashion. In this model, disruption of the Notch pathway would leave intact the oscillator but affect its outputs such as *c-hairy1* and *l-fng*.

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