Chapter 7

The small GTPase *Rap1* is an immediate downstream target for *Hoxb4* transcriptional regulation

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Abstract

The Hox genes are a family of homeodomain-containing transcription factors which determine anteroposterior identity early on in development. Although a lot is now known about their regulation and function, very little is known of their effector (downstream target) genes. Here we show that the small GTPase Rap1 is a direct, negatively regulated target of Hoxb4 and is excluded from Hoxb4 expressing cells. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

A number of molecular components employed in patterning the early embryo have been very highly conserved through evolution, and the Hox genes are a particularly noticeable example. They encode a family of homeodomain transcription factors that are expressed along the anterior to posterior (anteroposterior) axis from the gastrula stage onwards, in a spatial and temporal order that closely reflects their relative position within the chromosome. Their expression defines the anterior–posterior character of those cells which express them, and consequently their deletion or misexpression can cause the transformation of one part of the embryo into another (reviewed by Carroll, 1995; Gehring, 1998; Burke, 2000).

The control and function of Hox genes have been extensively studied in a wide range of species, and many of the molecular aspects of their regulation are now understood. However, far less is known about the actual molecular basis of Hox gene function. In vitro, most Hox proteins recognize the same four-base-pair consensus sequence that is actually repeated many times in the genome (Hayashi and Scott, 1990). Far greater binding specificity is achieved when Hox proteins bind as a complex with other proteins, including PBX (vertebrate homologues of Drosophila homeodomain-containing transcription factor extradenticle, Chang et al., 1996). Thus HOX proteins can recognize considerably more specific sites in vivo, the identity of which have now been tentatively established (Chang et al., 1995, 1996; Ryoo and Mann, 1999; White et al., 2000). In order to understand the molecular basis for Hox gene function, we need to know which genes are the immediate targets of transcriptional activation or repression. However, to date very few of the downstream targets of Hox genes are known.

For this reason we have looked for genes that are directly regulated by the Hox gene Hoxb4. This is the vertebrate homologue of the Drosophila deformed gene which is expressed in and required for the correct specification of a number of cephalic segments. Deformed mutants lack maxillary and mandibular structures, the head having been transformed to thoracic like structures dorsally and deleted ventrally (Merrill et al., 1987). Studies in the mouse and in the frog (Xenopus) have revealed that Hoxb4 is expressed in the hindbrain and spinal cord, with a sharp boundary in the hindbrain between rhombomeres 6 and 7 (Graham et al., 1988; Godsave et al., 1994). The homozygous null mutation of Hoxb4 in the mouse results in a homeotic transformation of the second cervical vertebrae from axis to atlas, and defective morphogenesis of the sternum (Ramirez-Solis et al., 1993). Ectopic expression of Hoxb4 in early Xenopus embryos results in the deletion of structures anterior to where Hoxb4 is usually expressed (i.e. the forebrain, midbrain and hindbrain anterior to rhombomere 7 (Hooiveld et al., 1999)).
Fig. 1. Differential display identifies Xrap1 as a possible downstream target of Hoxb4. Hoxb4GR (which confers dexamethasone dependence on Hoxb4 activity) was injected into fertilized eggs and activated at either stage 7 (blastula) or stage 10 (gastrula). Total RNA was extracted at the neural stage and randomly amplified. Identical but independent amplifications were performed to check for reproducibility. (+), Hoxb4 activated by dexamethasone at the stage indicated; (−), no dexamethasone added.

Here we report that the small GTPase Rap1 is a direct target of Hoxb4 regulation. Hoxb4 represses Rap1 expression in a manner that is independent of protein translation, and may bind to two putative HOXB4 protein binding sites located at the 3′ end of the Rap1 gene in order to mediate this inhibition.

2. Results

2.1. Xrap1 and Hoxb4 have complementary expression patterns in early development

In order to search for downstream targets of Hoxb4, we used a differential display technique to compare gene expression in embryos which had developed from eggs injected with Hoxb4 RNA to that in untreated controls. Our attention was drawn to one transcript in particular because it was absent in Hoxb4 injected embryos (Fig. 1). We therefore cloned and sequenced the corresponding cDNA from the untreated, control embryos. Conceptual translation of the partial open reading frame encoded in this clone gives a peptide which is 96% identical to the human RAP1 protein, a small GTPase (Pizon et al., 1988). The only differences in the amino acid sequence of the two proteins are ‘conservative’ changes (i.e. where the amino acids have very similar biochemical properties). We therefore conclude that the differentially expressed clone encodes the Xenopus homologue of the Human Rap1 gene, and we refer to it here as Xrap1 (accession number U05938).

In order to confirm that Xrap1 is indeed repressed by Hoxb4, we injected fertilized eggs with Hoxb4 mRNA and then examined the expression of Xrap1 later in development, at the neurula stage (Fig. 2). Using a similar approach, we also examined the effect that Hoxb1, Hoxb5 and Hoxb9 over-expression have on Xrap1 (Fig. 2). Both Hoxb4 and Hoxb5 result in a striking down-regulation of Xrap1, whilst both Hoxb1 (a more anteriorly expressed Hox gene) and Hoxb9 (the most caudally expressed Hox gene) have no apparent affect on its expression.

In order to determine whether the apparent mutually exclusive relationship between Xrap1 and Hoxb4 is reflected in their expression pattern in the embryo, we used whole-mount in situ analysis to study their expression at a number of different developmental stages (Fig. 3). The expression of Xrap-1 begins early in gastrulation (Fig. 3A) in the dorsal ectoderm (the future neural plate). Hoxb4 expression is detected slightly later in gastrulation in a more restricted pattern, being located at a more posterior and ventral position (Fig. 3B). Xrap1 remains confined to the neural tube during neurulation and is expressed throughout its length, with the exception of those cells that express Hoxb4. A sharp boundary between Xrap1 and Hoxb4 expression domains becomes apparent as neurulation proceeds. By the tailbud stage the Xrap1 and Hoxb4 domains of expression abut sharply both in the hindbrain just posterior to the otic vesicle (the boundary between rhombomeres 6 and 7, Fig. 3F), and in the head mesenchyme at the position of the fourth pharyngeal arch.

2.2. The repression of Xrap-1 by Hoxb4 is direct and independent of protein synthesis

The preceding results indicate that Hoxb4 represses Xrap1 expression, but they do not provide any indication as to whether this repression is direct (i.e. independent of further translation), or indirect. In order to address this, we used a fusion between Hoxb4 and the human glucocorticoid receptor (Hoxb4GR, Houivet et al., 1999). The glucocorticoid receptor binds the heat shock protein HSP90, preventing it from entering the nucleus. This steric hindrance of nuclear entry is relieved by ligand binding, in this case the glucocorticoid analogue dexamethasone (DEX), which by itself has no discernible effects on Xenopus development (Gammill and Sive, 1997). Hence the Hoxb4GR construct confers DEX dependence on the activity of Hoxb4 (Houivet et al., 1999).

We injected fertilized eggs with Hoxb4GR RNA and allowed them to develop to the mid-neural stage. The embryos were then treated with dexam in the presence or absence of cycloheximide (CHX), which blocks protein synthesis. We examined the expression of Xrap-1 and Hoxb4 by reverse transcription–polymerase chain reaction (RT–PCR) of RNA subsequently extracted from these
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embryos (Fig. 4). Hoxb4 positively autoregulates its own expression by a direct mechanism (Hooiveld et al., 1999), thus activating the Hoxb4/GR construct should upregulate Hoxb4 expression, even in the presence of cycloheximide, as indeed it does (Fig. 4).

The activation of Hoxb4/GR by DEX results in a strong down-regulation of XRap1, indeed none can be detected when dexamethasone is added. There is also a very strong down-regulation of XRap1 when DEX and CHX are added together, although some XRap1 transcript is still detectable. This implies that the down-regulation of XRap1 by Hoxb4 does involve a direct mechanism, at least in part. It should be noted though that this result is complicated slightly by the apparent upregulation of XRap1 by CHX (Fig. 4), which may counteract Hoxb4 repression to some limited extent.

2.3. A Hoxb4 consensus binding site present in the XRap1 gene can bind HOXB4 protein and mediate transcriptional repression by Hoxb4

The 3′ untranslated region (UTR) of the XRap1 gene contains two sites that are very similar to the consensus sequence determined for optimum binding of HOXB4 protein to DNA in vivo (White et al., 2000) (Fig. 5A). In order to determine whether these sites could mediate transcriptional repression by Hoxb4, we cloned them into a position immediately 3′ to a luciferase (luc) reporter gene, driven by a SV40 promoter (RP1B4+, Fig. 5A). This promoter drives expression of luc in Xenopus embryos (Fig. 5B; Eakin and Balcells, 1985). Co-injecting Hoxb4 RNA with the RP1B4+ construct results in a significant down-regulation of luc activity. As a control, we used site
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Hoxb4
XRap1
sof1a

\[ \frac{X R a p 1 / s o f 1 a}{\text{signal ratio}} \]

Fig. 4. RT-PCR analysis of RNA extracted from control (‘untreated’) or Hoxb4/GR expressing embryos. The embryos were treated with dexamethasone (DEX) and cycloheximide (CHX), either alone or in combination, as shown. E3/α is included as a loading control. – RT. PCR amplification without prior reverse transcription step. The \( \frac{X R a p 1 / E 3 / α}{\text{signal ratio}} \) is shown for each sample.

Fig. 5. The genomic region encoding the 3' UTR of XRap1 mRNA contains a putative HOXB4 binding site that can block the transcription of a luciferase (luc) reporter construct. (A) The reporter constructs were based on the pGL3 vector which contains the luc gene under the control of the ubiquitously active SV40 promoter. The putative HOXB4 binding site from XRap1 was cloned immediately 3' to the luc gene, as shown. The nucleotide sequence of the putative HOXB4 binding region (RPB4+) and a non-binding variant used as a control (RPB4−) are shown. (B) Co-injection of Hox4 mRNA with the RPB4+ reporter construct blocks its activity. The RPB4+ reporter construct was injected into fertilized eggs together with Hox4 mRNA (the amounts shown are in picograms (pg)), or with 1000 pg of a control Hox8b mRNA lacking the homeodomain region (RPB4+ + Hox8b). The RPB4− construct was co-injected with 1000 pg of Hox4 mRNA (RPB4− + B4). No reporter construct lacking the SV40 promoter. (C) Hox5, Hox6, Hox7 and Hox8 RNAs (100 pg each) were also co-injected with RPb4+ construct. The values represented in the figure are the means from three independent experiments, the error bars show the standard deviation. ‘No RNA’, RPB4+ construct injected alone.

directed mutagenesis to alter the HOXB4 consensus binding sequence in RPB4+. This second construct (RPB4−) is not affected by Hoxb4 co-injection. Additionally, we co-injected RPB4+ with RNA transcribed from a deletion construct of Hoxb4 that lacks its homeodomain (Hoxb4ΔHD) and has previously been shown not to upregulate Hoxb4 expression (Hooiveld et al., 1999). Hoxb4ΔHD has no significant affect on RPB4+ activity (Fig. 5B).

We also examined whether other Hox genes could also affect the expression of the reporter construct. To this end we also co-injected the RPB4+ construct with Hoxb1, Hoxb5 and Hoxb9 mRNAs (Fig. 5C). Of these, only Hoxb5 prevents the expression of the reporter gene, a finding that is in agreement with the data in Fig. 2.

Whilst the above data suggests putative HOXB4 binding site in the XRap1 gene can mediate Hoxb4 repression, they do prove that there is a direct interaction between them. We decided to examine this in vivo by using purified HOXB4 protein, linked to agarose beads via GST (glutathione-S-transferase). We incubated these beads with a PCR product derived from either the RPB4+ or the RPB4− construct,
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2.4. Blocking the expression of the endogenous Hoxb4 gene results in a significant increase in XRap1 transcription

The translation of specific mRNAs in the early Xenopus embryo can be prevented by the injection of short DNA sequences that are chemically modified to prevent their degradation by endogenous nucleases (‘morpholinos’). Morpholinos are designed to be complementary to the translation start site of the target mRNA, and have recently proved to be extremely effective at blocking the expression of specific target genes (Heasman et al., 2000; Ross et al., 2001). We designed a morpholino complementary to the translation start site of Hoxb4 mRNA, together with a control morpholino of the same length and base composition but with a scrambled sequence. These were injected into fertilized eggs, and total RNA was extracted from neurula stage embryos that subsequently developed from them. We used this to examine the expression of XRap1, Hoxb1, Hoxb4 and Hoxb5 by RT–PCR (Fig. 7). The Hoxb4 morpholino (B4morph) causes a significant down-regulation of the endogenous Hoxb4 gene (Fig. 7, lane 2), presumably as a result of blocking its auto-regulation (Hoolveld et al., 1999).

The control morpholino (cmmorph) has no effect on Hoxb4 expression. As an additional control on the specificity of the B4morph’s specificity we co-injected B4morph with (human) HOXB4 RNA. This prevents the B4morph mediated down-regulation of the endogenous HOXB4 gene (Fig. 7, lane 4). The B4morph, but not the cmmorph, causes a significant increase in the expression of XRap1. Again, this effect is prevented by co-injection of HOXB4 RNA (Fig. 7).

3. Discussion

3.1. Regulating XRap1

Here we have shown that the Hox gene Hoxb4 directly represses the expression of a small GTPase, XRap1. Furthermore, Hoxb4 and XRap1 have complementary expression patterns, with XRap1 being excluded from those cells which express Hoxb4. It is instead most strongly expressed in the anterior most part of the embryo which is fated to form the forebrain and midbrain.

XRap1 is a member of the Ras superfamily of small GTPases that cycle between a GTP bound (active) and a GDP bound (inactive) form (for a review see Bos et al., 2001). Their main function is to assemble and activate proteins at the cytoplasmic surface of membranes in response to specific stimuli. The most notable member of this family is Ras itself, with Ras mutations being present in 15% of all human tumours.

Rap1 antagonizes Ras signalling, probably by trapping one of its key effectors, the serine/threonine kinase Rap, in an inactive complex. Like Ras, Rap1 is activated by guanine exchange factors (GEFs) that catalyse the exchange of GDP for GTP, and is inactivated by GTPase activating proteins.

Fig. 7. Blocking Hoxb4 translation results in the increased expression of XRap1. Fertilized eggs were injected with either an antisense Hoxb4 morpholino (lane 2), a control (scrambled sequence) morpholino (lane 3), or both the antisense Hoxb4 morpholino and HOXB4 mRNA (lane 4). The untreated, non-injected control is shown in lane 1. Total RNA was extracted from the embryos at the mid-neurula stage and analysed for the expression of XRap1, Hoxb1, Hoxb4, Hoxb5 and efla (a loading control), as shown.
(GAPs), that stimulate the otherwise very slow enzymatic activity of Ras family proteins. Rap1-interacting GEFs are activated either directly or indirectly by a number of intracellular messengers such as eCAMP (de Rooij et al., 1998; Kawasaki et al., 1998a), Ca\(^{2+}\) and diacyl glycerol (DAG) (Kawasaki et al., 1998b; Yamashita et al., 2000; Ebini et al., 1998). Far less is actually known about how Rap1 is controlled at the transcriptional level, however. To our knowledge, its repression by Hoxb4 is the first example of control being exerted at the transcriptional level during development.

3.2. Rap1 in development

Rap1 mediates a number of cellular processes that are involved in brain development. Most notably, it is essential for neurite outgrowth (York et al., 1998) and integrin-mediated cell adhesion (reviewed by Bos et al., 2001). Furthermore, mutations in the Drosophila homologue of Rap1 disrupt the normal development of the eye (Karapilov et al., 1989). It is conceivable that there is a similar requirement for Rap1 in vertebrate eye development, a possibility that is supported by our observation that XRap1 is expressed in the developing eye (Fig. 3) and, further, by a previous study showing that antibodies to β-integrin (a Rap1 effector) block retinal development in Xenopus (Stone and Sakaguchi, 1996).

Why is XRap1 excluded from the more posterior regions of the embryo? Presumably XRap1 activity may disrupt the normal development of the spinal cord by blocking specific developmental events, or by activating inappropriate ones. Hopefully this will be addressed by XRap1 over-expression studies.

3.3. Transcriptional control of Rap1

As discussed above, Rap1 is subject to numerous, stringent controls at the post-translational level. What is the likely significance of the very stringent transcriptional control we observe in early development? This is another question which is worthy of investigation. One possibility though is that, unlike the situation in the adult organism (or the cell lines from which they are derived), the complex post-translational controls on Rap1 activity have yet to be established. It may be then, that they only way to ensure the ‘silence’ of XRap1 in Hoxb4-expressing cells is to prevent its transcription.

3.4. Hox downstream targets

Identifying the downstream targets of Hox genes is necessary if their function in development is to be understood in molecular terms. XRap1 joins a very short list as to date very few Hox targets have been identified, and those which have tend to come from some what disparate systems. Furthermore, it is not always clear whether the identified targets are actually under the direct control of the Hox gene in question. Table 1 lists some of the Hox gene targets that have been identified in vertebrates (excluding the Hox genes themselves, which are subject to extensive auto- and cross-regulation (Gerard et al., 1996; Gould et al., 1997; Nonechov et al., 1997; Sharpe et al., 1998; Hooijveld et al., 1999)). The data is striking only for the lack of any apparent sequence or functional similarity between targets, and hints at an enormous complexity of downstream target control. In the light of the recent development of far more powerful techniques for detecting changes in gene expression, such as microarray analysis, it is slightly surprising that the list of Hox targets has grown so little. It is our hope that future studies will continue to address this fascinating problem.

4. Materials and methods

4.1. Differential display analyses

Fertilized Xenopus eggs were injected with 500 pg of Hoxb4/GFP RNA. Dexamethasone was added at either stage 7 or 11, and the embryos were then allowed to develop until stage 17. At this point total RNA was extracted and

<table>
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<tr>
<th>Hox gene</th>
<th>Transcription repressed (+) or activated (+)</th>
<th>Target gene</th>
<th>Target gene class</th>
<th>Ref.</th>
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<td>Hoxb-1</td>
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<td>Collagen</td>
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<td>Small GTPase</td>
<td>This report</td>
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<td>+</td>
<td>p53</td>
<td>Tumour suppressor</td>
<td>Raman et al., 2000</td>
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<td>+</td>
<td>SFR2</td>
<td>Serine protease inhibitor</td>
<td>Safari, 1997</td>
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<td>+</td>
<td>Ren-1(c)</td>
<td>Rennin</td>
<td>Pan et al., 2001</td>
</tr>
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</table>
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1 μg was used to make cDNA by reverse transcription using a poly-deoxythymidine primer (T15). Two percent of this reaction was then randomly amplified by PCR using a single primer (5′-CAG ATT GTT GGT GAA TAT GC-3′), with two rounds of amplification at 94 °C for 30 s, 45 °C for 30 s and 72 °C for 60 s, and then 30 rounds of amplification at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 60 s. The PCR products were resolved by electrophoresis on 2% agarose for 4 h at 200 V (4 °C) and visualized by ethidium bromide and 4% acetic acid. Differentially displayed bands of interest were cut out the gel and the PCR products were extracted by Qiaquick PCR Purification kit (Qiagen) and eluted in 50 μl water. The purified PCR products were PCR-re-amplified and gel-purified if necessary, cloned into pGEM-T Easy vector (Promega), and sequenced.

4.2. RNA extraction and RT–PCR

Total RNA was extracted from whole embryos using the QuickPrep Total RNA extraction kit (Amersham Pharmacia Biotech Inc.). 3 μg of RNA was used in subsequent reverse transcription reactions. This was mixed with a poly T20 oligo to 5 μg/ml and heated to 75 °C for 5 min. After cooling on ice, the following additional reagents were added: dNTPs to 0.4 mM, RNase OUT (Promega) to 1.6 units/μl, Moloney Murine Leukemia Virus Reverse Transcriptase (M-MMLvRT) RNaseH – point mutant (Promega) to 8 units/μl and the appropriate buffer (supplied by the manufacturer) to 1X concentration. The mixture was incubated for 1 h at 37 °C, heated to 70 °C for 2 min and cooled on ice.

PCR reactions were all performed in a total volume of 40 μl. For each we used 1 μl of the M-MMLvRT reaction (as described above), 0.2 nmol of each primer and 20 μl of Redimix pre-mixed PCR components (Sigma). All reactions were cycled at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 60 s. Thirty cycles were used for all primer sets except those for c/f3a, for which 23 cycles were used. The primers used for Xrap1 amplification were: forward – XRAP1F: 5′-GAT ATG CTG GGG GTG AAG CC-3′ and reverse – XRAP1D: 5′-CTA TTG AAT GTT CTT CAG CAG-3′. The sequences of the other primer pairs can be found on the internet at www.sghms.ac.uk/depts/anatomy/pages/ichuang.htm/.

4.3. Embryo culture and microinjection

These were performed as described previously (Sive et al., 2000).

4.4. Whole-mount in situ hybridization

Xrap1 was cloned into vector pGEMT-easy (Promega), and this was linearized using HindIII. A fluorescein-labelled in situ probe was transcribed from this template using SP6 polymerase. A DIG-labelled Hoxb4 probe was transcribed as previously described (Godsave et al., 1994). Probe purification and subsequent double probe in situ analysis were performed as described (Sive et al., 2000), using BCIP alone for Xrap1 detection and then NBT/BCIP to detect Hoxb4.

4.5. Luciferase reporter constructs

The putative HoxB4 binding sites in the Xrap1 3′ UTR region were cloned into the Xhol site of the pG3 luciferase reporter construct (Promega), immediately 3′ to the luc reading frame (Fig. 5). In order to do this, the following oligos were synthesized: RP145 + U 5′-CTAG GTATG GTATG AAGTG-3′; RP14-D 5′-CTGC ACTTA ATCAC ATACA AATCA-3′; RP145 – U 5′-CTAG GCCCT GTATG GGACC AAGTG-3′; RP145 – D 5′-CTAGC ACTTG GTCCC ATACA GTGCC-3′. Each of these four oligos were phosphorylated in separate reactions using polynucleotide kinase (PNK), using the protocol recommended by the manufacturer, and then the two (+) and (−) oligos were annealed by mixing half of each PNK reaction together, heating to 90 °C for 5 min and then cooling on ice. The annealed RP145 + and RP145 – oligos were then ligated into pG3 which had been restricted with Xhol, dephosphorylated using calf intestinal phosphatase (Promega), and purified using the Concert PCR purification system (Life Technologies). RP145 + and RP145 – clones were selected that contained only one copy of the insert, and these were checked by sequencing. The chosen clones were then purified using the Plasmid Midi kit (Qiagen).

RP145 – and RP145 + were injected into fertilized Xenopus eggs (100 pg in 5 nl), using the further refinements described by Mayor et al. (1993). Luciferase activity was measured as previously described (Morgan et al., 1999).

4.6. In vitro HoxB4 protein/Rap1 RNA interaction

A HoxB4/GST fusion protein was made by cloning the full-length Hoxb4 reading frame into the pGEX-2TK vector (Amersham Pharmacia Biotech); further deletion constructs were then made from this base. The fusion proteins were produced and purified according to the manufacturer’s instructions (GST purification module, Amersham Pharmacia Biotech). Xrap1 RNA was added to the purified proteins (still attached to the agarose beads), and incubated for 5 min at room temperature in 50 mM NaCl, 2 mM KCl, 2 mM CaCl2, 1 mM MgCl2 and 5 mM HEPES buffer (pH 7.4). Each of the DNA samples were present at a final concentration of 1 pg/ml. yeast tRNA was also added to a final concentration of 1 mg/ml. The beads were washed ten times in binding buffer (5 min each) and the bound RNA eluted by adding TE buffer and heating for 5 min at 50 °C.

4.7. Cycloheximide and dexamethasone treatments of Hoxb4/GGR injected embryos

These were performed as described (Gammill and Sive, 1997). Embryos were incubated with cycloheximide for 30 min prior to the addition of dexamethasone. RNA was
extracted from embryos 2 h after desacemethasone treatment, by which point the untreated control embryos had reached stage 17.

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