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Citation for published version:

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Current biology : CB

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The *lunatic Fringe* gene is a target of the molecular clock linked to somite segmentation in avian embryos

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The most obvious segments of the vertebrate embryo are the trunk mesodermal somites which give rise to the segmented vertebral column and the skeletal muscles of the body. Mechanistic insights into vertebrate somitogenesis have recently been gained from observations of rhythmic expression of the avian *hairy*-related gene (*c-hairy1*) in chick presomitic mesoderm (PSM), suggesting the existence of a molecular clock linked to somite segmentation ([1]; reviewed in [2]). Here, we show that *lunatic Fringe (IFng)*, a vertebrate homolog of the *Drosophila* Fringe gene, is also expressed rhythmically in PSM. The PSM expression of *IFng* was observed as coordinated pulses of mRNA resembling the expression of *c-hairy1*. We show that *c-hairy1* and *IFng* expression in the PSM are coincident, indicating that both genes are responding to the same segmentation clock. The genes were found to differ in their regulation, however; in contrast to *c-hairy1*, *IFng* mRNA oscillations required continued protein synthesis, suggesting that *IFng* could be acting downstream of *c-hairy1* in the clock mechanism. In *Drosophila*, Fringe has been shown to play a role in modulating Notch–Delta signalling [3,4], a pathway which in vertebrates has been implicated in defining somite boundaries [5–9]. These observations place the segmentation clock upstream of the Notch–Delta pathway during vertebrate somitogenesis.

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Received: 6 July 1998
Revised: 4 August 1998
Accepted: 4 August 1998

Published: 17 August 1998

**Current Biology** 1998, 8:979–982  http://biomednet.com/elecref/0960982200800979
© Current Biology Publications ISSN 0960-9822

**Results and discussion**

Genetic studies in the mouse have implicated the Notch–Delta signalling pathway as playing a role in segmentation [10]. Mice harbouring mutations in the *Notch1*, *RBP-J kappa*, or Delta1 genes retain a basic metamerism of paraxial mesoderm derivatives [5–7]. The somites in these mutants are, however, uncoordinated and their anterior–posterior compartments are incorrectly specified. Thus, the Notch–Delta pathway is thought to act later than the segmentation clock to both coordinate somite formation and specify boundaries during the segmentation process. Therefore, genes in this pathway are potential downstream targets of the segmentation clock; however, the chick genes *c-Notch1* and *c-Delta1* do not display rhythmic expression in the PSM [11]. A link between the two systems has yet to be established. To explore such a link, we analysed the expression of *IFng* during somitogenesis in the chick embryo. The *IFng* gene is expressed in the PSM [12–15] and has recently been shown to play a role in mouse somitogenesis [8,9]. We show that *IFng* transcripts are expressed in a rhythmic fashion in the PSM and thus appear to be directly regulated by the segmentation clock.

**Expression of IFng is cyclical in the PSM**

To analyse the expression of *IFng* in the PSM, a series of embryos (*n* = 49) were hybridised with an *IFng* probe. Expression of *IFng* was found to vary considerably even among embryos of the same somite number (Figure 1). Some embryos showed expression in two domains: a broad caudal domain extending from the tail bud region up to the rostral third of the PSM, and a narrower band located more rostrally in the PSM. In other embryos, the broad expression domain appeared to narrow, by becoming progressively downregulated caudally, while moving anteriorly. Concomitantly, the rostral-most domain became narrower and progressively faded in intensity. Weak expression was also observed within the anterior part of newly formed somites. Therefore, in the chick PSM, *IFng* displays a dynamic expression pattern.

In order to demonstrate the cyclical nature of this expression profile, the caudal portion of chick embryos was divided into two halves by sectioning along the midline; one half was fixed immediately, while the other half was cultured *in vitro* (Figure 2). When the experimental half was cultured for 30 minutes (*n* = 15), a very different profile of *IFng* expression was observed in each of the two halves (Figure 2a). This analysis enabled us to order the expression sequence of *IFng* in the PSM (Figure 1).

When the experimental half was incubated for 90 minutes (*n* = 5), a new somite was generated in the explant, and the two halves showed identical *IFng* expression patterns (Figure 2b). These experiments demonstrate that, like *c-hairy1*, *IFng* expression appears as a cyclical wavefront that sweeps along the PSM once during the formation of each somite.
Cyclical expression of ifng is an autonomous property of the PSM

In order to analyse further the regulation of this expression pattern, we tested whether progression of the wavefront results from the propagation of a signal in the PSM. To that end, the expression of ifng was assayed in caudal-half embryo explants in which, on one side, the posterior part including the tailbud was surgically ablated. Both sides were then cultured for the same time period. In all cases (n = 7), the same expression pattern of ifng was observed in the operated and control sides, indicating that the movement of the ifng wavefront does not rely on signal propagation (Figure 2c). This suggests that cyclical expression of ifng is an intrinsic property of the PSM. To verify this, we compared ifng expression in caudal-half embryo explants (n = 10) in which, on one side, the PSM was microdissected and isolated from the surrounding tissues. After culture under these conditions, the expression pattern in the isolated PSM was found to be the same as that of the intact side (Figure 2d). Therefore, like c-hairy1, rhythmic expression of ifng is an autonomous property of the PSM.

Tandem oscillation of ifng and c-hairy1 in the PSM

A precise comparison of the ifng expression domain with that of c-hairy1 in the PSM was performed in the caudal halves of chick embryos. Except in the rostral-most PSM, the expression domain of ifng on one side of the embryo was similar to that of c-hairy1 on the other side (n = 20, Figure 3a–c). These findings indicate that the oscillations of both c-hairy1 and ifng are coordinated in both time and
space and, thus, are controlled by the same segmentation clock. In the rostral-most PSM, in contrast, the expression domains of these two genes progressively diverge at the forming border of somite S0 (Figure 3d,e).

**Cycloheximide blocks IFng oscillations**

The c-hairy1 gene encodes a transcription factor of the basic helix-loop-helix (bHLH) family whereas IFng encodes a secreted molecule. As both genes are expressed with similar dynamics in PSM, one hypothesis might be that IFng acts downstream of c-hairy1. Such a model implies that translation of the c-hairy1 product will be required in order to drive IFng expression. To address this idea, we examined whether blocking protein synthesis with cycloheximide affects IFng expression. Progression of the IFng wavefront was found to be retarded in cycloheximide-treated explants compared with the controls (10 out of 15 cases, Figure 4a). To confirm that the dynamic expression of IFng in PSM was indeed blocked, one explant half was fixed immediately while the other half was cultured for 30 minutes in the presence of cycloheximide. The expression pattern of IFng was similar on both sides, indicating a rapid and effective block of the IFng wavefront (9 out of 10 cases, Figure 4b). In similar experiments, c-hairy1 expression was not affected (n = 7; data not shown, and [1]). These experiments show that the regulation of c-hairy1 and IFng differ: both genes appear to be downstream of the segmentation clock, but only IFng requires protein synthesis to drive its dynamic expression. It is therefore possible that IFng acts downstream of c-hairy1 in the PSM, although we cannot rule out the possibility that these genes are regulated by parallel pathways.

Our results demonstrate that IFng is a target of the recently identified segmentation clock, leading to periodic expression of IFng in the region where the prospective somite boundaries will form. In the fly, Fringe acts to differentially modulate Notch reception of Delta and Serrate ligands [3,4]. During vertebrate somitogenesis, Notch–Delta signalling is required for specification of the anterior and posterior somitic compartments, which occurs in the rostral-most PSM [10]. The IFng gene has also been shown to be essential for this process in the mouse [8,9]. The discrete expression domains of Notch and its ligands in this region where IFng oscillates suggests that, in the chick rostral PSM, IFng also modulates Notch signalling to establish the somite boundary. Thus, clock control on this local modulation of the Notch–Delta signalling pathway would confer the periodic arrangement of the boundaries that underlie the segmental body plan.
Materials and methods

Eggs, embryos and somite nomenclature
Fertilised chick eggs (Gallus gallus, JA57, Institut de Sélection Animale, Lyon, France), obtained from commercial sources, were incubated for up to 48 h in a humidified atmosphere at 38°C. The embryos were staged by the number of somite pairs formed. Chick embryos at stages ranging from 15 to 20 somite pairs were used throughout this study. The somite staging system [16] has been used for numbering somites. In this system, the newly formed somite is referred to as somite $S_0$. We refer to somite $S_0$ as being the forming somite whose caudal clefts are not yet completely formed.

In vitro culture and cycloheximide treatment of explants
Microsurgical operations on the embryonic halves and in vitro culture were carried out as described [1]. The caudal portion of chick embryos was separated along the midline. The experimental half was either cultured for the same period of time or fixed immediately. In all series, explants were processed for whole mount in situ hybridisation with c-hairy1 or lFng probes. Efficiency of protein synthesis inhibition in these conditions has been previously established [1].

In situ hybridisation
The c-hairy1 probe has been described [1]. The lFng/c-Fringe1 probe was produced as described [15]. Whole-mount in situ hybridisation was performed as described [17]. Embryos were photographed as whole mounts using a Leica MZ10 stereomicroscope. In situ hybridisations on 20 μm cryosections were carried out as described [18].

Acknowledgements
We thank Ken-Ichi Katsube for the kind gift of the lFng/c-Fringe1 probe, Stephen Kerridge and David Ish-Horowicz for critical reading of the manuscript. M.J.M. is the recipient of a postdoctoral EMBO fellowship. J.K.D. is a recipient of a Wellcome Travelling Research Fellowship. Financial support was provided by the Centre National de la Recherche Scientifique (CNRS), the Association Française contre les myopathies (AFM), the Association pour la Recherche contre le Cancer (ARC) and the Fondation pour la Recherche contre le Cancer (ARC) and the Fondation pour la Recherche contre le Cancer (ARC).

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