Erratum

Identification of minimal enhancer elements sufficient for Pax3 expression in neural crest and implication of Tead2 as a regulator of Pax3

Rita C. Milewski, Neil C. Chi, Jun Li, Christopher Brown, Min Min Lu and Jonathan A. Epstein *Development* **131**, 829-837.

An error in this article was not corrected before going to press.

The first two lines of the Summary should read:

Pax3 is a transcription factor that is required by pre-migratory neural crest cells, which give rise to the peripheral nervous system, melanocytes, some vascular smooth muscle, and numerous other derivatives. Both mice and humans with Pax3 deficiency exhibit neural crest-related developmental defects.

We apologise to the authors and readers for this mistake.

Identification of minimal enhancer elements sufficient for Pax3 expression in neural crest and implication of Tead2 as a regulator of Pax3

Rita C. Milewski, Neil C. Chi, Jun Li, Christopher Brown, Min Min Lu and Jonathan A. Epstein*

Cardiovascular Division, Department of Medicine, University of Pennsylvania, Philadelphia, PA, USA
*Author for correspondence (e-mail: epsteinj@mail.med.upenn.edu)

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Summary

Pax3 is a transcription factor that is required by Pre-migratory neural crest cells give rise to the peripheral nervous system, melanocytes, some vascular smooth muscle, and numerous other derivatives. These cells require the transcription factor Pax3, and both mice and humans with Pax3 deficiency exhibit neural crest-related developmental defects. Pax3 is also expressed in the dorsal neural tube, and by myogenic progenitors in the presomitic mesoderm and the hypaxial somites. Molecular pathways that regulate *Pax3* expression in the roof plate probably represent early upstream signals in neural crest induction. We have identified an enhancer region in the *Pax3* genomic locus that is sufficient to recapitulate expression in neural crest precursors in transgenic mice. We show that Tead2, a member of the Tead box family of transcription factors, binds to a neural crest enhancer and activates *Pax3* expression. Tead2, and its co-activator YAP65, are co-expressed with *Pax3* in the dorsal neural tube, and mutation of the Tead2 binding site in the context of *Pax3* transgenic constructs abolishes neural expression. In addition, a Tead2-Engrailed fusion protein is able to repress retinoic acid-induced *Pax3* expression in P19 cells and in vivo. These results suggest that Tead2 is an endogenous activator of *Pax3* in neural crest.

Key words: Pax3, Neural crest, Tead2, Myogenesis, Neurogenesis

Introduction

Pax3 is a transcription factor that is first expressed at E8.5 in the dorsal neural tube and by pre-migratory neural crest cells (Goulding et al., 1991). Mouse embryos lacking Pax3 suffer from spina bifida and display multiple neural crest-related defects including abnormal dorsal root ganglia, deficient enteric ganglia, cardiac outflow tract abnormalities and defective melanocyte development (Auerbach, 1954). Heterozygous *Pax3* mutant *Splotch* mice are viable, but display a characteristic white belly spot caused by deficient melanocyte migration. In cell culture, *Pax3* is induced by retinoic acid in P19 embryocarcinoma cells (Natoli et al., 1997; Pruitt, 1992), but little is known about other upstream regulators of *Pax3* expression during neural crest induction.

Pax3 plays a distinct role during development in myogenic precursors where it is thought to function upstream of MyoD (Maroto et al., 1997; Tajbakhsh et al., 1997). Pax3 is expressed in the presomitic mesoderm and becomes restricted to the ventrolateral region of the somites that gives rise to hypaxial derivatives (limb muscle, tongue, diaphragm and ventral body wall muscle). Pax3-deficient embryos lack limb musculature (Bober et al., 1994; Franz et al., 1993), and embryos lacking both Pax3 and Myf5 have no musculature whatsoever below the neck (Tajbakhsh et al., 1997).

Humans with heterozygous *PAX3* mutations suffer from Waardenburg syndrome which is characterized by pigmentation defects, including a characteristic white forelock, and deafness due to defective melanocyte contribution to the inner ear (Baldwin et al., 1992; Tassabehji et al., 1992). Some Waardenburg patients with *PAX3* mutations also suffer from limb muscle defects (Hoth et al., 1993).

Pax3 is one of the earliest markers of neural crest induction. Tissue-tissue interactions between neural ectoderm and epidermal tissues result in expression of early neural crest markers including *Slug*, *Snail*, *Pax3* and *Wnt1* (Garcia-Castro and Bronner-Fraser, 1999; LaBonne and Bronner-Fraser, 1999). However, specific signaling pathways necessary and sufficient to result in neural crest induction and *Pax3* expression have not been elucidated in detail. One approach for the identification of molecular cascades resulting in *Pax3* expression by neural crest precursors is to identify cis-acting regulatory sequences that are capable of mediating *Pax3* expression, and subsequently using this information to identify upstream trans-acting regulatory factors. Therefore, we and others have attempted to identify crucial regulatory elements mediating *Pax3* expression (Li et al., 2000; Li et al., 1999; Natoli et al., 1997).

Previous studies have identified a region of the proximal upstream genomic *Pax3* locus that is capable of mediating expression of reporter genes in appropriate developmental locations (Li et al., 2000; Li et al., 1999; Natoli et al., 1997). The 1.6 kb proximal *Pax3* upstream region is sufficient to mediate *lacZ* expression in the dorsal neural tube. Moreover, we have described mice expressing Cre-recombinase in this domain, and we have utilized these mice to fate-map *Pax3*-

expressing precursors during development (Epstein et al., 2000; Li et al., 2000). By crossing P3proCre mice with R26R Cre reporter mice, we have demonstrated that Pax3-expressing neural precursors become the enteric ganglia, the peripheral nervous system, and form significant portions of the aortopulmonary septation complex. Moreover, Pax3-expressing neural crest precursors become the smooth muscle cells of the aortic arch artery and major cranial vessels.

We have also utilized the proximal 1.6 kb *Pax3* genomic region to direct expression of Pax3 itself in neural crest (Li et al., 1999). Over-expression of Pax3 in this region is well tolerated, without overt abnormalities. Transgenic expression of Pax3 in this region is sufficient to rescue all of the neural crest defects, including lethal cardiac defects, normally found in Pax3-deficient *Splotch* embryos. Rescued pups succumb at birth because of defective musculature, which requires Pax3 and is not rescued by transgenic neural crest expression. Hence, these results made it clear that myogenic and neural crest expression of *Pax3* were separable, and that the proximal 1.6 kb upstream *Pax3* genomic region contained sequences sufficient to mediate functional expression of Pax3 in neural crest.

We have identified enhancer elements sufficient to mediate neural crest expression of *Pax3*, and have utilized these discrete sequences to identify an upstream trans-acting regulator of *Pax3* in neural tissues.

Materials and methods

Transgenic mice

Pax3 upstream genomic sequence was amplified by PCR and sequence verified. Serial deletion constructs, P3proLacZ construct 1 through construct 10 (Fig. 1), were prepared by PCR using oligonucleotides that contained either *Asc*I or *Bgl*II restriction sites, or unique restrictions sites within the endogenous *Pax3* sequence. Regions of endogenous sequence contained in each construct are summarized below; base pair numbering corresponds to GenBank AC084043.

Construct 1: 25751 bp – 19591 bp
Construct 2: 21447 bp – 19591 bp, 19430 bp – 18253 bp
Construct 3: 21447 bp –19591 bp
Construct 4: 21231 bp –19591 bp
Construct 5: 20853 bp –19591 bp
Construct 6: 21447 bp –20853 bp, 19930 bp – 19591 bp
Construct 7: 21231 bp –19591 bp, 19930 bp – 19591 bp
Construct 8: 21231 bp –20621 bp, 19930 bp – 19591 bp
Construct 9: 21231 bp –21009 bp, 20853 bp – 20621 bp, 19930 bp – 19591 bp
Construct 10: 21231 bp – 21009 bp, 20853 bp – 20703 bp, 19930 bp – 19591 bp

For the production of transgenic mice, constructs were restriction digested with *Asc*I and *Bgl*II to remove vector sequences and gel purified. To evaluate the requirement of the Tead site in the *Pax3* neural crest enhancer region 2 (NCE2) we mutated the Tead site (TGAATGT to TCCATGG) in construct 9 (Fig. 1). We also created transgenic mice in which 15 kb of *Pax3* upstream genomic sequence was cloned upstream of *lacZ*, and an additional construct in which a distinct *Pax3* somite-specific enhancer, located within this 15 kb region (C.B.B. and J.A.E., unpublished), was subcloned upstream of NCE1 and the mutated NCE2. The somite enhancer acted as an internal control for *lacZ* expression. Constructs were injected into the male pronucleus of B6SJLF1/J zygotes. Embryos were fixed in 2% paraformaldehyde for 2 hours and incubated in 0.1% X-gal solution at 37°C to assess expression of β-galactosidase activity.

To create the Tead2-Engrailed transgenic construct, the *Wnt1* promoter-enhancer construct (Lee et al., 1997) in pWEXPZ (kindly provided by D. Epstein) was modified to drive expression of a Tead2-Engrailed fusion protein that was encoded by bp 1-453 of GenBank D50563 (Tead2) and bp 169-1071 of GenBank M10017 (*Drosophila* Engrailed). This construct was digested with *Aat*II to remove vector sequences prior to oocyte injection.

P19 cell culture and differentiation

P19 embryonal carcinoma cells were cultured on glass coverslips coated with 0.1% gelatin in DMEM with 10% fetal bovine serum and co-transfected at 50% confluency with 2.5 μg pCMV-GFP and either 25 μg pcDNA3 or 25 μg pcDNA3-Tead2-Engrailed with 75 μl Fugene (Roche). Twelve hours post-transfection, 1 μM retinoic acid was added to induce Pax3 expression (Natoli et al., 1997). After 5 days, cells were fixed in 4% paraformaldehyde for 10 minutes and then dehydrated to 100% methanol. The cells were then rehydrated and incubated in 5% goat serum blocking agent for 1 hour and incubated overnight with a rabbit polyclonal anti-Pax3 antibody at a 1:1000 dilution at 4°C. The cells were then incubated in goat anti-rabbit secondary antibody (GAR-Alexa 594 IgG, Molecular Probes) for 1 hour and examined by dual fluorescent microscopy for GFP and Pax3 expression.

Yeast one-hybrid assay

Yeast one-hybrid assay was performed using the MATCHMAKER One Hybrid system (Clontech) according to manufacturer's instructions. The target-reporter construct ('bait') included the 232 bp NCE2 (corresponding to GenBank AC084043 bp 20621-20853). Yeast colonies with integrated target-pHISi-1 (pHISi-1P3proNCE2) were tested for background expression and showed no growth on synthetic defined agar plates lacking histidine (SD/-HIS) at <7.0 mM 3-amino-1,2,4-triazole (3AT). A dual reporter yeast strain was made containing integrated pHISi-1P3proNCE2 and pLacZ P3proNCE2. An E10.5 mouse AD fusion library was screened using 10 mM 3AT on Ura/His/Leu-deficient plates. Initially, 1.4 million clones were screened and 120 positive clones were picked by nutrient growth selection. Twenty of these clones stained positive with X-gal. Plasmid was isolated from these clones and sequenced.

In situ hybridization and immunohistochemistry

Radioactive in situ hybridization was performed as described previously (Wawersik and Epstein, 2000). cDNAs for *Tead1, 3* and *4* (provided by Iain Farrance, University of Maryland) were subcloned into the pcDNA3 expression vector (Invitrogen) and used as template for riboprobe synthesis. *Tead2* (GenBank D50563 bp 1-2115) was obtained from the yeast one-hybrid screen. *YAP65* cDNA (GenBank NM_00934 bp 1-4125) was subcloned into pCMVSport6 (Invitrogen) prior to linearization and riboprobe synthesis. Immunohistochemistry was performed using polyclonal rabbit Pax3 antibodies prepared in our laboratory (Li et al., 1999) or monoclonal anti-neurofilament antibodies (2H3, Hybridoma Study Bank) using standard techniques. Details are available at www.uphs.upenn.edu/mcrc/histology.

Electrophoretic mobility shift assay (EMSA)

Tead2 cDNA (GenBank D50563 bp 1-2115) was cloned into pcDNA3 and *YAP65* (GenBank NM_009534 bp 1-4125) was cloned into pCMVSport-6 for in vitro transcription and translation (TNT, Promega). Production and size of the protein products were confirmed by SDS-PAGE. Radioactive DNA probes were generated by annealing complimentary oligonucleotides and filling in remaining overhangs with Klenow DNA polymerase or by end labeling of oligonucleotides. EMSA reactions were performed as described previously (Epstein et al., 1994). Probe sequences are summarized below.

Tead site, forward GCGGATCGGGGATGAATGTGTACGTG-GAGA

Tead site, reverse GCGGTCTCCACGTACACATTCATCCCCGAT

Tead site mutation, forward GCGGATCGGGGATCCATGGGTACGTGGAGA

Tead site mutation, reverse GCGGTCTCCACGTACCCATGGATCCCCGAT

Luciferase co-transfection assays

The pGL2P3proNCE2 construct was generated by cloning NCE2 into pGL2-basic (Promega). Derivative constructs contained the identical mutations in the Tead binding site as described above for transgenic constructs and EMSA experiments. NIH-3T3 cells were transfected using FuGENE 6 (Roche). All transfections included equal amounts of total DNA and results were corrected for transfection efficiency.

Results

Identification of minimal neural crest enhancer elements in the proximal Pax3 promoter

To delineate specific regions within the 1.6 kb proximal Pax3 promoter that regulate neural crest expression of Pax3 in vivo (Li et al., 1999), we constructed a series of deletion mutants of the 1.6 kb promoter construct (Fig. 1, see Materials and methods for sequence information). We also tested additional upstream sequence including the proximal 6.1 kb region, and the 1.1 kb intron 1 sequence that contains regions of sequence conservation between mouse and human genomes. The 10 constructs tested in transgenic mice are shown in Fig. 1. Inclusion of the entire 6.1 kb upstream genomic region produced transient transgenic mice with β-galactosidase expression evident in the hindbrain, dorsal neural tube and dorsal root ganglia (construct 1, Fig. 1, Fig. 2A). This expression pattern, in eight independent transgenic embryos, was very similar to what we have previously observed using only the 1.6 kb upstream region (construct 3, Fig. 1). Addition of the 1.1 kb intron 1 sequence did not significantly alter expression at E10.5 (construct 2, Fig. 1 and Fig. 2B). This expression pattern recapitulates some, but not all, endogenous *Pax3* expression domains. *Pax3* is normally expressed in the somites, an area of expression completely absent in the transgenic mice that we generated. Also, endogenous *Pax3* is expressed uniformly in the dorsal neural tube along the rostral-caudal axis of the embryo. Expression in our transgenic mice was weak or absent in cervical and caudal hindbrain regions, suggesting that *Pax3* expression in cranial neural crest is mediated by enhancer regions outside of the regions that we examined. Also, β-galactosidase expression was evident in dorsal root ganglia even after endogenous *Pax3* mRNA expression had subsided, presumably because of perdurance of the β-galactosidase protein.

Sequence comparison of mouse and human genomes identified two regions of significant conservation within the proximal 1.6 kb region. We refer to these regions as neural crest enhancer 1 and 2 (NCE1 and NCE2; Fig. 1). Deletion of sequence 5′ to these conserved elements had no effect upon expression in transgenic mice (construct 4, Fig. 1 and Fig. 2C). Likewise, progressive deletion of sequence 3′ to these conserved elements did not alter expression (constructs 7, 8, Fig. 1 and data not shown). However, deletion of either NCE1 (construct 5, Fig. 1) or NCE2 (construct 6, Fig. 1) destroyed activity, suggesting that both conserved elements are required for transgenic expression. A 610 bp fragment containing NCE1 and NCE2 (construct 8, Fig. 1) was sufficient to direct *lacZ* expression in a pattern similar to the entire 6.1 kb upstream

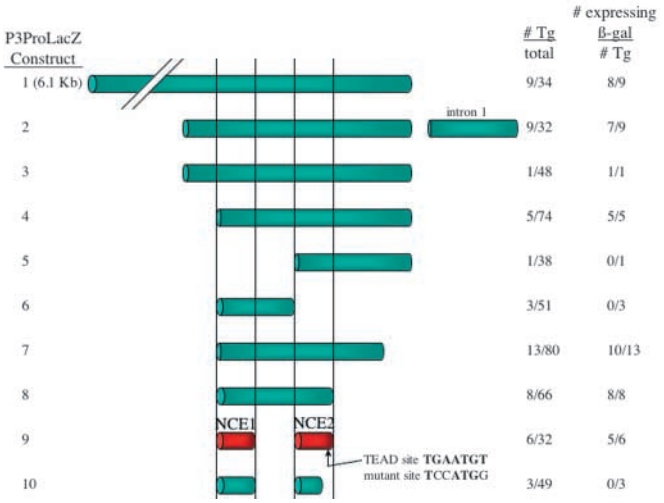

Fig. 1. Identification of minimal cis-acting neural crest enhancer regions in the proximal *Pax3* genomic region. Constructs 1-10 used for the creation of transgenic mice (not to scale, see Materials and methods for construct details). Neural crest enhancer elements, NCE1 and NCE2, are in red. The Tead binding site within NCE2 is indicated and the mutant version of this site used in subsequent experiments is shown. The number of genotype positive E10.5-11.5 embryos obtained from injection of each construct is shown and those expressing β-galactosidase are indicated.

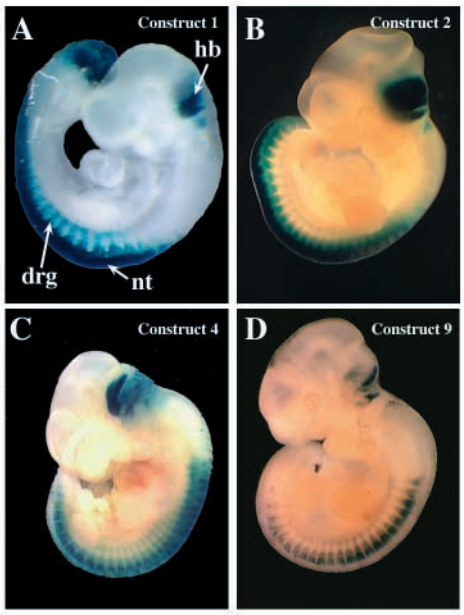

Fig. 2. β-galactosidase expression in *Pax3* transgenic mice. (A) The 6.1 kb upstream region of the proximal *Pax3* genomic sequence (construct 1) partially recapitulates *Pax3* expression in the hindbrain (hb), the dorsal root ganglia (drg), and the neural tube (nt). (B) Transgenic expression from the 1.6 kb upstream region and intron 1 (construct 2) yields a similar expression pattern to the 6.1 kb construct. (C) Deletion of 5′ upstream region (construct 4) maintains the neural crest expression pattern. (D) Deletion of sequences 5′ and 3′ to NCE1 and NCE2 as well as the 156 bp internal region (resulting in construct 9) maintains the expression pattern seen in A, although hindbrain and neural tube expression is somewhat weaker.

region, and deletion of the non-conserved 156 bp sequence between NCE1 and NCE2 (construct 9, Fig. 1 and Fig. 2D) had little effect on the pattern of *lacZ* expression, though hindbrain and dorsal neural tube expression was somewhat weaker and more patchy in these embryos. Further deletion of 82 bp at the 3′ end of NCE2 resulted in complete loss of expression in 3 transgenic embryos. Hence, NCE1 and NCE2 together are sufficient to direct *lacZ* expression to the dorsal neural tube, dorsal root ganglia, and hindbrain region in transgenic embryos. Subsequent analysis focused on the requirement for specific sequences within NCE2, as described below; further studies will be required to determine the necessity of NCE1 sequences for *Pax3* expression, especially because the number of informative transgenic embryos in which NCE1 was specifically deleted was low.

Tead2 binds to a Pax3 neural crest enhancer

Having identified discrete enhancer sequences, NCE1 and NCE2, that are sufficient to mediate neural tube and neural crest expression of *Pax3*, we sought to utilize these elements to identify trans-acting factors that regulate *Pax3*. We performed a yeast one-hybrid assay using a mouse E10.5 cDNA library and either NCE1 or NCE2 as bait. We were unable to obtain informative results from experiments using NCE1 as bait owing to high background, presumably because endogenous yeast activators of transcription were able to bind to this reporter construct. However, utilizing NCE2 as a bait, a total of ~1.4 million clones were screened and 20 positive clones were identified by dual selection criteria. Of these, 10 positive clones encoded portions of the transcription factor protein, Tead2. These non-identical clones all contained the coding region for the DNA binding domain of Tead2, the Tead-box. We expressed the full length Tead2 protein and confirmed the specific interaction with NCE2 in yeast (data not shown).

Analysis of the sequence of NCE2 reveals a consensus Tead binding motif (Davidson, 1988; Jacquemin, 1996; Xiao, 1991) within this enhancer (Fig. 1). Interestingly, this potential Tead binding site is located within a region of NCE2 that is required for enhancer activity in transgenic mice, since deletion of this region abolished reporter activity (compare constructs 9 and 10, Fig. 1). We tested whether Tead2 could bind to the putative binding site present in NCE2 by performing electrophoretic mobility shift assays (EMSA). As shown in Fig. 3, Tead2 is able to bind to the NCE2 binding site (Fig. 3, lane 1) and specific binding is dramatically reduced when a mutation is introduced into the putative Tead site (Fig. 3, lane 2). An antibody specific for Tead2 was not available for supershift experiments. However, Tead2 is known to bind directly to a transcriptional co-activator, Yes-associated protein (YAP65/Yap) (Vassilev, 2001). Addition of in vitro-translated/transcribed YAP65 to the binding reaction resulted in a supershift of the Tead2/DNA complex (Fig. 3, lane 3) consistent with the ability of Tead2, and a Tead2/YAP65 complex, to bind to the Tead binding site in NCE2. YAP65 alone was unable to bind to NCE2 (data not shown).

Tead2 and Pax3 are co-expressed in the dorsal neural tube

Our *Pax3* promoter analysis and yeast one-hybrid studies suggest that a Tead binding site in the *Pax3* promoter is crucial for neural crest and dorsal neural tube expression, and that a

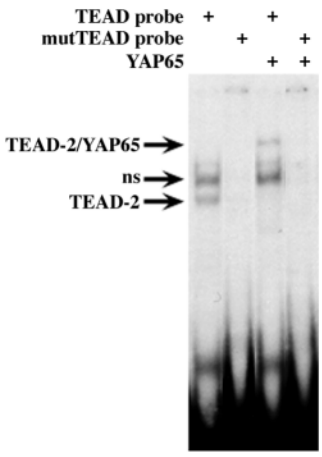

Fig. 3. Tead2 binds to a Tead binding site within the *Pax3* neural crest enhancer. An electrophoretic mobility shift assay is shown with Tead2 protein and an oligonucleotide derived from the NCE2 sequence. Tead2 binds to this site as seen in lane 1. Mutation of the Tead binding site abolishes binding (lane 2). When the known Tead co-factor YAP65 is added to the reaction, a supershift of the Tead2/DNA complex is observed (lane 3). Mutation of the Tead site abolishes the observed supershift (lane 4). All samples were run on the same EMSA gel.

Tead2/YAP65 complex is able to bind to this site. Next, we sought to determine if *Pax3*, *Tead2* and *YAP65* are co-expressed in the dorsal neural tube. We performed in situ hybridization analysis, and we examined the expression pattern of other members of the Tead-box family including *Tead1*, *3* and *4*. [See Table 1 for clarification of Tead nomenclature; adapted from Stewart et al. (Stewart et al., 1998).] The DNA binding domains of Tead-box family members are highly conserved and these factors have over-lapping DNA binding properties (Davidson, 1988; Jacquemin, 1996; Xiao, 1991). In situ analysis reveals remarkable overlap of *Pax3*, *Tead2* and *YAP65* in the ventricular zone of the dorsal neural tube (compare Fig. 4A, B and C). *Tead1* and *Tead3* are expressed weakly in a pattern that overlaps *Tead2* (Fig. 4D,E). *Tead4* is not expressed in the neural tube (Fig. 4F). Expression of *Tead2* and *YAP65* extended ventrally further than that of *Pax3*, which is restricted to the dorsal half of the neural tube (Fig. 4A, arrow). It is worth noting that dorsal restriction of *Pax3*

Table 1. Tead gene family nomenclature*

Tead gene	Alternative or previous name
Tead1	*mTead1*
	TEF-1
Tead2	*mTead2*
	TEF-4
	ETF
Tead3	*mTead3*
	TEF-5
	ETRF-1
	DTEF-1
Tead4	*mTead4*
	TEF-3
	ETRF-2
	RTEF-1

*Adapted from Stewart et al., 1998.

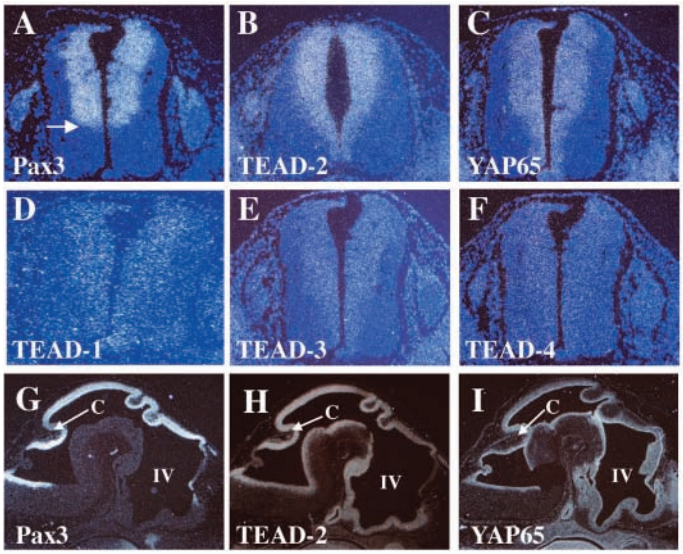

Fig. 4. *Tead2* and *Pax3* are co-expressed in the dorsal neural tube. In situ hybridization was performed at E11.5. (A) *Pax3* expression is dorsally restricted in the neural tube (arrow). (B) *Tead2* expression overlaps that of *Pax3* and extends ventrally. (C) *YAP65* expression mimics that of *Tead2*. (D,E) *Tead1* (D) and *Tead3* (E) are expressed weakly in the neural tube. (F) *Tead4* expression is not detected in the neural tube. (G-I) Sagittal sections through the brain showing expression of *Pax3* in the ventricular zone (G), overlapping with that of *Tead2* (H) and *YAP65* (I). c, cerebellar pimordium; IV, fourth ventricle.

expression is known to be due to repressive signals, including Sonic hedgehog (Shh), emanating from the floorplate and notochord (Goulding et al., 1993). Surgical extirpation of these structures results in ventral expansion of *Pax3* such that it resembles the endogenous *Tead2* and *YAP65* expression domains. These results suggest that Tead2 and YAP65 may act to positively regulate *Pax3* via the NCE2 Tead binding site, while additional negatively acting regulators downstream of Shh act to dorsally restrict *Pax3* expression.

Tead2 activates transcription via the NCE2 Pax3 enhancer

We tested whether Tead2 and the co-activator, YAP65, could interact with the NCE2 enhancer region of the proximal *Pax3* promoter to activate the expression of a reporter construct in a eukaryotic system. To test this hypothesis, the proximal *Pax3* promoter including the NCE2 enhancer region was subcloned into the pGL2 luciferase reporter vector. This pGL2Pax3proNCE2 construct was co-transfected with either full-length Tead2 alone, or together with the YAP65, into NIH-3T3 cells. The pSV-β-galactosidase control vector was used to normalize for transfection efficiency. As seen in Fig. 5, transfection of the pGL2Pax3proNCE2 construct with Tead2 alone resulted in minimal luciferase reporter activity above baseline. Addition of YAP65 activated luciferase reporter activity by 3.5 fold. YAP65 alone had no effect. The ability of Tead2 and YAP65 to activate reporter gene expression was specific, since mutation of the Tead binding site abolished activation. Thus, in a eukaryotic system, Tead2 and its co-activator, YAP65 can activate the NCE2 Pax3 enhancer.

Mutation of the Tead2 binding site in the NCE2 enhancer abolishes transgenic expression in neural tube and neural crest

In order to determine if the Tead2 binding site located in the *Pax3* neural crest enhancer NCE2 is required for transgenic expression of reporter genes in the neural crest, we engineered a mutation in this site in the context of construct 9 (Fig. 1). The mutation that we introduced was identical to the mutation used in EMSA experiments that demonstrated abrogation of interaction with Tead2. We examined 45 embryos at E10.5 and identified three that carried the mutated transgene. None of these embryos expressed detectable levels of β-galactosidase activity in the neural tube. We engineered the identical mutation in a construct that contained NCE1 and NCE2 in addition to a more upstream element that mediates expression in the somites (C.B.B. and J.A.E., unpublished). Seven of 126 E10.5 embryos carried this transgene, and two embryos expressed *lacZ* in the somites. No expression was detected in the neural tube of transgenic embryos carrying the Tead mutation (Fig. 6A) in contrast to transgenic embryos with

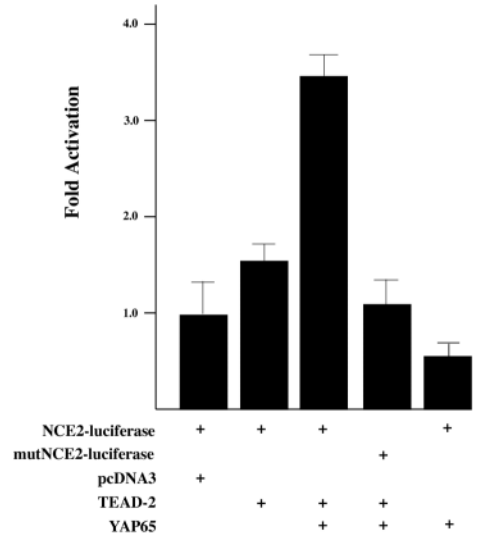

Fig. 5. Tead2 activates transcription of a reporter construct containing NCE2. Co-transfection of a reporter construct containing NCE2 with Tead2 results in minimal activation (bar 2) over baseline transfection with pcDNA3 (bar 1). Addition of the Tead co-activator YAP65 (bar 3) results in a 3.5-fold activation over baseline. Mutation of the Tead binding site in NCE2 (bar 4) abolishes activation. YAP65 alone (bar 5) is unable to activate luciferase expression. Results are normalized for transfection efficiency and expressed as the mean ± standard deviation of three experiments, each performed in triplicate.

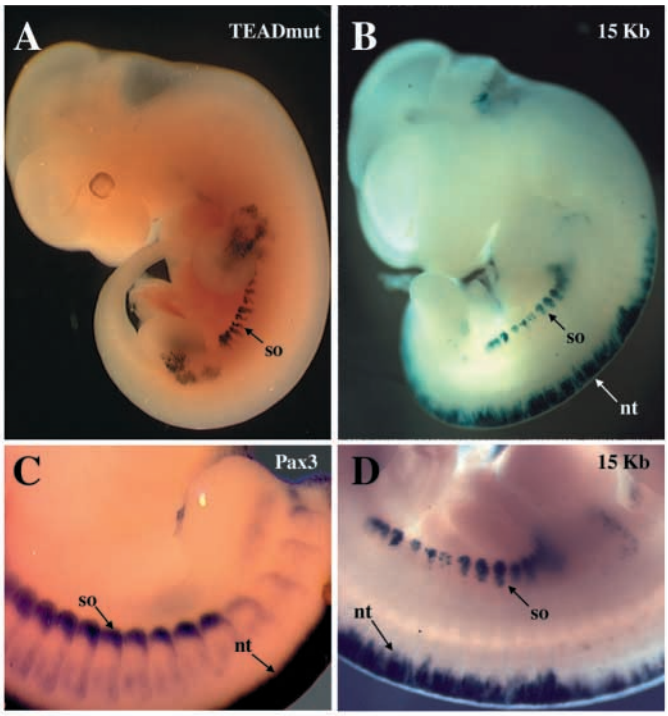

Fig. 6. Mutation of the Tead2 binding site in the NCE2 enhancer abolishes transgenic expression in the neural tube and neural crest. (A) Transgenic embryo carrying a construct that includes a somite-specific *Pax3* enhancer and the neural crest enhancers with a mutation in the Tead site of NCE2 is shown. No expression is evident in the dorsal neural tube or neural crest, while somite expression is maintained. (B) Transgenic embryo with the proximal 15 kb proximal *Pax3* region including intact somites and neural crest enhancers demonstrates expression in both the somites and neural crest. (C) Whole-mount in situ hybridization demonstrating the wild-type *Pax3* expression pattern. (D) Magnification of transgenic embryo shown in B.

intact somite and neural crest enhancer sequences (Fig. 6B,D) which recapitulated wild-type *Pax3* expression (Fig. 6C). These results demonstrate that an intact Tead2 binding site in the context of our transgenic constructs is required for neural tube expression.

Dominant negative Tead2 reduces Pax3 expression and affects development of neural crest derivatives

Tead2 is expressed broadly during development and is likely to affect development of numerous tissues. In order to test specifically if Tead2 is required in vivo for *Pax3* expression, we created transient transgenic mice in which a dominant negative Tead2 protein was expressed in neural crest. We constructed a Tead2 fusion protein incorporating the potent Engrailed repression domain downstream of the Tead2 DNA binding domain. We used the *Wnt1* promoter, which has previously been well characterized and expresses in the dorsal neural tube and neural crest progenitors (Jiang et al., 2000; Serbedzija and McMahon, 1997), to direct expression in neural crest. Fate-mapping studies indicate that this *Wnt1* promoter expresses in a population of neural crest cells that overlaps with that expressing *Pax3* and our *Pax3* transgenic constructs (Brown et al., 2001; Jiang et al., 2000). We obtained four transient transgenic embryos at E10.5, of 74 embryos examined. Pax3 expression, assessed by immunohistochemistry, was detected in the dorsal neural tube, the dorsal root ganglia and the somites of wild-type embryos (Fig. 7A,B). Transgenic littermates, however, had abnormally small dorsal root ganglia and reduced Pax3 expression in the dorsal neural tube, which displayed abnormal morphology (Fig. 7C,D). Pax3 expression in the somites, a region that does not overlap with *Wnt1* expression, was grossly unaffected.

We examined neurofilament expression by immunohistochemistry in wild-type and transgenic embryos. Neurofilament expression by axons emerging from the dorsal root ganglia was significantly reduced in transgenic embryos compared to wild-type embryos (Fig. 7E,F). At this time point, differentiation of enteric ganglia, derived from neural crest, is just initiating and faint neurofilament expression was seen in the hindgut of wild-type embryos (Fig. 7E). Neurofilament expression was absent from the hindgut of transgenic embryos (Fig. 7F). These data support the hypothesis that the Tead2 binding site in the *Pax3* enhancer is functional in vivo.

In order to verify that the Tead2-Engrailed fusion protein was capable of inhibiting Tead2 and YAP65-mediated activation of NCE2, we performed co-transfection assays in NIH-3T3 cells with pGL2Pax3proNCE2, Tead2 and YAP65, with or without increasing amounts of a Tead2-Engrailed expression vector. Tead2 and YAP65 were able to modestly activate reporter gene expression (Fig. 7G), and this activation was inhibited in a dose-dependent fashion by Tead2-Engrailed.

We also tested the ability of Tead2-Engrailed to inhibit endogenous Pax3 expression in cultured cells. P19 embryocarcinoma cells can be induced to adopt neuronal features, and to express Pax3, by addition of retinoic acid (Pruitt, 1992). Retinoic acid also induces activation of reporter gene activity in P19 cells when genomic fragments containing our Tead2 binding site are included in the reporter construct (Natoli et al., 1997). Hence, we investigated whether Tead2-Engrailed could prevent retinoic acid-induced Pax3 expression in P19 cells. We co-transfected a GFP expression vector with control vector, or with a Tead2-Engrailed expression vector, and subsequently added retinoic acid. More than half of the GFP-expressing transfected P19 cells also expressed Pax3 in control experiments (92 of 167 cells counted, 55%) (Fig. 7H). However, fewer than 10% of cells co-transfected with GFP and Tead2-Engrailed expressed Pax3 (15 of 158, 9.5%) (Fig. 7I). Hence, Tead2-Engrailed significantly inhibited Pax3 expression both in vitro and in vivo.

Discussion

Pax3 has crucial effects in two distinct domains during development. In the dorsal neural tube, Pax3 is required for proper development of the neural crest. In the somites, Pax3 is vital for normal myogenesis. In this report, we have characterized the upstream genomic sequences sufficient for *Pax3* expression in the dorsal neural tube and neural crest. We have utilized this information to identify an upstream regulatory factor, Tead2, and we provide evidence to support the conclusion that Tead2 can regulate *Pax3* expression.

Previous studies from our laboratory have demonstrated that sequences within the proximal 1.6 kb *Pax3* promoter are sufficient to recapitulate *Pax3* expression in the dorsal neural

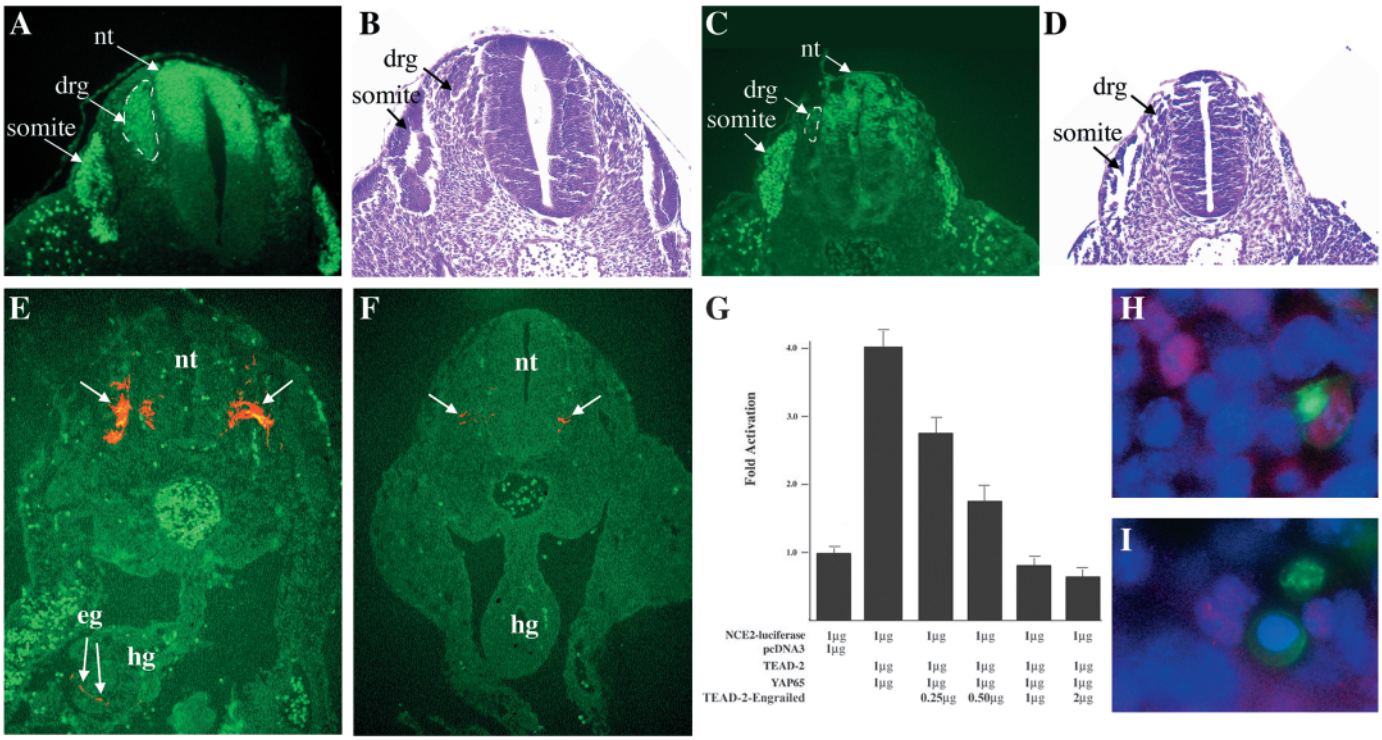

Fig. 7. Expression of dominant negative Tead2 affects Pax3 expression. A Tead2 fusion protein including the Engrailed repressor domain was expressed in the neural tube using the *Wnt1* promoter. (A) Wild-type embryo with normal morphology and Pax3 protein expression (green) in the dorsal neural tube (nt), dorsal root ganglia (drg, dotted line) and somites. (B) Adjacent Haematoxylin and Eosin-stained wild-type section. (C) Transgenic embryo with an abnormal dorsal neural tube (nt) and small dorsal root ganglia (drg, dotted line) with diminished Pax3 expression in neural tube and drg. Pax3 protein expression in the somites is unaffected. (D) Adjacent Haematoxylin and Eosin stained transgenic section. (E) Wild-type E10.5 embryo stained with anti-neurofilament antibody (arrows) showing strong expression adjacent to neural tube (nt) and dorsal root ganglia and weaker expression by enteric ganglia (eg) in the hindgut (hg). (F) Neurofilament expression in transgenic embryo is weak in regions adjacent to dorsal root ganglia (arrows) and absent from hindgut. (G) Tead2-Engrailed inhibits Tead2 and YAP65-mediated activation of a luciferase reporter construct containing NCE2 (NCE2-luciferase) in a dose-dependent manner. Fold activation of luciferase activity is corrected for transfection efficiency and performed as in Fig. 5. (H) Retinoic acid induces endogenous Pax3 protein expression (red; detected by immunohistochemistry) in P19 cells transfected with a GFP expression vector (green). (I) Co-transfection of a Tead2-Engrailed expression vector with GFP inhibits Pax3 expression such that those cells expressing GFP do not express Pax3. Nuclei are stained blue.

tube, and we have shown that transgenic expression of Pax3 in this tissue is sufficient to rescue neural crest defects in Pax3-deficient *Splotch* mice. In the work reported here we identified crucial enhancer regions located within the proximal 1.6 kb upstream region. We identified two conserved regions, each approximately 200 bp in length, that are separated by a dispensable 156 bp linker region. Previous studies, using cell culture-based assays, have implicated similar genomic regions as being important for Pax3 expression.

Although the minimal neural crest enhancers are sufficient to direct reporter gene expression to the dorsal neural tube, this expression does not entirely recapitulate endogenous Pax3 neural expression. Notably, in all the transgenic embryos that we examined, reporter gene expression was diminished in the cervical regions and was absent from the majority of domains within the CNS in which endogenous Pax3 is expressed. In addition, we noted variable ectopic expression in the most caudal regions of the embryo, lateral to the neural tube. Also, expression within the neural tube was more dorsally restricted than that of endogenous Pax3, and the border between dorsal expressing cells and ventral non-expressing cells was less sharp. These results indicate that regulatory sequences outside of the regions examined contribute to the regulation of Pax3 neural expression. Transgenic analysis of sequences within the proximal 15 kb of upstream sequence, and including the first intron, do not suggest the presence of additional neural regulatory elements within these regions.

Pax3 is an early marker of the neural crest lineage (Goulding et al., 1991). It is expressed prior to *Wnt1* and is thought to function upstream of *Foxd3* during neural crest induction (Dottori et al., 2001). Neural crest induction requires tissue-tissue interaction between neural and epidermal ectoderm. Secreted growth factors of the BMP, Wnt and FGF families have been implicated in neural crest induction (Garcia-Castro and Bronner-Fraser, 1999; LaBonne and Bronner-Fraser, 1998), but how they integrate to result in gene-specific induction, including activation of *Pax3*, remains unknown. The identification of specific enhancer regions capable of mediating neural crest expression of *Pax3* should aid in the identification of these signaling pathways. The observation that Tead2 can bind to one of the *Pax3* neural crest enhancers and mediate activation suggests that Tead2 may function as an intermediary in a signaling cascade required for neural crest induction.

The Tead (TEA Domain) family of proteins contain a highly

conserved 72 amino acid DNA binding domain, which is evolutionarily conserved between yeast, *Drosophila*, rat, chick, mouse and human (Jacquemin, 1996; Kaneko, 1997; Kaneko, 1998). There is at least one Tead protein family member expressed in all tissue in the developing embryo as well as in most adult tissues (Kaneko, 1997; Vassilev, 2001). The Tead proteins were initially identified as transcription factors that activated the divergent GT-IIC (TGGAATG), SphI (AAGCATG), and SphII (AAGTATG) enhansons of the SV40 enhancer (Davidson, 1988; Xiao, 1987; Xiao, 1991) and the polyoma virus enhanson (TAGAATG) and its mutated form (TGGAATG) (Davidson, 1988; Xiao, 1987), as well as a muscle-specific M-CAT (CATTCCT) enhanson (Farrance, 1992; Larkin, 1996). The Tead family of proteins (Tead 1-4, Table 1) are known to all bind these divergent sequences with varying affinities (Davidson, 1988; Kaneko, 1998; Xiao, 1991). The binding site identified in the *Pax3* NCE2 enhancer, TGAATG, most closely resembles the GT-IIC binding site. Tead2 is able to bind to the NCE2 DNA binding site with high affinity. The other Tead family members are also able to bind the Tead site in NCE2, although with lower affinity (data not shown). Sequences flanking the core binding site are known to modulate the affinity of Tead proteins for Tead binding motifs (Larkin, 1996), which may be an important factor for determining isoform specificity (Farrance, 1996).

Each of the four Tead protein family members, Tead 1-4, displays an overlapping, yet distinct spatiotemporal expression pattern (Jacquemin, 1996; Jacquemin, 1998; Kaneko, 1998; Vassilev, 2001; Yasunami, 1996; Yockey, 1996). Tead2 is the earliest Tead family member to be expressed in the murine embryo with initial appearance at the two cell stage (Kaneko, 1997; Kaneko, 1998). Tead2 was first isolated from neural precursor cells (Yasunami, 1995). By mid-gestation the Tead2 expression pattern includes the ventricular layer of the neuroepithelium in the developing brain and spinal cord and by late gestation persists in the ventricular zone of the CNS, as well as facial and gut mesenchyme, cortical layer of kidney and lung (Jacquemin, 1996). Tead1 and Tead2 have overlapping patterns of expression in early gestation with patterns diverging at mid-gestation. However, both Tead1 and Tead2 continue to be co-expressed in neuroepithelium. The expression data suggests that Tead2, amongst the Tead family of proteins, is most strongly expressed in regions of neural *Pax3* expression. However, other Tead members, specifically Tead1, are co-expressed although less intensely in the dorsal neural tube, suggesting possible functional redundancy (Jacquemin, 1996; Jacquemin, 1998). The dominant negative Tead2-Engrailed construct that we employed in transgenic mice is likely to antagonize transcriptional activation mediated by various members of the Tead family because of their overlapping DNA binding characteristics.

In summary, our studies identify enhancer elements located in the proximal *Pax3* upstream genomic region that together are sufficient to recapitulate neural expression of *Pax3*. One of these enhancer regions is capable of binding Tead2, and Tead2, together with the co-activator YAP65, is able to activate this enhancer. Molecular pathways involved in neural crest induction are likely to converge upon these *Pax3* neural enhancers, suggesting that identification and analysis of additional trans-acting regulators will provide further insights into crucial developmental processes required for specification and maturation of the neural crest.

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