

# Molecular characterization of the TrkA/NGF receptor minimal enhancer reveals regulation by multiple *cis* elements to drive embryonic neuron expression

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## SUMMARY

Neural development relies on stringent regulation of key genes that mediate specialized function. TrkA is primarily expressed in neural crest-derived sensory and sympathetic neurons where it transmits critical survival information. We have identified a 457 base pair sequence upstream of the murine first *TrkA* coding exon that is conserved in human and in chick, and is sufficient for expression in the correct cells with appropriate timing. Mutation analysis of consensus transcription factor binding domains within the

minimal enhancer reveals a complex positive regulation that includes sites required for global expression and sites that are specifically required for DRG, trigeminal or sympathetic expression. These results provide a foundation for identification of the transcriptional machinery that specifies neurotrophin receptor expression.

Key words: TrkA, Neurotrophins, Enhancer, Promoter, Transgenic, Mouse

## INTRODUCTION

Neural development is controlled by accurate temporal and spatial gene expression. Among the many critical genes involved in this process are those encoding the NGF family of neurotrophins and their cognate Trk family receptor tyrosine kinases (Levi-Montalcini, 1987; Snider, 1994). The neurotrophins are widely distributed during development with expression found in neural tissues but also in diverse non-neural innervation target tissues where they fulfill their role in the 'Neurotrophic Hypothesis'. In contrast, Trk family receptors are largely expressed in neurons and, in the peripheral nervous system (PNS), are confined to complementary restricted subsets of neuronal subpopulations (Martin-Zanca et al., 1990; Tessarollo et al., 1993). Among them, the receptor for NGF, TrkA, is the most spatially restricted with embryonic expression confined to subsets of sensory neural crest-derived neurons and sympathetic trunk neurons. Gene knockout experiments have demonstrated the requirement for accurate TrkA expression during development (Liebl et al., 2000; Smeyne et al., 1994). In the absence of TrkA, sensory ganglia lose a significant proportion of their neurons via apoptosis and studies have demonstrated that these are largely pain transmitting (nociceptive) neurons (Liebl et al., 2000, 1997; Smeyne et al., 1994). In addition, absence of TrkA function results in loss of virtually all sympathetic neurons (Smeyne et al., 1994). Thus, in the embryonic PNS, TrkA receptor expression defines a subpopulation of neurons that rely on its function for survival.

The study of organisms ranging from *Drosophila* and *C. elegans* to mammals has resulted in the identification of transcription factor encoding gene families that are critical for specifying domains of cellular differentiation and specialization. For example, the *myoD* gene family controls early myogenic differentiation (Olson, 1990; Weintraub et al., 1991). In the nervous system, the *achaete-scute* related gene family governs many aspects of neurogenesis in *Drosophila* and in the mammalian peripheral nervous system (Anderson, 1994; Jan and Jan, 1990, 1993). The current challenge is to determine the physiologically relevant target genes through which these and other important transcription factors mediate gene regulation.

Despite the importance of Trk family receptor expression during peripheral neurogenesis, relatively little is known about the underlying mechanisms that direct their restricted and complementary expression patterns. The neurogenin family of transcription factors appears to be required for the emergence of peripheral neurons (Anderson et al., 1997; Lee, 1997; Sommer et al., 1996), but current understanding indicates a role for these transcription factors in neuronal precursor expansion rather than directly on *Trk* gene expression. In a series of elegant studies, the Pou related Brn3a transcription factor has recently been identified as a likely regulator of Trk family gene expression (Huang et al., 1999; McEvelly et al., 1996; Xiang et al., 1996, 1995). The mechanism whereby this regulation takes place remains to be determined.

In the present study, we have undertaken to identify the *cis* genomic elements that define murine *TrkA* gene expression as

a means of identifying the critical transcription factors that target upon *TrkA*. We have chosen this neurotrophin receptor because of its particularly restricted gene expression profile during embryogenesis (Martin-Zanca et al., 1990; Tessarollo et al., 1993). Through transgenic analysis using a  $\beta$ -galactosidase reporter assay, we have defined a 457 base pair sequence that is sufficient to drive accurate *TrkA* expression in embryogenesis. Survey of the human and chick *TrkA* genes has confirmed the conservation of this enhancer region in approximately the same domain. Mutational dissection of this minimal enhancer further reveals the importance of previously identified regulatory elements as essential for *TrkA* expression in trigeminal, dorsal root and sympathetic neurons. These studies provide the first information about essential transcriptional target domains for the neurotrophin receptors and form a basis for identification of the factors whose *in vivo* targets are the Trk family genes.

## MATERIALS AND METHODS

### Constructs and primers for mutagenesis

Constructs in Fig. 1 were made as follows: plasmid pBS-Hsp68-lacZ-pA (Kothary et al., 1989; Verma-Kurvari et al., 1998) was digested with *Sma*I and treated with calf alkaline phosphatase (GibcoBRL). The linearized plasmid was ligated to different *TrkA* enhancer DNA fragments. Constructs with inserts were sequenced.

### Pronuclear injection

Pronuclear injection and oviduct transplantation of injected embryos were performed as described by Hogan et al. (1986) using either CD2 or B6D2F1 females. The DNA for pronuclear injection was first digested with *Sal*I restriction enzyme and then purified from 1% agarose gels with QIAGEN gel purification kit (QIAGEN). After injection, the eggs were transferred back to the oviducts of pseudopregnant CD2 female mice for development. The recipient females were sacrificed around embryonic day (E)13 or on the day of birth for recovery of embryos or postpartum (P)0 pups. Embryos or P0 pups were genotyped (see below) and all transgene-positive embryos or P0 pups were used for  $\beta$ -galactosidase staining (see below). Three stable transgenic lines were established by standard procedures (Hogan et al., 1986).

### Histology

Embryos or P0 pups were fixed in 4% paraformaldehyde for 30 minutes at 4°C. Staining for  $\beta$ -galactosidase was carried out in 1× PBS, 2 mM MgCl<sub>2</sub>, 50 mM potassium ferri/ferrocyanide, 1 mg/ml X-gal at 25°C for 48 hours or at 37°C for 24 hours. After whole-mount analysis, specimens were either embedded in 3% agar, chicken albumin or OCT for either vibratome sectioning at 100 to 200  $\mu$ m (agar or chicken albumin embedded) or cryostat sectioning at 50  $\mu$ m (OCT embedded). The sections were then counterstained with Eosin and embedded in DPX-mountain dew for long term storage.

### Electrophoretic mobility shift assay

EMSA was carried out as described by Edmondson et al. (1992). Briefly, 10000 to 40000 cts/minute <sup>32</sup>P-labeled oligonucleotide probes were mixed with 1  $\mu$ g DRG nuclear extract, 5× gel shift buffer, 1  $\mu$ g poly(dI-dC) (Boehringer) and water to a final volume of 20  $\mu$ l. The mix was incubated at 25°C for 30 minutes. For supershift assay with antibody (Wang et al., 1996; rabbit anti-mouse ikaros), the antibody was added 10 minutes after the mix was prepared. The mix was incubated for a further 20 minutes. Electrophoresis was done with 5% polyacrylamide gels in 0.5 × TBE buffer at 25°C for 2 to 3 hours. The

gel was then dried and exposed to Kodak X-ray film or to phosphorimager (BioRad).

### Genotyping

Genotyping of embryos or adult mice for *lacZ* transgene was carried out using the following PCR conditions: 94°C, 30 seconds; 65°C, 30 seconds; 72°C, 30 seconds; 30 cycles. The primers for *lacZ* PCR are: 5'-AACTGGAAGTCGCCGCGCCACTGGTGTGGG-3' and 5'-TGAAGTCCAGCTGGCGCAGGTAGCAGAGC-3'. *lacZ*-positive embryos or adults are considered as transgenic positive.

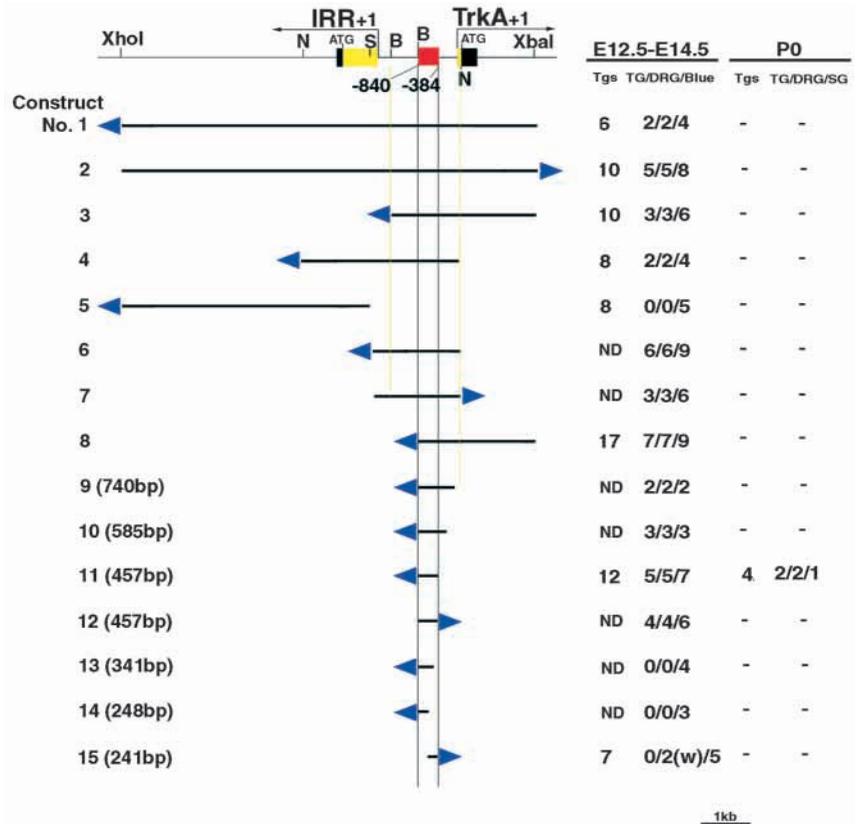
## RESULTS

### Identification of *TrkA cis* regulatory elements

The *TrkA* gene maps to mouse chromosome 3 and human chromosome 1 (Tessarollo et al., 1993). In studying the *TrkA* genomic structure, we uncovered the presence of a gene encoding the insulin receptor-related receptor (*IRR*) located in the opposite transcriptional orientation with its start site approximately 2 kb upstream of the first *TrkA* coding exon (Bates et al., 1997; Kelly-Spratt et al., 1999). Analysis of human, rat and chicken genomic DNA indicated conservation of linkage for the two receptor encoding genes (Fig. 7). Previous studies had demonstrated coexpression of *IRR* and *TrkA* *in vivo* (Reinhardt et al., 1993, 1994). These investigators demonstrated a per cell coexpression for the two genes in the PNS and basal forebrain, whereas the gene encoding *IRR* has additional sites of expression (Bates et al., 1997; Reinhardt et al., 1993, 1994). We reasoned it likely that *TrkA* and *IRR* share enhancer elements directing neuronal coexpression and that such an enhancer would lie proximal to both genes. We therefore began analysis using a 9 kb mouse genomic DNA fragment that spanned the first exon and part of the first intron of the *TrkA* gene and the first four exons and three introns of the *IRR* gene (Fig. 1; and not shown). To facilitate the identification of enhancer activity, we used a  $\beta$ -galactosidase (*lacZ*) reporter gene linked to an *Hsp68* minimal promoter (Kothary et al., 1989; Verma-Kurvari et al., 1998). In the present study we performed enhancer analysis using transgenic animals as we were unable to identify cell lines that expressed both the *TrkA* and *IRR* genes leading to concerns about the potential lack of promoter/enhancer fidelity in available cell lines.

*TrkA* is primarily expressed in neural crest derived sensory neurons during embryogenesis (Martin-Zanca et al., 1990). After E17, sympathetic neurons also express *TrkA* (Tessarollo et al., 1993; Wyatt and Davies, 1995). As described in Fig. 1, the *Hsp68/lacZ* reporter gene was placed on either end of the 9 kb fragment to test the directional specificity of the fragment (constructs no. 1 and 2). These linearized constructs were microinjected into fertilized eggs and the recipient pseudopregnant females were sacrificed around E13 to analyze sensory expression by X-gal staining. As outlined in Fig. 1, out of a total of 16 transgenic embryos obtained with these two constructs, 12 expressed the transgene (Fig. 1, Blue) and 7 exhibited strong and specific expression of the *lacZ* reporter gene in trigeminal (TG) and dorsal root ganglion (DRG). The remaining 5 embryos showing  $\beta$ -gal activity varied in their expression probably resulting from either positional effects of chromosomal integration or rearrangement of the *TrkA* sequences upon integration. Fig. 2A shows a representative

**Fig. 1.** Diagram of the fusion constructs used for defining the TrkA minimal enhancer. ATG above the yellow-black box is the start codon for *TrkA* and *IRR* genes, respectively. The yellow-black box and the red box represent the positions of the first exons of *TrkA* and *IRR* genes and the minimal enhancer, respectively. The direction of the arrows and the arrowheads from construct No.1 to No.15 indicates the transcriptional orientation and positions of the Hsp68-*lacZ* reporter gene, respectively. For constructs 9 to 15 the length of the *TrkA* enhancer fragments fused to the *lacZ* reporter gene is given in parentheses. Mouse embryos from E12.5 to E14.5 or at birth (P0), were used for analysis of trigeminal (TG), dorsal root (DRG), and sympathetic (SG) ganglion *lacZ* reporter gene expression. Tgs, the number of transgenic embryos regardless of transgene expression for each construct; TG/DRG/Blue, the number of embryos expressing the *lacZ* reporter gene (Blue) in TG, DRG; TG/DRG/SG, the number of P0 transgenic pups expressing the *lacZ* reporter gene in TG, DRG and/or SG, respectively. N, *NarI*; B, *BglIII*; S, *SalI*. ND, the total number of transgenic embryos was not determined; w, weak expression of the *lacZ* reporter gene in DRG. Scale bar, 1 kb.



transverse section from a construct 2 derived *lacZ*-positive transgenic embryo. The major sites of expression are the prominent TG and in the upper region, the DRG (arrows). At the apex (roof plate) of the spinal cord, X-gal staining reflects DRG afferent innervation into superficial laminae of the spinal cord (see below). Expression in the retinal epithelium is not reproducible and therefore likely a site of ectopic transgene expression (Fig. 2A). From this initial analysis, we concluded that the 9 kb region contained sufficient information to drive *TrkA* expression at the appropriate sites in sensory neurons. In addition, the 9 kb fragment fulfills the requirement of an 'enhancer' by retaining reporter gene activity when placed in either orientation.

### A 457 bp fragment is sufficient for TrkA-specific expression

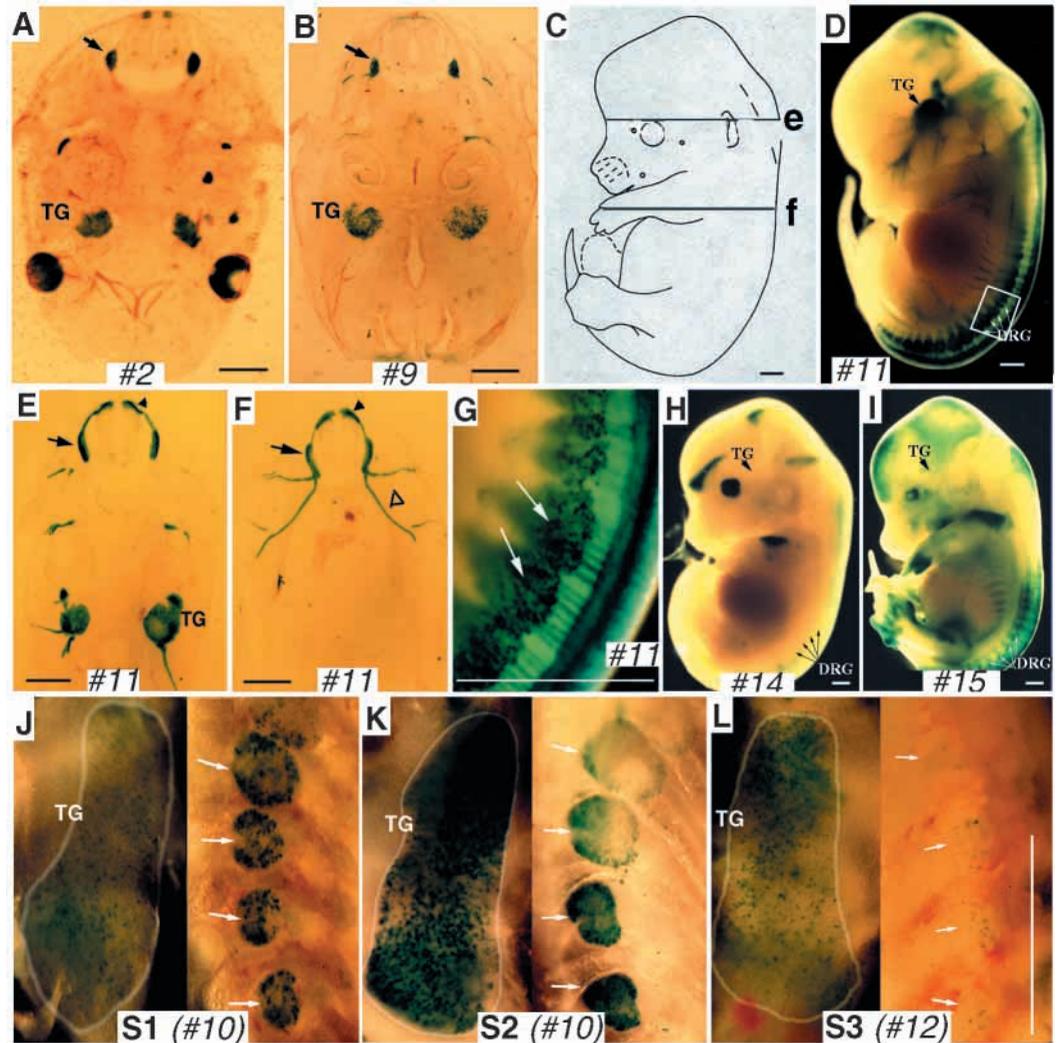
On the basis of the preceding data, we adopted a strategy of microinjecting progressively smaller genomic fragments together with the minimal promoter and reporter gene. As outlined in Fig. 1, transgenic constructs 3, 4, 6, 7 and 8 each provided specific and reliable *lacZ* transgene expression in TG and DRG at E13.5. Construct 5, which contained about 5.5 kb of the original 9 kb enhancer, did not produce any *TrkA*-like activity (data not shown). The blue embryos derived from construct 5 exhibited random and varying *lacZ* expression. Taken together, the data derived from constructs 1-8 (Fig. 1) indicated that the enhancer activity was likely contained within a 740 bp DNA sequence shared by the active fragments, upstream of the first *TrkA* exon and excluding a role for intronic sequences within the *TrkA* and *IRR* genes (Fig. 1).

To further delineate the *TrkA* specific enhancer, we tested

construct 9, which retained approximately 740 bp of sequence in common with the preceding active fragments. Construct 9 again provided sufficient *TrkA* enhancer information with 2 of 2 *lacZ*-expressing embryos showing both TG and DRG expression (Fig. 1). As indicated in Fig. 1, constructs 9-12 further refine the boundaries of the enhancer to a minimal fragment of 457 base pairs that has *TrkA*-specific expression activity in either orientation. Fig. 2B shows a representative transverse section through embryonic brain showing expression in DRG and TG generated with construct 9. Fig. 2C-G illustrates the *lacZ* expression pattern driven by the minimal 457 bp fragment (construct 11) in the TG and DRG, including central and peripheral projections in an E13.5 embryo. Fidelity of *TrkA* expression is further exemplified by X-gal labeling of all DRG along the embryonic spinal cord. Afferent projection into the superficial laminae of the dorsal horn spinal cord (Fig. 2E) is consistent with appropriate nociceptive afferent innervation, where *TrkA*-expressing neurons are normally found (Liebl et al., 1997). This is further supported by the punctate expression pattern seen in DRG (Fig. 2G), reflecting *TrkA* expression only in a subset of DRG neurons, similar to the expression pattern documented for embryonic *TrkA* expression (Fig. 2G; Martin-Zanca, 1990; Tessarollo, 1993). Thus, a 457 bp *cis* genomic fragment exhibits the hallmarks of a minimal enhancer capable of driving *TrkA* expression in DRG and TG neurons in vivo.

Attempts to further refine the size of the minimal enhancer were unsuccessful (Fig. 1, constructs 13-15). Constructs 13 and 14, lacking progressive sequences of the 3' enhancer region did not exhibit *lacZ* expression in DRG or TG (Fig. 1; Fig. 2H for construct 14). Construct 15 included the 3'-most 250 bp of the

**Fig. 2.** Transgenic embryos expressing *lacZ* under control of the TrkA enhancer recapitulate endogenous expression in TG and DRG. Transverse vibratome sections of E13.5 transgenic embryos generated with constructs 2 (A) and 9 (B), respectively. Note strong and specific expression in trigeminal ganglion (TG) and dorsal root ganglion (arrows). (C) Graphic representation of an E13.5 mouse embryo and the relative positions of the sections in A, B, E and F. (D) A typical E13.5 transgenic embryo generated with construct No. 11 (Fig. 1) expresses *lacZ* reporter gene in TG and DRG. (G) Higher magnification of the boxed area in D. Note the punctate *lacZ* expression in DRG. (E) and (F) Transverse sections as indicated in (C). (H, I) Transgenic embryos generated with constructs 14 and 15 (Fig. 1), respectively. Notice the absence of *lacZ* expression in TG (H, I), DRG (H) and weak expression of *lacZ* in DRG (I). (J-L) Whole-mount TG and DRG *lacZ* expression by the three stable TrkA enhancer transgenic strains at P0. Strain 1 (construct 10) is shown in J, strain 2 (construct 10) in K and strain 3 (construct 12) in L, respectively. TG, trigeminal ganglion; Arrows indicate dorsal root ganglion; Arrowhead indicates efferents from DRG to spinal cord; Open arrowhead, afferents from DRG to target area. Scale bars, 1 mm.



enhancer but excluded the 5' 200 bp. This construct was insufficient for appropriate expression, however it did produce weak staining in DRG but not in TG (Fig. 2I). Thus, sequences in the 3' half of the enhancer are absolutely required for minimal expression and may contain regulatory sequences that are sufficient to drive DRG but not TG expression.

### Stable transgenic lines retain TrkA-specific *lacZ* expression

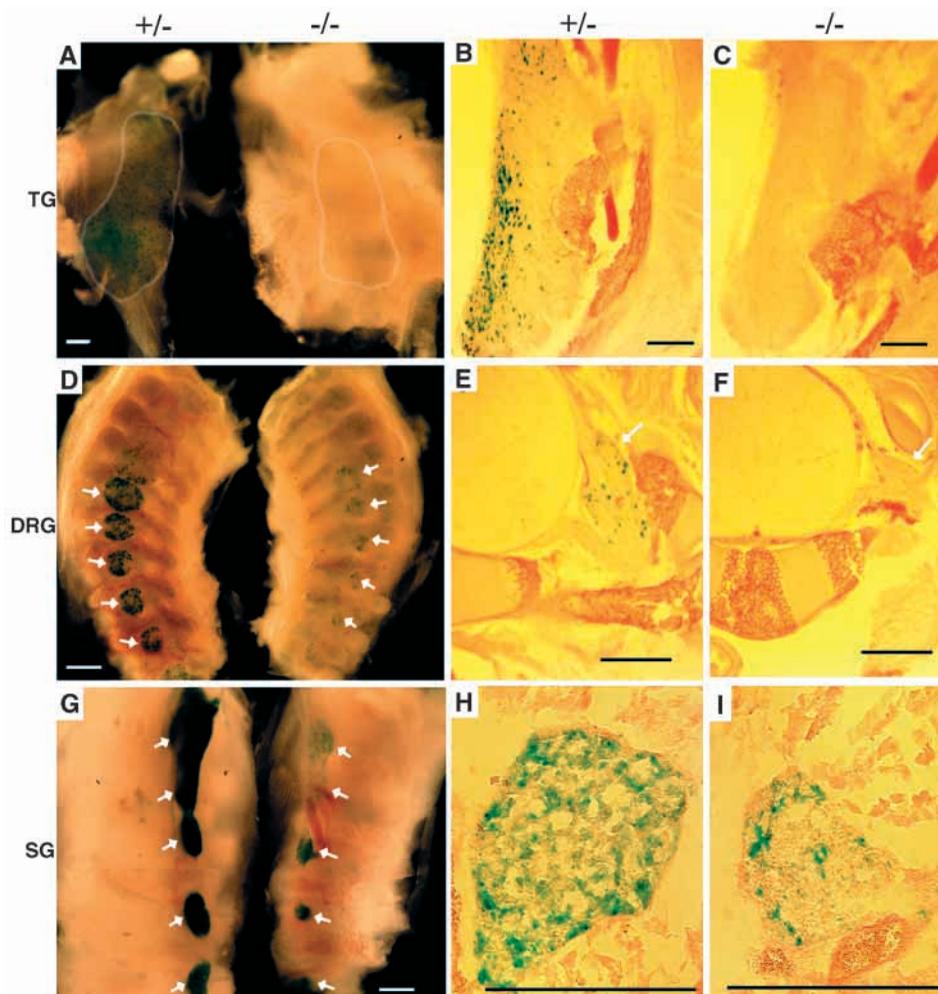
To examine, in greater detail, the properties of the TrkA enhancer, we generated three independent stable mouse transgenic strains using either constructs 10 or 12 (see Materials and Methods for details). All three transgenic strains exhibited specific reporter gene expression in TG and DRG at P0 (Figs 2J-L, 3). In addition, a degree of variable, non-specific expression was observed from strain to strain (data not shown).

TrkA is essential for the survival of nociceptive sensory neurons (Liebl et al., 2000; Smeyne et al., 1994). We therefore crossed the three stable TrkA enhancer transgenic lines into the TrkA null genetic background (Liebl et al., 2000) to determine whether *lacZ* expression was confined to TrkA-positive, NGF-

dependent neurons. Fig. 3A and D show representative whole-mount staining of TG and DRG, respectively, for crosses of the *lacZ* transgene into TrkA heterozygotes (+/-; left), or TrkA null P0 pups (-/-; right). Whereas the compound enhancer transgenic/heterozygotes express *lacZ* appropriately, in the null background, X-gal staining was abolished (compare also Fig. 3B-F). These data confirm the fidelity of the 457 bp enhancer in TrkA-expressing sensory neurons.

### Identification of potentially important *cis*-elements

The highly stereotypic expression of the *TrkA* gene during development in subsets of sensory neurons and in sympathetic neurons in late development suggests that regulation may be mediated by diverse regulatory elements. Therefore, we surveyed the mouse sequence for known consensus transcription factor binding sites (Fig. 4A). This analysis identified 15 different types of highly conserved *cis*-elements within the minimal enhancer: E4 BP4 (Cowell et al., 1992); Nkx2.5 (Chen and Schwartz, 1995); DeltaEF (Funahashi et al., 1993; Remacle et al., 1999; Sekido et al., 1994, 1997); e/d Hand (Cserjesi et al., 1995; Hollenberg et al., 1995); Ets (Ahne



**Fig. 3.** The *lacZ* reporter gene is expressed specifically in TrkA neurons in TG, DRG and SG. (A) TG (encircled area) from compound TrkA enhancer and TrkA null heterozygous (left) or null pups (right) stained for *lacZ* expression. Cryostat sections of (B) TG from TrkA heterozygous or (C) TrkA null pups (C). (D-I) As A-C, but showing DRG (D-F) and SG (G-I, white arrows). TG, trigeminal ganglion; DRG, dorsal root ganglion; SG, sympathetic ganglion. Scale bars, 100  $\mu$ m.

and Stratling, 1994; Chen et al., 1996; Pastorcic and Das, 1999); MZF (Hromas et al., 1996; Hui et al., 1995; Morris et al., 1994); Ikaros (Molnar and Georgopoulos, 1994); Ebox (Olson, 1993; Weintraub et al., 1994); CACCC box (Cassard-Doulcier et al., 1994); AP1FJ1 (Kouzarides and Ziff, 1988; Neuberg et al., 1989; Rauscher et al., 1988); Sp1 (Dyban and Tjian, 1983a,b; Schmidt et al., 1989); NF1 (Osada et al., 1996, 1999); Cux (Andres et al., 1994; Harada et al., 1995); Gfi-1 (Zweidler-Mckay et al., 1996); and S8 (de Jong et al., 1993). These *cis*-elements are spread throughout the 457 bp minimal enhancer and form small clusters of 1 to 3 *cis*-elements with 0 to 10 intervening nucleotide spaces (Fig. 4A).

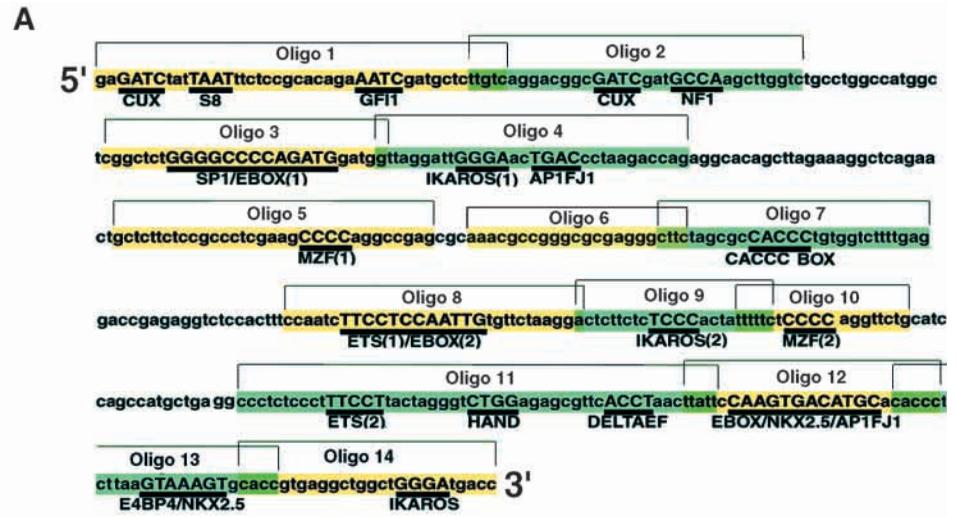
As a first approach toward determining whether the identified consensus binding sites are required for DRG TrkA expression, we generated 14 oligonucleotides that spanned the consensus binding domains (Fig. 4A), and subjected them to gel mobility shift assays with embryonic DRG nuclear extracts (Fig. 4B,C). Strong mobility shift was observed with five of the oligomers (Fig. 4B; oligos 4, 5, 9, 10, 11). One oligomer shifted moderately weakly (oligo 8; Fig. 4B), and weak or no shifts were observed with eight of the oligomers (oligo 14, Fig. 4C; and oligos 1, 2, 3, 6, 7, 12 and 13, not shown). The mobility shift data provided evidence for the presence of binding factors in DRG extracts. While the negative results do not exclude the presence of weakly binding elements, the binding data

provided a basis to pursue a functional analysis of the minimal enhancer.

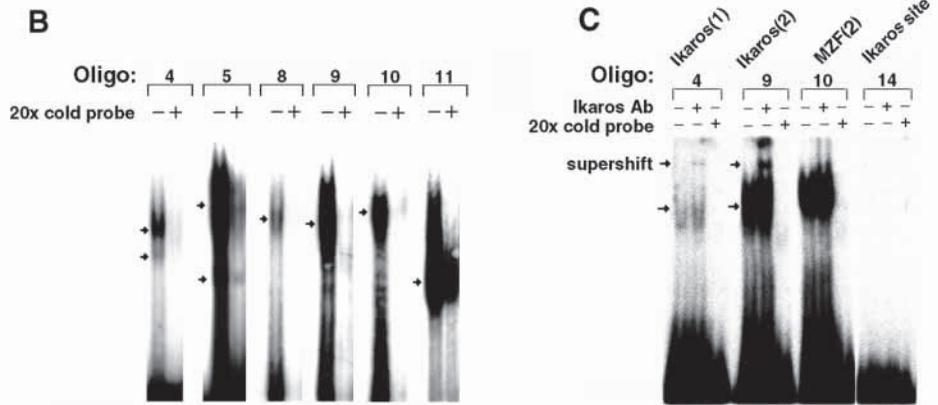
#### Mutational analysis of the minimal enhancer

As illustrated in Fig. 5, eleven core transcription binding elements contained within gel-shifted oligonucleotides were selected for functional analysis, including an Ebox (Fig. 5, Ebox(1)) within oligo 3 that did not shift as a comparison to the selected Ebox (Fig. 5, Ebox(2)). On the basis of these criteria, the NF1, S8, Gfi-1, Cux, CACCC box, Nkx2.5, and E4BP4 sites were excluded from the present studies. The 457 bp fragment contained in construct 11 (Fig. 1) was subjected to in vitro mutagenesis designed to alter at least four of the consensus core nucleotides for each of the core consensus sequences (Fig. 5, 'Mutation'). Replacement mutagenesis was selected to avoid disruption of possible spacing requirements for a functional enhancer. Each mutation described in Fig. 5 was employed to generate transgenic embryos. *lacZ* expression in TG and DRG was analyzed from E12.5 to E14.5. The results of this analysis are summarized in Fig. 5, illustrated in Fig. 6, and described below.

**DeltaEF:** DeltaEF belongs to the homeobox-zinc finger family of transcription factors (Funahashi et al., 1993; Sekido et al., 1994; Sekido et al., 1997). Oligonucleotide 11 containing the DeltaEF site in the TrkA enhancer shifts strongly with DRG



**Fig. 4.** Sequence of the TrkA minimal enhancer and gelshift analysis with DRG nuclear extracts. (A) The complete sequence of the TrkA minimal enhancer (457 bp). Oligo 1 through oligo 14 were used in a gel shift assay with E13.5 DRG nuclear extract. Below the sequence, highly conserved *cis*-binding elements for known transcription factors are indicated. The core sequences of the *cis*-elements are listed. (B) 6 of 14 oligos tested that shift strongly or weakly (oligo 8) with E13.5 DRG nuclear extracts. Arrows indicate visible shifted bands. In C, an Ikaros polyclonal antibody (Wang et al., 1996) was used in a gel shift assay with E13.5 DRG nuclear extract. Lower arrow indicates normal shift with nuclear extract; upper arrow indicates supershift by the Ikaros polyclonal antibody. Oligo 10 (MZF(2)) was used as a control for antibody specificity.



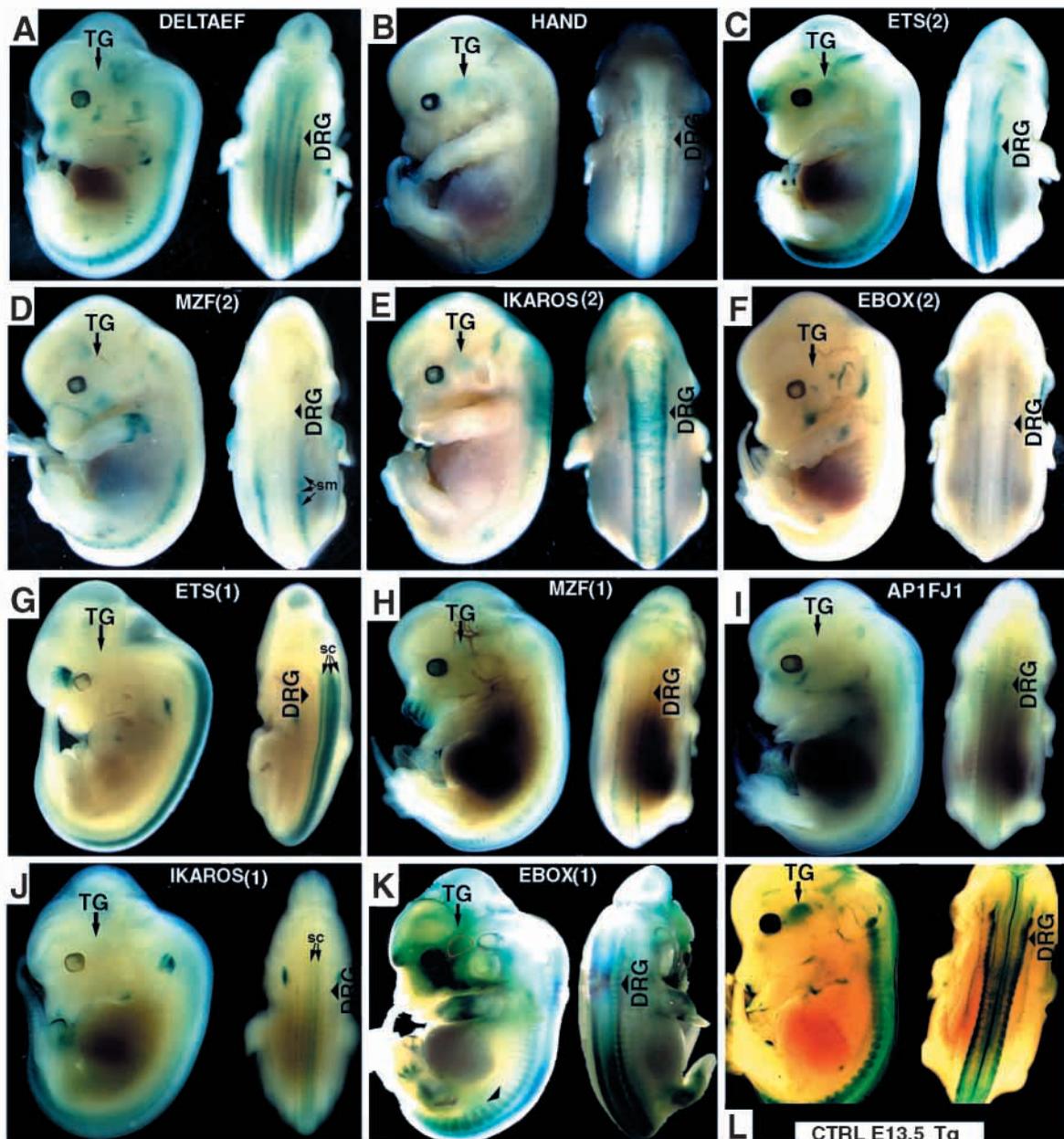
**Fig. 5.** Identification of *cis*-elements required for normal TrkA minimal enhancer function. The yellow column represents the full minimal enhancer from 5' to 3' in accordance with the sequence shown in Fig. 4. The blue boxes along the enhancer represent the *cis*-elements chosen for mutational analysis. Eleven *cis*-elements contained within the shifted oligos (Fig. 4) were mutated in the core sequence (indicated in the Mutation column). The upper black and lower red oligonucleotide sequences are the wild-type and mutant core sequences, respectively. Transgenics are the number of transient transgenic embryos obtained for each mutated *cis*-element. In the TG and DRG columns the expression of the *lacZ* reporter is indicated; (w) weak or (n) normal.

cis-elements	MUTATION	TRANSGENICS				
		BLUE	TG	DRG		
DELTAEF HAND ETS(2)	ACCT (w) CTTG (M)	11	3	0	3	Required for TG
	CTGG (w) TCTT (M)	11	5	2(w)	2(w)	Involved in TG/DRG
	TTCCT (w) CCAAC (M)	5	3	2	2	Not Required
MZF(2) IKAROS(2)	CCCC (w) TTTT (M)	10	4	0	0	Required for TG/DRG
EBOX(2) ETS(1)	TCCC (w) CTTT (M)	ND	4	3(w)	4(w)	Involved in TG/DRG
	CAATTG (w) TGATGT (M)	5	3	0	1(w)	Required for TG Involved in DRG
MZF(1)	TTCCT (w) CCAAC (M)	11	5	0	0	Required for TG/DRG
	CCCC (w) TTTT (M)	9	2	0	2(w)	Required for TG Involved in DRG
AP1FJ1 IKAROS(1)	TGAC (w) CCCT (M)	6	3	0	2(w)	Required for TG Involved in DRG
	GGGA (w) AAAG (M)	6	4	0	2(w)	Required for TG Involved in DRG
EBOX(1)	CAGATG (w) TGAGAT (M)	3	2	2 (1w/1n)	2 (1w/1n)	Not Required

nuclear extracts (Fig. 4B, oligo 11). Mutation of the DeltaEF site specifically ablated TG expression while retaining normal DRG expression (Figs 5, 6A).

**Hand:** Oligonucleotides containing a highly conserved non-canonical e/d-Hand transcription factor binding site (Cserjesi et al., 1995; Hollenberg et al., 1995) shifted strongly with DRG nuclear extract (Figs 4A; 4B, oligo 11). The TrkA minimal enhancer with mutation of this site maintained the ability to drive *lacZ* reporter expression, albeit reduced, in both TG and DRG (Fig. 5; compare Fig. 6B and L). In addition, mutation of the e/d-Hand site ablated sympathetic chain expression (see below).

**Ets:** Two highly conserved Ets transcription factor binding sites were also found in the minimal enhancer (Fig. 4A; Ahne and Stratling, 1994; Chen et al., 1996; Pastorcic and Das, 1999). Oligonucleotides containing either Ets site were shifted with DRG nuclear extracts (Fig. 4B, oligos 8 and 11, respectively). Mutation of the second Ets site (Fig. 5, Ets(2)) did not affect minimal enhancer function (Figs 5, 6C). Two of three blue embryos generated with the Ets(2) mutation showed normal TG and DRG. However, mutation of the first Ets site (Fig. 5; Ets(1)) completely abolished *lacZ* expression in both TG and DRG (Figs 5, 6G and data not shown). Ectopic *lacZ* transgene expression was observed in several of the embryos.



**Fig. 6.** Transient transgenic embryos provide important information about the function of each *cis*-element in the minimal enhancer. Representative whole-mount *lacZ* pattern for the TrkA minimal enhancer bearing the *cis*-element mutation described in Fig. 5. (A) DeltaEF *cis*-element mutation; (B) Hand; (C) ETS(2); (D) MZF(2); (E) Ikaros(2); (F) Ebox(2); (G) ETS(1); (H) MZF(1); (I) Ap1FJ1; (J) Ikaros(1); and (K) for Ebox(1). A control E13.5 TrkA enhancer transgenic embryo is shown in L. Arrows indicate trigeminal ganglion; arrowheads dorsal root ganglion; sm, somites showing *lacZ* expression (D); sc, spinal cord expressing *lacZ* (G,J).

Thus, one of two Ets sites appears required for TrkA expression in sensory neurons.

**MZF:** The MZF family of mouse zinc finger transcription factors is homologous to *Drosophila* Kruppel (Hromas et al., 1996; Hui et al., 1995; Morris et al., 1994). Two highly conserved MZF binding *cis*-elements (Morris et al., 1994; Shields and Yang, 1998) are found in the minimal enhancer (Fig. 4A) and oligonucleotides including each MZF site shift strongly with DRG nuclear extracts (Fig. 4B, oligos 5 and 10). Mutation of the 5' MZF(1) site, like the DeltaEF site, ablated expression in TG but retained expression in DRG (Fig. 5; Fig. 6H) while mutation of the 3' MZF(2) site ablates *lacZ* reporter gene expression in both TG and DRG (Fig. 5; Fig. 6D),.

**Ikaros:** The Ikaros family of mouse zinc finger transcription factors is homologous to *Drosophila* Hunchback (Georgopoulos et al., 1992). Three highly conserved Ikaros binding *cis*-elements (Molnar and Georgopoulos, 1994) are found within the minimal TrkA enhancer (Fig. 4A), however we discounted the 3'-most site since it is contained within an oligomer that was not shifted by DRG extracts (Fig. 4C). To examine if any Ikaros-like transcription factors bind the second and third Ikaros *cis*-elements, we performed gel shift assays using an Ikaros polyclonal antibody (Wang et al., 1996) (Fig. 4C, oligos 4 and 9, second lane). Both the Ikaros(1) and Ikaros(2) *cis*-elements were 'supershifted' with addition of antibody, while neither the 3'-most Ikaros site (Fig. 4C, oligo 14) nor the MZF(1) *cis*-element containing oligomer (Fig. 4C, oligo 10) showed evidence of a supershifted band.

Mutation of the first Ikaros(1) site completely ablated expression in TG while retaining weak DRG expression (Figs 5, 6J). Mutation of the Ikaros(2) site attenuated *lacZ* expression in both TG and DRG compared with wild-type enhancer (Figs 5, 6E). Thus, Ikaros-mediated transcription is an important element for *TrkA* regulation and the Ikaros(1) site appears to primarily be required for TG expression as were the MZF(2), and DeltaEF sites described above.

**EBox:** Three EBox *cis*-elements were found in the minimal enhancer (Fig. 4A; Olson, 1993; Weintraub et al., 1994). Oligonucleotides containing the 5'-most (Ebox(1)) and third EBox (3'-most) sites showed no shift in DRG nuclear extract assays (data not shown), while oligonucleotides containing the second Ebox (Ebox (2)) site were shifted in the assay (Fig. 4B, oligo 8). The Ebox(2) site is only one nucleotide removed from the Ets(1) site described above (Fig. 4A), suggesting that interaction could exist (Sieweke et al., 1998). As shown in Figs 5 and 6F, mutation of Ebox(2) resulted in complete ablation of enhancer function in TG while still maintaining weak activity in DRG. Mutation of the 5'-most Ebox(1) site contiguous with a consensus SP1 site had no apparent effect on sensory neuron expression (Figs 5, 6K), consistent with the gelshift data that oligonucleotide 11 containing this Ebox did not shift with DRG nuclear extract.

**APIFJ1:** Two AP1 *cis*-elements are found in the TrkA minimal enhancer (Kouzarides and Ziff, 1988; Neuberger et al., 1989; Rauscher et al., 1988) (Fig. 4A). The 3'-most APIFJ1 *cis*-element, present in oligo 12, was not shifted by DRG nuclear extracts (data not shown). The core sequence of the 5'-most APIFJ1 *cis*-element is only 2 nucleotides removed from the core sequence of the Ikaros(1) site whose mutation ablated TG expression while retaining weak DRG expression (Fig. 4A, see above). Mutation of the 5'-most APIFJ1 *cis*-element also

ablated *lacZ* reporter gene in the TG while retaining weak DRG expression (Figs 5, 6I).

In summary, regulation of TrkA expression by the 457 bp minimal enhancer is complex and contains *cis* elements required for expression in both TG and DRG, while additional elements that are particularly important for TG expression.

### Evolutionary conservation of the TrkA enhancer

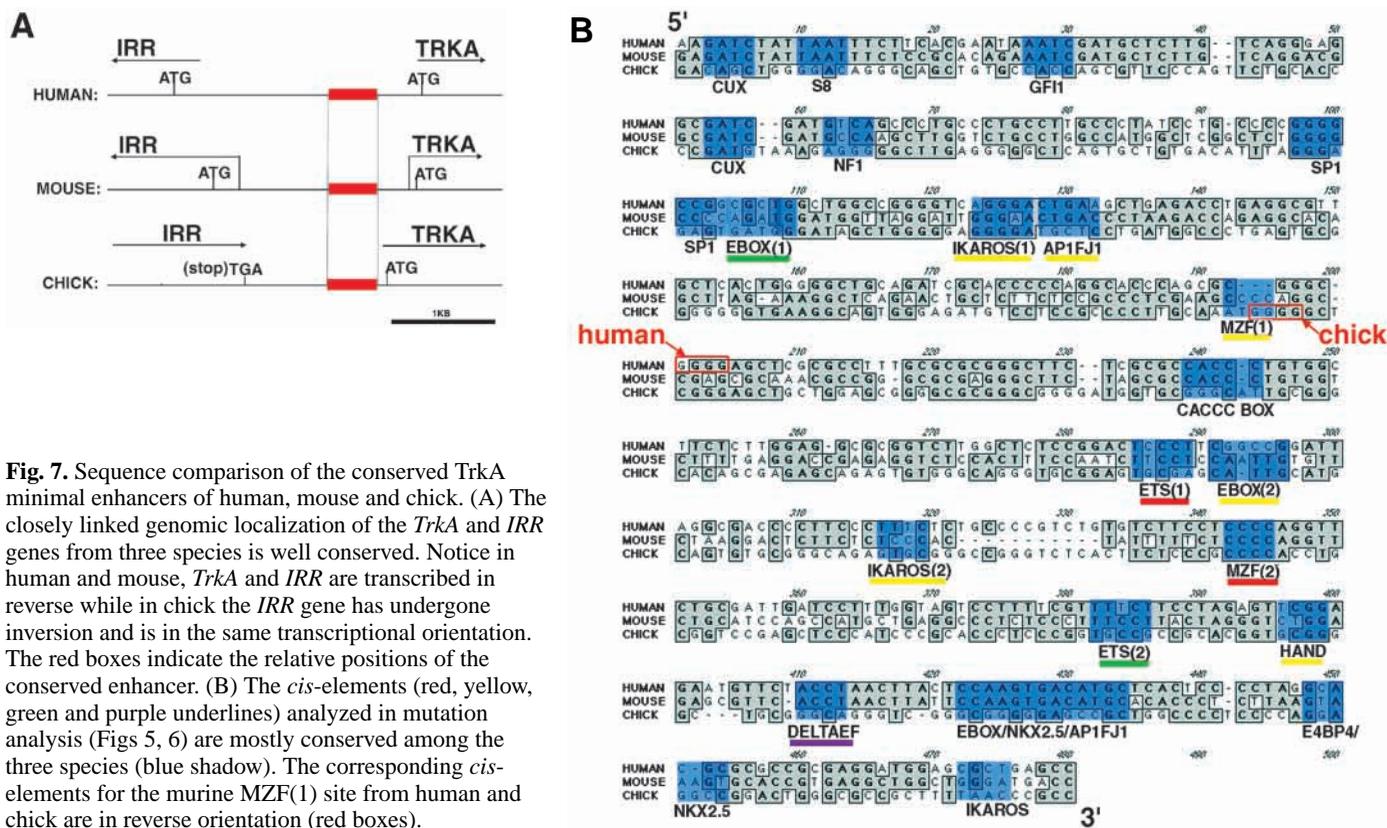
The intervening genomic sequence between the TrkA and IRR genes from human and chick was sequenced and surveyed for regions of homology with the murine enhancer. As schematically represented in Fig. 7A, highly conserved domains were found in all three species. Overall nucleotide conservation was 65% between mouse and human and 42% between mouse and chick. Strikingly, the clusters of transcriptional binding sites that affected enhancer function (Fig. 7B, red, yellow and purple), are highly conserved. As noted in Fig. 7B, the MZF(1) site that is required for trigeminal expression appears absent in human or chick. However, inspection of the human and chick sequences reveals MZF binding sequences within a few nucleotides, just 3-prime (Fig. 7B, red boxes). The Ets(2) site (nucleotide 382) is not required for function although conserved and present in an oligomer that was shifted (Fig. 4, oligo 11). We provisionally attribute the shift to Hand and DeltaEF sites present in the same oligomer and required for global and transgenic expression respectively (Figs 5, 6).

The regions spanned by oligomers 1-3, and 12-14 (Fig. 4), were not shifted with DRG extracts but exhibit high sequence conservation (Fig. 7B). Accordingly, mutation of the conserved Ebox(1) domain within oligo 3 (Fig. 4) did not affect enhancer function in DRG. It is likely that these conserved sites, may serve to specify TrkA expression in locations other than DRG such as the superior ganglion, cholinergic basal forebrain neurons, and selected spinal cord neurons (Martin-Zanca et al., 1990; Liebl et al., unpublished). We did not prepare an oligomer to span nucleotides 140-180 (Fig. 7B) as we did not discern consensus transcriptional binding sites. However the high degree of evolutionary conservation suggests a possible functional domain (see Discussion).

### The 0.45 kb minimal enhancer is sufficient for sympathetic ganglion expression

In addition to expression in neural crest-derived sensory neurons, TrkA function is present and essential for the survival of late embryonic and postnatal sympathetic neurons. In mice, sympathetic expression of TrkA begins at E17.5 and reaches highest levels several days after birth (Dixon and McKinnon, 1994; Tessarollo et al., 1993). To examine whether the minimal enhancer determined for sensory neuron expression was also sufficient for sympathetic expression, we tested construct 11 (Fig. 1, far right column) by allowing the microinjected zygotes to develop to term prior to analysis. Of four transgenic pups, two expressed *lacZ*. Each embryo exhibited TG and DRG expression and one of two embryos also exhibited expression in neurons of the sympathetic trunk (Fig. 1 and not shown). These results indicate the presence of regulatory elements within the 457 bp minimal enhancer that specify sympathetic neuron expression.

Sympathetic neurons require NGF signaling through the TrkA receptor for survival (Smeyne et al., 1994; Snider, 1994). At P0, *TrkA* null pups have severe loss of sympathetic neurons



**Fig. 7.** Sequence comparison of the conserved *TrkA* minimal enhancers of human, mouse and chick. (A) The closely linked genomic localization of the *TrkA* and *IRR* genes from three species is well conserved. Notice in human and mouse, *TrkA* and *IRR* are transcribed in reverse while in chick the *IRR* gene has undergone inversion and is in the same transcriptional orientation. The red boxes indicate the relative positions of the conserved enhancer. (B) The *cis*-elements (red, yellow, green and purple underlines) analyzed in mutation analysis (Figs 5, 6) are mostly conserved among the three species (blue shadow). The corresponding *cis*-elements for the murine MZF(1) site from human and chick are in reverse orientation (red boxes).

and the remaining populations progressively disappear in the ensuing days. Two of the three derived stable *TrkA* minimal enhancer transgenic lines exhibited *lacZ* expression in sympathetic ganglia (SG) at P0 (Fig. 3G; and data not shown). When we examined the expression of *lacZ* in the compound *TrkA* heterozygotes (+/−) and homozygotes (−/−; Fig. 3G), in the homozygotes both the size of the ganglia and the degree of *lacZ* expression was considerably diminished. Comparison of Fig. 3H and I illustrates the reduction of *lacZ* expression in dying neurons. These results provide further evidence for the specific expression of the *lacZ* transgene in sympathetic neurons mediated by the 457 bp *TrkA* enhancer.

The eHand and dHand transcription factors are strongly expressed in sympathetic chain during embryonic development (Cserjesi et al., 1995; Hollenberg et al., 1995). We therefore examined whether the site encompassed by oligo 11 (Fig. 4) contributes to function of the minimal enhancer in sympathetic neurons. Of three *lacZ*-expressing transgenic mouse pups (P0 stage) generated with this site mutated in the minimal enhancer, none showed *lacZ* expression in sympathetic ganglion, while one showed weak expression in TG and DRG (data not shown). These data support the idea that the e/d Hand site is required for appropriate *TrkA* expression in sympathetic neurons. Thus, a 457 bp DNA fragment upstream of the *TrkA* gene is sufficient to support appropriate expression in neural crest derived sensory and sympathetic neurons.

## DISCUSSION

The molecular forces that regulate development include a

nexus of extracellular stimuli, intracellular signaling and transcriptional modulation, leading to coordinate expression of key target genes that deploy specialized functions. Multiple examples of identified transcription factor families that are essential for normal development exist, including *mash* (Anderson et al., 1997; Guillemot et al., 1993), the neurogenins (Anderson et al., 1997; Fode et al., 1998; Lee, 1997; Sommer et al., 1996) and *otx* (Shimamura et al., 1995). In each instance, genetic analysis has confirmed the requirement of these transcription factors for neural development as was initially suspected from stereotypic expression profiles and/or relatedness to gene products with important regulatory functions in other organisms. A major challenge remains in the understanding of the complex coordinate regulation by which transcription factors specify the networks of physiologically relevant target genes.

*TrkA* is an example of an essential developmental target gene that specifies survival of nociceptive sensory and sympathetic neurons (Liebl et al., 2000; Smeyne et al., 1994). The highly stereospecific profile of this gene has made it particularly suitable for seeking *cis*-transcriptional regulators. Molecular and transgenic dissection of the genomic region 5-prime to the first coding exon has uncovered a contiguous 457 base pair sequence that contains sufficient information to drive appropriate *TrkA* expression in sensory and sympathetic neurons. We have restricted the present analysis to consensus transcription factor binding sequences contained in oligomers that could be detected in gel shift assays using embryonic DRG nuclear extracts. Thus, one limitation of the present study is the requirement for strong binding under the conditions used, and the requirement that the transcription factors be expressed

in embryonic DRG at E13.5. In consequence, our functional analysis would only be expected to identify enhancer sequences that are required for either global *TrkA* expression or those that impinge on DRG expression.

The results obtained by mutation analysis are consistent with the preceding deletion analysis of the minimal enhancer. Construct 14 (Fig. 1), lacking 200 bp of 3-prime sequence, lost all enhancer activity, whereas the 3-prime 250 bp region retained weak DRG activity (construct 15). Consistent with this observation, mutation of the MZF(1), AP1FJ1 and Ikaros(1) sites, all located in the 5-prime 200 bp region, ablated expression in TG while retaining weak expression in DRG. These data support the model that the three sites mentioned are crucial for the function of the 5-prime 200 bp region of the enhancer. Conversely, mutations of the MZF(2) and ETS(1) sites in the 3-prime 200 bp region ablated both TG and DRG expression, suggesting these two sites are absolutely required for the 3-prime 200 bp function. Of note, mutation of the overlapping consensus Ets(1) and Ebox(2) sites yielded very similar effects on enhancer function causing loss of TG expression. We cannot rule out that mutation of either core consensus sequence may in fact impair the whole region. However, it is interesting to note that possible interactions between these two different types of transcription factors have been proposed (Sieweke et al., 1998).

There is increasing evidence for remarkable conservation of enhancer sequences across species (Aparicio et al., 1995; Brenner et al., 1993; Elgar et al., 1996). For example, such homology has assisted in the identification and dissection of regulatory sequences for *wnt* genes, *neurogenin*, *nestin* and *Ddx* in the nervous system (Helms et al., 2000; Josephson et al., 1998; Rowitch et al., 1998; Zerucha et al., 2000). In the present study we find conservation of the 457 bp enhancer region in mouse, human and chick. This finding provides strong support for the physiological relevance of the domain and of the identified consensus binding sites within.

A likely component of *TrkA* regulation that we have not so far examined or experimentally addressed, is the activity of repressors that may act to confine its expression to neurons and, in DRG, to nociceptive neurons (Chen et al., 1998; Chong et al., 1995; Kraner et al., 1992). Of note, among the consensus sites not analyzed in this study because of absence of gel shift, two Cux consensus sites were identified. Products of this family are known to act as transcriptional repressors (Higgy et al., 1997). Activity of repressors would be anticipated in non-*TrkA*-expressing cells, thus, utilization of the Cux containing oligonucleotides (Fig. 4, oligos 1 and 2) in gel shifts assays employing extracts either from non-neural tissues, or neurons that do not express *TrkA*, may provide evidence for action of *cux* genes in suppression of *TrkA* expression outside its tightly restricted realm. Thus, continued analysis of the 457 bp minimal enhancer may yet reveal additional information about gene repression.

The *Brn3a* gene, a member of the POU domain subclass of transcription factors, is strongly expressed in sensory neural crest-derived neurons (Ninkina et al., 1993; Xiang et al., 1995). Genetic ablation of *Brn3a* function results in loss of sensory neurons, and recent work by Huang and colleagues has demonstrated a significant attenuation of *Trk* family gene expression specifically in trigeminal ganglion (Huang et al., 1999). We have scrutinized the 457 bp minimal *TrkA* enhancer

and fail to detect well conserved *Brn3a* consensus binding sites (not shown). Thus, the action of this factor may lie outside the key determinants for *TrkA* expression but may rather exert its influence from a genomic location that can impinge on the activity of all three *Trk* family genes (Huang et al., 1999). Alternatively, *Brn3a* binding sites may lie within the 457 bp minimal enhancer, but of necessity would diverge from the reported consensus binding domains (Gruber et al., 1997; Xiang et al., 1995). We do not rule out this possibility since in this study, other identified domains, when mutated, either attenuate or abrogate *TrkA* expression in the trigeminal ganglion.

A recent study has examined the promoter elements that drive *TrkA* gene expression in neural cell lines (Sacristan et al., 1999). This work has defined a promoter region proximal to the 5' exon for the *TrkA* gene that is sufficient to elicit reporter gene activity. This promoter activity lies downstream (3') from the minimal enhancer described in the present study. Our analysis was designed to uncover enhancer sequences in the presence of a minimal (*Hsp68*) promoter in vivo and did not address minimal promoter function.

In conclusion, we identify an enhancer that contains key contiguous *cis* sequences required for in vivo, global, DRG-specific, TG-specific or sympathetic neuron expression. The data reveal a coordinated regulation of *TrkA* gene expression involving multiple regulatory sites. While this provides a complex picture, it is one that most likely conforms with physiological gene regulation, rather than the existence of a single 'master regulator'. Moreover, this information, provides the first tools to identify the specific transcription factors whose physiological targets include the *TrkA* gene. In DRG, *TrkA* is only expressed in nociceptive neurons while *TrkB* and *TrkC* are found in mechanoreceptive and proprioceptive neurons respectively. It is therefore likely that common elements of regulation for the three genes may be shared while unique components may specify modality associated neuronal subsets. Complementary approaches to those described here including yeast 1-hybrid and expression cloning using the defined consensus sites in the minimal enhancer have yielded sensory neuron-specific transcription factors consistent with the present study (Lei Lei, L. M. and L. F. P., unpublished data). Thus, continued analysis of the *TrkA* minimal enhancer may lead to identification of the key transcription factors that determine neurotrophin receptor expression.

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