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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
The primary accomplishments of the previous funding period were: 1) RACE analysis of the 5′ end of $Na_V1.8$/Scn10a murine DRG cDNA confirmed the presence of one intron splice site in the ~200 bp 5′UTR of the Scn10a transcript; 2) Three BAC clones were isolated from a genomic library. All three contain at least the entire cds containing portion of the Scn10a gene plus the 5′UTR and an additional >4.0kb of upstream sequence; 3) Homologous recombination (conversion) of all three BAC clones to EGFP containing reporter constructs has been completed; 4) LM-PCR upstream from the putative transcription start site (5′RACE end) produced 4.0kb of sequence that may contain some/all of the elements required for the exquisitely controlled expression of $Na_V1.8$, the Scn10a gene product; 5) The putative promoter appears to contain several known consensus binding sequences for transcription factors; 6) Putative neuronal-specific silencer elements may be present. Deletion analysis of the 4 kb promoter fragment indicates activating elements between ≈ -3200 and -2500 bp. These latest findings will allow us to extend our analysis of the regulation of the Scn10a gene and efficiently focus our efforts during the final year of this project.

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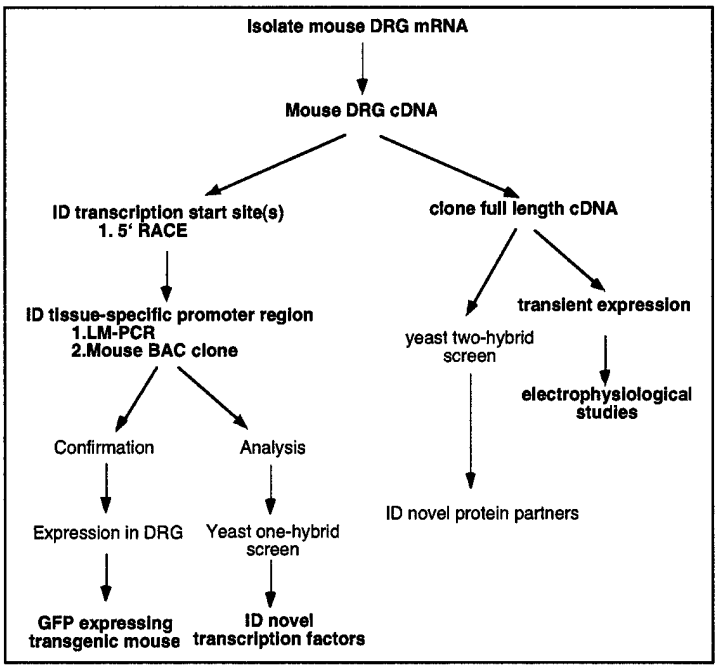
Introduction

The Scn10a gene product encodes a tetrodotoxin-resistant sodium channel (SNS/PN3) expressed exclusively in a subset of primary sensory neurons (e.g., dorsal root and nodose ganglia) believed to be involved in pain transmission (Akopian et al., 1996). Thus, it is important to understand mechanisms contributing to both the function of the protein and the exquisite specificity of gene expression. The overall research plan is detained in the flowchart depicted to the right. Significant progress was made during the latest funding period on both the genomic (left branch) and proteomic (right branch) sections of the research plan.



Specifically, we have isolated three bacterial artificial chromosome (BAC) clones that contain the coding sequence for Scn10a and extensive 5'-flanking DNA that contains some/all of the *cis* regulatory elements that control expression of this gene. The BAC clones have been engineered to now contain the coding sequence for enhanced green fluorescent protein (EGFP) immediately downstream of the 5'-flanking sequence that will allow an analysis of Scn10a regulation in microinjected rat sympathetic neurons. In parallel, the putative transcriptional start site for Scn10a was identified by 5' rapid amplication of cDNA ends (RACE) which led to the isolation of 4.0 kb of genomic sequence immediately upstream of the transcriptional start site by ligation-mediated PCR (LM-PCR). Expression of EGFP protein was detected in rat sympathetic neurons microinjected with a fusion construct of this 4.0 kb genomic DNA to the EGFP gene. Deletion analysis of this 4.0 kb genomic sequence further localized essential *cis* regulatory elements. The BAC clones also contain this 4.0 kb of genomic sequence. Extensive sequence analysis of this 4.0 kb region and the flanking sequences contained on one of the BAC clones revealed consensus binding sites for essential transcription factors.on the genomic sequence

Body

1. Construction of Scn10a-Enhanced Green Fluorescent Protein (EGFP) Fusions in BAC (bacterial artificial chromosome) clones harboring the Scn10a gene

We reasoned that the Scn10 gene promoter was likely very large since other Na^+ channel genes are regulated by promoters that span greater than 50 kb of genomic DNA. We, therefore, chose to screen a mouse genomic BAC library to increase the likelihood of isolating the entire promoter. Three BAC clones obtained from Incyte Genomics (#26462, #26463, and #26464) were identified in a PCR-based screen using oligonucleotide primers specific to exon 1 of the Scn10a gene. Restriction analysis of the clones shows that they are similar and carry extensive overlapping stretches of mouse genomic DNA (Figure 1.1). Extensive sequence analysis has been performed which confirms that the BAC clones contain extensive 5' flanking sequence to the Scn10a gene (Figure 1.2 and appended sequence).

Figure 1.1 Digestion of alkaline lysis miniprep isolated BAC clones with EcoRI

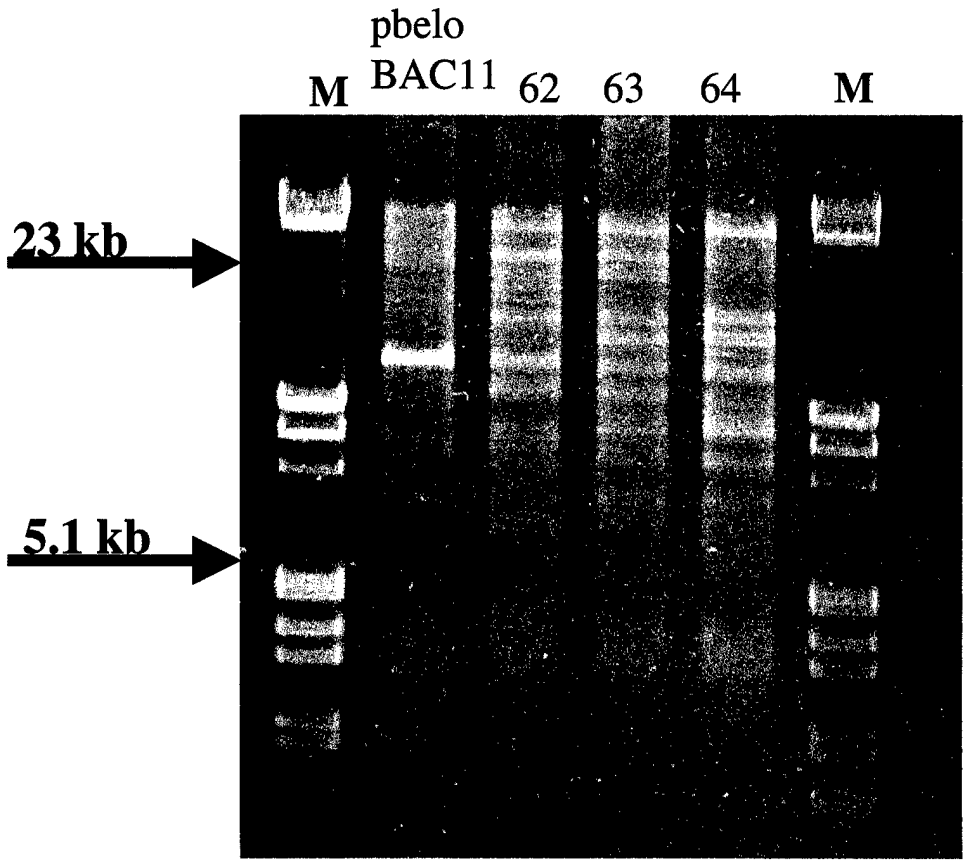
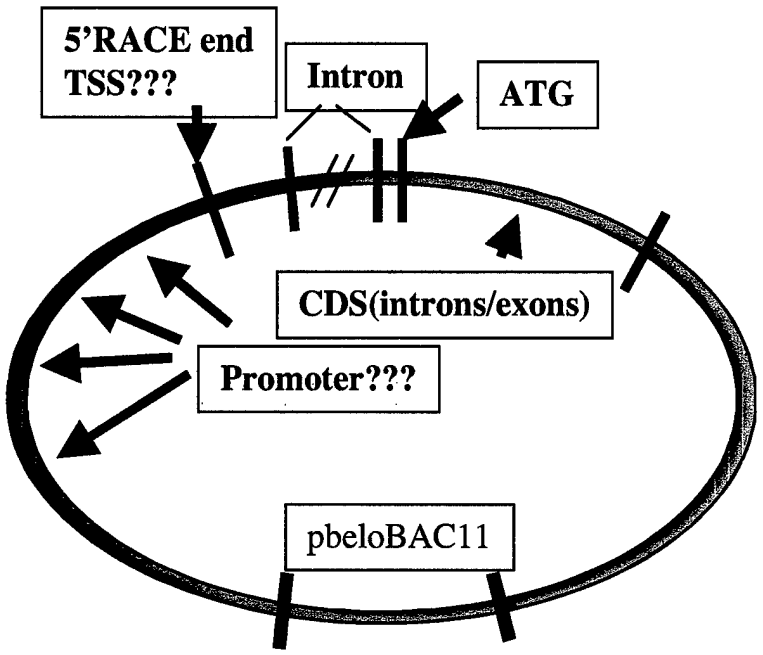
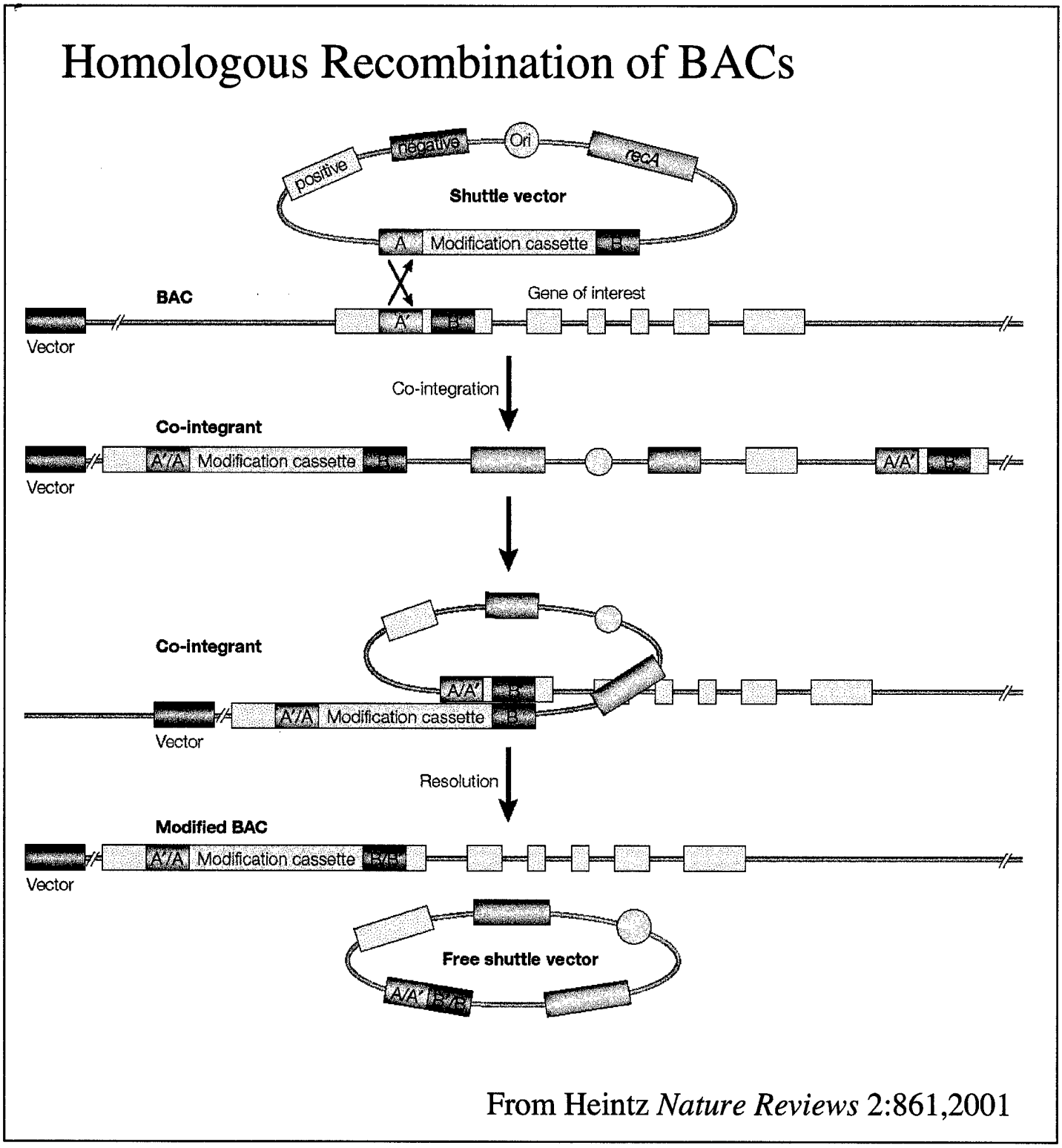


Figure 1.2 Direct BAC sequencing shows the presence of a large portion of the Scn10a gene in all three BAC clones.



Use 5' RACE data to design sequencing primers
Assess extent of genomic insert

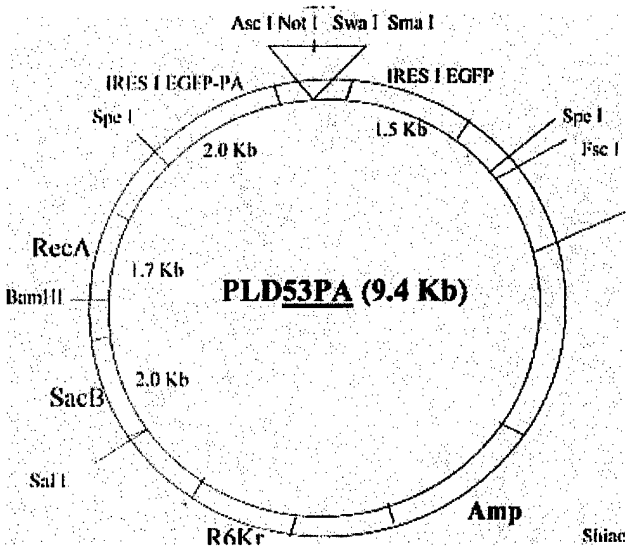
We have modified the BAC clones by integrating the coding sequence for the enhanced green fluorescent protein (EGFP) downstream of the 5'-flanking sequence of the Scn10a gene contained in the BACs. This modification of the BAC clones will also allow us to examine the Scn10a gene promoter in transfected primary neurons. Expression of the EGFP gene under control of the Scn10a gene promoter is expected to provide a very sensitive read-out of this promoter's activity. The technique for inserting EGFP downstream of any DNA sequence contained on a BAC clone was first described by Yang et al. (1997) and the general scheme is shown in Figure 1.3 on the following page.



In this protocol, a specialized shuttle vector was first constructed *in vitro* that carries a 600 bp segment of the 5'-flanking sequence of the Scn10a gene fused to the EGFP gene in the shuttle vector PLD53PA (Figure 1.4).

This 600 bp subfragment was subcloned into the multiple cloning site of the shuttle vector via the NotI and SwaI sites incorporated into the forward and reverse primers. Homologous recombination between the 600 bp Scn10a fragment contained on the shuttle vector with the same region contained on the BACs was performed *in vivo* resulting in co-integrates (Figure 1.6, next page). The co-integrates contain the 5'-flanking sequence of the Scn10a gene on the BAC clone fused to one of the two IRES-EGFP sequences of the shuttle plasmid. The co-integrates were resolved by utilizing the SacB gene on the shuttle plasmid to sucrose-counterselect for BAC recombinants that have undergone a recombination event resulting in a final BAC clone containing a stably-integrated Scn10a promoter-EGFP fusion. Recombination can take place either between the two IRES-EGFP sequences or between the two 600 bp Scn10a regions contained on the co-integrates. Only recombination between the IRES-EGFP sequences will result in a final BAC recombinant containing the proper fusion (Figure 1.5).

Figure 1.4 Creation of a reporter construct by recombinationof a BAC clone



New version: Homologous recombination shuttle vector

Confirmation that the final resolved BAC clones contained properly integrated IRES-EGFP sequence was done by PCR using specific primers that were expected to yield a 3 kb PCR product with the resolved BAC recombinant as template if the fusion was present. If the fusion was not present, the expected PCR product would be 1 kb in size. The results confirmed that the BAC clones contained the correct fusion (Figure 1.6).

Figure 1.5 Construction of Scn10a-IRES-EGFP BAC Recombinants

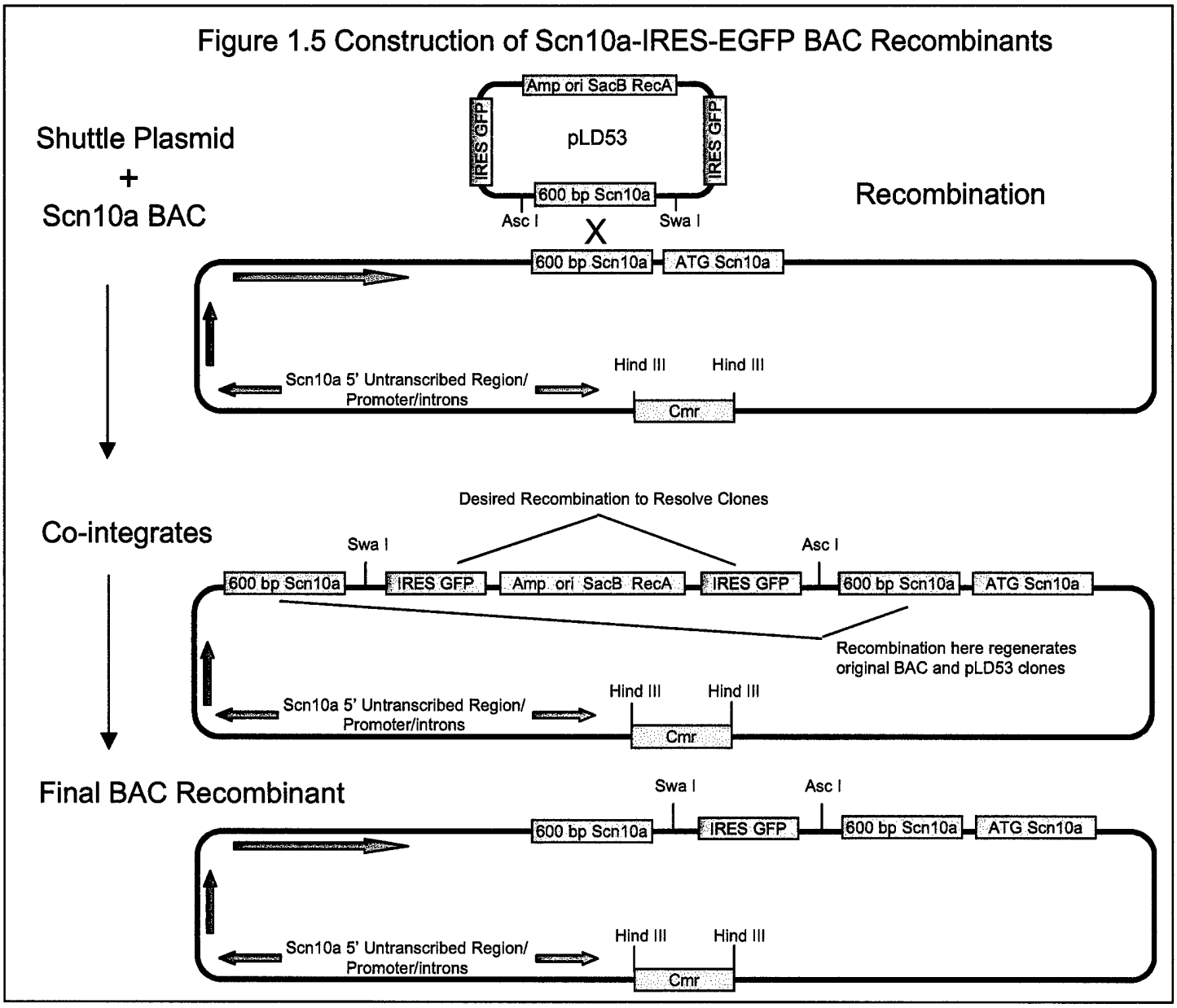
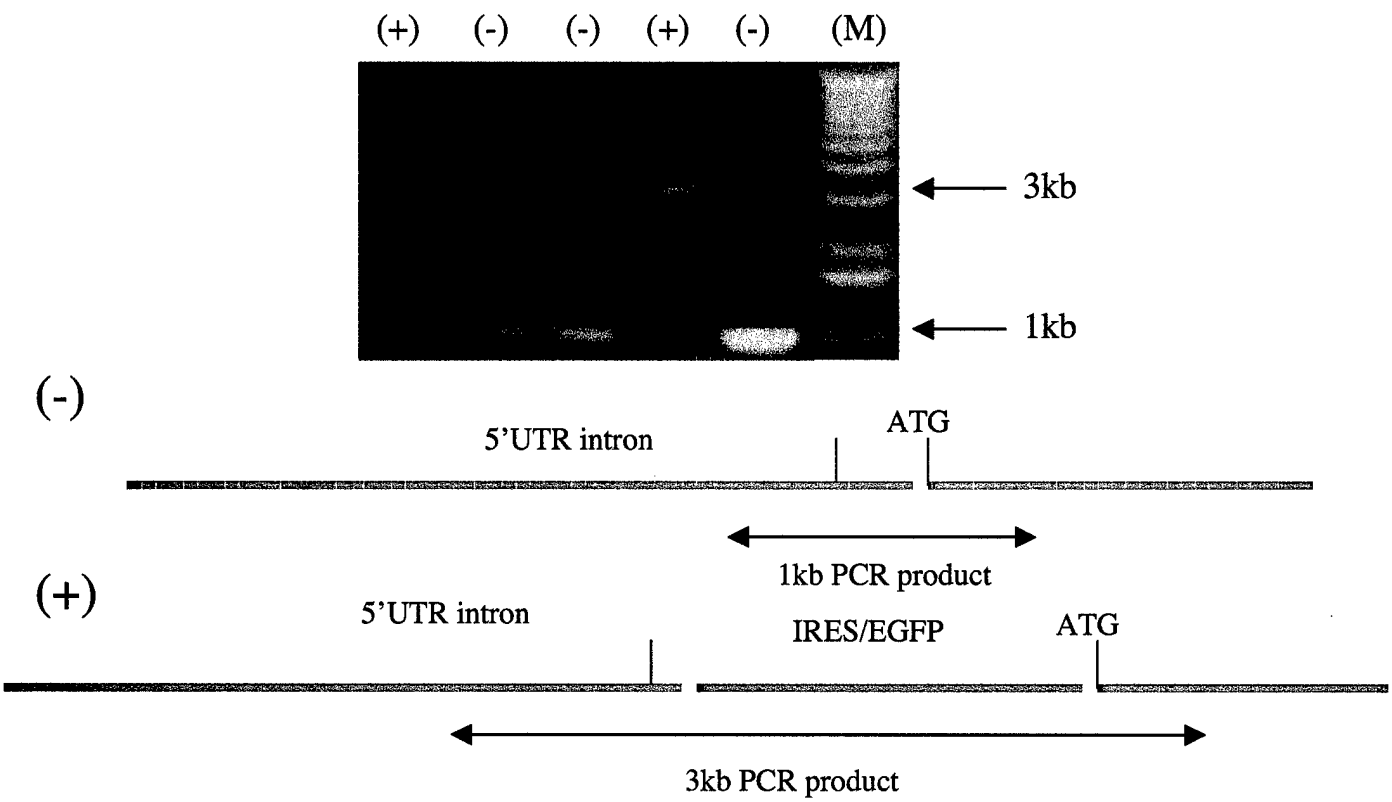


Figure 1.6 Homologous Recombination Results



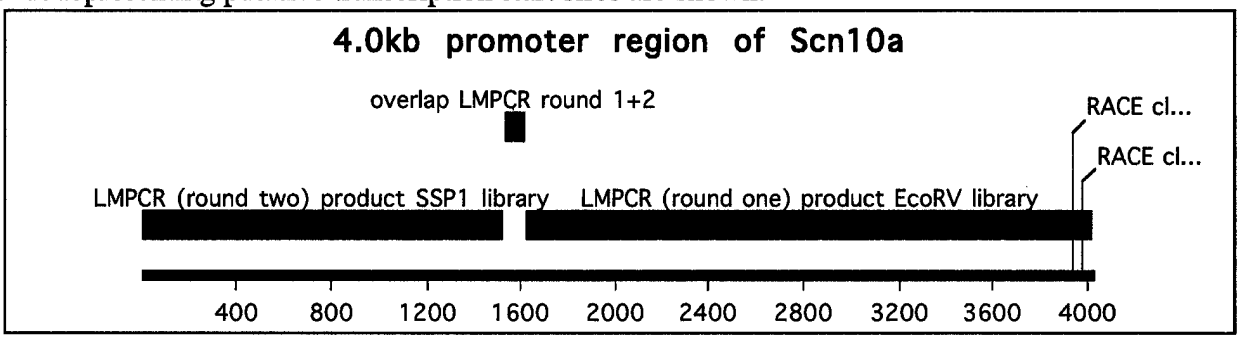
Reference

Yang, X.W., Model, P. and Heintz. Homologous recombination based modifications in *Escherichia coli* and germline transmission in transgenic mice of a bacterial artificial chromosome. *Nature Biotechnology* **15**:859-865, 1997.

2. Expression studies with 4.0kb and 2.5kb LM-PCR products:

Two successive rounds of LM-PCR yielded sequence information corresponding to approximately 4 kb of genomic sequence flanking the 5' end of the Scn10a transcriptional start site. The sequence and structure of the two LM-PCR fragments are shown in figure 2.1 below. Sequences corresponding to each fragment and the region of overlap are indicated. The 2.5kb product of the first round of LM-PCR and the combined 4kb fragment were found in all three BAC clones by the PCR.

Figure 2.1. Schematic of 4.0kb fragment generated by two rounds of LM-PCR. 5' RACE ends representing putative transcription start sires are shown.



Sequence Range: 1 to 4032

>LMPCR_(round_two)_product_SSP1_library

```
          |       10        20        30        40        50        60
ATTCCAGTTGCTGAGTGGAGAGAGCACTGTAGGGTCATGGAAGGACAGTGGGGAGGTCTG

          70        80        90       100       110       120
TTAGAGGTCCTTGAAATTATATAGTGACCTCGCCATGATGGTGGTCTCAGAGATCGAGAG

         130       140       150       160       170       180
ATGATGTAATCAGGAGGACTCTAGGAATTCAAGTTAGAGGCCCCAGAAAGAGGGCTGTGG

         190       200       210       220       230       240
ACGAGGGACGGCTCTTGGATTACCTCTAGATGCTGGGCTTGTGAGTCCAGGCAAGCAGAG

         250       260       270       280       290       300
TGTTCTTGGAGAGGCTTCTCTGGGGGAGGATCATTCTGAGCAGGGCACAGGCACAGAAAT

         310       320       330       340       350       360
CATTAGTCCATCTGTAAACATGTCTGAGATGTTAGTGGAGTGTCCATGAAGGGAAATTCA

         370       380       390       400       410       420
GGCTTCTACCACATTAGTGTATATTTAAATCTGACACCAGGAGAGAGATTTATGATGGAG

         430       440       450       460       470       480
CTGACAGACTCCGGTGCCATGTCAGGTAGGTGACTGAAGCCCTGGGGAAGGAGAGGCGTA

         490       500       510       520       530       540
GGATGGAATCTTAAAACGATTCTCCAACTACTTCCAGGTGGCAGAGGAGGAGGCAGCCCA

         550       560       570       580       590       600
GGCCAGAGAAGCTCCTCTGAAAACAGAAGTCAAGAGGGTGGAGTGTGGTGCAAGGACCAT

         610       620       630       640       650       660
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GCAGCTAATCCTGCGGAGCCCCTAGGATGAGAGCGCCAGAGAGGAGACACATGACACAGG

       670        680        690        700        710        720
GAGACCAGTAGAAACCTGTTAAGATTCCGGGTGTCTCAGGACTGCCTCTGGATGCACACT

       730        740        750        760        770        780
TCTTCCTTCTTGGGAAGTTACTTTTCTGTCACTGTGATGAAATACCTTAACCAAGGTGAC

       790        800        810        820        830        840
TCAAAGAAGAGAGGGTTTATCTGGGCTCACGGGTCCAGAGGTAGAGGAACACATGGAGAT

       850        860        870        880        890        900
CGTGGTGGGGAACCAGTGTAGCAGGCAAGCATGGTGGCTGGGGCTGAGGCTGAGAGCTTA

       910        920        930        940        950        960
TATCTTTGTCTGTATACAGAAAGCAGAGAGAGCCAACTGGGAATGACTTGTGGCTTTTGG

       970        980        990       1000       1010       1020
AACCTGAAACCTGTCTTCGGTGACATGCTCCCTCCAGCGAAGGCAATGCCTCCTCAAACT

      1030       1040       1050       1060       1070       1080
CCCCAAAGGGCACCACAAACTAGGAACCAAGCACTCAGATGCCCGAGACTATGAGCGACA

      1090       1100       1110       1120       1130       1140
TCTCCTTCAGATCACCACACTTGGGTACACCCATTCTCCTGTCTCATCCAGTTTGCTCTT

      1150       1160       1170       1180       1190       1200
CTGGAAGGGTGGTGAGAGGGATGACAGCTAGTGACAAGTTGGAGAGACTTTAGAATAATT

      1210       1220       1230       1240       1250       1260
GCCATCACACAAAGCCTACCCTATCAGTTAGTGGCTGGCACGCTATCCCAACAGCTTGAG

      1270       1280       1290       1300       1310       1320
TCTGAACTTGCCAGAAATGGCCTCCGTCTCACCTCTCCCAGGCTCCCCAGCACCCACAGG

      1330       1340       1350       1360       1370       1380
TGCCCTCCCCCAAGACCTGACATCATCGGAGCACTGAAGAGATGCCTCCTCTGCCCCTTT

      1390       1400       1410       1420       1430       1440
CTCCCCTGGTCTGATTGCTACCAGGCAGCTGATCCACATGCCCTGCTCCAAGTTTGACCC

      1450       1460       1470       1480       1490       1500
CAGTCAGCAGGCTTCTCTGAAGAAGAGGGTCTGTTAGCATGACACACAGCATTTCCCATG

                          >overlap_LMPCR_round_1+2
                                      |
      1510       1520       1530     | 1540       1550       1560
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      1570       1580       1590       1600       1610       1620
TGTACCCTTACACACCTGAACGTGCATATACACACGTACACTTGTACACACACTAAAATA

>LMPCR_(round_one)_product_EcoRV_library
      |
      1630       1640       1650       1660       1670       1680
ACTGTGGTGCCAGGAGTGATCAGGGTACTGAGGTGACAGTCATTCACAAGGCTTGCCTTT

      1690       1700       1710       1720       1730       1740
CTCCACGTGACACACTTTGACCTTACCACCTGTTTCCACCTTGCTCTGGCATTTTAAAAA

      1750       1760       1770       1780       1790       1800
CATGACATTTTTAGTAAATTCTTTGAATTTTTTTGAGACAGGGTCTTACACTACAGCTCA

      1810       1820       1830       1840       1850       1860
AGCTGATCTTGGGTTTGCAGCAATCCTCTTCCCTCGACCACCCCCCCAACTAGGATGTGA
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      1870       1880       1890       1900       1910       1920
GCTGCCATGCCCAGTTTGACTCTTTCCCAGATGTTTGTTTTTATTCTGTATGTATGAGTG

      1930       1940       1950       1960       1970       1980
TTCTCACTGTATGTATGTCTGTATATGCACCATGTAGAGCCCAAAAGCAGTTGCTGAATG

      1990       2000       2010       2020       2030       2040
CTCCGGAGCTTGGAGCTGTGGGTGGCTGTGAGCTGCCACGTGGGGTGCCAAGAATAGAAC

      2050       2060       2070       2080       2090       2100
ACAGGTCCTCTGCAAGAGCATCGAATGCTCTTAACCACTGAGCTATCCCTCCAGGTATTA

      2110       2120       2130       2140       2150       2160
AAACATAAAATAAGCTTGACTTTTTACTCTGACATAATTTAAAATTCACAAAAAGTTTTA

      2170       2180       2190       2200       2210       2220
AAAAAACGTAACAGCTTCCGTATACTCCCAATCCCATTTCCCCAGTTAGGATATTCTTTA

      2230       2240       2250       2260       2270       2280
ACCATAGTACATTGTCAAATGAGAAAACTAACATTCATACAACACGACTGATTTTGGTGA

      2290       2300       2310       2320       2330       2340
AAATCCTATTTGGAGTACACTACCTTTGACTGTGATTTCTTTTTGCACTATGGCCCAGGC

      2350       2360       2370       2380       2390       2400
TAGCCTGCAACTATTTATCATTATTTATCCCAGGGTGACTTGAACTTAGGGCAATTCTTC

      2410       2420       2430       2440       2450       2460
TACCTCAGCTCTGCCCCCACTCCCACCTCAACTCCAGTCCTGGGGTTACAGGAGCAAGCC

      2470       2480       2490       2500       2510       2520
ATCAAGTTCTATAACATTTAATACACAAGGACACTGGTTAAACTCAGAAGGACCTAAATT

      2530       2540       2550       2560       2570       2580
AGCATAAGACTATGGGGACCAGAGAAGTGAGAAGTGAGGACAGGGGAGGAGGGCAGGGGA

      2590       2600       2610       2620       2630       2640
GGGAAGATGGGAGGAATGATGGGAAGAGAATGAGAGAAGGCAGGGAGGGAGAGGAGAAGG

      2650       2660       2670       2680       2690       2700
CCAGTGAAGGGAGAATGGGAAGGGAGGGAGTTGAGAGAAGGCAGGATCGGGAGCCATAGA

      2710       2720       2730       2740       2750       2760
ATGTCTGTAGGAAACCATCAAAGGCATTTAATTTAATAAAGCAACCAGGATTGTACATAA

      2770       2780       2790       2800       2810       2820
TTCTACTGTGTACATACAAACACTCAAGTTTTGGGAGCAAGAATTTTAGCTTTCCTTCCC

      2830       2840       2850       2860       2870       2880
CTGCCCCTTTATGATTCAGTCTCTGCTAGAAAAAGTGGAGCCTTGCAGGGTGTGGTGGTG

      2890       2900       2910       2920       2930       2940
CACGCCTTTAATTCCAGCGTTTGGGAGGCAGAGGCAGGTGGATTTCTGTGAGTTCCCGGT

      2950       2960       2970       2980       2990       3000
CAACCAAATCTCCATAGTATGATCCTTCGTGGAATACCGGCCAACCAAACAAGCAAACAA

      3010       3020       3030       3040       3050       3060
ACAAACAAAAATCCCAAACAAACCCCACCCCCACCCAAATAGAGGGGATTATTGACTCAA

      3070       3080       3090       3100       3110       3120
AGAAGCCAATAATTTTGAGTTGGTTTGGGACATTTGAGTAAATGAAGCTGTAATGGGCAA

      3130       3140       3150       3160       3170       3180
```

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GCATGGGCCCTCGACAGTTTCCTGCAGTATAGCATGGCTTCCTAAGGCTGCGTGGGTTGC

      3190       3200       3210       3220       3230       3240
ACTGTTACGGAGGGCTCAGCTCAGACAGGGGGTTCCCTGTGCAACCTCCTTTCTTATGGT

      3250       3260       3270       3280       3290       3300
CCCACAACCCCACAGATAGGGCACTTTCCCTACCCAGCTCCCTTCTCGGCTCTCACTGGG

      3310       3320       3330       3340       3350       3360
GTCGGAGAACATTTTTGTTTCAGCATTTCATCTGAAGCCACGGTTTCACATCATCAAGTC

      3370       3380       3390       3400       3410       3420
TGCAAAAAACCGTTCACAAACCACACCAGAACTTCTCGGTAAAGAACTCCTAAGACCAAA

      3430       3440       3450       3460       3470       3480
GAGGGAGACTGGGTAGATTGTTTTTAATTTGTTTCTTTTTGTCAAAGGGGGACAAAACAC

      3490       3500       3510       3520       3530       3540
GCTTTGGTGAGTGCGAGTGTTTATTCTGGGACACAAACCCAGAGTCTGGAAGGGAGCATT

      3550       3560       3570       3580       3590       3600
CAACGGGTGCTGCTCTGCCACGCAGGGGCAGCGGTGGGACTCAGCCCATCCTGCTAAGGA

      3610       3620       3630       3640       3650       3660
CGGGCAGCCTGAGCCAGGCTTGGGAGTCTGTCATGGCTGCCAGACGAATCATTATCTAAT

      3670       3680       3690       3700       3710       3720
TGCAGCCTTTTCTCTTCCTTAGGTTTCAGCAGGTCCCGAGAGAGCATTTAAAATCGCATT

      3730       3740       3750       3760       3770       3780
TACTACTTTACCATCTAATCACACATAAGCCTCTCCCTATACCCTCCACCCTCCTTCCAT

      3790       3800       3810       3820       3830       3840
TCAGAGTGTACTTTCTGGAGCCCATCCAGCAAGCAGGGTGGAACTCATGACGGGAAATGG

      3850       3860       3870       3880       3890       3900
GAACGGCGCCCACGAAGGCGTGATTCCTTGTAGATCCTTGAGTGATGGACGGGTGAGGTT

                                >RACE_clone_B_end
                                        |
      3910       3920       3930       3940       3950       3960
TCCGTCAGGCAAGCCCAGCCACCTTCGTGGAGGAGCCCCGGACAAGTGTAAGTTTCGCAG

          >RACE_clone_I_end
                  |
      3970       3980       3990       4000       4010       4020
AGCTGGGGTCTCCAGCTTACTTCTGCTAATGCTACCCCAGGCCTTTAGACGGAGAACAGA

      4030
TGGCAGATGGAG
```

The PCR products corresponding to these regions were clone into the pEGFP-1 vector from Clontech (see figure 2.2 below). This vector contains the coding region of the enhanced green fluorescent protein down stream from a multiple cloning site. The vector allows the analysis of sequences for promoter activity by their ability to drive expression of the EGFP protein product.

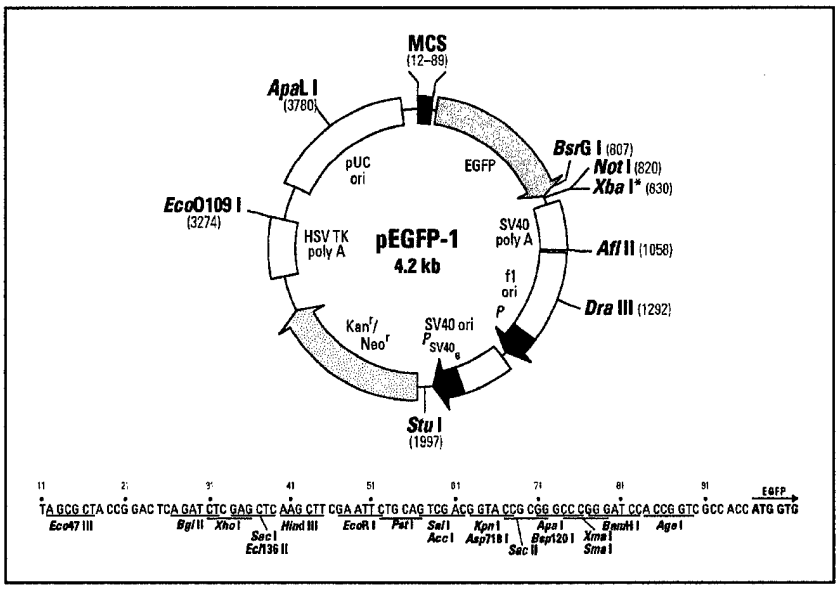
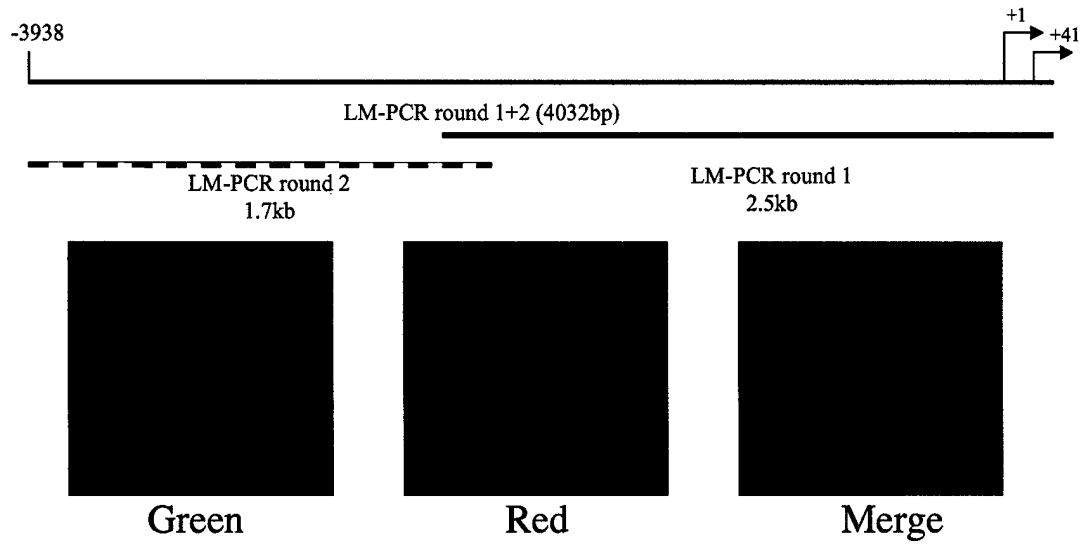


Figure 2.2

The resulting expression constructs were microinjected into the nuclei of neurons from primary cultures of dorsal root ganglia. A nuclear targeted dsRED construct was coinjected as a positive control. The presence of visibly red nuclei indicated a successful injection yet would not interfere with the detection of the EGFP signal which was predominantly cytoplasmic. The neurons were dissociated with collagenase and trypsin and cultured for two days in the presence of nerve growth factor and glial derived neurotrophic factor. The construct containing the 2.5kb fragment failed to produce visible EGFP production as shown in figure 2.3. The 4.0kb fragment successfully drove expression in a majority of but not all injected cells.

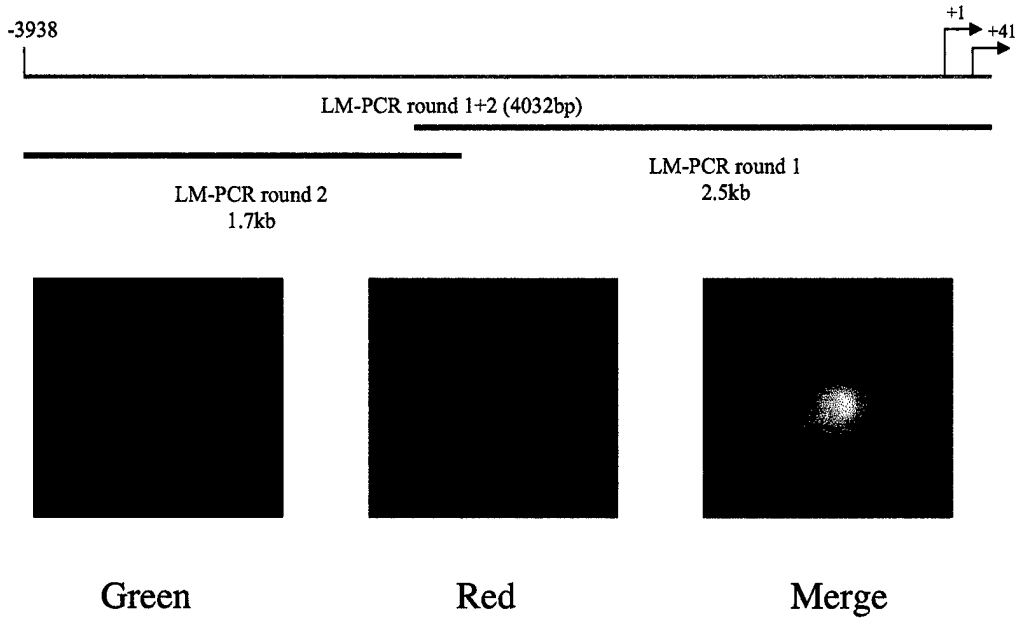
2.5kb fragment does not drive EGFP expression in mouse DRG neurons
Figure 2.3



Mouse DRG in culture 2 days (NGF+GDNF)
0.1 μg/μl 4kb + and 0.005 μg/μl dsRed2-nuc

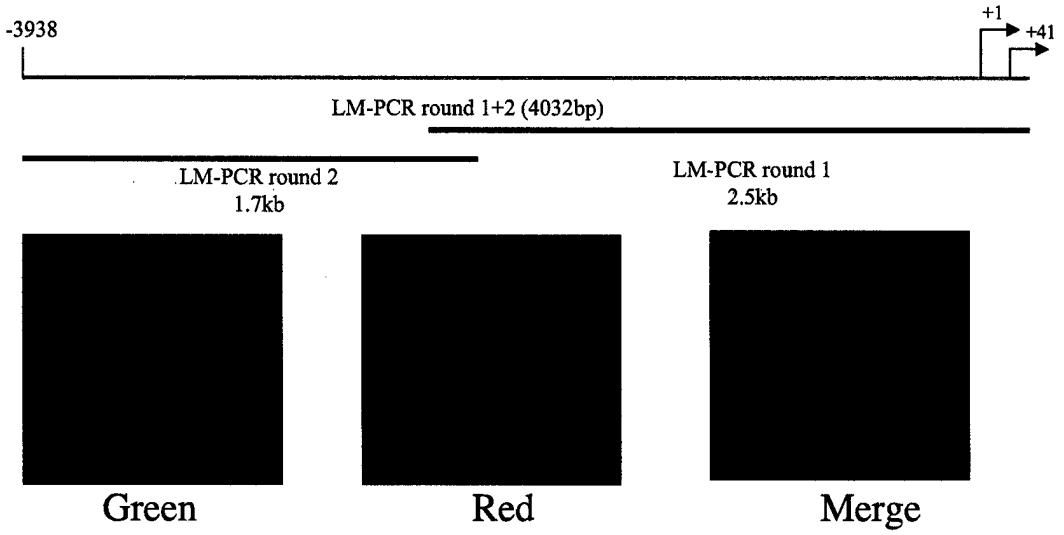
Figure 2.4 shows cells expressing EGFP following injection. Figure 2.5 shows an example of a successful injection, as viewed by dsRED production, with no EGFP production. The expression of Scn10a in only a subset of small diameter neurons in DRGs may account for the failure of this construct to express in all injected cells.

4kb construct drives expression of EGFP in DRGs
Figure 2.4



Mouse DRG in culture 2 days (NGF+GDNF)
0.1 $\mu g/\mu l$ 4kb + and 0.005 $\mu g/\mu l$ dsRed2-nuc

Negative DRG neuron with 4kb construct (Not all DRG neurons express $Na_V1.8$)
Figure 2.5

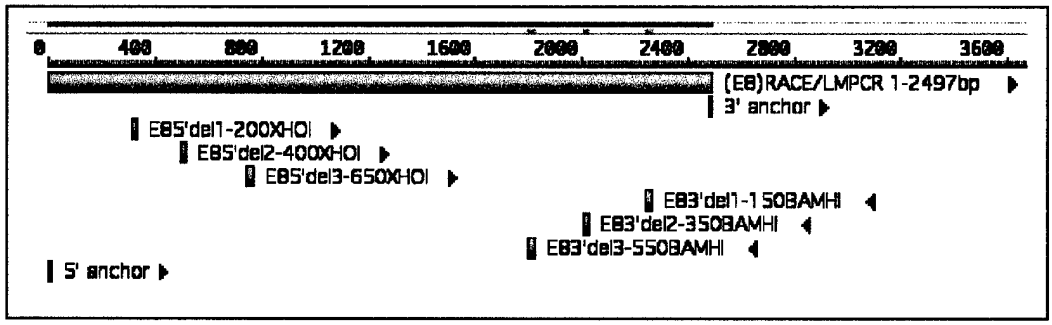


Mouse DRG in culture 2 days (NGF+GDNF)
0.1 $\mu g/\mu l$ 4kb + and 0.005 $\mu g/\mu l$ dsRed2-nuc

Injection of all constructs into sympathetic neurons isolated from superior cervical ganglia failed to produce visually detectable levels of EGFP. The positive control nuclear directed dsRED construct produced red nuclei in all cases. Scn10a is not expressed in these neurons and therefore this experiment serves as a negative control. Additionally, reporter constructs were electroporated into the human neuroblastoma cell line SK-N-SH. No green cells were visible even after three days in culture. Separate electroporations with SK-N-SH cells and a CMV driven GFP construct produced green cells.

Deletion analyses of the 2.5kb and 4.0kb fragment have begun. Six deletion constructs (three from each end) have been cloned and await analysis. The primer scheme is shown in figure 2.6. Constructs were generated by PCR with deletion and anchored primers from each end. Fragments were cloned via restriction endonuclease sites incorporated into the primers. Three deletion fragments of the 5' end of the 4.0kb fragment have also been generated by the PCR and cloned into pEGFP-1. These constructs designated S, M, and L were generated by designing primers to various positions of the parent 4.0kb fragment as shown in figure 2.6. The PCR was performed with an anchored primer at the 3' end of the 4.0kb sequence (Not Shown). Preliminary injection experiments performed as described above with the M or medium sized construct produced visibly green cells. The injection of the L (large) and S (small) fragments have not been performed.

Figure 2.6: A. 5' and 3' deletion strategy and results for 2.5kb LM-PCR round one fragment:



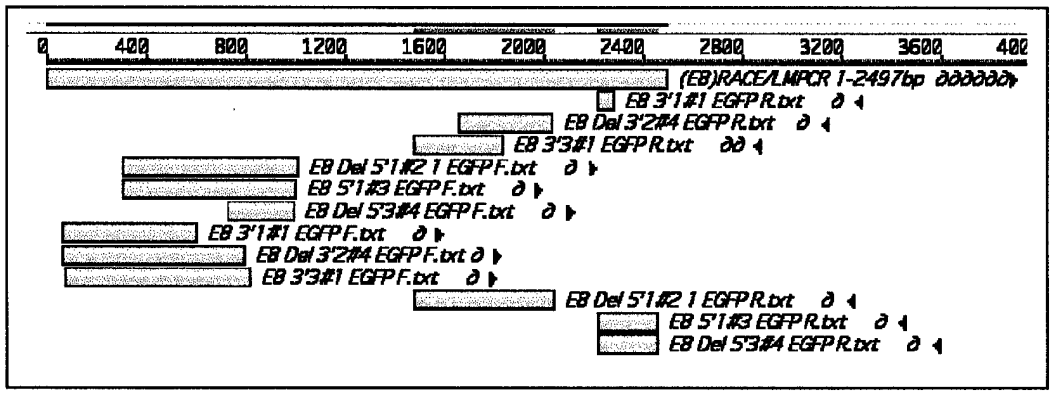
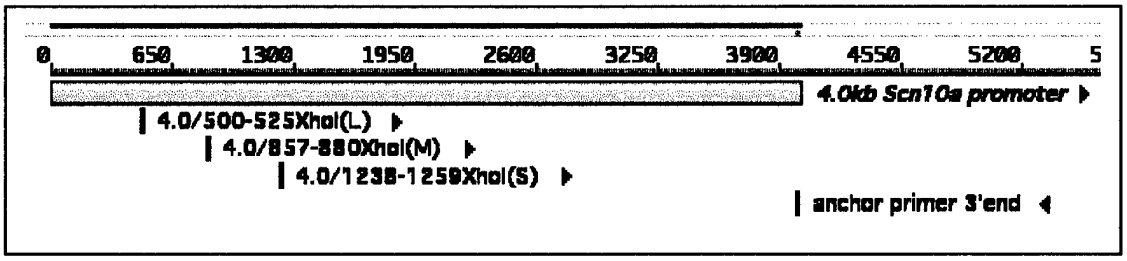
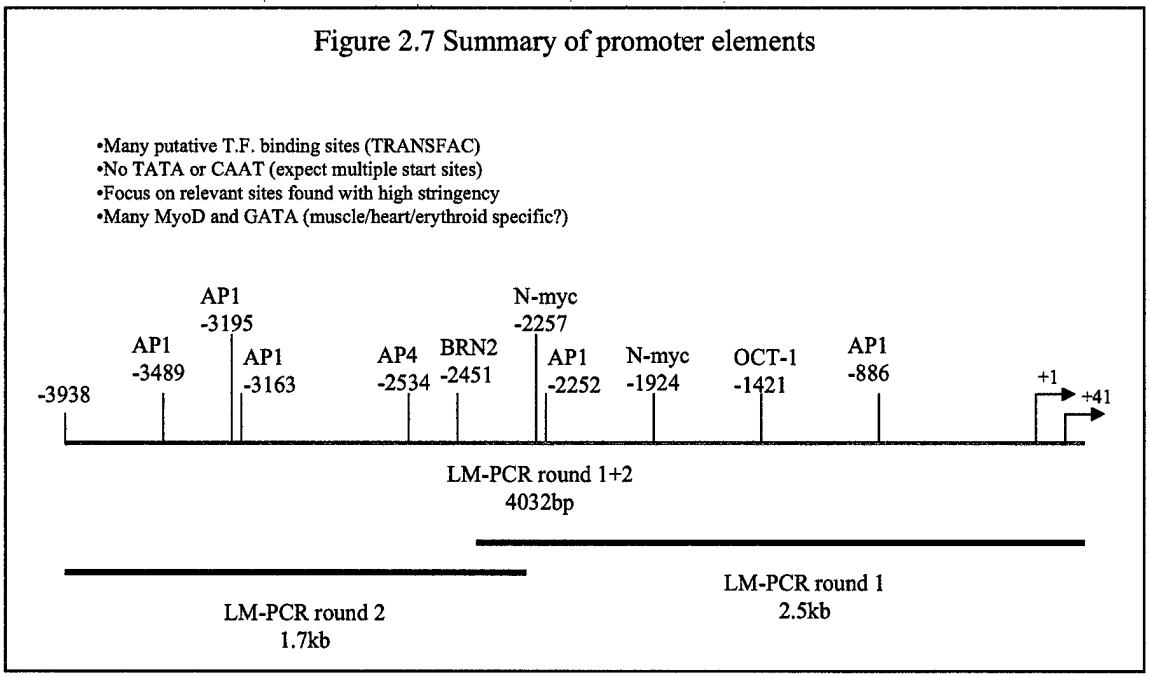


Figure 2.6B. 5' Deletion strategy for 4.0kb deletion constructs.



The results of this portion of experiments indicate that important factors involved in the expression of the Scn10a gene may lie in the region from the M construct to the point of origin for the 2.5kb fragment containing construct generated from the first round of LM-PCR. Sequence analysis of the entire 4.0kb fragment is shown in figure 2.7.

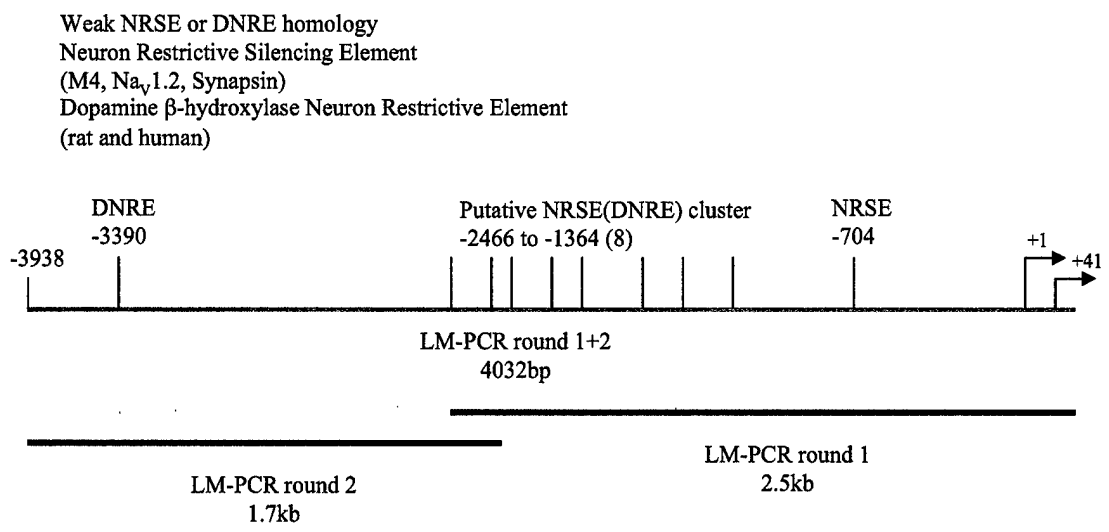
Figure 2.7 Summary of promoter elements



Our sequence was compared to the TRANSFAC database. This database searches input sequences for putative transcription factor binding sites. The results were extensive and therefore only a partial list of potential sites is shown in figure 2.7. This list includes several factors involved in expression of other neuron specific genes such as AP1, AP4, BRN2 and Oct1 (both POU factors) and N-myc.

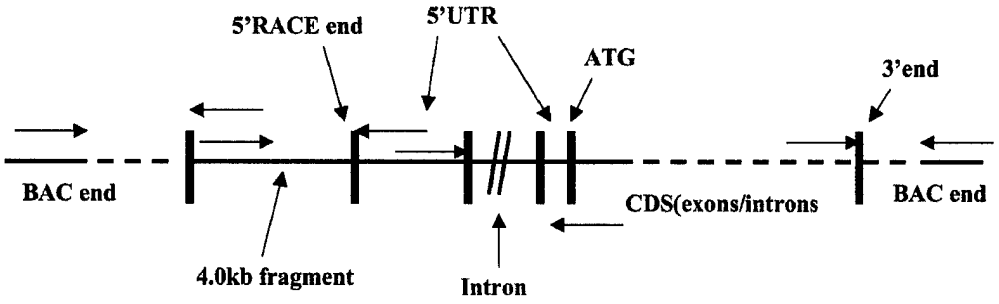
Figure 2.8 shows the presence of several sites with weak homology to a variety of silencer elements such as NRSE (Neuron restrictive silencer element) and DNRE (Dopamine beta-hydroxylase neuron restrictive element). These elements have been shown to be potent silencers capable of completely abolishing expression of genes carrying these sequences. The expression of genes associated with such sequences can occur only if the silencer protein is not expressed in a particular cell type.

Figure 2.8 Summary of promoter elements: silencers



Bacterial artificial chromosome direct sequencing has continued with a focus on clone #26463. This clone has been partially sequenced following the strategy outlined in Figure Z below. The illustration includes the known structure of the genomic fragment and shows, via horizontal arrows, the direction of successive sequencing reactions. The goal has been to span unknown region within the BAC constructs. The procedure has been successful in verifying the presence and sequence of the entire 4.0kb LM-PCR derived fragment. Sequence data from reactions with primers directed out from the 5' and 3' most coding exons have confirmed the presence of all coding exons for the Scn10a gene on the BAC clone #26463. The regions between the BAC ends and the start of the known Scn10a sequence have not been fully determined as these regions appear to be larger than the amount of sequence obtained thus far.

Figure Z: Illustration of genomic structure of Scn10a BAC clones. Horizontal arrow represent point of origin for successive rounds of sequencing



Additional information on the structure of the BAC clones is presented as Appendix material.

Key Research Accomplishments

1. RACE analysis of the 5' end of $Na_V1.8$/Scn10a murine DRG cDNA confirmed the presence of one intron splice site in the ~200 bp 5'UTR of the Scn10a transcript.

2. Three BAC clones were isolated from a genomic library. All three contain at least the entire cds containing portion of the Scn10a gene plus the 5'UTR and an additional >4.0kb of upstream sequence.

3. Homologous recombination (conversion) of all three BAC clones to EGFP containing reporter constructs has been completed.

4. LM-PCR upstream from the putative transcription start site (5'RACE end) produced 4.0kb of sequence that may contain some/all of the elements required for the exquisitely controlled expression of $Na_V1.8$, the Scn10a gene product.

5. The putative promoter appears to contain several known consensus binding sequences for transcription factors. In addition, putative neuronal-specific silencer elements may be present.

6. Deletion analysis of the 4 kb promoter fragment indicates activating elements between $\approx$ -3200 and –2500 bp.

Reportable Outcomes

1. Ikeda, S.R., King, M.M., Aronstam, R.S. and Puhl, H.L. Cloning and expression of cDNA encoding a tetrodotoxin-resistant (TTX-R) sodium channel (Scn10a) from mouse dorsal root ganglion neurons. *Experimental Biology Meeting*, 2001.

2. Puhl, H. L., King, M.M. and Ikeda, S.R. Identification of the promoter region of the mouse scn10a gene encoding the tetrodotoxin-insensitive voltage gated sodium Channel $Na_V1.8$, *Soc. Neurosci Abstr.* 2002, in press.

3. Puhl, H. L., King, M.M., Aronstam, R.S. and Ikeda, S.R. Cloning and functional characterization of mouse cDNA encoding a tetrodotoxin-resistant (TTX-R) sodium channel (Scn10a). *Soc. Nerosci. Abstr.* 2001.

Conclusions

Four major objectives were achived during the previous funding period that extend our understanding of the regulation of the Scn10a gene.

1. The three BAC clones containing the entire coding sequence of the Scn10a gene were converted into EGFP reporter constructs by homologous recombination of the EGFP coding sequence downstream of the 5-flanking sequences contained on each BAC. These constructs will be analyzed by intranuclear injection of rat sympathetic neurons to determine whether essential *cis* regulatory elements for Scn10a are present.

2. The putative transcriptional start site for the Scn10a gene was determined by RACE analysis beginning at the ATG start codon. The presence of an intron splice site ~200 bp upstream from the ATG codon was found which suggested that the promoter region for Scn10a was interrupted by at least one intron of undetermined size. The genomic sequence immediately upstream of the RACE product was identified by LM-PCR which produced 4.0 kb of sequence that may contain some/all of the *cis* elements required for expression of Scn10a.

3. The 4.0 kb putative Scn10a promoter was fused to the EGFP reporter gene and injected into the nuclei of rat sympathetic neurons. The detection of EGFP expression in these neurons indicates that some/all of the *cis* elements comprising the Scn10a promoter are present within the 4.0 kb sequence. An EGFP reporter containing a deletion of the 5'-end of the 4.0 kb sequence (-3200 to -2500) did not express EGFP in rat neurons. This shows that essential activating *cis* elements are present within the -3200 to -2500 region. Sequence analysis also indicates the presence of consensus binding sites for transcription factors. The BAC-EGFP reporter constructs described above contain the 4.0 kb genomic sequence.

4. Extensive sequence analysis of one the BAC clones was done. This will complement the efforts described above to localize essential *cis* elements for the Scn10a gene.

These latest findings will allow us to extend our analysis of the regulation of the Scn10a gene and efficiently focus our efforts during the final year of this project.

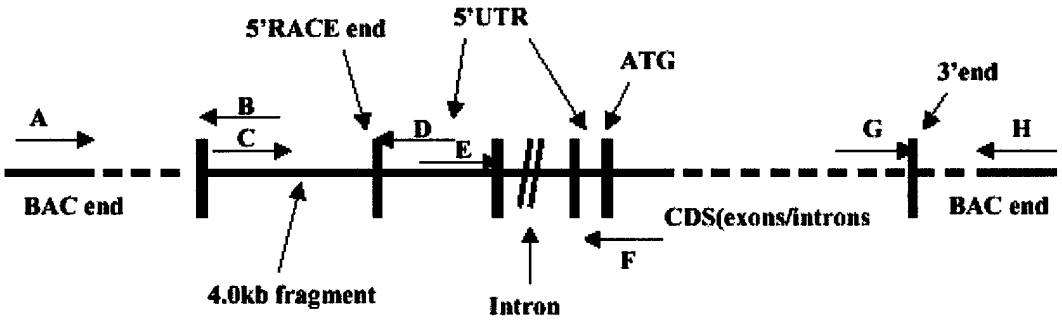
References

Akopian, A.N, Sivilotti, L. and Wood, J.N. A tetrodotoxin-resistant voltage-gated sodium channel expressed in sensory neurons. *Nature* **379**:257–262, 1996.

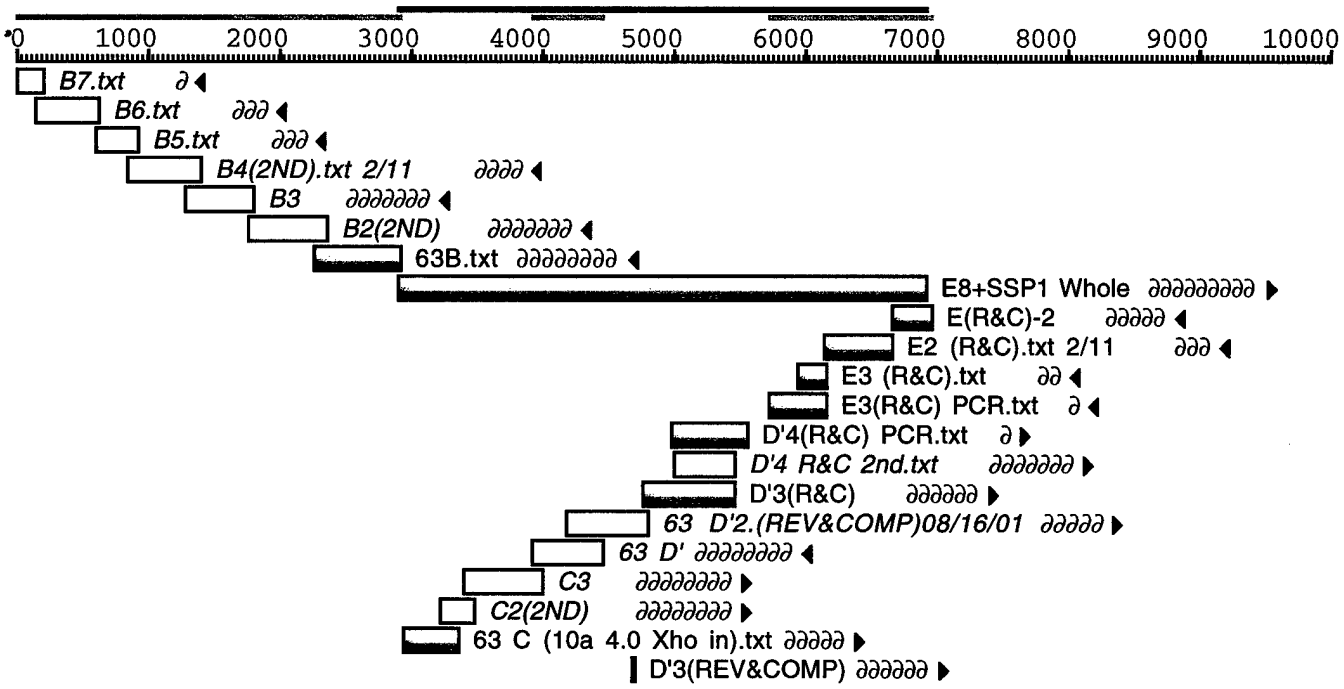
Heintz, N. BAC to the future: the use of BAC transgenic mice for neuroscience research. *Nat Rev Neurosci.* 2:861-870, 2001.

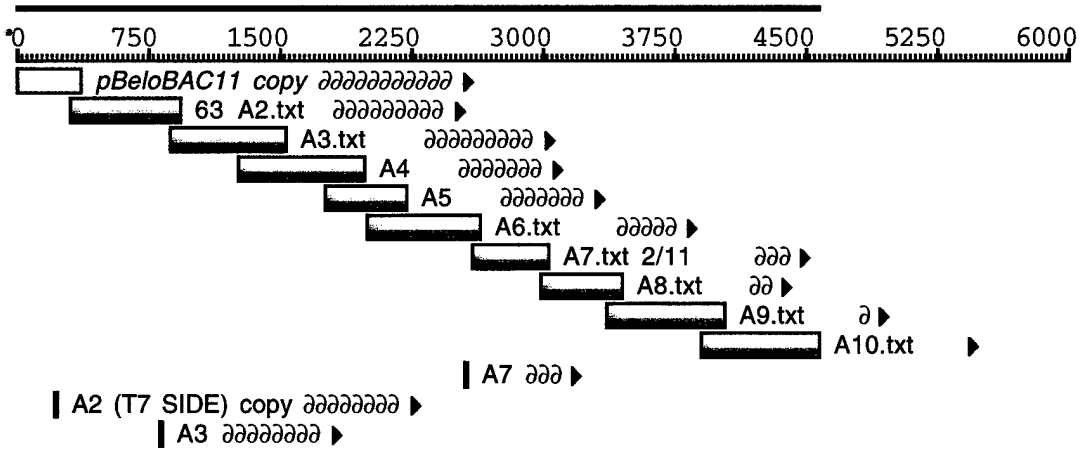
Yang, X.W., Model, P. and Heintz. Homologous recombination based modifications in *Escherichia coli* and germline transmission in transgenic mice of a bacterial artificial chromosome. *Nature Biotechnology* **15**:859-865, 1997.

Appendix A: Sequencing analysis of Scn10a BAC clone.



The alignment contigs are in relation to their point of origin as indicated by the horizontal arrows in the above schematic. The alignment data was generated by successive rounds of DNA sequencing.


0
1000
2000
3000
4000
5000
6000
7000
8000
9000
10000
B7.txt
B6.txt
B5.txt
B4(2ND).txt 2/11
B3
B2(2ND)
63B.txt
E8+SSP1 Whole
E(R&C)-2
E2 (R&C).txt 2/11
E3 (R&C).txt
E3(R&C) PCR.txt
D'4(R&C) PCR.txt
D'4 R&C 2nd.txt
D'3(R&C)
63 D'2.(REV&COMP)08/16/01
63 D'
C3
C2(2ND)
63 C (10a 4.0 Xho in).txt
D'3(REV&COMP)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1500
2250
3000
3750
4500
5250
6000
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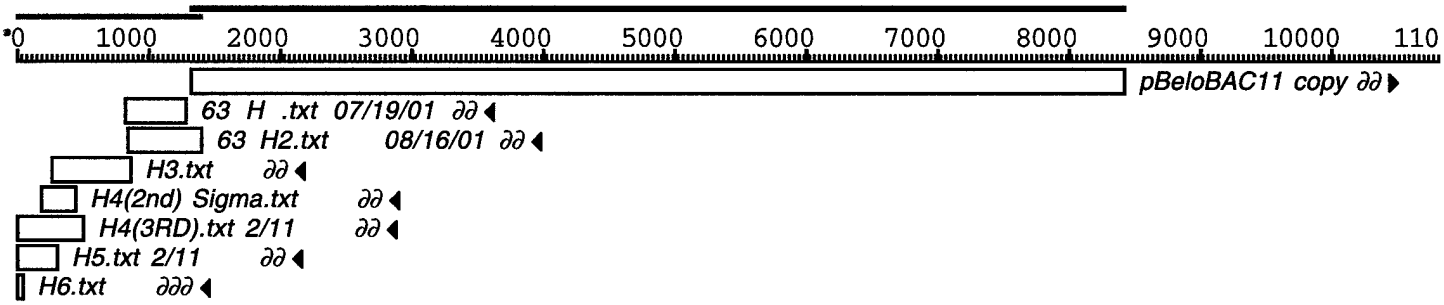
A3.txt
A4
A5
A6.txt
A7.txt 2/11
A8.txt
A9.txt
A10.txt
A7
A2 (T7 SIDE) copy
A3


0
1000
2000
3000
4000
5000
6000
7000
8000
9000
10000
110
pBeloBAC11 copy ∂∂
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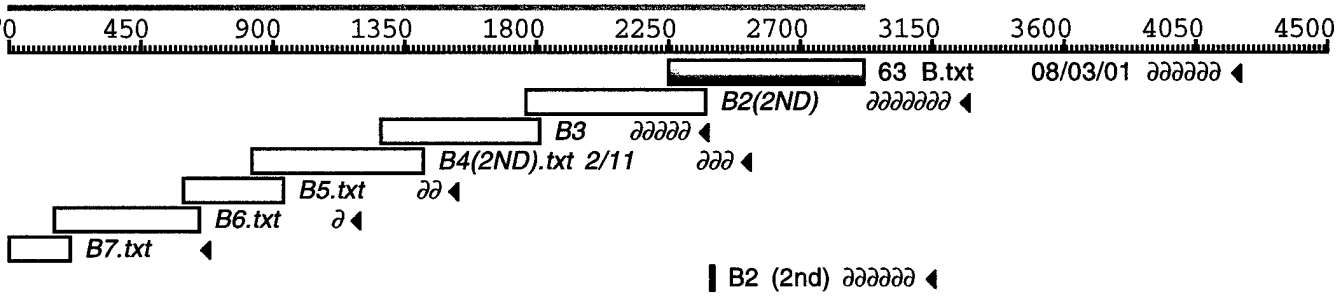
63 H2.txt 08/16/01 ∂∂
H3.txt ∂∂
H4(2nd) Sigma.txt ∂∂
H4(3RD).txt 2/11 ∂∂
H5.txt 2/11 ∂∂
H6.txt ∂∂∂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450
900
1350
1800
2250
2700
3150
3600
4050
4500
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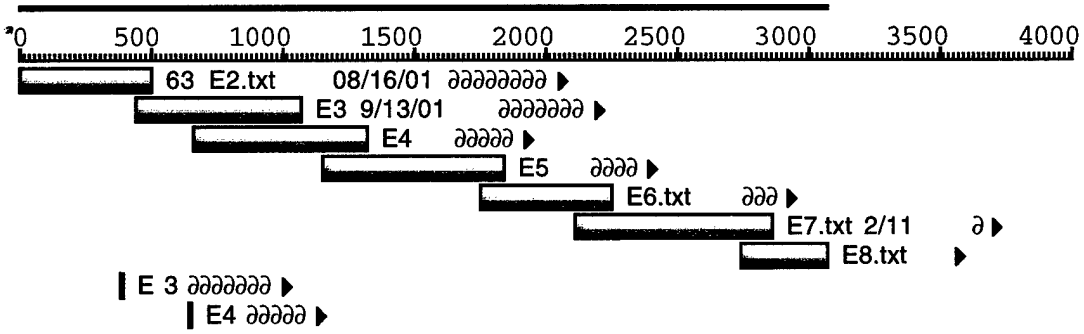
B2(2ND) ∂∂∂∂∂∂∂
B3 ∂∂∂∂∂
B4(2ND).txt 2/11 ∂∂∂
B5.txt ∂∂
B6.txt ∂
B7.txt
B2 (2nd) ∂∂∂∂∂∂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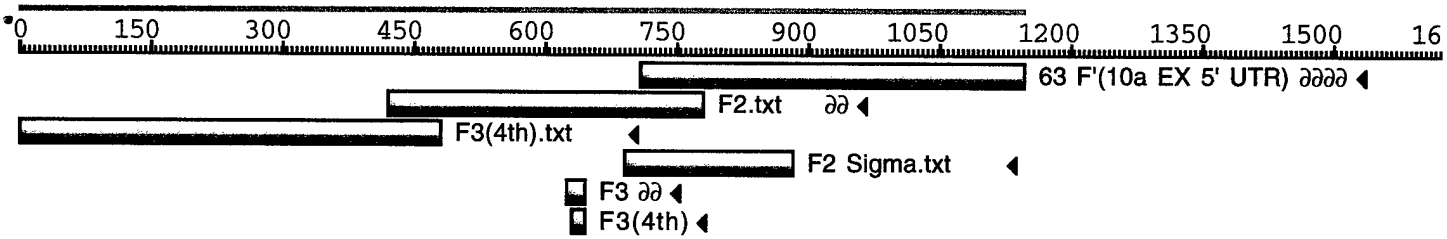
500
1000
1500
2000
2500
3000
3500
4000
63 E2.txt 08/16/01 ∂∂∂∂∂∂∂∂
E3 9/13/01 ∂∂∂∂∂∂∂
E4 ∂∂∂∂∂
E5 ∂∂∂∂
E6.txt ∂∂∂
E7.txt 2/11 ∂
E8.txt
E 3 ∂∂∂∂∂∂∂
E4 ∂∂∂∂∂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300
450
600
750
900
1050
1200
1350
1500
16
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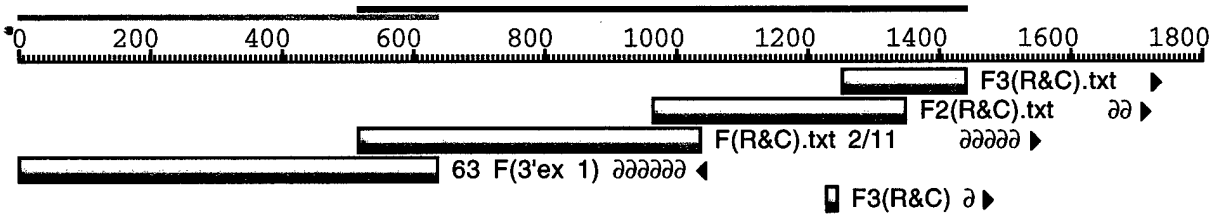
F3(4th).txt
F2 Sigma.txt
F3 ∂∂
F3(4th)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800
1000
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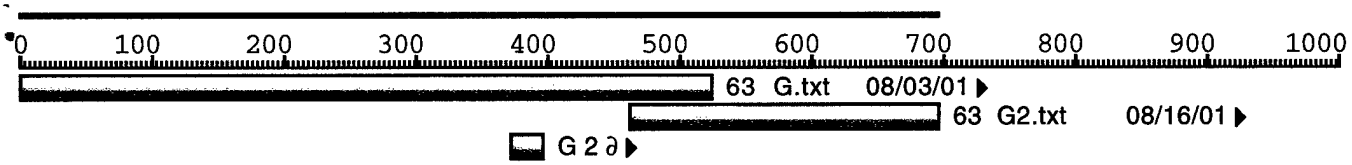
F(R&C).txt 2/11
63 F(3'ex 1)
F3(R&C)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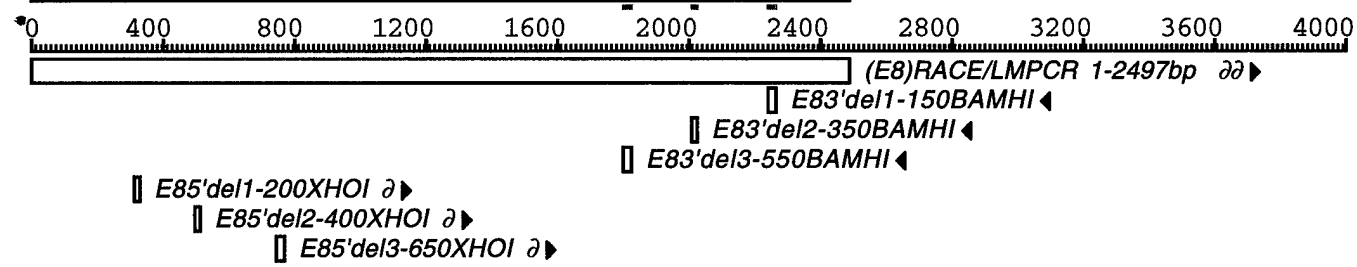
G 2 ∂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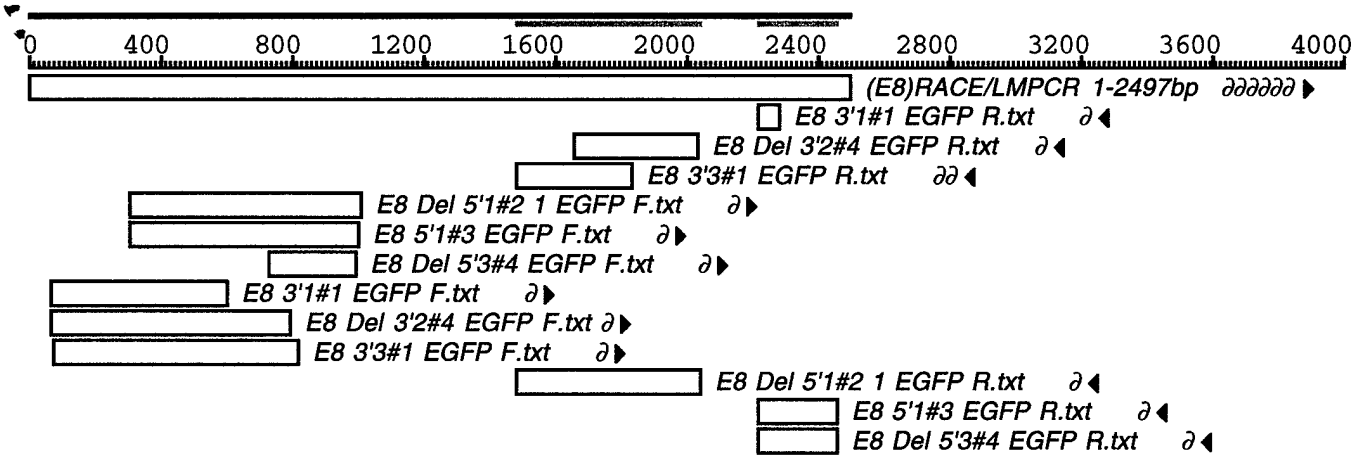
400
800
1200
1600
2000
2400
2800
3200
3600
4000
(E8)RACE/LMPCR 1-2497bp
E83'del1-150BAMHI
E83'del2-350BAMHI
E83'del3-550BAMHI
E85'del1-200XHOI
E85'del2-400XHOI
E85'del3-650XHOI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1200
1600
2000
2400
2800
3200
3600
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E8 Del 3'2#4 EGFP R.txt
E8 3'3#1 EGFP R.txt
E8 Del 5'1#2 1 EGFP F.txt
E8 5'1#3 EGFP F.txt
E8 Del 5'3#4 EGFP F.txt
E8 3'1#1 EGFP F.txt
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E8 3'3#1 EGFP F.txt
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