CHAPTER V

RESULTS

Identification of inversion breakpoint-spanning BACs by FISH

The cytogenetic and molecular studies were performed to characterize the breakpoints at Xp22.2 and Xq13.1 in detail. Fluorescence *in situ* hybridization (FISH) was first carried out on metaphase chromosomes of the patient and his mother to investigate the breakpoints involved in inv(X). 4 BAC clones were used as FISH probes. BAC clone RP11-351K23 containing the human *EDA* gene was used to examine the breakpoint on Xq13.1 and other 3 BAC clones RPCI11-42N20 containing the *IL1* gene, RPCI11-188G3 containing the *RSK2* gene and RPCI1-218N20 containing the *ARX* gene were used to investigate the opposite breakpoint in the region of interest at Xp22.2 (Fig. 4). FISH results demonstrated the signal from probe containing the *EDA* gene was split on inv(X) in both the patient and his mother. When FISH analysis was performed using BAC that contained the human *IL1*, *RSK2* and *ARX* genes, no split hybridization signal was detected on inv(X) (Fig. 5).

Subsequently, a chromosome walking strategy was used across the Xp22.2 region to narrow down the breakpoint. Finally, BAC clone RP11-804N7 was identified to span the breakpoint at Xp22.2.

Fine mapping of the breakpoints by polymerase chain reaction (PCR)

To further refine the breakpoints, PCR was carried out using several primer pairs spanning the BAC RP11-804N7 and RP11-351K23. The primers were prepared according to the genomic DNA sequence database for this portion of the X chromosome. PCR amplification was performed with genomic DNA as a template and with primer-pairs (Table 1) designed to amplify the

sequences of *EDA* gene between positions 87483 to 183744 within BAC RP11-351K23. All the regions of the *EDA* gene were amplified and checked for their presence (Fig. 7-9 and Table 1). Only a ~5.5 Kb interval between the position chr. X: 68,984,906-68,990,401 (UCSC Genome Browser, May 2004) could not be amplified by PCR in both the patient and normal individuals. This region localized within the intron 3 of the *EDA* gene and contained a long interspersed nuclear element (LINE), L1PA2, which was difficult to amplify by PCR.

For the breakpoint on Xp, amplified products were obtained from almost every primer pair, except there was no PCR products generated from the genomic DNA of the patient at the position chr. X: 13,939,284-13,940,183 (UCSC Genome Browser, May 2004). These results indicated that the boundary of the Xp22.2 breakpoint of inv(X) lay between position chr. X: 13,939,284-13,940,183 (UCSC Genome Browser, May 2004). The results delimit the breakpoint region at Xp22.2 to a ~900 bp interval between the position chr. X: 13,939,284-13,940,183 (UCSC Genome Browser, May 2004) (Fig. 11-14). This region could not be amplified in the patient. The interval spanning the breakpoint mapped to a long interspersed nuclear element (LINE), L1MC5.

The sequence of both breakpoint-spanning fragments was compared with each other. The breakpoint within Xp22.2 occurred within a region rich in the long interspersed nuclear elements. Immediately at the position chr. X: 13,938,480-13,939,103 (UCSC Genome Browser, May 2004) distal to the p-arm-specific breakpoint, a 624-bp L1PA7 sequence with 85% similarity to the L1PA2 is found (Fig. 12). The inversion breakpoint on Xq13.1 was assigned to a ~5.5 kb region within intron 3 of *EDA* gene, which is also included in a repeat-rich region (Fig. 10).

Expression analysis of the gene flanking the inversion breakpoint on Xp

Sequence analysis and database searches of the Xp breakpoint-covering fragments did not give any hint that a gene is directly affected by the inversion breakpoint. Analysis of the sequenced BAC RP11-804N7 showed that the breakpoint on Xp located proximal to the 5' end of the *FAM51A1* gene (Fig. 15). Expression of the *FAM51A1* gene flanking the Xp breakpoint in lymphoblastoid cell line was tested. The gene was expressed highly enough in the cell lines available to use to quantify the relative amount by real-time RT-PCR and the expression of *FAM51A1* in the patient found to be indistinguishable from the expression in normal controls.

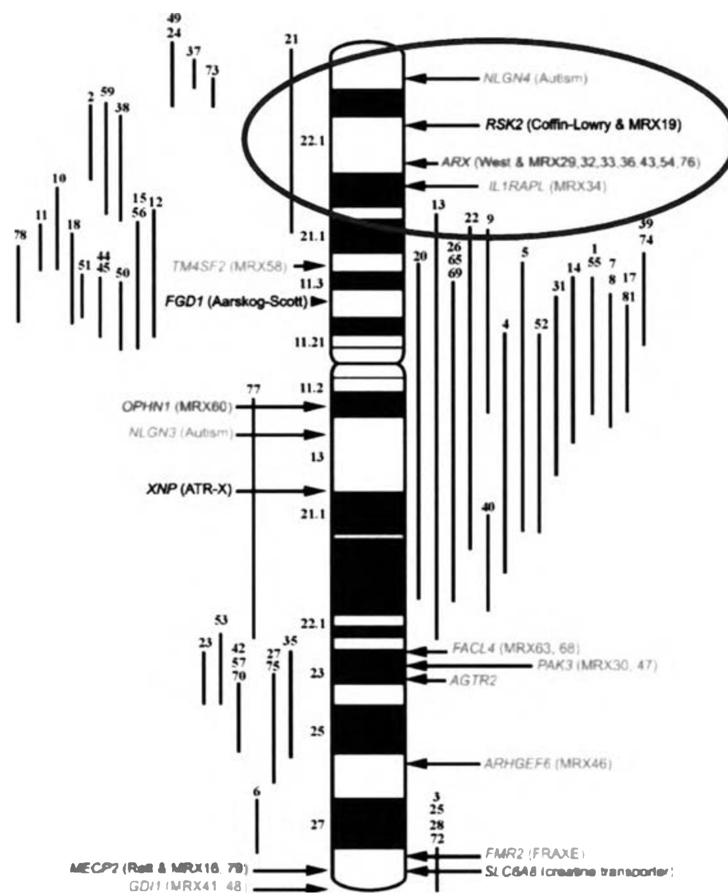


Figure 4. The MRX genes in the region of interest on Xp22.2

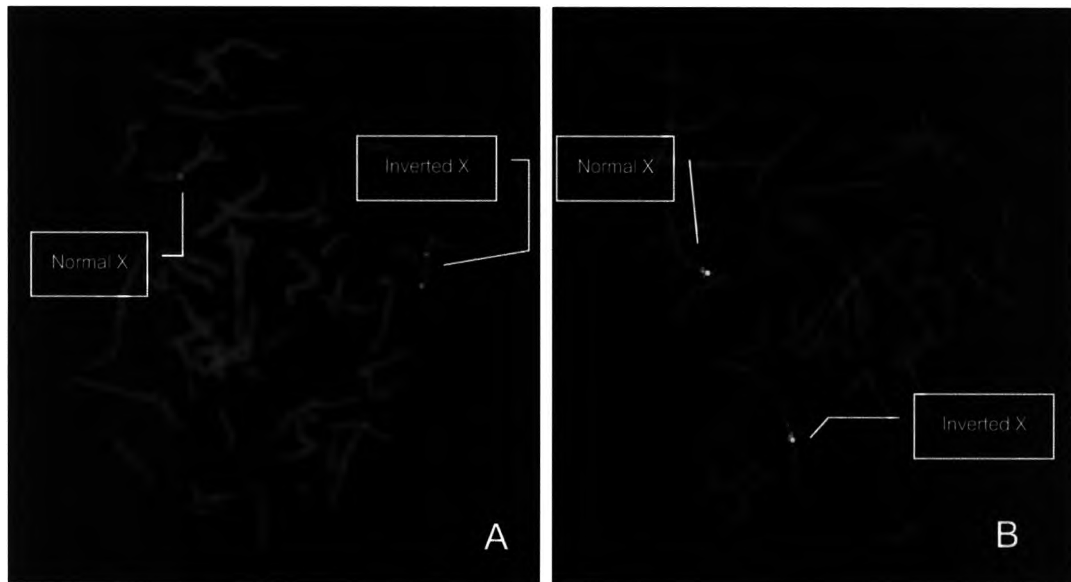


Figure 5. FISH results in the patient's mother probe from BAC clone RP11-351K23 containing the human *EDA* (A) and probes that contained human *IL1*, *RSK* and *ARX* genes (B)

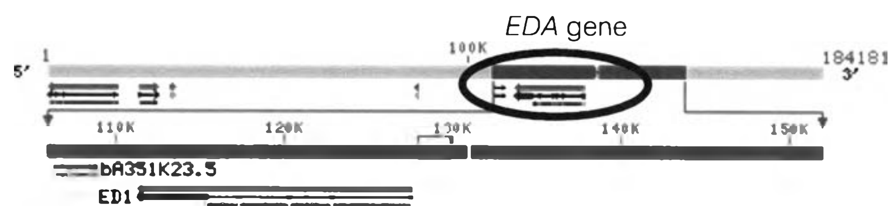


Figure 6. Mapping of *EDA* gene (exon 4-9) on BAC clone RP11-351-K23

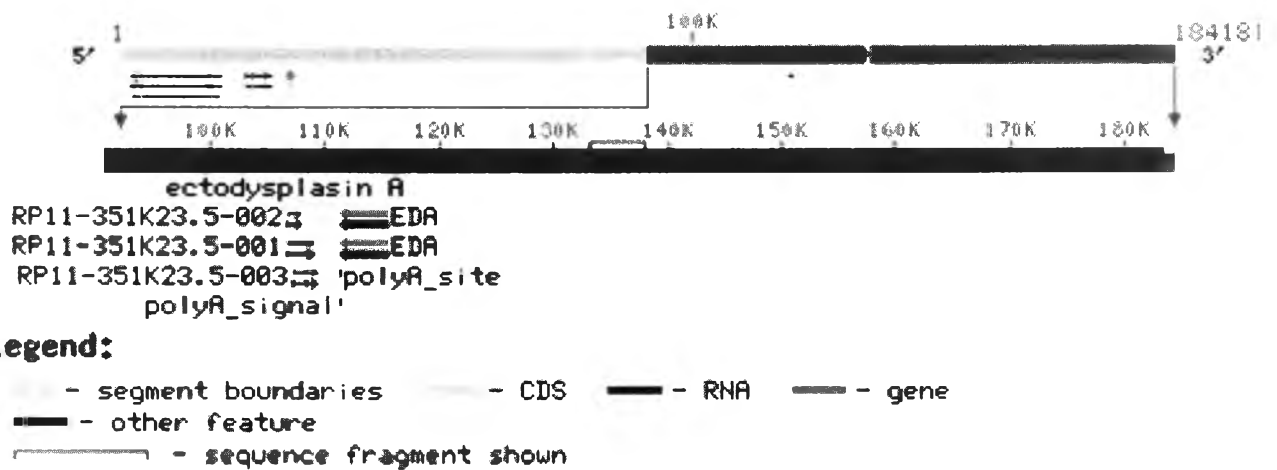


Figure 7. The diagram illustrates the sequence on BAC clone RP11-351K23 which were amplified by PCR using primer pairs listed in the Table 1 (Red line)

Pr9 = 124972-129432

Pr10 = 129332-134765

Pr11 = 134221-140495

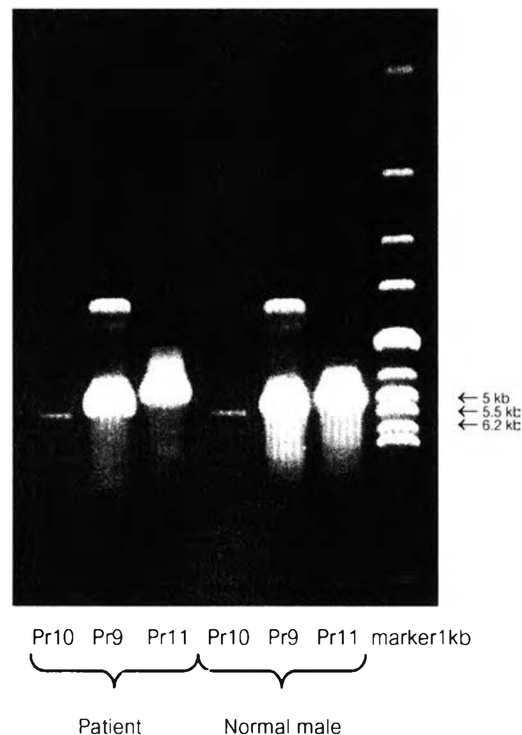


Figure 8. Long PCR results on BAC RP11-351K23 using Primer pair 9, 10 and 11 of the patient and normal male. The size of PCR products were 5.4 kb, 6.2 kb and 5 kb respectively.

Table 1 Sequence of Primers on BAC Clone RP11-351K23

Primer pair	Primer	Sequence 5'⟶3'	Position
1	87K-88KF	caggatgatcggcttcttgac (21)	87483-87503
	93589R	cagagggacctgtcattccttc (22)	93904-93883
2	93916F	ccattgtgtgggtttgtttttgt (23)	93916-93938
	100013R	tacgtttcactgcctgtggattt (23)	100013-99991
3	99983F	tcgaggtaaaatccacaggcagt (23)	99983-100005
	100571R	tgtgatgaaaccctggtgtgaaa (23)	100571-100549
4	100571F	aggcctcttgtcagctgcacg (21)	100517-100537
	106988R	tgcccaatgtgcaactccagg (21)	106988-106968
5	106787F	cggtttacctgtgtgaggcactg (23)	106787-106809
	115256R	caagatggtgcacgctgacatc (22)	115256-115235
6	115235F	gatgtcagcgtgcaccatcttg (22)	115235-115256
	118942R	gcaagctagccaagatgccag (21)	119286-119266
7	119254F	gctctgtaagcatctggcatc (21)	119253-119273
	120142R	acgaagtcattctttgctcca (21)	120140-120120
8	118-140F	tgctcaggacagccagtag (19)	120092-120110
	119-140R	gagaagacagtgcacataggc (21)	125854-126188
9	124972F	gaggtttctctgcacttagcagc (23)	124972-124994
	129435R	acttggtccgggccagcacac (21)	129432-129415
10	129332F	agctctatcagccagctagccac (23)	129332-129354
	134765R	agggatggagaaaatctgccaag (23)	134765-134743
11	134221F	ctgtggtttgacagtgtggttgg (23)	134221-134243
	140495R	tctgttgtcaggcacgcatacat (23)	140495-140473
12	140473F	atgtatgcgtgcctgacaacaga (23)	140473-140495
	145478R	ttagcatgactgtgattcaatgg (23)	145478-145456

Primer pair	Primer	Sequence 5'⟶3'	Position
13	145289F	gagggcaagggctgtgaattact (23)	145289-145311
	146721R	acacgcttatgactttgggaaca (23)	146721-146699
14	146421F	cccatcccatcccaatctcaagt (23)	146421-146434
	147989R	ctcagctcccctctttgtgttga (23)	147989-147967
15	147967F	tcaacacaaagaggggagctgag (23)	147967-147989
	149621R	tccatcctagggcaggcactaat (23)	149621-149599
16	149426F	tggctctagattgggtcccagaa (23)	149426-149448
	150981R	tccaaagcagcaccagaaagaca (23)	150981-150959
17	150959F	tgtctttctggtgctgctttgga (23)	150959-150981
	151615R	tgtagctgagtggactgagaaaa (23)	151615-151593
18	151543F	tgtgtgacactttcttgagagac (23)	151543-151565
	151992R	aaggggaaacatgtgttgcttgg (23)	151992-151970
19	151689F	gtctctagaggcatcagaggtg (22)	151689-151710
	153912R	acatgtttcgcttgactgtcatt (23)	153912-153890
20	151671F	tcaggcaccaatgttattgtctc (23)	151671-151693
	154479R	cttacaggcactgcccatgtgtt (23)	154479-154457
21	154145F	aatgtgggagttgggaaaggaaa (23)	154145-154167
	155660R	ctggaaagccaaaatccctgcta (23)	155660-155638
22	155636F	attagcagggattttggctttcc (23)	155636-155658
	157235R	acacagcaggttggacagagctt (23)	157235-157213
23	157142F	tgagctttccttggaagcagtgt (23)	157142-157164
	158748R	ctgggtaagggtttccctgctaa (23)	158748-158726
24	158698F	attggaaaaaggaaagcccaaca (23)	158698-158720
	160262R	tggccttctctgataccacatca (23)	160262-160240
25	160159F	cccattcacacaaacaaccaaca (23)	160159-160181
	161711R	aactgaaccaccacctccaatca(23)	161711-161689
26	161689F	tgattggaggtggtggttcagtt (23)	161689-161711
	163247R	tcccagacagggcactacagaag (23)	163247-163225

Primer pair	Primer	Sequence 5'→3'	Position
L1PA2			
27	168736F	tgccattttctggtccttaaacg (23)	168736-168758
	170356R	taggccctagcaacctgtgcttt (23)	170356-170334
28	173252F	tagagtttggggcaaatgggttc (23)	173252-173274
	174774R	aaacccagctgttttcaggctta (23)	174774-174752
29	174758F	tgaaaacagctgggtttacacat (23)	174758-174780
	177656R	tcttttcctccacccccagataa (23)	177656-177634
30	177383F	ggtatagccaggccctactgctg (23)	177383-177405
	180412R	accattgcaggcaacataacagg (23)	180412-180390
31	180093F	atcaccatgaagatcccgttctg (23)	180093-180115
	181718R	tgcgagccagaggtctaatcaac (23)	181718-181696
32	181699F	gattagacctctggctcgcagta (23)	181699-181721
	183744R	atcgattaccttgggtggaagag (23)	183744-183722

F=Forward primer

R=Reverse primer

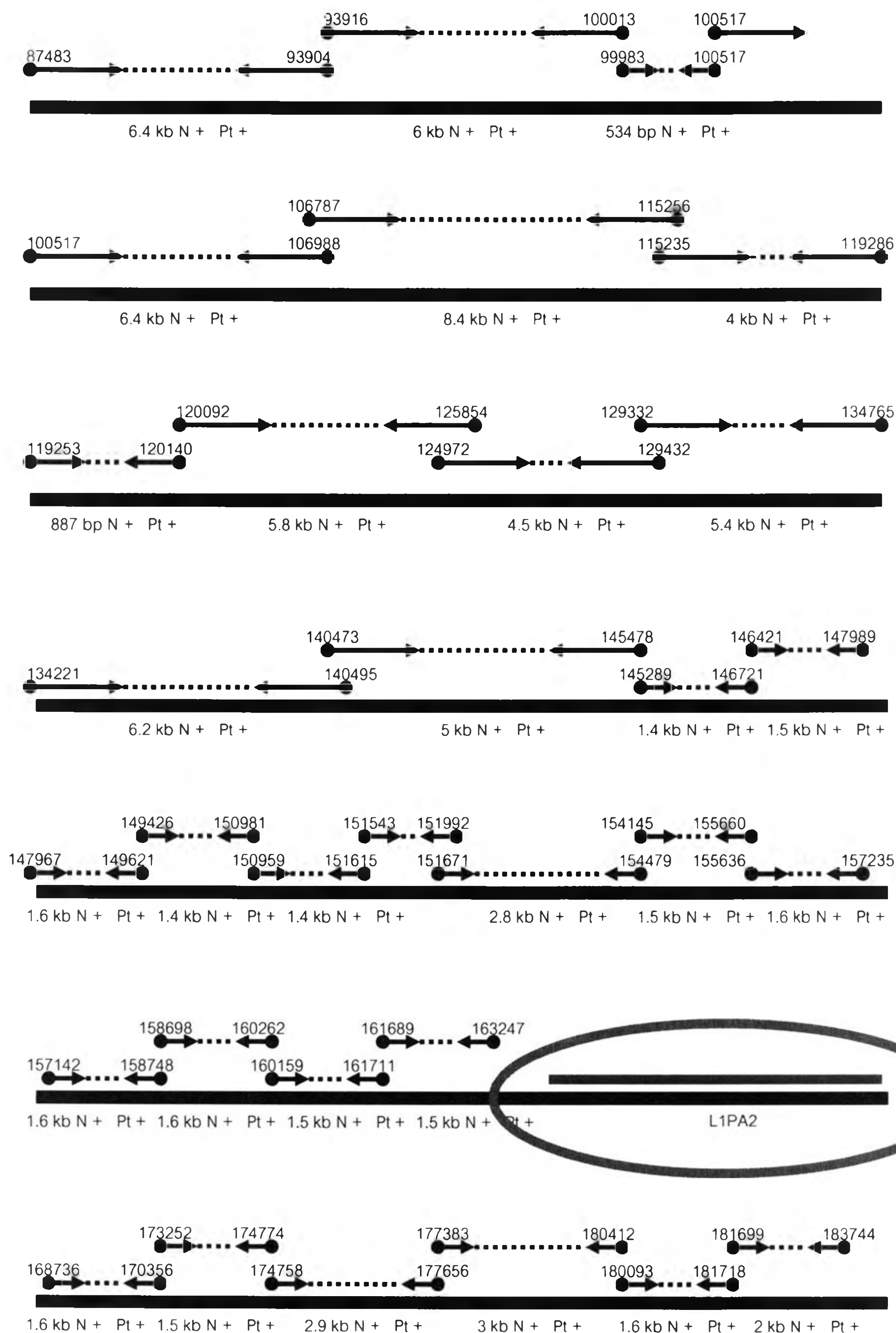
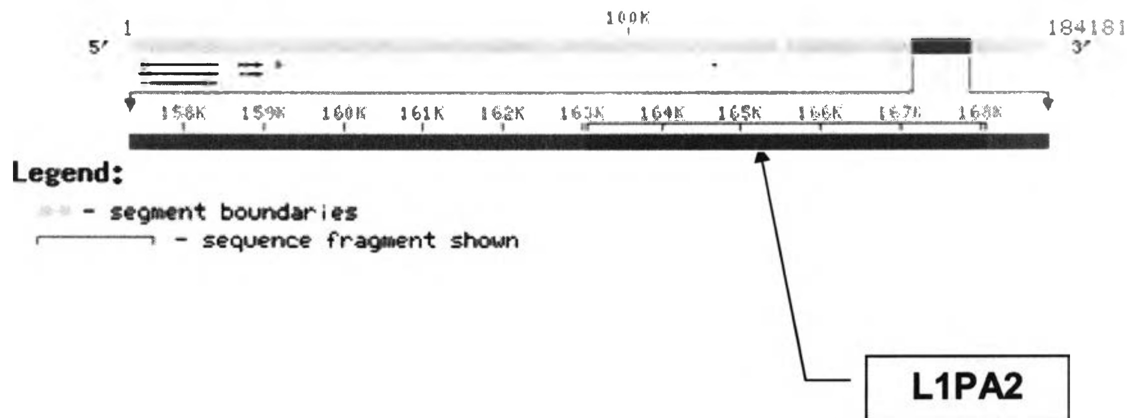
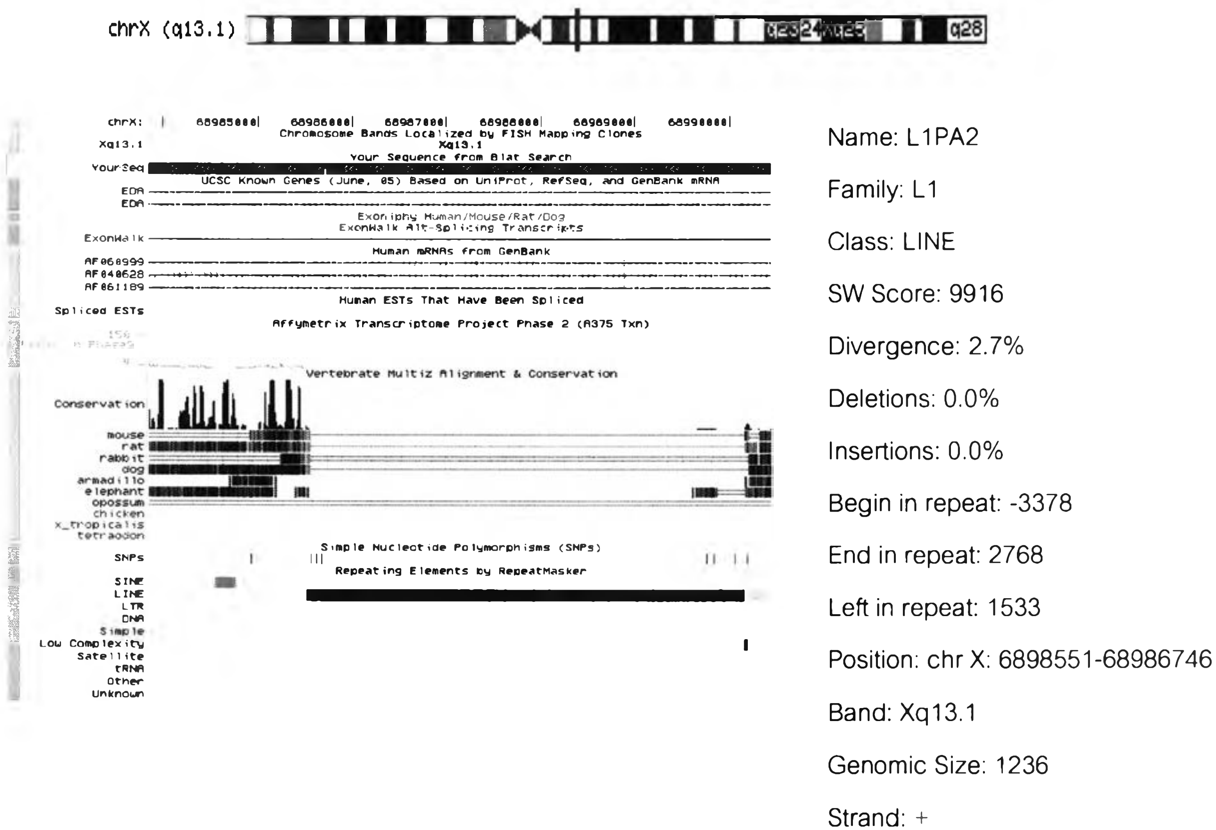


Figure 9. Mapping of the primers and PCR results on BAC clone RP11-351K23





Name: L1PA2

Family: L1

Class: LINE

SW Score: 9916

Divergence: 2.7%

Deletions: 0.0%

Insertions: 0.0%

Begin in repeat: -3378

End in repeat: 2768

Left in repeat: 1533

Position: chr X: 6898551-68986746

Band: Xq13.1

Genomic Size: 1236

Strand: +

Figure 10. The position of L1PA2 in BAC clone RP11-351K23

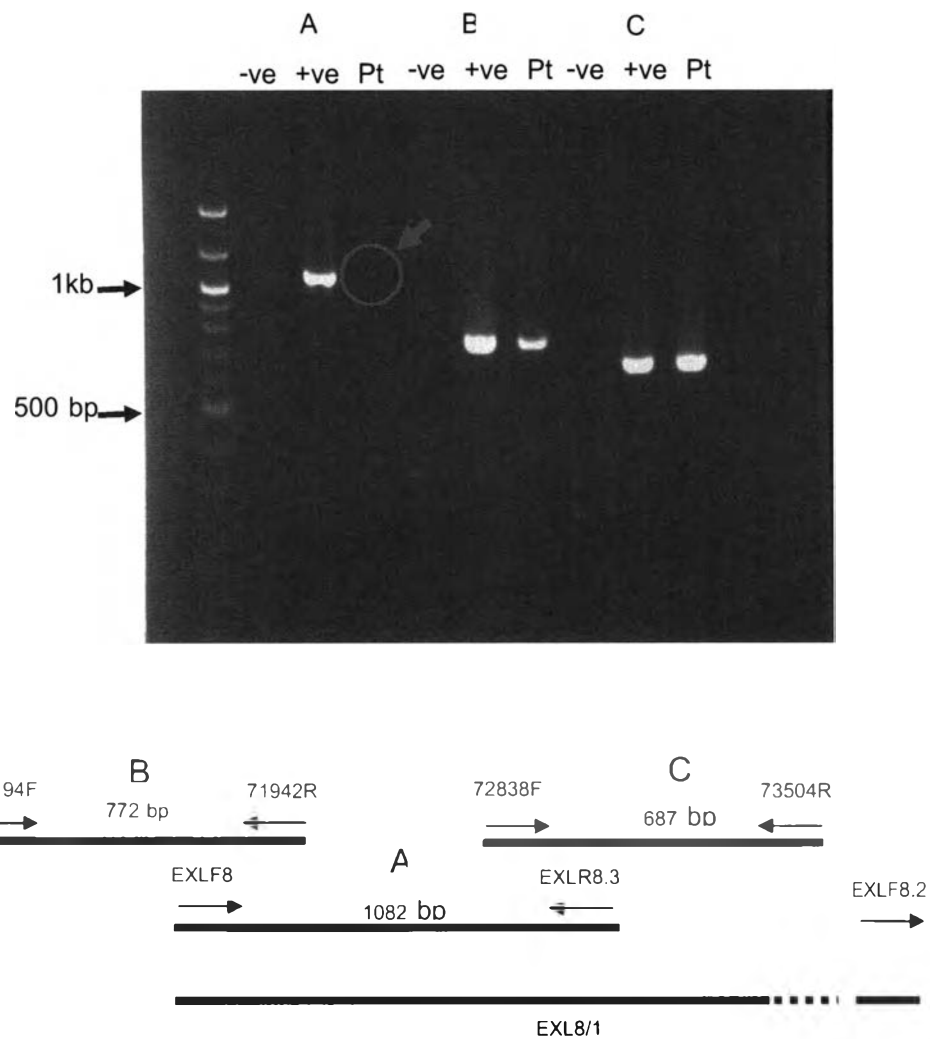


Figure 11. The PCR results using primer EXLF8/EXLR8.3 (fragment A), there was no PCR product from the patient's genomic DNA. While the fragment B and C could be amplified using primers 71194F/71942R (fragment B) and 72838F/73504R (fragment C). Thus, the breakpoint should be about 860 bp between primer 71942R and 72838F

repeat_region 71109..71122
/rpt_family="AT_rich"
repeat_region
complement(71137..71760)
/rpt_family="L1PA7"
repeat_region
complement(71765..72844)
/rpt_family="L1MC5"
repeat_region
complement(73140..73842)
/rpt_family="HAL1"
repeat_region
complement(76063..77170)
/rpt_family="L1M4"
repeat_region
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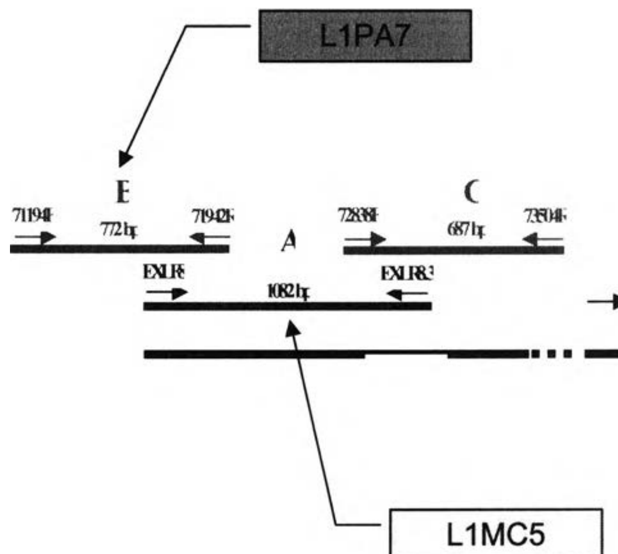
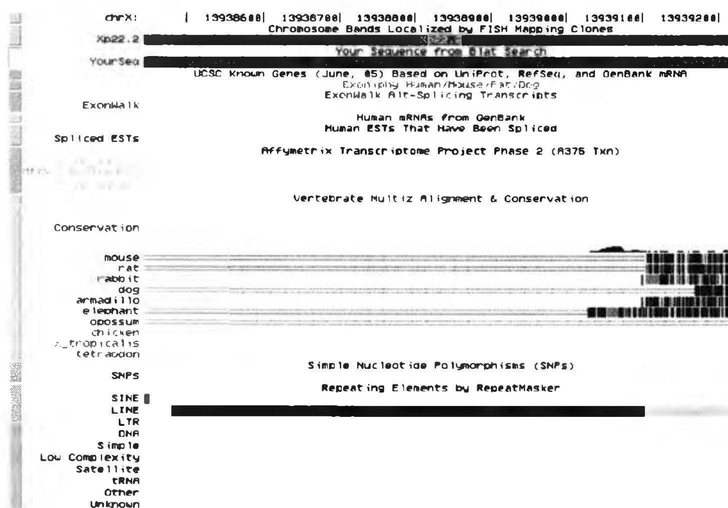


chrX (p22.2)



Name: L1PA7

Family: L1

Class: LINE

SW Score: 4874

Divergence: 7.1%

Deletions: 0.0%

Insertions: 0.2%

Begin in repeat: -13

End in repeat: 6141

Left in repeat: 5519

Position: chr X: 13938480-139391033

Band: Xp22.2

Genomic Size: 624

Strand: -

Figure 12. The position of L1PA7 in BAC clone RP11-804N7

EXLF8

71821 cctacagttt acctcttttg acctatagaa tttcacatat tcagattttg ctg***atggcat***
71881 ***actcttggta caggtca***ata tgtttctctg tttgctatat ttcctacgaa ttgacagttg
71941 aatccagagg tttgatcaga ctcaggttaa atctctttga caaagccaga gagaatgatg
72001 tcttctttct tcaggaggca catggttttc tgtgattttt gtgatgttaa caactgttgt
72061 ttctcagtgt agatccatta gtcccaatgg ggttgcaaaa tggcgttatt ttaacttatt
72121 tatttttcat tcattaattt gaacactttt acaaataatt acttcttatc tattctggtt
72181 acccagtggt acagttcata caggaaaggt aggataaatg cttgattccc ttcatttaca
72241 aaggaatgga tgttaattca tctcaaagca atgaattggc tcattatcat cctgatcatt
72301 caaaattgac atgagatttt aaaaaaatat tttgtgctca tggattccaa catatttgat
72361 ggctttcaat taattgcaac tgttattctt ttaaaactca aattgtccca tctttggcta
72421 gtggaagcct cctcgagtta gctcctgaga ccttttggct tgacccttgt agtctttgat
72481 agctccctca gtatctggtg taataaaata ttcccagatc atcatatgca tttctaatcc
72541 cagacctaga atcatccact tctcccaatt gccctggttt cttttaatgg gaaatgatat
72601 ttaaggaccc aaattgggta ccatcactat tgggtttgcc attgtttcta agcctcttca
72661 gtggacagag ctcagaaata catgtatcta tgcagttaaa aaaaaaaaag ataaaacgcc
72721 tcatcaggtg ttactgatac tttcatttca aattcagcaa tacagttttt tgttttgttt
72781 tgttttatgc tttacctgtc ccttattaca tcttggttcc tttcttccac acagagaaac
72841 ctggtcttca aggacatgag aaattgtaga atgcaatgtt ccataattgt ttatattttt
72901 tactccacat ttcacacata caagtctaaa taacgacact gatactcaac caccaacata
72961 attgccaaaa acagttaaaa agtttgcct atgttttccc ccttccattc taaatagttg
73021 aactatatgc acattatctg agcatgtaga tgttacacac aacaatcttt cccttctaac
73081 cctcatttaa tatttattct aaaagtaact atatatatgt ttaagtcttg tcagcagtcc
73141 ttatgtcaat gtctttctag ttttttttgg ttgtctgaag tttattgtct agtggatttt
73201 tcaggcaagt tcatgagaac aacattccct gttcttgcat gttgataaca atttttgccc
73261 tttacactgg aaagtcaatt ttgctggata taaaatcttt gtctcacatc ttctttcctt
73321 gagaatctta aatataccta tttccttctg gcttaaagca ttgatgttga aaatgaagct
73381 aatttgttca ttataattca caggtttttt tttttgccta catgaacaaa ggattttttt
73441 ctttatattt aaagtctagt tatgttatta aaatatatct tgatgttagt aatcccaggt
73501 cagtattctc aggtaagcag tgtgctccta tagtttcaaa tctttaaaaa cattattatt
73561 acagtacagt tttcttaata ataatttata gtatttgttc tttctttgct tagttttctt
73621 cattaggaat ttccattatt cataggttga attttctttg catatcttca atatctgact
73681 ctctcaaatt cttgttatct cttcattcat tgtaaaaata tttctccctg tttactctcc
73741 atttcattta aggaattgta tgttgtattt attcattctt gatttccttc tggtttcacc
73801 tttatttttg aagtaaagtt tttttatttt taattccttg ttgagtttaa tcacactttt
73861 gtgtttttct aactctgatc ttttcatgtg ttttatcttt ttcttaatgc ctttaagccc
73921 gttttgaaaa agttttgacc tgatctatgg gcacgtcttt ctaatgattg cattctctgt
73981 gggaatgtta ttctgttcct tattcttttt ctcatttttt aagtggcatt tggccttgat
74041 aatttttggt tacttatctt tatgtgaaat atattgtcct gaacttttca ggagaaggtg
74101 ggattcatta tagcttttct agcttcatag agctcctttg tgggttagtt ctatgtagtg
74161 ttcaagacta tggtagtttg ctttttgcaa tttctagatt ctgttcctct ccttcctttt
74221 atctagactc tctctttcct gcatctctgt tgcccctatc ctcatccgtg tttattctac
74281 tcctagcaat ttgtctaaat ttgagggtct tgtcctggaa aggagtccta gtatgtcaat
74341 tttgagagtc catagggcct taactgctct acctcctgta gacattctta ccaagggccc
74401 ttgtcctcat ttcttaatgg agtgggcaac ctcctgcagt ttcagctgat gttctcaaat
74461 tgatccatac tgttttcagg ggagcacctt ggctagtttg gcattttcct gttctctggt
74521 ccttcagata tgaatacctc tttctgcttt ctcccacaca gaaactatta ccactcaggt
74581 gtcagggttt tggtgacttt ttcatctctg cttgtacttt tgtgttccaa gtgatacctt
74641 atcaggtagt ttgctggaaa atattgtcta tgaggtttct attttgctct ctaatgattc
74701 tttttatgt gagaatttga agagactcag aaatgat***gtc actgtgtgac tgtcatcttc***
74761 ***g***cagcttttt ccattctttc gaacattaat tttcaaagtg ctcttttaga gagtgaaaat

EXLF8.2

Figure 13. The position of primer EXLF8 and EXLR8.2 on BAC clone RP11-804N7

Table 2 Sequence of Primers on BAC Clone RP11-804N7

Primer pair	Name	Sequences 5'→3'	Position
1	EXLF1	tagtaatgggaggggggttacagaa (24)	668-691
	EXLR1	cagacaagggtcagatggtgttac (24)	11058-11035
2	EXLF2	atagtcctgggcaagtacaggaag (24)	10243-10266
	EXLR2	gtggctcaagttgtgattatggac (24)	20695-20672
3	EXLF3	gaccccacctcttaataccatcac (24)	21262-21285
	EXLR3	aaggtcctgcaagtaaatctgtcc (24)	31591-31568
4	EXLF4	ggacagatttacttgcaggacctt (24)	31568-31591
	EXLR4	atcctctcagcatgagtcaaacac (24)	42157-42134
5	EXLF5	gaggaagtttctcaagaggtcagc (24)	42369-42392
	EXLR5	tggtcagaatctagagcattccag (24)	52547-52524
6	EXLF6	tagaccaggtcagtcagtgtctcc (24)	50072-50095
	EXLR6	aactggaacttaatcccactgagg (24)	60679-60656
7	EXLF7	gctttagcttccatagccagagag (24)	60129-60152
	EXLR7	gtaacagaccaagactccgtctca (24)	70606-70583
8·	EXLF8	atggcatactcttggtacaggtca (24)	71874-71897
	EXLR8	gtaaccatggtgcataaactgctc (24)	82362-82339
9_1·	EXLF8	atggcatactcttggtacaggtca (24)	71874-71897
	EXLR8_2	agaccagcacttactagcacatgg (24)	77996-77973
9_2	EXLF8_2	gtcactgtgtgactgtcatcttcg (24)	74738-74761
	EXLR8	gtaaccatggtgcataaactgctc (24)	82362-82339

Primer pair	Name	Sequences 5'→3'	Position
10_1	71194F	tacatgtgccctggtggttttctg (24)	71194-71217
	71942R	ctgagtctgatcaaacctctggat (24)	71965-71942
10_2*	EXLF8	atggcatactcttggtacaggtca (24)	71874-71897
	EXLR8_3	ttggtggttgagtatcagtgtcgt (24)	72956-72933
10_3	72838F	aacctggtcttcaaggacatgaga (24)	72838-72861
	73504R	cacactgcttacctgagaatactg (24)	73524-73501

F=Forward primer

R=Reverse primer

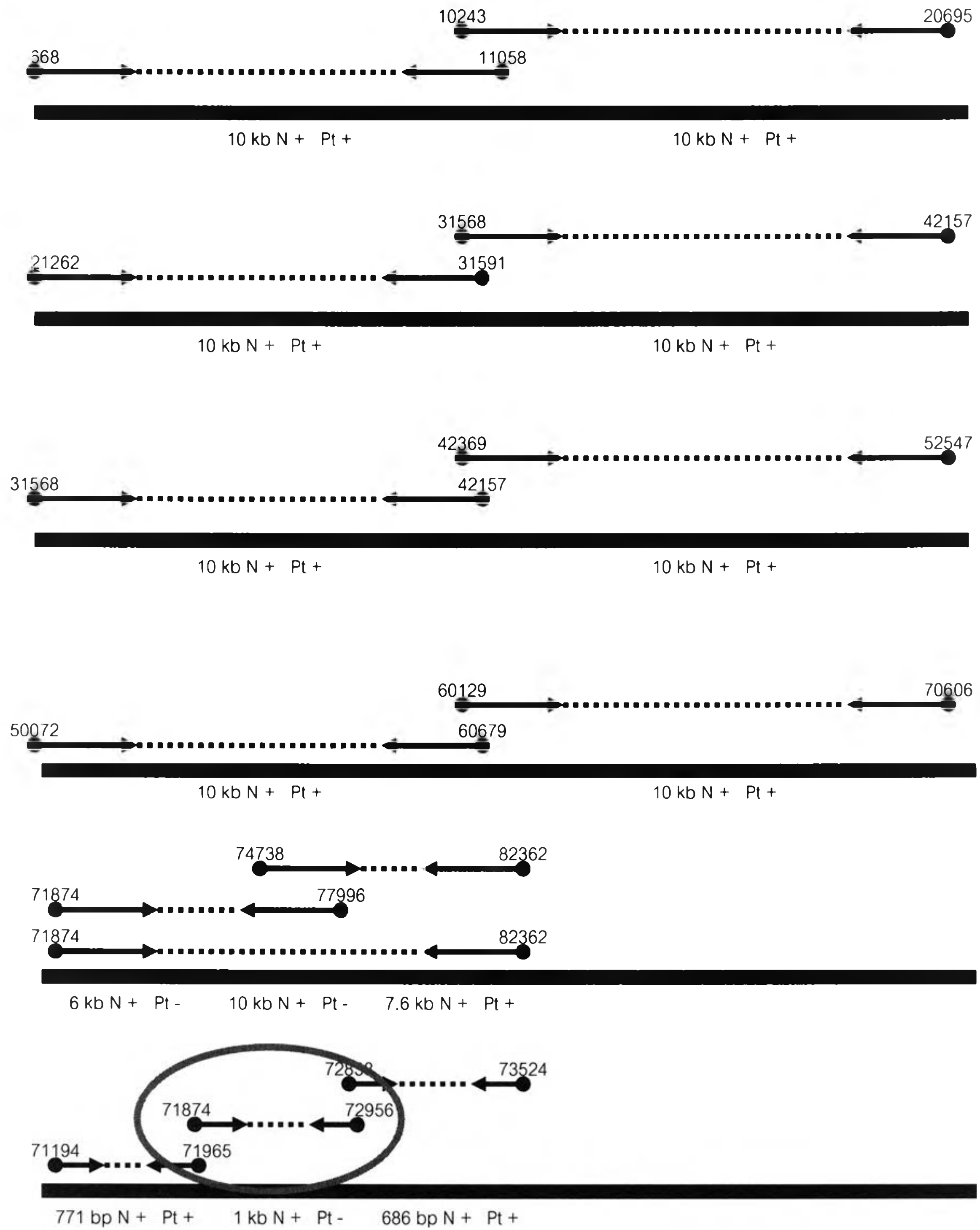


Figure 14. Mapping of the primers and PCR results on BAC clone RP11-804N7

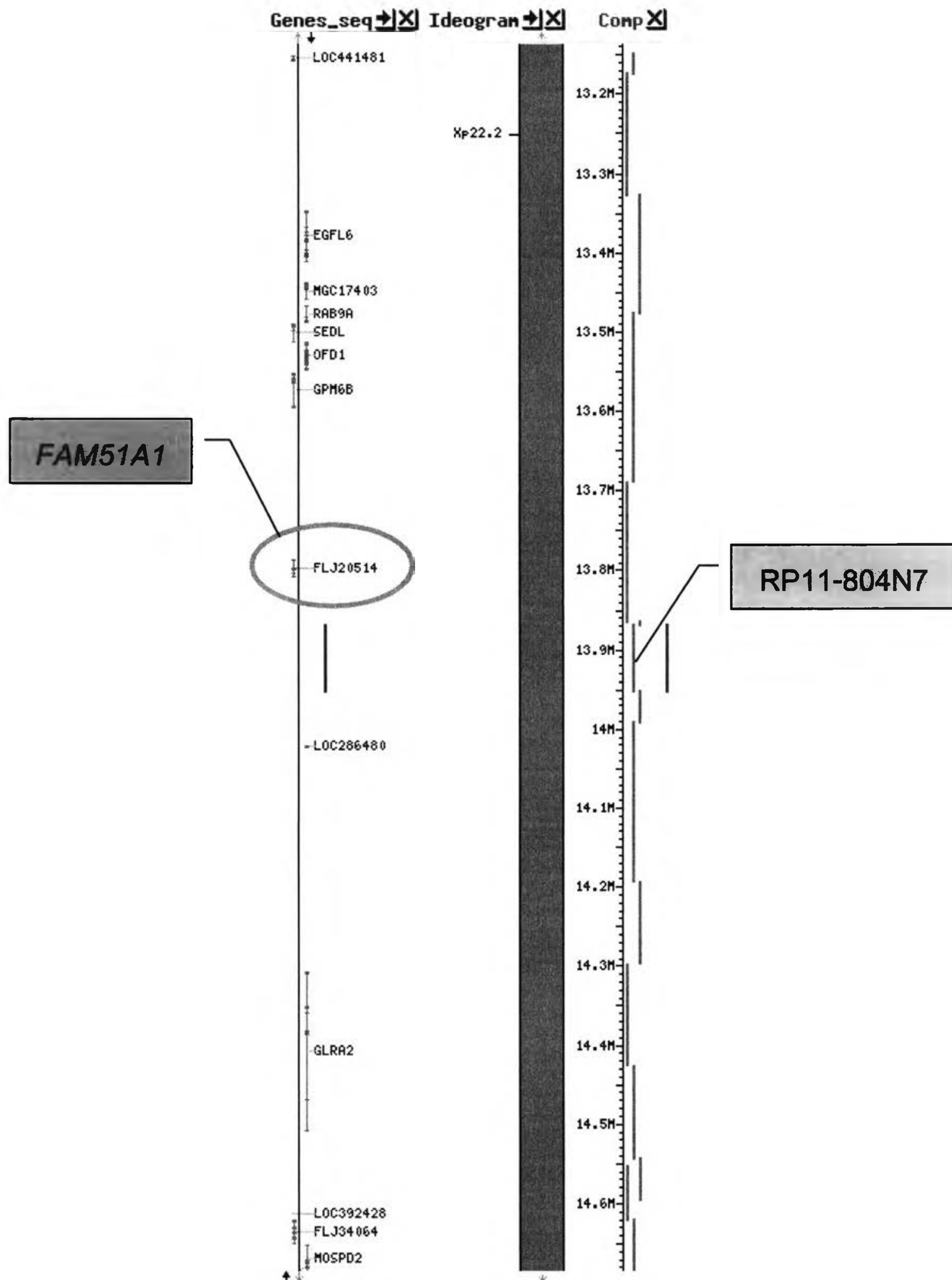


Figure 15. Map viewer of the BAC clone spanning breakpoint on Xp22.2