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Imprinted Expression of *Neuronatin* from Modified BAC Transgenes Reveals Regulation by Distinct and Distant Enhancers

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***Neuronatin* (*Nnat*) is an imprinted gene that is expressed exclusively from the paternal allele while the maternal allele is silent and methylated. The *Nnat* locus exhibits some unique features compared with other imprinted domains. Unlike the majority of imprinted genes, which are organised in clusters and coordinately regulated, *Nnat* does not appear to be closely linked to other imprinted genes. Also unusually, *Nnat* is located within an 8-kb intron of the *Bc10* gene, which generates a biallelically expressed, antisense transcript. A similar organisation is conserved at the human *NNAT* locus on chromosome 20. *Nnat* expression is first detected at E8.5 in rhombomeres 3 and 5, and subsequently, expression is widespread within postmitotic neuronal tissues. Using modified BAC transgenes, we show that imprinted expression of *Nnat* at ectopic sites requires, at most, an 80-kb region around the gene. Furthermore, reporter transgenes reveal distinct and dispersed *cis*-regulatory elements that direct tissue-specific expression and these are predominantly upstream of the region that confers allele-specific expression.**

Key Words: **Genomic imprinting; *Neuronatin*; BAC transgenesis; enhancers; regulatory elements.**

INTRODUCTION

Genomic imprinting is a unique mode of transcriptional regulation in which the expression of specific mammalian genes is dictated by epigenetic modifications established in the male or female germ line (Surani, 1998). Approximately 40 imprinted genes have been identified, some of which play an essential role in embryonic development (DeChiara *et al.*, 1991; Guillemot *et al.*, 1994; Yan *et al.*, 1997; Zhang *et al.*, 1997) and behaviour (Lefebvre *et al.*, 1998; Li *et al.*, 1999). Many imprinted genes are located within specific chromosomal regions which were identified by genetic noncomplementation experiments (Beechey and Cattanach, 1996; Cattanach and Jones, 1994). Studies of human genetic disorders and experiments in mice suggest that imprinted genes are usually found in clusters and coregulated within these domains (Buiting *et al.*, 1995; Hoovers *et al.*, 1995; Leighton *et al.*, 1995; Wutz *et al.*, 1997). A number of imprinted but noncoding transcripts have also been identified within these regions which may be mechanistically involved in the reciprocal imprinting of oppositely expressed genes (Lee, 2000; Lee *et al.*, 1999; Smilinich *et al.*, 1999; Wutz *et al.*, 1997).

We, and others, initially identified *neuronatin* (*Nnat*) in a screen for paternally expressed genes and showed that, like the majority of imprinted genes studied, the silent (maternal) allele was methylated (Kagitani *et al.*, 1997; Kikyo *et al.*, 1997). *Nnat* was also identified in other screens for genes involved in neural development and differentiation (Joseph *et al.*, 1994). Expression of *Nnat* is first detected at 8.5 days of embryonic development (E8.5) with segmental expression in the developing hindbrain and subsequently throughout the postmitotic central nervous system (Dou and Joseph, 1996; Joseph *et al.*, 1994; Wijnholds *et al.*, 1995). *Nnat* is also expressed in nonneuronal tissues derived from the mesoderm: the limb mesenchyme, the pancreas, the

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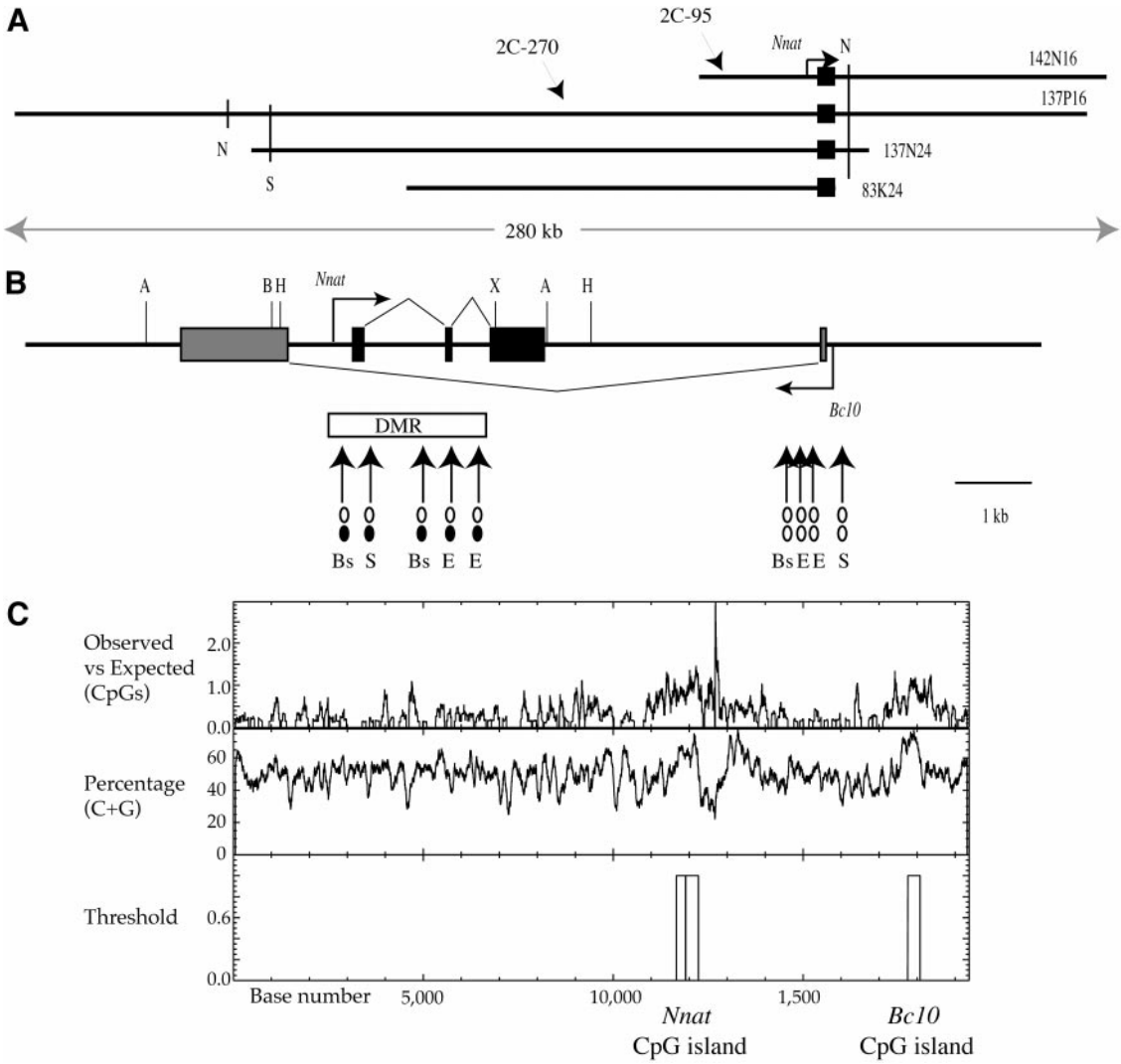

FIG. 1. Organisation of the *Nnat* locus. (A) Physical map of *Nnat* BACs. The *Nnat* gene is indicated by the filled black box with direction of transcription shown by the arrow. The contig spans approximately 280 kb of genomic DNA. N, *Not*I; S, *Srf*I. Modified BACs are indicated by 2C-95 and 2C-270. (B) Structure of the *Nnat* locus. The *Nnat* gene spans 2.4 kb of genomic DNA and three exons (black boxes). *Bc10* (grey boxes) spans the *Nnat* gene. The active paternal *Nnat* allele is unmethylated (open circles), and the silent maternal allele is methylated (closed circles) at the sites indicated. The DMR is shown as an open box. The CpG island overlaying exon I of *Bc10* is unmethylated on both alleles. B, *Bam*HI; Bs, *Bss*HII; E, *Eag*I; S, *Sac*II; H, *Hin*dIII; A, *Avr*II; and X, *Xho*I. (C) CpG plot analysis of 20 kb of sequence around the *Nnat* gene. Two islands are present, one associated with *Nnat* and a second island associated with the *Bc10* gene.

paraxial mesoderm, and in the somites. *Nnat* encodes three putative proteins with an amino acid composition suggesting an association with transmembrane complexes but the precise function of *Nnat* is unknown.

Nnat lies on distal mouse chromosome 2 approximately 18 cM proximal to the breakpoint T2Wa. *Nnat* is therefore located outside a previously defined imprinted region between T2Wa and T28H (Kikyo *et al.*, 1997; Williamson *et al.*, 1998). This region contains a cluster of imprinted transcripts, *Gnas*, *Gnasxl*, and *Nesp* (Peters *et al.*, 1999). *Nnat* marks the position of a novel imprinted region which is distinct from this cluster. It is therefore unlikely to contribute to the developmental defects and lethality associated with either maternal or paternal disomy distal to T2Wa (Cattanach and Kirk, 1985). Differences in *Nnat* expression may instead be involved in other aspects of development, including a role in cerebellar folding (Kikyo *et al.*, 1997).

One of our objectives has been to determine how various imprinted genes are regulated. Previously, we showed that a 130-kb yeast artificial chromosome (YAC) spanning the imprinted genes *H19* and *Igf2* contained the *cis*-regulatory

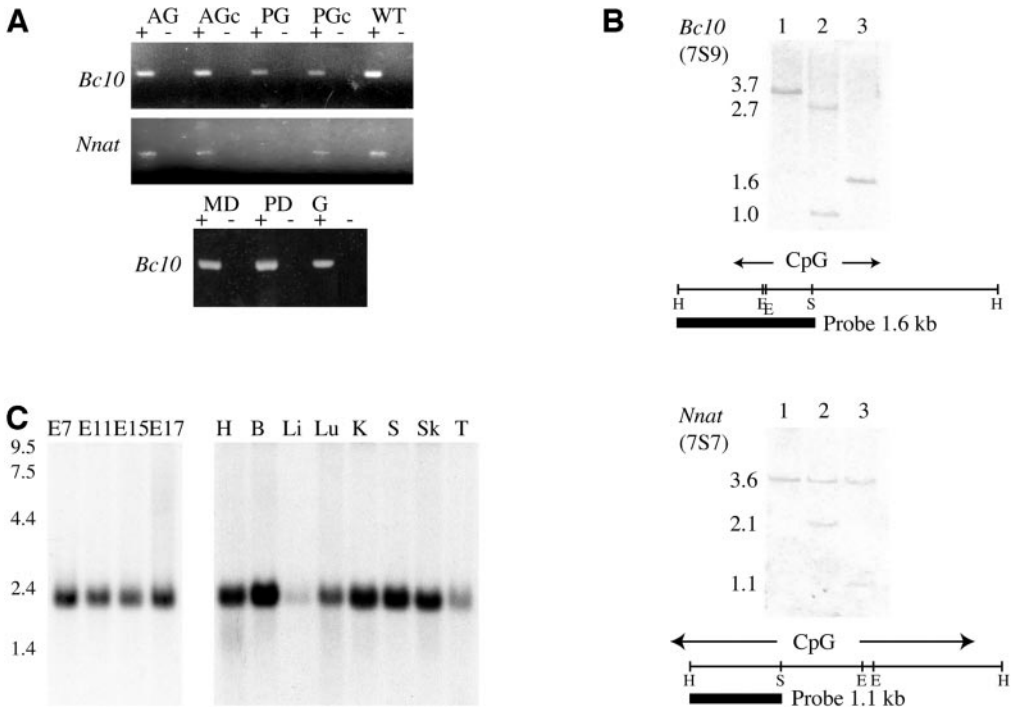

FIG. 2. Characterisation of the *Bc10* gene. (A) RT-PCR analysis of *Bc10* expression. The upper panel shows expression of *Bc10* in androgenetic (AG), parthenogenetic (PG), controls (AGc and PGc, wild-type embryos generated by pronuclear transfer), and control wild-type embryos (WT, CBA × C57Bl6). The middle panel shows expression of *Nnat* in AG, AGc, PGc, and wild-type RNA but not in PG RNA as expected. The lower panel shows shows expression of *Bc10* in maternal disomy (MD), paternal disomy (PD) embryos, and control embryos (WT). *Bc10* is present in all samples at levels similar to the control samples, indicating biallelic expression. (B) Methylation analysis of the *Bc10* and *Nnat* CpG islands. The *Sac*II and *Eag*I sites within the *Bc10* CpG island are unmethylated. The upper panel shows hybridisation of a 1.6-kb *Sac*II–*Hin*dIII probe isolated in the CpG island screen to genomic DNA digested with *Hin*dIII (lane 1) and either *Eag*I (lane 2) or *Sac*II (lane 3). All three sites spanned by this probe are unmethylated. The lower panel shows the differential methylation obtained when a 1.1-kb *Sac*II–*Hin*dIII fragment originating at the *NNat* CpG island was used as a probe. (C) Northern analysis of *Bc10* expression. The left and right panels show Northern blots of poly(A)$^+$ RNA from embryonic and adult tissues, respectively. The embryonic blot has lanes for E7, E11, E15, and E17 of gestation. The adult blot contains: H, heart; B, brain; Li, liver; Lu, lung; K, kidney; S, spleen; Sk, skeletal muscle; T, testis. There is a strong expression of a 2.2-kbp transcript seen in most tissues and developmental stages.

elements that are required for the initiation and maintenance of parent-of-origin-dependent monoallelic expression at ectopic sites (Ainscough *et al.*, 1997). One other region has been explored in a similar way by using a 300-kb YAC spanning the 100-kb *Igf2r* gene (Wutz *et al.*, 1997). In both these cases, genes expressed from the maternal and the paternal allele are present on the transgenes. Imprinting of the mouse *Igf2r* gene is apparently dependent on the presence of an intergenic CpG-rich sequence which shows parental-specific methylation. This marks the start position of the oppositely imprinted transcript which is untranslated and antisense to *Igf2r* (Wutz *et al.*, 1997). Much smaller transgenes have been used to identify *cis*-control elements in the immediate vicinity of single genes, particularly *H19* (Bartolomei *et al.*, 1993; Brenton *et al.*, 1999; Elson and Bartolomei, 1997; Pfeifer *et al.*, 1996), none of which imprint reliably. However, analysis of imprinted genes such as *Nnat*, which apparently exist as single loci and not as part of a cluster, may provide further insight into the mechanism of imprinting.

MATERIALS AND METHODS

Isolation of Genomic Clones Containing the Murine Nnat Locus

Four BAC clones, 83K24, 142N16, 137P16, and 137N24, were isolated by screening a Genome Systems 129 gridded pBeloBAC library with a 1.4-kb fragment from the *Nnat* cDNA (gift of F. Ishino). BACs were characterised by *Not*I and *Srf*I digestion of BAC DNA, pulse-field gel electrophoresis (Pharmacia Gene Navigator), and standard Southern blotting. BAC and plasmid DNA were isolated from liquid culture by alkaline lysis (Sambrook *et al.*, 1989). Genomic Southerns were prepared and hybridised as previously described (Kikyo *et al.*, 1997). Sequence was analysed by using the CpG plot of EMBOSS software available from the Sanger Centre.

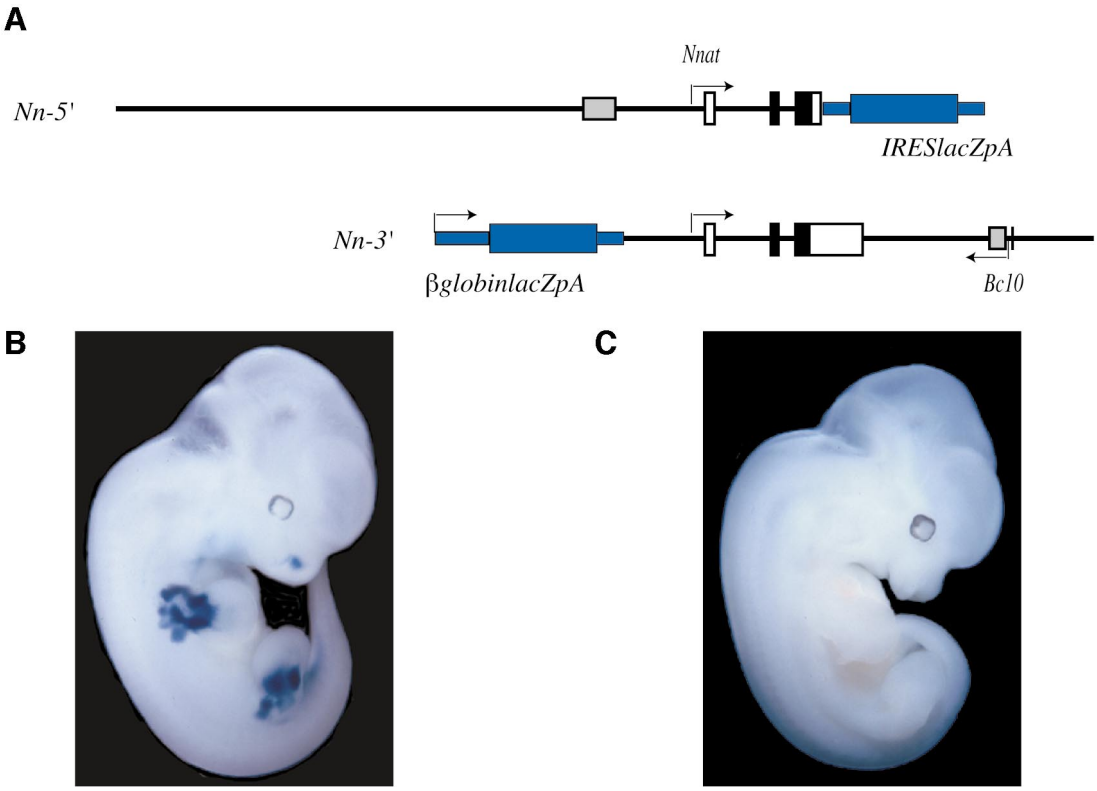

FIG. 3. *Nnat* expression in limb buds is controlled by proximate enhancers that lie outside the *Bc10* gene. (A) Representation of the *Nn*-5′ construct (top) and the *Nn*-3′ construct (bottom). The black (coding) and white (untranslated) boxes represent the *Nnat* gene and the grey boxes indicate the position of *Bc10*. The blue boxes indicate the reporter genes. (B, C) Whole-mount lacZ stains of transgenic E11.5 embryos. *Nn*-5′ contains enhancers for *Nnat* expression in the limb buds and the epithelium over the nasal cavity at E11.5 and the palatal shelves, the submandibular glands and the genital tubercle at E13.5 (not shown). No expression was observed in 11 embryos containing the *Nn-3′* transgene. The nearest *Nnat* enhancers for expression at this stage of development lie between −1.55 and −13.0 kb of the *Nnat* promoter.

Bc10 Expression Analysis

Embryos containing maternal-only or paternal-only chromosomes were generated by pronuclear transfer (Barton, 1987). Embryos disomic for murine chromosome 2 proximal to T2Wa were generated as previously described (Kikyo *et al.*, 1997) from mice carrying a Robertsonian translocation of T2Wa to chromosome 8. RT-PCR was performed as previously described (Ainscough *et al.*, 1997). *Bc10* primers for the androgenetic and gynogenetic RT-PCR were 5′-GTCTTGTCTGAACCACCTTGG and 5′-TGGATACAGTGTAGTTCAATGTC which produce an 808-bp product. PCR conditions: 1 min denature at 95°C and (95°C for 15 min/60°C for 15 min/72°C for 45 min) over 25, 30, and 35 cycles. *Bc10* primers for the disomy RT-PCR were 8648: 5′-CGCTGACACTAGTGCACACA and 9148: 5′-TCACTTCCAACCCCTTTCCT which produce a product of 0.5 kb. PCR conditions: 1 min denature at 95°C and (95°C for 60 min/60°C for 60 min/72°C for 60 min) for 30 cycles. *Nnat* primers were CGGCAGAACTGCTCATCATCG and 5′-CTCCAGGAGCTTACAATCTAG. PCR conditions: 1 min denature at 95°C and (95°C for 15 min/60°C for 15 min/72°C for 45 min) over 25, 30, and 35 cycles. The Northern blots were obtained from Clontech and hybridised under standard conditions to a 2.0-kb *Bc10* cDNA fragment (Sambrook *et al.*, 1989).

Construction of Plasmid-Based Transgenes

Nn-5′ and *Nn*-3′: *IRESlacZpolyA* (Li *et al.*, 1997) was blunt ligated into the *Xho*I site of a 13-kb *Eco*RI–*Xho*I genomic fragment from upstream of the *Nnat* gene to generate *Nn*-5′. The insert was isolated after *Not*I digestion, gel purified (QIAquick, Qiagen), and injected at 0.5 ng/μl. A 7.6-kb *Bam*HI–*Not*I genomic fragment was blunt ligated into the *Spe*I site downstream of *globin-lacZpA* to generate *Nn*-3′, and a *Bam*HI fragment was used to generate transgenic embryos. Copy number was determined by comparing the intensity of hybridisation of a 0.5-kb*Nnat* cDNA probe from the 5′ end of the 1.4-kb cDNA to the endogenous locus (two copies) and the transgene by using a PhosphorImager.

Modification of BAC-Based Transgenes

The two-colour *IRESβgeo-loxP-Plap* cassette was constructed as follows: *IRESβgeo* was excised from *pIRES-βgeo* (Mountford *et al.*, 1994) and cloned into an *Eco*RV site of pPolyIIID. A loxP linker was cloned into the unique *Not*I site in the polylinker (GAGCTCACCTAGGTATCTAGCCTAGGATAACTTCGTATAGCATACATTATACGAAGTTATCTAGAACCGGTGACGTCACCATGGGAAGCTTCGTGGATCCATAACTTCGTATAGCATACATTATACGAAGTTATCGCCTAGGAATTCC). A 2.5-kb human

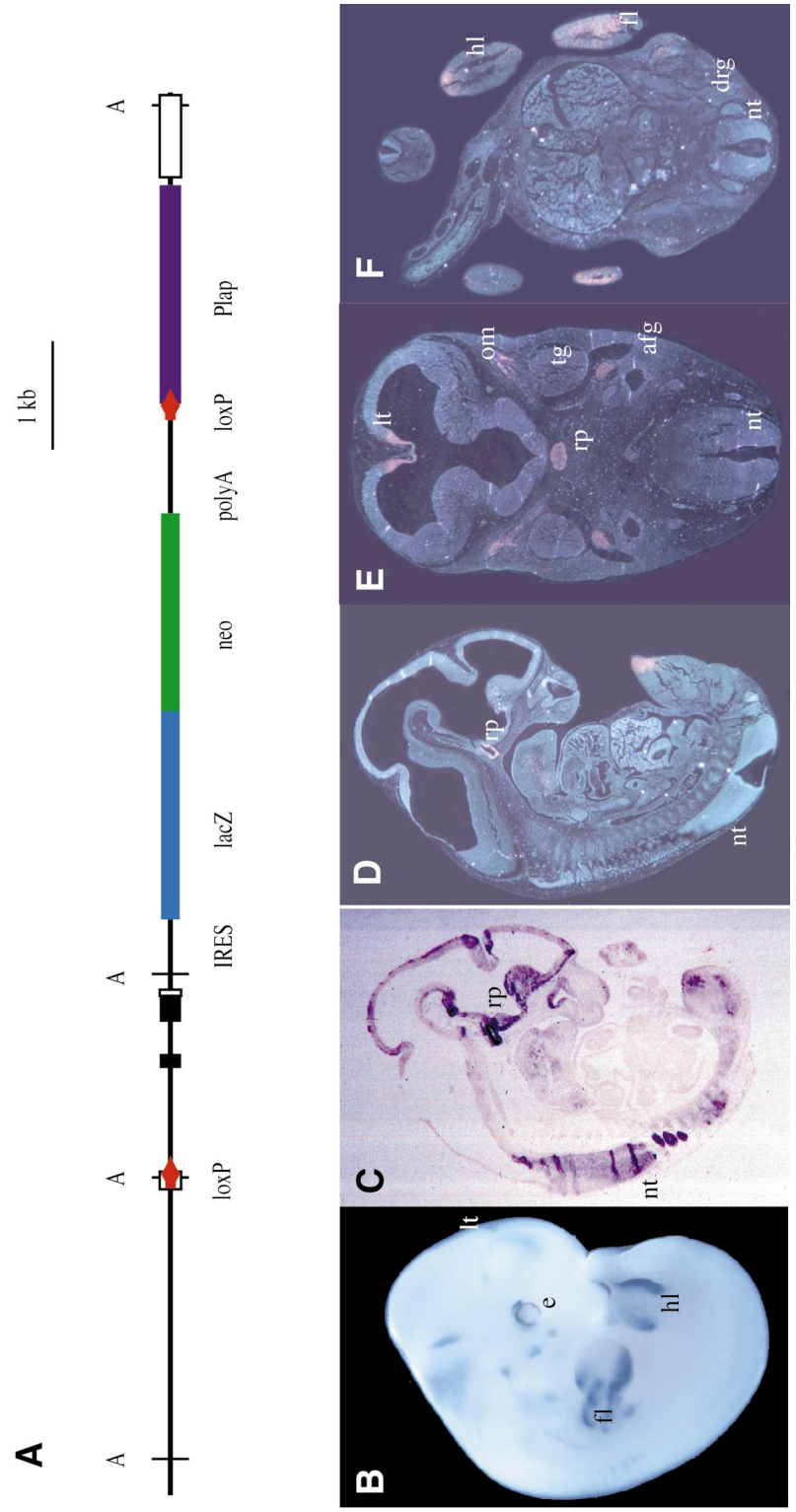

FIG. 4. Restricted *Nnat* expression from a 95-kb modified BAC transgene. (A) The modification construct. The black (translated) and white (UTR) boxes represent *Nnat* as in Fig. 3. A loxP-*Avr*II site (red arrow) was inserted in the 5′UTR of *Nnat*, a *lacZ-neomycin* fusion reporter gene (blue and green boxes) in the 3′UTR, a second loxP site, and a human *placental alkaline phosphatase* reporter gene (purple box). A, *Avr*II. (B) Whole-mount lacZ stain at E11.5 of line 2C-95B which carries four copies of the modified 95-kb BAC. Marked expression is seen on the limbs (fl, hl), around the eye (e), and in the lamina terminalis (lt). (C) *In situ* hybridisation with the 1.4-kb *Nnat* cDNA of a midsaggital section at E11.5 of a nontransgenic embryo. (D) Comparable midsaggital section at E11.5 of a lacZ-stained 2C-95B embryo as a dark field image. Reporter expression (pink) is visible in Rathke's pouch (rp) but not in the neural tube (nt). (E) Transverse midbrain section at E11.5. Rathke's pouch, the lamina terminalis, the preocular muscle mass (om), and the facial component of the acoustico-facial (VII-VIII) ganglion (afg) express the reporter but not the trigeminal ganglion (V, tg). Widespread expression in the neural tube and other neural tissues was absent. (F) Expression in the limb buds is detectable but not in the dorsal root ganglia (drg) or the neural tube.

placental alkaline phophatase cDNA with an upstream IRES sequence was ligated into a unique *Sal*I site provided by the loxP linker. A 2.7-kb *Nnat Spe*I fragment was cloned into pBS II SK and a loxP-*Avr*II linker (oligonucleotide 1, ACTATAACTTCGTATAGCATACATTATACGAAGTTATCCTAGG and oligonucleotide 2, AGTCCTAGGATAACTTCGTATAATGTATGCTATACGAAGTTTAT) cloned into a unique *Hin*fI site. A 2.7-kb *Spe*I fragment from the 5′ region of *Nnat* was cloned upstream to extend 5′ homology. An *Xho*I–*Spe*I digest of this clone was ligated into the *Not*I site in *IRESβgeo-loxP-IRESPlap* cassette. A *Xho*I–*Bam*HI (5.8 kb) from downstream *Nnat* was ligated into the *Srf*I site downsteam of *Plap* to provide 3′ homologous sequence. The *Nnat-IRESβgeo-loxP-IRESPlap* construct was cloned into the *Sal*I site of pSV1.*RecA* which was transformed into chemical competent bacteria containing the target BAC clone (Yang *et al.*, 1997). Cointegrant and resolved clones were detected by Southern analysis of *Avr*II-digested DNA obtained by alkaline lysis from 2-ml cultures.

Analysis of Transgenic Animals

Transgenic founders were generated by pronuclear injection of the *Srf*I (2C-95) or λterminase (2C-270) linearised DNA or linearised *Nn-5′* and *Nn-3′* as previously described (Ainscough *et al.*, 1997). Genomic DNA was extracted as described (Hogan *et al.*, 1994) from yolk sacs, tail tips, or whole embryos. Transgenic lines were characterised by hybridisation of Southern blots of *Avr*II-digested genomic DNA with a 1.5-kb *Bam*HI–*Bss*HII fragment which lies 5′ to exon I of *Nnat* and 5′ to the loxP-*Avr*II site in the transgene. Copy number was determined by comparing the intensity of hybridisation of this fragment to the endogenous locus (two copies) by using a PhosphorImager. A comparison of hybridisation signal of transgene end probes derived from the BAC vector was made to confirm the integrity of the transgene integrations. Fluorescent *in situ* hybridisation was used to map the integration sites of the BAC transgenes. Splenocytes were derived from heterozygous adult transgenic animals. Metaphase chromosome spreads were prepared from splenocytes by standard procedures and FISH analysis performed as described (Ainscough *et al.*, 1997) by using BAC clone 142N16. FISH signals were visualised by confocal fluorescent microscopy.

In Situ Hybridisation and Histology

In situ hybridisation was performed by using a 1.4-kb fragment of the mouse *Nnat* cDNA. Sense and antisense RNA probes were prepared by *in vitro* transcription using the DIG RNA labelling kit (Boehringer Mannheim). Saggital and transverse sections (10 μm) from transgenic mouse embryos at E11.5 and E13.5 were used for *in situ* hybridisation. Briefly, embryos were fixed in 4% paraformadehyde at 4°C overnight, sections made, and hybridised with the probes overnight at 65°C. The sections were washed at 65°C and incubated with preadsorbed alkaline phosphatase-conjugated anti-DIG antibody overnight at 4°C. Alkaline phosphatase activity was detected by using BM purple AP substrate (Boehringer Mannheim) and counterstained with 0.5% eosin. No signal was detected with the sense probe. For whole-mount lacZ staining, embryos were dissected free of extraembryonic tissues and fixed for 1–3 h in formaldehyde (2%), glutaraldehyde (0.2%), NP-40 (0.02%), $MgCl_2$ (1 mM), and sodium deoxycholate (20 mM) at 4°C and washed three times in PBS before staining for 16 h at room temperature in β-galactosidase (0.4 mg/ml), potassium ferricyanide (4 mM), potassium ferrocyanide (4 mM), $MgCl_2$ (2 mM) in PBS. At E13.5 and E15.5, a midsaggital incision was made midway through fixation to facilitate penetration of the stain. Embryos were washed extensively in PBS poststaining, fixed overnight in 4% formaldehyde, and cleared in 70% ethanol. LacZ-stained embryos were dehydrated through ascending alcohol series, cleared in xylene, and embedded in fibrowax (BDH). Saggital and transverse sections were made at 8 μm thick, mounted on slides, dewaxed, and rehydrated in water through descending alcohol series and counterstained with 4% eosin. Mounted sections were photographed under dark field where the lacZ signal is pink.

RESULTS

We previously identified *Nnat* as an imprinted gene that marked the position of an unexplored imprinted region outside the previously defined domains (Kikyo *et al.*, 1997). We therefore physically characterised the *Nnat* locus and initiated a transgenic analysis to identify the *cis*-elements responsible for tissue-specific and allele-specific regulation of this gene.

Neuronatin Lies within the Intron of a Second, Biallelically Expressed Gene, Bc10

We initially constructed a physical map of *Nnat* region. Figure 1A shows four overlapping mouse bacterial artificial chromosomes (BACs) which were isolated and characterised by pulse-field gel electrophoresis (PFGE). The exon–intron boundaries for *Nnat* were determined from 20 kb of sequence surrounding the gene (Fig. 1B; GenBank Accession No. AF303656). We previously described the maternal allele-specific methylation of this gene. We now show that the differentially methylated region (DMR) of the *Nnat* gene lies within a CpG island spanning exon I and extending into intron 2 (Fig. 1B).

Our sequence analysis of the region revealed an unusual arrangement between *Nnat* and a second gene, *Bc10* (Gromova *et al.*, 1999). *Nnat* was contained within the 8-kb intron of the *Bc10* gene and this was transcribed in the opposite direction to *Nnat* (Fig. 1B). We submitted our 20 kb of sequence to a CpG prediction program (CpG plot) which revealed that both genes were associated with CpG islands (Fig. 1C). Given the close proximity of these two genes, we first examined the expression of *Bc10* in embryos with a paternal-only genome (androgenetic), a maternal-only genome (parthenogenetic), and in embryos with reciprocal maternal or paternal translocations of proximal chromosome 2 (T2Wa; Fig. 2A). The *Bc10* transcript was present in all the samples. *Nnat*, as expected, was present only in androgenetic cDNA. These results suggest that, unlike *Nnat*, *Bc10* is bilallelically expressed. In addition, the CpG island spanning exon I of the *Bc10* gene was found to be unmethylated at all the restriction enzyme sites we tested within the predicted island (Fig. 2B, upper panel; *Cfo*I sites, data not shown) in contrast to the differential methylation seen at the *Nnat* CpG island (Fig. 2B, lower panel). A 2.1-kb *Bc10* transcript was detected by Northern analysis from

embryonic day 7 (E7) onwards with expression continuing into adulthood where expression was detectable in a wide range of tissues (Fig. 2C). This contrasted with *Nnat*, whose expression is limited from an early stage to neural tissue, including the pituitary, with postnatal downregulation of expression (Kikyo *et al.*, 1997). Therefore, despite the intimate arrangement of these two genes, they do not appear to share regulatory elements either for tissue-specific expression or for allele-specific expression.

Neuronatin Appears to Be an Isolated Imprinted Gene

In order to identify additional DMRs and their imprinted genes, a CpG-island cloning strategy was applied within the 280-kb BAC contig (John *et al.*, 1994). These CpG-rich DMRs resemble CpG islands and are characteristic of imprinted genes (Razin and Cedar, 1994). CpG islands have been found to almost always contain one or more *Eag*I and *Sac*II restriction enzyme sites (Bickmore, 1992). Therefore we isolated 14 unique genomic clones adjacent to *Eag*I and *Sac*II restriction enzyme sites from the BAC clones. The *in vivo* methylation status of these sites was determined by Southern analysis and the clones were partially sequenced (data not shown). Only two sites showed differential methylation (example in Fig. 2B, lower panel). The sequence of these clones showed that both were derived from the *Nnat* DMR. A cluster of three clones detected unmethylated sites within a second CpG island (example in Fig. 2B, upper panel). This island was associated with the 5′ exon of the *Bc10* gene. Sequence analysis of the remaining nine clones did not reveal the presence of any additional CpG islands or matches any known genes or expressed sequence tags (ESTs) in any database suggesting that no other CpG island associated genes were present in the 280-kb region scanned.

This approach does not rule out the presence of genes that lack CpG islands. However, while approximately 40% of nonimprinted genes lack CpG islands, all imprinted genes seem to be associated with at least one CpG island (Onyango *et al.*, 2000). A recent comparative analysis between mouse and human of a 1-megabase imprinted region (mouse distal 7/human 11p15) revealed the presence of one or more CpG islands associated with all nine imprinted genes in the region. Indeed, eight of the nine imprinted genes were associated with two or more CpG islands sequences. This also appears to be the case for imprinted genes on other chromosomes (Brandeis *et al.*, 1993; Shemer *et al.*, 1997; Wutz *et al.*, 1997). As we identified two clones from the *Nnat* CpG island and three clones from the *Bc10* CpG island, we believe we have identified all the possible CpG islands in the 280-kb region scanned. In addition, other systematic studies have failed to identify additional imprinted genes in the mouse distal chromosome 2 region based on the methylation status of additional restriction enzyme sites (Kelsey *et al.*, 1999). Therefore, *Nnat* does not appear to be closely linked to other imprinted genes.

While this work was in progress, 200 kb of sequence around the human *NNAT* locus became available (AL109614). This sequence, which extends 62 kb upstream of *NNAT* and 130 kb downstream, had previously been scanned for EST matches and CpG islands. This revealed the conserved organisation of the *NNAT* and *BC10* genes and their CpG islands. In addition to *NNAT* and *BC10*, two other gene-like sequences were present, neither of which are associated with CpG islands. The first sequence lies 2.2 kb upstream of *BC10* and has homology to *peptidyl prolyl isomerase A* (*PP1A*). The second sequence, 36 kb upstream of *BC10*, has homology to *glutaredoxin* (*GLRX*). Neither of these loci have matching EST transcripts within the public databases and they both contain frameshift/stop codons. Both appear to be nontranscribed pseudogenes. In addition, an equivalent mouse homologue of the *PP1A* psuedogene does not appear to be present on the mouse *Nnat* BAC contig. Low-stringency hybridisation was performed to the level at which we could detect *Bc10* and *Nnat* homologues across species but no signal was seen on BAC Southerns with a mouse *Pp1a* probe (data not shown). Although we cannot absolutely exclude the presence of imprinted genes further away, this work does suggest that *Nnat* is the only imprinted gene within the region we have analysed.

Elements External to the Bc10 Gene Regulate Nnat Expression

We initiated our characterisation of the *Nnat* gene by asking whether its regulatory elements were in close proximity, perhaps even within the *Bc10* intron. Two reporter transgenes, *Nn*-5′ and *Nn*-3′, were constructed (Fig. 3A). *Nn*-5′ contained a 13.6-kb *Eco*RI–*Xho*I genomic fragment from upstream of the *Nnat* gene which was placed upstream of an *IRESlacZpolyA* reporter. *Nn*-3′ contained a 9.1-kb *Bam*HI genomic fragment which was placed downstream to a *globin-lacZpA* reporter. A transient transgenic assay was performed with *Nn*-5′ and reporter expression was examined at E11.5. One embryo showed marked expression in the limb buds characteristic of the endogenous expression pattern of *Nnat* (Fig. 3B). We went on to generate eight transgenic lines with this construct. Only three of these lines showed expression of the reporter and this was consistent with the expression seen in the transient assay (see later). One of these was a single copy line and two had integrated multiple copies of the transgene. At E13.5, expression was additionally observed in the submandibular glands, part of the palatal shelf and the genital tubercle. By contrast, no specific expression was observed with *Nn*-3′ in a transient transgenic assay with 10 independent integration events (Fig. 3C). Therefore, the nearest *Nnat* enhancers lie in a region between 1.55 and 13 kb upstream of the promoter and are external to the *Bc10* gene.

Expression of Nnat from Modified BACs Reveals Distinct and Dispersed Enhancers

The small *Nnat* transgenes were prone to silencing and ectopic expression which is known to occur with this size

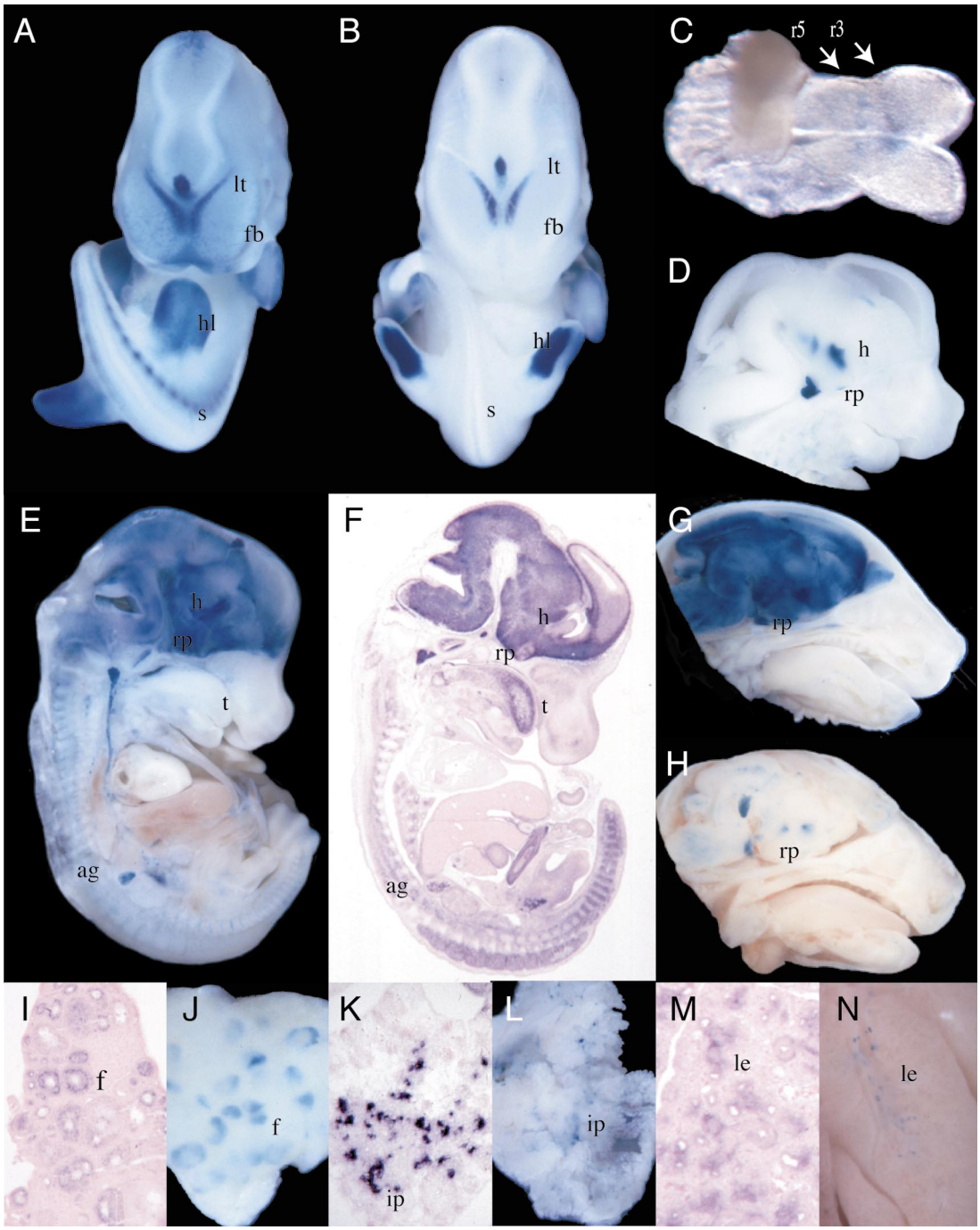

FIG. 5. Nnat enhancers lie more than 25 kb upstream of the gene. (A) Ventral view of whole-mount lacZ-stained pregerm line E11.5 embryo, 2C-270P. (B) Ventral view of E11.5 whole-mount lacZ-stained embryo from line 2C-95B. Expression in the fore and hind limbs (fl and hl) and in the lamina terminalis (lt) is seen with both transgenes but only 2C-270 directs widespread expression in the forebrain (fb) and strong expression in the somites (s). (C) Whole-mount lacZ stain at E8.5 (7 somites) of line 2C-270A revealing expression in rhombomeres 3 and 5 (r3 and r5) and in Rathke's pouch. (D) Midsaggital head section of lacZ-stained line 2C-95B embryo at E13.5. (E) Midsaggital full section of lacZ-stained line 2C-270A embryo at E13.5. There is overlapping expression from both transgenes within Rathke's pouch (rp) and the hypothalamus (h). 2C-270 additionally directs expression in all postmitotic tissues of the central nervous system, in the medulla of the adrenal gland (ag), and the tongue. (F) *In situ* hybridisation with the 1.4-kb *Nnat* cDNA of a midsaggital section at E13.5 of a nontransgenic embryo. (G) Midsaggital head section of lacZ-stained embryos at E17.5 of line 2C-270A and line 2C-95B (H). (I) Endogenous expression of *Nnat* in the follicular cells (f) surrounding the maturing oocytes in the adult ovary. (J) Expression of lacZ reporter in the follicular cells in line 2C-95B. Expression was also observed with line 2C-270A. (K) *Nnat* endogenous expression in the future islet cells of the pancreas (ip). (L) Expression of lacZ reporter in the pancreas, line 2C-270A. (M) Endogenous expression in the primordial epithelium of the lung (le). (N) Expression of lacZ reporter in the lung, line 2C-270A.

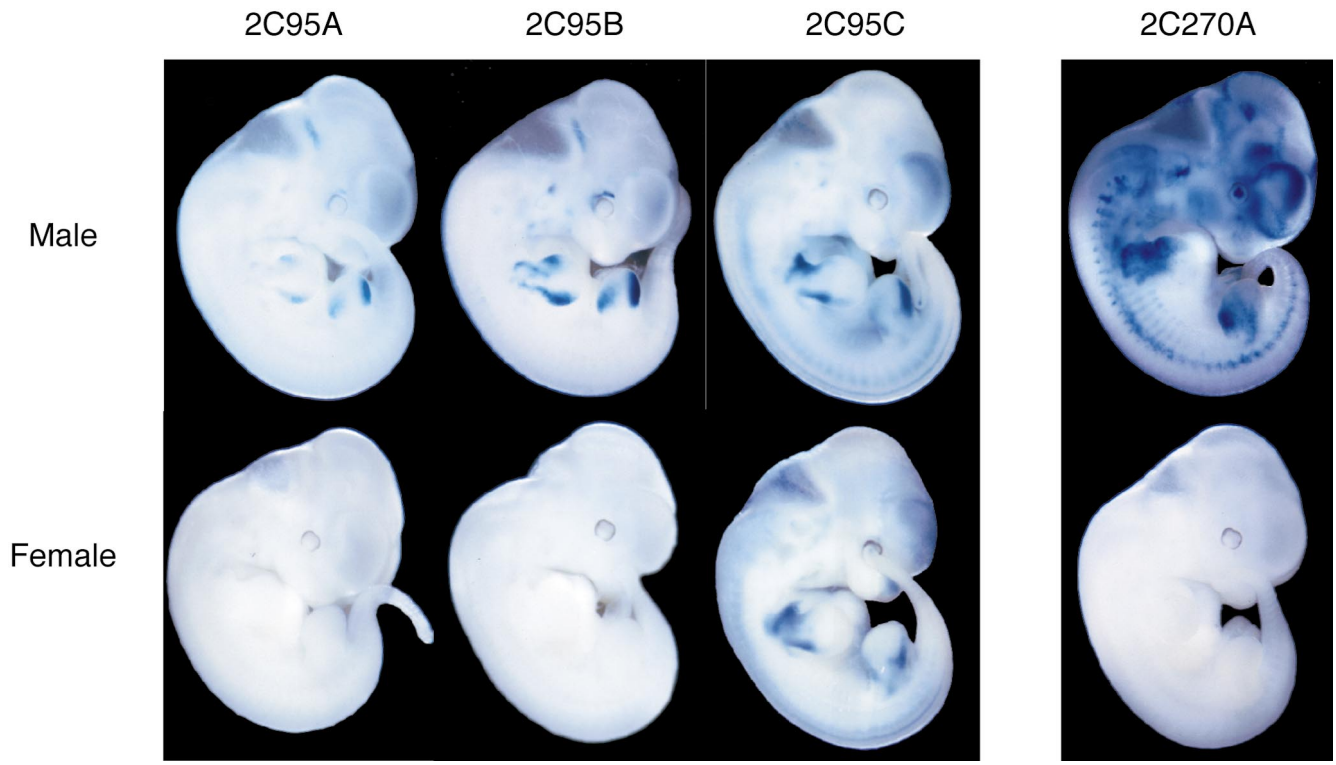

FIG. 6. A maximal region of 80 kb is required for paternal-specific expression of *Nnat*. Whole-mount lacZ stain at E11.5 of lines 2C-95A, 2C-95B, 2C-95C, and 2C-270A after paternal (top row) or maternal (bottom row) transmission.

of construct. We therefore went on to use the larger BAC transgenes to generate further transgenic lines, in order to identify additional enhancers and possibly the imprinting element(s) for *Nnat*. A 95-kb BAC clone, 142N16 (Fig. 1A), was modified to include a 5′UTR loxP-*Avr*II sequence and a reporter gene in the 3′ UTR of *Nnat* (Fig. 4A). Three lines, 2C-95A, 2C-95B, and 2C-95C with one, four, and five copies of the transgene, respectively, were generated (not shown). With these larger clones, all the transgenic lines showed a consistent expression pattern (Fig. 4B, 2C-95A). A comparison of saggital and transverse sections of lacZ-stained embryos with matched *in situ* sections revealed that this was a subset of the endogenous expression pattern (Figs. 3C–3F, data not shown). Expression of the transgene was seen in Rathke's pouch (Figs. 3D and 3E), the preocular muscle mass, the facial component of the acoustic-facial ganglion, the lamina terminalis (Fig. 3E), and the limbs (Fig. 3F) consistent with the endogenous expression pattern. However, expression was absent in substantial areas, such as the neural tube and the somites where the endogenous *Nnat* gene is expressed.

All Nnat Enhancers Lie Upstream of the Gene

As 95 kb around the gene was not sufficient to drive full expression of *Nnat*, the largest BAC clone, 137P16, spanning 270 kb of the *Nnat* locus, was modified. Prior to generating transgenic lines, we examined expression in a transient transgenic assay at E11.5. One embryo with four copies of the trangene was obtained. This showed expression consistent with the presence of all the *Nnat* enhancers within BAC 137P16 (Fig. 5A). Widespread expression in the forebrain and developing somites was seen in this embryo which was absent in the embryos carrying the 95-kb transgene (Fig. 5B). Subsequently, one seven-copy founder line, 2C-270A, was generated. At E11.5, embryos from this line showed an identical expression pattern to the first integration event in the transient assay. We went on to examine expression at other stages of development to confirm the presence of all the enhancers within this transgene. At E8.5, *lacZ* expression was seen in rhombomeres 3 and 5 and Rathke's pouch (Fig. 5C). Saggital sections of E13.5 embryos from line 2C-95B revealed that *lacZ* expression was restricted to Rathke's pouch and hypothalamus (Fig. 5D). Expression from the larger BAC, in line 2C-270A, was widespread throughout all neural tissues (Fig. 5E). This expression was comparable to expression from the endogenous *Nnat* gene (Fig. 5F). Differences between 2C-270A and 2C-95B were most apparent at E17.5 (Figs. 5G and 5H).

In addition to tissues of neural origin, *Nnat* is also expressed in the follicular cells surrounding the maturing oocytes (Fig. 5I). This expression was observed with both

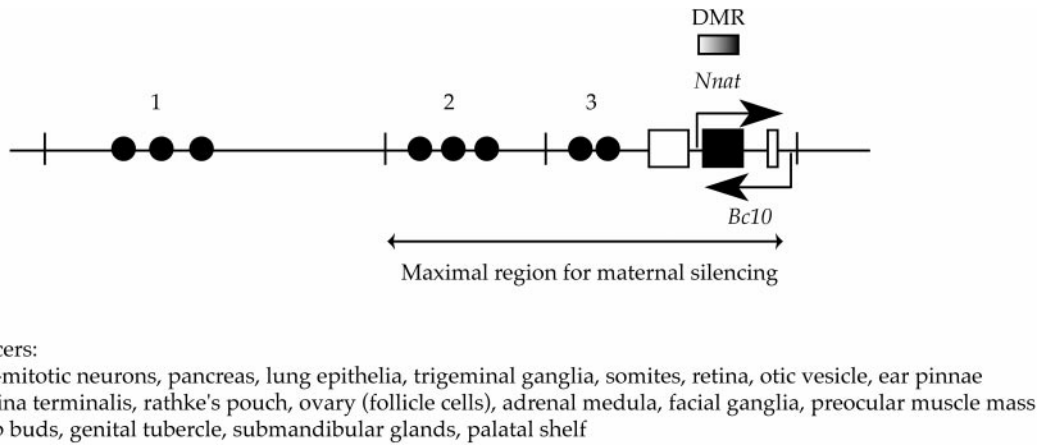

FIG. 7. Overview of the position of regulatory elements directing tissue-specific and allele-specific expression of the *Nnat* gene.

the 2C-95 and the 2C-270 transgenes (Fig. 5J). Expression in the future islet cells of the pancreas (Fig. 5K), the primordial epithelium of the lung (Fig. 5M), the medulla of the adrenal gland (Fig. 5F), and the retina, otic vesicle, and ear pinnae (data not shown) was only seen with the 270-kb transgene (Figs. 5L, 5N, and 5E).

These analyses clearly demonstrate that discrete upstream enhancers regulate tissue-specific expression of *Nnat*. Furthermore, expression from the minitransgenes and the modified BACs suggested that all the enhancers regulating *Nnat* expression were situated upstream of the *Nnat* promoter. We confirmed this by examining pregermline expression of a 30-kb *Not*I fragment obtained from the 2C-95 construct. This fragment contained 25 kb of sequence upstream to the gene and extended to the *Not*I site within the *Bc10* promoter. An identical pattern of expression was observed for this transgene and the full-sized 2C-95 transgene for two independent intergration events (data not shown).

Imprinting of Nnat at Ectopic Sites

Finally, we examined the imprinting capabilities of the *Nnat* transgenic lines. The endogenous *Nnat* gene is only active after paternal transmission and no expression is seen after maternal transmission. We therefore examined expression in all the transgenic lines after both paternal and maternal transmission. The three transgenic lines with the *Nn-5′* mini transgene lines showed expression of the transgene after both paternal and maternal transmission (data not shown). Hence, this 13.6-kb fragment was not able to respond to an imprinting signal. In contrast, three of the BAC transgenic lines (2C-95A, 2C-95B, and 2C-270A) showed no expression of the transgene after maternal transmission, similar to the endogenous locus (Fig. 6). All the BAC integration sites were mapped outside regions currently defined as containing imprinted genes (2C-95A, middle chromosome 2; 2C-95B, proximal chromosome 14; 2C-95C, distal third of chromosome 17; 2C-270A, centromeric chromosome 15). Therefore, the BAC transgenes were able to respond to imprinting signals independently of their site of integration. The *cis*-regulatory elements for generating allele-specific expression of *Nnat* must lie within the minimal overlap between the two BAC clones, a region of 80 kb (Fig. 7).

One BAC transgenic line (2C-95C) showed expression after both maternal and paternal transmission. Variability in the imprinting of much larger clones has been reported. One out of four lines for the 300-kb *Igf2r* YAC transgene did not imprint (Wutz *et al.*, 1997). We also reported loss of silencing of high copy lines for the 120-kb *Igf2*/*H19* YAC transgenes (Ainscough *et al.*, 1997). Copy number also appears to be important in the imprinting of the *H19* mini transgenes. In this case, only the multicopy lines imprint (Bartolomei *et al.*, 1993; Brenton *et al.*, 1999; Elson and Bartolomei, 1997; Pfeifer *et al.*, 1996). However, imprinting of the *Nnat* BAC transgenic lines was independent of copy number. The seven-copy line, 2C-270A, was always efficiently silenced after maternal transmission. Also, we did not observe imprinting for either the single copy or the two multicopy *Nn-5′* mini transgene lines. Copy number does not appear to be a factor in the imprinting of *Nnat*. We are unable to explain why this one BAC transgenic line did not imprint. Although large insert transgenes may sometimes undergo rearrangement or deletion of small fragments of DNA, Southern blotting shows no evidence of this in our case. Given the size of these transgenes, it might suggest that the imprinting control regions (ICR) are generally more susceptible to chromatin context than enhancers. It is therefore interesting to note that the sequence of known ICRs is less well conserved across species compared with enhancers (John and Surani, 2000).

DISCUSSION

How specific epigenetic states like imprinting originated during evolution remains central to our understanding of the mechanism of imprinting and its role in mammals. Detailed examination of imprinted loci such as *Nnat* may provide some understanding of this process. One mechanism for generating novel epigenetic states is by retrotransposition. This has been observed with insertion of the intracisternal A particles (IAP), as seen at the *agouti* and *fused* loci (Morgan *et al.*, 1999). Both of these loci exhibit variable penetrance and differential methylation that, at least in part, is dependent on their parental origin. The imprinting of the mouse *U2af-rs1* gene on proximal chromosome 11, which is thought to have occurred by retrotransposition, appears to be of this type (Kitagawa *et al.*, 1995). *U2af-rs1* shares some similarities with the imprinting of *Nnat*, since *U2af-rs1* is also a single imprinted gene located within an intron of the biallelically transcribed, antisense *Murr1* gene. However, unlike *Nnat*, *U2af-rs1* is intronless, and possibly neomorphic in the mouse, since none of the three human *U2AF-RS1* homologues are imprinted and none reside on chromosome 2 where the human *MURR1* gene is located (Nabetani *et al.*, 1997). *Nnat*, in contrast to *U2af-rs1*, does contain introns and the overall organisation of the *Nnat* locus relative to the other genes in the region is conserved between mouse and humans. The human locus is also differentially methylated, indicating a conservation of imprinting across species (our unpublished data). This suggests that imprinting of this locus is well established amongst eutherians and also that it is not imprinted as a result of a retrotransposition event.

Further insight into the mechanism of imprinting comes from comparisons between marsupials and eutherian mammals which diverged some 130 million years ago (John and Surani, 2000; Killian *et al.*, 2000). The *Igf2r* gene imprinting depends on an intronic, differentially methylated CpG island, and its paternal antisense transcript in mice (Wutz *et al.*, 1997). However, this island (and presumably the antisense transcript) is absent from the imprinted marsupial *IGF2R* locus and therefore it is apparently not obligatory for imprinting (Killian *et al.*, 2000). Here, we have demonstrated that *Nnat* is apparently an isolated imprinted gene. We suggest that imprinting of single, isolated genes could be viewed as a prototypic state. If so, progression towards the more familiar domains with multiple imprinted genes may have occurred in a stepwise fashion.

We have used a transgenic approach to identify the *cis* elements regulating tissue-specific expression of *Nnat* and those controlling allele-specific expression. The enhancers for tissue-specific expression of *Nnat* gene are distinct and dispersed. Some lie more than 25 kb upstream of the *Nnat* promoter. In fact, it appears that all the enhancers lie upstream of the gene (Fig. 7). This arrangement may be a common feature of imprinted loci since enhancers for the *Igf2* gene (Kaffer *et al.*, 2000) and for $p57^{Kip2}$ (R.M.J., manuscript in preparation) are also located to one side of these genes. In the case of *Igf2*, all the enhancers apparently lie downstream of the gene and are functionally separated from the *Igf2* promoter by a boundary element active on the maternal allele (Bell and Felsenfeld, 2000; Hark *et al.*, 2000; Schmidt *et al.*, 1999). However, there is no evidence for a similar boundary at the *Nnat* locus.

Both the 95-kb BAC transgene and the 270-kb transgene can exhibit imprinting at ectopic sites (Fig. 6). Therefore, most of the significant imprinting elements for *Nnat* must be within the minimal overlap between these transgenes, an 80-kb region. Preliminary data for the 30-kb transgene excludes a role for the *Bc10* transcript and sequences downstream of *NNat* in the imprinting of this gene. The differential methylation of the *Nnat* parental alleles (DMR) is confined close to the gene and does not extend to any neighbouring regions or genes, such as *Bc10*. This argues for a localised effect of a putative ICR. The 13-kb transgene immediately proximate to the gene is not sufficient for imprinting. However, it is the case that other small transgenes imprint poorly at ectopic sites, even those for *H19* where there are known ICRs present (Bartolomei *et al.*, 1993; Brenton *et al.*, 1999; Elson and Bartolomei, 1997; Pfeifer *et al.*, 1996). It seems that appropriate chromatin context is essential for imprinting, a condition that is met with the larger BAC or YAC transgenes. This may reflect the greater degree of susceptibility of ICRs to nearby *cis*-sequence as compared, for instance, with enhancer sequences. However, it may equally suggest that ICRs extend over larger regions than currently thought and that the sequence content of the region is important in establishing an efficient imprint. For *Nnat*, the presence of an ICR in close proximity to the gene is supported by our preliminary studies which have shown the presence of allele-specific *DNaseI* hypersensitive sites closely linked to the *Nnat* gene.

By virtue of its isolation from other imprinted genes, the *Nnat* locus represents a simple but effectively imprinted domain with conserved genomic organisation in mouse and man.

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