Chapter 4

MiR-10c acts as an autoregulatory microRNA on HoxB3a

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submitted

Abstract
In Zebrafish the miR-10 microRNAs target the more anterior HoxB1a and HoxB3a genes. The HoxBa cluster has a complex transcription profile including long range and polycistronic transcription. We show that miR-10c which is located on a polycistronic transcript together with HoxB5a, HoxB4a and HoxB3a also targets the specific HoxB3a splice isoforms produced from this transcript. The locus therefore encodes an essentially autoregulatory transcription unit containing both a microRNA and a target gene. This is of interest because this HoxB3a splice isoform has an expression pattern that falls completely within the miR-10c expression domain. Since the gene is downregulated by miR-10 within its entire expression domain a function for the transcription of this specific splice isoforms is difficult to envisage. For the Hox related miR-196 microRNAs we identify polycistronic transcripts that contain both the microRNA and a target gene; these loci can therefore also act in an autoregulatory fashion. We present evidence for a link between clustering and posttranscriptional gene regulation. We suggest that clustering of the genes places constraints on the Hox regulatory system and explains why posttranscriptional silencing of nearby genes is used as a second layer of control in addition to the primary transcriptional regulation.
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Introduction

The Hox genes encode a family of conserved homeodomain transcription factors and are the main genes responsible for patterning the embryonic anterior-posterior axis. The genes find their evolutionary origin in cis-duplications of a single ancestral gene (1) and the clustered configuration of the Hox genes that was thus created has been preserved in vertebrates and many other metazoans till the present day.

Hox genes are expressed along the main body axis in a sequential fashion and the order in which they are expressed corresponds to their sequence within a Hox cluster; the more 3’ in the cluster a gene is located the more anterior its expression domain. This phenomenon is commonly referred to as ‘spatial colinearity’. The genes have sharply defined anterior boundaries of expression but their posterior boundaries are in general less clear and overlap with the expression of more posterior genes.

Apart from the Hox coding genes the miR-10, miR-196 and miR-615 microRNA gene families are present within the vertebrate Hox clusters (2, 3, 4). MicroRNAs are small (~22 nt) non-coding RNAs which are produced from stemloop containing precursor transcripts through processing by the RNase III enzymes Dicer (5) and Drosha (6). In Zebrafish, a germline Dicer mutant has revealed an important function for microRNAs in the coordination of normal embryonic development (7, 8).

MicroRNAs function in post-transcriptional gene silencing by binding to imperfect target sites in messengerRNA and thereby induce translational inhibition and messengerRNA destabilization (9, 10). Specificity of recognition is determined by nucleotide 2-7 of the microRNA, called the seed, which needs a perfect match with the target site to allow interaction. Point mutations in the seed sequence in either microRNA or target mRNA sequence will abolish the interaction and prevent silencing.

In the Hox clusters, miR-10 is closely associated with the position of the Hox-4 paralogue members, miR-196 is located between Hox-9 and Hox-10 paralogues group genes and the more recently cloned miR-615 is located in the HoxC5 intron in mammals but appears to be absent from Teleosts and Xenopus tropicalis and may therefore be restricted to either mammals or amniotes (JMW & AJD Blast results, data not shown).
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Recent functional studies also have identified *HoxA7* and *Hox-8* paralogous genes as targets of *miR-196* (11, 12, 13) and in chicken the interaction with *HoxB8* has been implicated in the mechanism that abolishes the competence of posterior lateral plate mesoderm for limb induction by Retinoic acid (13). In Drosophila a conserved interaction has been shown for the *miR-196* homologue *IAB-4* which turns out to target the *Ubx Hox* gene (14).

*MiR-10* microRNAs are expressed in the posterior hindbrain and spinal cord with their anterior boundaries at rhombomere 6/7 (chapter 3, this thesis). We have recently shown that in Zebrafish *miR-10* targets the anterior *HoxB1a* and *HoxB3a* genes and that it is involved in the proper migration of the Xth nerve axons into the posterior branchial arches (chapter 3, this thesis).

The anterior *Hox* genes *HoxB1a* and *HoxB3a* that we identified as *miR-10* targets have a dominant anterior domain of expression in which they have known selector functions and a much weaker posterior domain where their action is blocked by the *miR-10* microRNA. The restriction of the high level anterior domain appears to be primarily regulated at the transcriptional level. But why then do these genes have a presumably a-functional weak posterior domain of expression and is repression in this domain regulated at the posttranscriptional rather than at the transcriptional level?

As with the majority of microRNA/target interactions there is yet no satisfactory explanation why in the case of the *Hox* clusters mechanisms of posttranscriptional regulation have arisen rather than arranging things similarly by transcriptional regulation. A possible answer to this question may lie in the complexity of the *Hox* expression patterns which is reflected by the presence of extensive control mechanisms involving multiple global and local transcriptional elements. The high selective pressure to maintain the clustered genomic organization of vertebrate *Hox* genes results from the presence of global enhancers located outside the clusters and from dependence on sharing of local enhancers (15). As a result the *Hox* clusters consist of closely spaced transcription units and enhancer regions. The high density of transcription units could easily cause them to interfere with one another and make the system prone to inappropriate enhancer sharing, leading to ectopic expression. The silencing of nearby genes may suggest that the clustering of the *Hox* genes places constraints on the level of transcriptional control that can be achieved within the locus and requires the involvement of posttranscriptional gene silencing to appropriately define functional domains of gene activity. The posterior expression
domains of the anterior Hox genes could well be a consequence imposed on the transcriptional process by the clustered nature of the genes and do not necessarily serve any function. We suggest that an inability to separate the transcriptional controls of several Hox genes is at the basis of the post-transcriptional gene silencing relationships within the Hox clusters. Here we will investigate this issue further by looking more closely at some aspects of microRNA Hox relationships and considering these in the context of clustering.

**Results**

*The HoxB3a splv 2 primary transcription unit is an autoregulatory locus*

An interesting aspect of the Hox clusters is the existence of long range polycistronic transcription where multiple Hox genes are transcribed on one primary transcript. One example is in the human HoxC cluster (16) and another is in the Zebrafish HoxBa cluster (17), but this phenomenon seems to be much more widespread. The Zebrafish HoxBa cluster transcript contains both the miR-10c microRNA and HoxB3a splv2 which is interesting since our previous data indicate an interaction between these two genes. The zebrafish polycistronic transcript starts just 3’ of HoxB5a, takes two exons 3’ of HoxB4a, and is fused to the main HoxB3a open reading frame (fig.3a). This long transcript contains both a target gene (HoxB3a) and a microRNA (miR-10c).

In situ hybridization with a 5’ UTR probe shows that its expression obeys spatial colinearity, corresponding to its transcriptional start site (i.e. expression similar to HoxB5a) (fig.3b and reference 18) and that it is expressed more posteriorly than the main HoxB3a expression domain. The shared expression patterns are consistent with the fact that the primary HoxB3a splv2 transcript overlaps with the miR-10c microRNA.

This transcript at least contains the full HoxB3a open reading frame including the 3 miR-10 target sites previously reported in chapter 3. Morpholino knockdown of the microRNA as described in chapter 3 leads to its upregulation (fig.3c) showing that the transcript is indeed targeted by miR-10 in vivo.

The combination of this expression pattern with the fact that the transcript is a miR-10 target indicates a paradoxical situation; an ORF containing transcript targeted by a microRNA is *exclusively* expressed within the domain of this microRNA itself. Although
the transcript contains an ORF, it is probably never translated in vivo and it is also not meant to be translated. Of course this observation inevitably raises the question as to what could be the raison d’être of this transcript in the first place. One possible explanation is that this transcript has no function at all and that its existence, as would be the case for the inappropriate enhancer sharing, is simply an obligate consequence of the clustered nature of the Hox genes.

**Polycistronic transcripts containing both miR-196 and target Hox genes**

Strikingly we find that miR-10 targets the HoxB3a splv2 transcript while the primary unspliced RNA for this transcript contains the sequences for both the HoxB3a splv2 transcript and the miR-10c microRNA. The fact that this transcript is targeted by miR-10 means that the microRNA is in fact autoregulatory. These findings prompted us to search for other microRNA target pairs. The more posterior Hox-9 associated microRNA miR-196 has in multiple species been shown to target HoxA7, HoxB8, HoxC8 and HoxD8 (11, 12, 13). Inspection in the human genome Ensembl assembly of the transcription profile associated with the genomic region surrounding the miR-196 genes reveals the presence of ESTs spanning the region of miR-196a-1 to HoxB7 and miR-196a-2 to HoxC6 (fig.4e). These primary transcripts contain both the miR-196 microRNA and the targeted HoxB8 and HoxC8 genes.

An apparent question in the case of these polycistronic transcripts containing both a target gene and a microRNA is what the reason could be for including both on the same transcript; since the target genes are actively repressed by the accompanying microRNA. Wherever this combination is transcribed they could as well have been omitted from the transcript.

We interpret the existence of these apparently ‘non-functional’ transcription combinations in the light of the above stated considerations, leading to the conclusion that not all transcripts in the Hox cluster necessarily do serve a function and that some transcripts may arise from restrictions imposed on the transcriptional control within the Hox clusters.
Figure 1) a) Schematic representation of the transcript structure of HoxB3a splv2 with ‘posterior’ exons indicated in orange. b) Double in situ hybridization using HoxB3a exon 1 coding sequence probe (red) and a probe derived from the ‘posterior’ exons (marked orange in fig. 4a) of HoxB3a splv2 (purple). NB despite similar length of the two probes the exon 1 probe gives a much stronger signal (~4 hr staining time shown with Fast Red substrate which would equal about 2 hr staining with BM-purple) compared to the ‘posterior’ exons probe (staining with BM-purple overnight) indicating a much higher level of transcript presence in the anterior domain of HoxB3a. c) Response of HoxB3a splv2 to inhibition of miR-10, in injections with both morpholinos the signal for the 5 ‘exons’ is upregulated. Embryos were stained equally long and stopped before signal saturation could be reached. d) Polycistronic transcription comprising both human miR-196 and targeted Hox-8 genes.

**Hox related microRNA genes are absent from species that have lost Hox gene clustering**

In vertebrates and Drosophila the Hox genes have stayed clustered throughout evolution while in Ciona the Hox clusters have broken up and are present in separate regions of the genome. If the selective forces maintaining the microRNAs within the Hox clusters indeed result from genomic clustering the microRNA genes would be expected to be absent from these species. The genomic databases at the NCBI and Ensembl were searched with the miR-10 and miR-196 sequences. Both microRNAs were found in all vertebrates and arthropods (here IAB-4 replaces miR-196) and miR-10 also in Sea urchin (*Strongylocentrotus purpureus*) and amphioxus (*Branchiostoma floridae*). Both microRNAs are however absent from *Ciona intestinalis* and *Ciona savignyi*. These
observations provide in the case of the Hox clusters a phylogenetic link between posttranscriptional gene silencing and gene clustering.

Discussion

In general it is poorly understood why under some circumstances mechanisms of posttranscriptional gene regulation have evolved instead of arranging things similarly at the transcriptional level. In the case of the miR-10/Hox interaction it is striking that a transcript targeted by miR-10 is restricted to the miR-10 expression domain itself, raising the question why transcription of this gene has not simply been shut off. In the case of the miR-10c/HoxB3a splv2, miR-196a-1/HoxB8 and miR-196a-2/HoxC8 interactions there are polycistronic transcripts containing both the microRNA and the target gene. This situation is counterintuitive in the sense that one wonders why the target gene is not simply omitted from the transcript since it is silenced by the accompanying microRNA anyway.

Both miR-196 and miR-10 target anterior Hox genes located within a remarkably close genomic proximity; miR-10 is ~25 kb from the target sites in HoxB3a and ~48 kb from HoxB1a and in human miR-196is ~18 kb from HoxB8 and HoxC8 and ~14 kb from HoxA7. It appears that the Hox related microRNAs function to repress the activity of nearby anterior genes when they are expressed in posterior domains. Our observations with respect to the short genomic distances involved, the occurrence of polycistronic transcripts containing both the microRNA and the target genes and the phylogenetic comparison, all strongly suggest that at these short genomic distances it may not be possible to accurately separate transcriptional controls and transcription units. We suggest that the involvement of ‘autoregulatory’ gene silencing basically arises from a conflict between the need for well defined Hox expression zones and a clustered genomic context. It would be interesting to see whether it is possible to extrapolate these observations to other microRNA / (predicted) target pairs and see if similar constraints can be identified that can account for the involvement of posttranscriptional gene regulation.
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Material and Methods

In situ hybridization, morpholino injections and embryo manipulations were performed as described in chapter 3. HoxB3a splv2 was synthesized from PCR product from a partial cDNA cloned in pGEM-TE.

References


