

Disruption and sequence identification of 2,000 genes in mouse embryonic stem cells

Brian P. Zambrowicz, Glenn A. Friedrich, Eric C. Buxton, Stan L. Lilliberg, Christophe Person & Arthur T. Sands

Lexicon Genetics, 4000 Research Forest Drive, The Woodlands, Texas 77381, USA

The dramatic increase in sequence information in the form of expressed sequence tags (ESTs)¹ and genomic sequence has created a 'gene function gap', with the identification of new genes far outpacing the rate at which their function can be identified. The ability to create mutations in embryonic stem (ES) cells on a large scale by tagged random mutagenesis provides a powerful approach for determining gene function in a mammalian system; this approach is well established in lower organisms^{2,3}. Here we describe a high-throughput mutagenesis method based on gene trapping that allows the automated identification of sequence tags regardless of their expression status in ES cells. To facilitate the study of gene function on a large scale, we are using these techniques to create a library of ES cells called Omnibank, from which sequence-tagged mutations in 2,000 genes are described.

Gene trapping is a method of random insertional mutagenesis that uses a fragment of DNA coding for a reporter or selectable marker gene as a mutagen^{4,5}. The marker can only be expressed if it inserts within an intron, an exon or a promoter of a transcriptionally active gene. Although such a strategy allows for the selection of insertion events in genes and tags the genes for subsequent cloning, active genes are targets, restricting the set of genes that can only be mutated in any particular cell type. To date, most trap designs have not been optimized to allow automation of the trapped gene identification⁶⁻⁹, and the sequence tags often correspond to non-transcribed or 5' untranslated regions. Such sequence information is underrepresented in genomic, complementary DNA and EST databases and is less conserved between species, making it difficult to match the mutated gene to a known gene. To overcome these limitations, we developed gene trap vectors (Fig. 1a; VICTR3 and VICTR20) based on two functional units: (1) a sequence acquisition component consisting of a promoter (from the mouse gene encoding phosphoglycerate kinase-1) active in ES cells fused to the puromycin N'-acetyltransferase gene coding sequence that lacks a polyadenylation sequence but is followed by a synthetic consensus splice donor sequence (PGKpuroSD); and (2) a mutagenic component that consists of a splice acceptor sequence fused to a selectable, colorimetric marker gene followed by a polyadenylation sequence (SABgeobpA or SALRESβgeobpA).

We anticipated that puromycin resistance could be achieved only by splicing into exons downstream of a trapped gene and addition of a polyadenylation sequence (Fig. 1b). In this design, a gene need not be transcribed to be trapped and the sequence of the trapped gene can be obtained by rapid amplification of cDNA ends (3' RACE), a simple, automatable procedure. The resulting sequence of the fusion transcript is informative in database searches because it is likely to contain coding regions. To determine whether FGKpuroSD could trap a gene as predicted, it was targeted by homologous recombination to the second intron of the *Hprt* gene. Sequencing of the 3' RACE products from puromycin-resistant colonies demonstrated that these colonies were the result of successful targeting events (targeting of *Hprt* was confirmed by Southern blot analysis; data not shown).

Our gene trap strategy relies on the effective mutagenesis of the

reactions, each of which contained 100 ng DNA, PCR buffer (50 mM Tris-HCl (pH 9.2 at 25 °C), 14 mM (NH₄)₂SO₄, 1.75 mM MgCl₂), 350 μM of each dNTP, 0.5 μM of each primer and 0.35 U expand long *Taq* polymerase (Boehringer). PCR conditions were 35 cycles at 94 °C for 30 s, 50–53 °C for 30 s, 68 °C for 30 s–1 min. Cycle sequencing used the appropriate PCR primers and a commercial kit.

Southern and northern blot analysis. Genomic DNA was extracted from the blood leukocytes of the Family 1 members and from FLEB 14–14 cells. Three micrograms of each genomic DNA were digested with *EcoRI*, electrophoresed and blotted onto nylon membrane. ³²P-labelling of cDNA clones was done by random primer extension. Hybridization was done as described²⁷. Multiple-tissue northern blots were purchased (Clontech) and hybridized as directed.

RT-PCR. Total cellular RNA was isolated from the brain tissue of patient II-2 in family 2 according to the acid guanidinium thiocyanate-phenol-chloroform procedure²⁷. Reverse transcription of 1 mg total RNA was primed at 50 °C for 30 min using a Titan one-tube RT-PCR system kit (Boehringer Mannheim), and the reaction mixture was directly used for PCR with forward primer (nucleotides 351–371) 5'-GGAAGCGACGACGCCA GAAAC-3' and reverse primer (nucleotides 963–983) 5'-GGGACAGCCAGCCACAGAC-3'. PCR was done at 94 °C for 30 s, 56 °C for 30 s, 68 °C for 1 min and repeated for 45 cycles.

Received 30 October 1997; accepted 10 February 1998.

1. Parkinson, J. *An Essay on the Shaking Palsy* (Whittingham and Rowland, London, 1817).
2. Yamamura, Y., Aizawa, K., Kobayama, T. & Nakamura, S. Early-onset parkinsonism with diurnal fluctuation: clinical and pathological studies. *Chin. Neurol. (Tokyo)* 33, 491–496 (1993).
3. Ishikawa, A. & Tanji, S. Clinical analysis of 17 patients in 12 Japanese families with autosomal recessive juvenile parkinsonism. *Neurology* 47, 160–166 (1996).
4. Matsunaga, H. et al. Localization of a gene for autosomal recessive form of juvenile parkinsonism to chromosome 6q25.2–27. *Am. J. Hum. Genet.* 60, 588–596 (1997).
5. Matsunaga, H. et al. Evidence for a novel mutation of D6S305 in a family of autosomal recessive juvenile parkinsonism (JRP). *Genomics* (in press).
6. Aizawa, S. et al. Human BAC library: Construction and rapid screening. *Gene* 191, 69–79 (1997).
7. Finley, D. & Chao, Y. Ubiquitin-mediated degradation of proteins. *Annu. Rev. Biochem.* 70, 421–452 (1997).
8. Sauter, A. J., Borden, T. B., Boddy, M. N. & Freeman, P. S. Does this have a familiar RTN? *Trends Biochem. Sci.* 21, 208–214 (1996).
9. Aizawa, S., Gibson, T. J. & Stewart, A. P. The PHD finger: implications for chromatin-mediated transcriptional regulation. *Trends Biochem. Sci.* 20, 56–59 (1995).
10. Takahashi, H. et al. Familial juvenile parkinsonism: clinical and pathologic study in a family. *Neurology* 44, 437–441 (1994).
11. Cairns, D. B. & Langston, J. W. Aetiology of Parkinson's disease. *Lancet* ii, 1457–1459 (1983).
12. Jenner, P., Schapira, A. H. V. & Marsden, C. D. New insights into the cause of Parkinson's disease. *Neurology* 42, 2241–2250 (1992).
13. Schapira, A. H. V. et al. Mitochondrial complex I deficiency in Parkinson's disease. *J. Neurochem.* 54, 823–827 (1990).
14. Mizuno, Y. et al. Role of mitochondrial dysfunction in the etiology and pathogenesis of Parkinson's disease. *Biochim. Biophys. Acta* 1271, 265–274 (1995).
15. Spillmann, M. G. et al. Mutation in α-synuclein in Lewy bodies. *Nature* 388, 839–840 (1997).
16. Polymeropoulos, M. H. et al. Mutation in α-synuclein gene identified in families with Parkinson's disease. *Science* 276, 2045–2047 (1997).
17. Mori, H., Konoy, J. & Iwata, Y. Ubiquitin is a component of paired helical filaments in Alzheimer's disease. *Science* 275, 1641–1644 (1997).
18. Morishima-Kawashima, M. et al. Ubiquitin is conjugated with amino-terminally processed tau in senile plaques. *Neuron* 18, 1151–1160 (1997).
19. Gengen, L., Puch, C., Figueiredo-Pereira, M. E., Nostrom, W. E. V. & Goldberg, D. Amyloid β-protein inhibits ubiquitin-dependent protein degradation in vitro. *J. Biol. Chem.* 270, 19702–19708 (1995).
20. Love, S., Satoh, T., Qujada, S., Cole, G. M. & Terry, R. D. Abz-50, ubiquitin and Tau immunoreactivity of neurofibrillary tangles, Pick bodies and Lewy bodies. *J. Neurochem. Exp. Neurol.* 47, 393–405 (1998).
21. Gallwey, R. G., Malvar, O. & Terry, G. Filaments of Lewy bodies contain insoluble cytoskeletal elements. *Am. J. Pathol.* 140, 809–822 (1992).
22. Iwasaki, T. et al. Purification and characterization of Lewy bodies from the brains of patients with dementia. *Am. J. Pathol.* 140, 809–822 (1992).
23. Hochstrasser, M. Ubiquitin, proteasomes, and the regulation of intracellular protein degradation. *Cell* 79, 13–21 (1994).
24. Hochstrasser, M. Ubiquitin, proteasomes, and the regulation of intracellular protein degradation. *Cell* 79, 13–21 (1994).
25. Geyer, L., Puch, C., Figueiredo-Pereira, M. E., Nostrom, W. E. V. & Goldberg, D. Amyloid β-protein inhibits ubiquitin-dependent protein degradation in vitro. *J. Biol. Chem.* 270, 19702–19708 (1995).
26. Finley, D., Bartel, B. & Varshavsky, A. The tails of ubiquitin precursor are ribosomal proteins whose fusion to ubiquitin facilitates ribosome biogenesis. *Nature* 338, 394–401 (1989).
27. Sambrook, J., Fritsch, E. F. & Maniatis, T. *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989).

Supplementary information is available on Nature's World Wide Web site (<http://www.nature.com>) or as paper copy from Mary Sheehan at the London editorial office of Nature.

trapped genes. Previous studies of gene trap vectors using SA β geobpA have shown that it can disrupt endogenous messages and create null alleles^{6,7,10,11}. We tested both VICTR20 and PGKpuroSD independently for their ability to mutagenize the *Hprt* locus. Gene targeting of VICTR20 to the second intron of *Hprt* resulted in colonies resistant to 6-thioguanine, indicating disruption of wild-type *Hprt*. In contrast, targeting PGKpuroSD alone to the same position in *Hprt* did not yield colonies resistant to 6-thioguanine. Targeting for all constructs at the *Hprt* locus was confirmed by Southern analysis (Fig. 2). Although these data do not guarantee that VICTR20 will be mutagenic at all loci, they support previous data that most gene trap events containing SA β geobpA in a retrovirus will result in a null allele.

Having established the basic functionality of our gene trap constructs, we expanded the application to a large-scale mutagenesis program. ES cells were electroporated with a PGKpuroSD plasmid or infected with recombinant VICTR3 or VICTR20 retrovirus produced from a MoMLV-based packaging cell line. After transduction of the gene trap vectors, puromycin selection produced clonal lines. Lysis of cells from each clone provided RNA for 3' RACE. Direct sequencing of 3' RACE products yielded high-quality sequence 80–700 nucleotides long from 75% of the clones when the process was performed manually, and from 60% when performed robotically. The sequence tag from each clone, termed an Omnibank sequence tag (OST), was entered into a relational database for analysis and archiving. The first 3,000 OSTs were compared to GenBank (version 100.0) and SwissProt (version 34) databases using the BLASTN or BLASTX algorithm¹², respectively, to search for sequence similarities. Compiling all matches that result in a BLASTN probability score of 10^{-30} or less, we found that 18% of OSTs match known genes (see Supplementary Information), 10% match human and rodent ESTs, 10% match repeats such as B1, B2 or transposable elements, and 61% do not match any sequences in the databases.

To demonstrate that OSTs can identify the gene that is trapped

and determine the position of retroviral integration, we carried out Southern blot analysis on DNA isolated from a gene trap event at the Bruton's tyrosine kinase (*btk*) locus¹³. The *btk* OST sequence began with exon 2, suggesting that the insertion occurred in the first intron of the *btk* gene. Southern blot analysis using several different restriction enzymes confirmed the location of the retroviral insertion in the first intron of the gene (Fig. 3). Comparing OST sequences to cDNA sequences for 108 known genes revealed that 44% of the viral integration events were near the 5' ends of genes (within the first 350 nucleotides of the 5' end of the encoded cDNA), 44% were in the middle of genes, and 12% were near the 3' end of genes (within 350 nucleotides of the 3' end of the encoded cDNA). Our data suggest that, although there may be some bias towards 5' integrations, VICTR trap events integrate throughout genes.

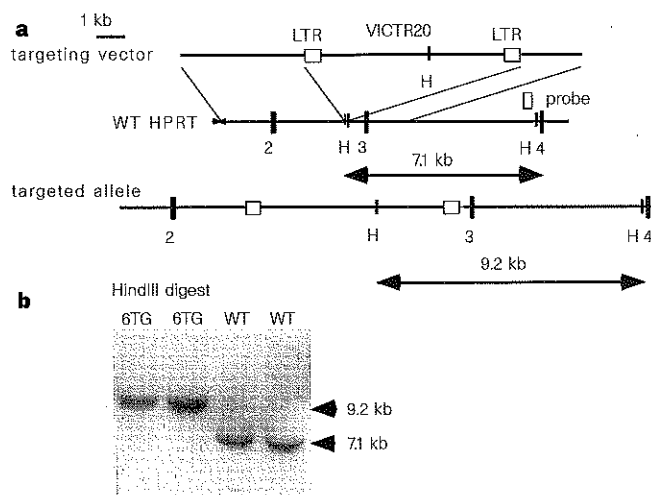


Figure 2 Targeting of VICTR20 to the second intron of the *Hprt* locus. *Hprt* targeting experiments were carried out as previously described²⁰. PGKpuroSD and VICTR20 were placed into the *Eco*RI site of pRIV6.9I. **a**, The targeting vector, wild-type *Hprt* allele and targeted allele are shown. Open rectangles denote the retroviral LTRs and the black numbered rectangles denote exons 2, 3 and 4 of *Hprt*. (H) *Hind*III. **b**, Southern blot of the targeted 6-thioguanine resistant (6-TG) clones versus non-targeted wild-type (WT) clones. The blot was probed with sequences 5' of exon 4.

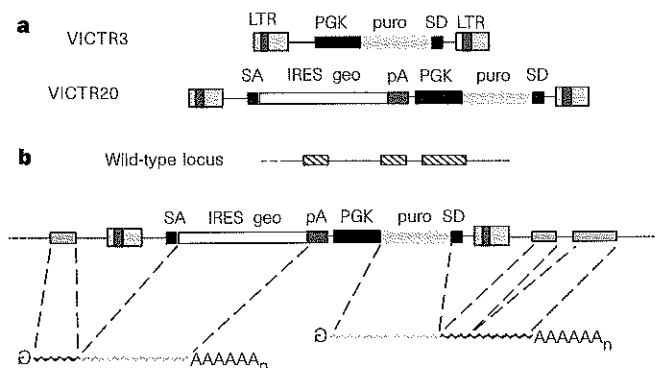


Figure 1 Vector design for 3' trapping. **a**, The retroviral constructs VICTR3 (for viral construct for trapping) and VICTR20 are drawn schematically. The splice donor of PGKpuroSD in VICTR3 and VICTR20 is preceded by stop codons in all three reading frames to prevent expression of fusion transcripts as well as unique sequences for nested 3' RACE and direct sequencing. The splice donor was added to puromycin using a PCR primer containing a unique sequence for 3' RACE, the sequence of the OBS sequencing primer, and a consensus splice donor sequence (5'-CCG CTC GAG ACT TAC CTG ACT GGC CGT CGT TTT AA GAC GAG CTC CCT AGC TAG TCA GGC ACC GGG CTT-3'). LTR, long terminal repeat; PGK, phosphoglycerate kinase-1 promoter; puro, puromycin *N*-acetyltransferase gene; SD, splice donor sequence; SA, splice acceptor sequence; IRES, internal ribosome entry site; β geo, galactosidase/neomycin phosphotransferase fusion gene; pA, polyadenylation sequence. **b**, A representation of a VICTR20 retroviral insertion into a three-exon gene. The resulting integration would produce two fusion transcripts, one containing puro sequences at the 5' end and sequences from the trapped gene at the 3' end, and the other containing sequences of the trapped gene at the 5' end and β geo sequences at the 3' end.

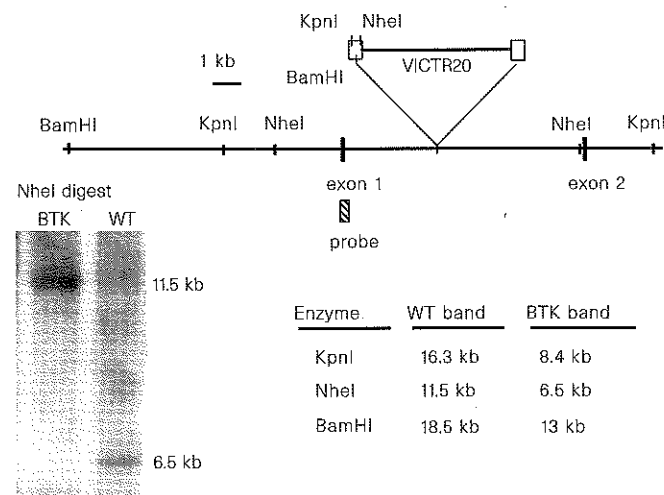


Figure 3 VICTR20 has integrated into the first intron of the *btk* gene. The diagram shows the position of retroviral integration into the *btk* locus. The Southern blot shows the *Nhe*I restriction-fragment length polymorphism produced by VICTR20 integration and the table shows the fragment sizes observed after digestion of DNA from wild type versus the *btk* gene trap ES cells.

We predicted that the gene trap design should not rely on expression of the target gene. The current set of OSTs contains many genes that are known to have restricted patterns of expression and were thus unlikely to be expressed in ES cells. We detected *btk* gene known to be expressed in B lymphocytes¹⁴. We detected *btk* expression in the spleen by an RT-PCR (polymerase chain reaction after reverse transcription of RNA) assay but were unable to detect expression in ES cells (Fig. 4a). Similarly, several OSTs with unique sequences could not be detected in ES cells (data not shown), confirming the ability of the gene trap vectors to trap genes that are not expressed in ES cells.

Sequence analysis of over 3,000 OSTs has demonstrated a high stringency for splicing to generate drug-resistant colonies, as only 10 of the OSTs contain vector sequence that follows the synthetic splice donor. Nonetheless, the large fraction of new OSTs (61%) made it important to demonstrate that they represented transcribed sequences. We chose 37 of the novel or unknown OSTs at random (17 produced by electroporation and 20 produced by retroviral infection) for expression analysis using RT-PCR (Fig. 4b). Of the 37 OSTs, 34 were expressed in brain, testis, ES cells or day-10.5 embryos. To demonstrate further that the OSTs correspond to exonic sequence and not unspliced nuclear RNAs, we probed

northern blots of poly(A)⁺ RNA from multiple tissues with 16 unknown OSTs (Fig. 4c). Of 16 of the OST probes, 14 hybridized to discrete messenger RNAs at high stringency, suggesting that the unknown OST sequences are present in mRNA and represent exonic sequences of genes. Because the 3' RACE products tend to be longer than the initial OST obtained, additional sequence was obtained from the 3' RACE products of 290 unknown OSTs. This provided 70–700 base pairs (an average of 263 bp) of additional sequence per OST, and BLASTN searches revealed that 27% of these expanded OSTs matched sequences in GenBank (34 ESTs, 43 known genes). These results provide further support that VICTR gene trap vectors are trapping transcribed sequences. The expanded OST sequences average about 500 bases in length, yet 74% remain unknown and may represent newly discovered genes.

Our expression-independent method of sequence acquisition is unlikely to suffer from the same biases inherent to EST acquisition methods that typically result in underrepresentation of genes that are expressed at low levels, transiently or only in limited cell types. Gene trap methods, however, are not without selective pressures affected by vector design, delivery, target gene size and gene accessibility, which are likely to produce some level of bias in the relative representation of trapped genes. It is encouraging that

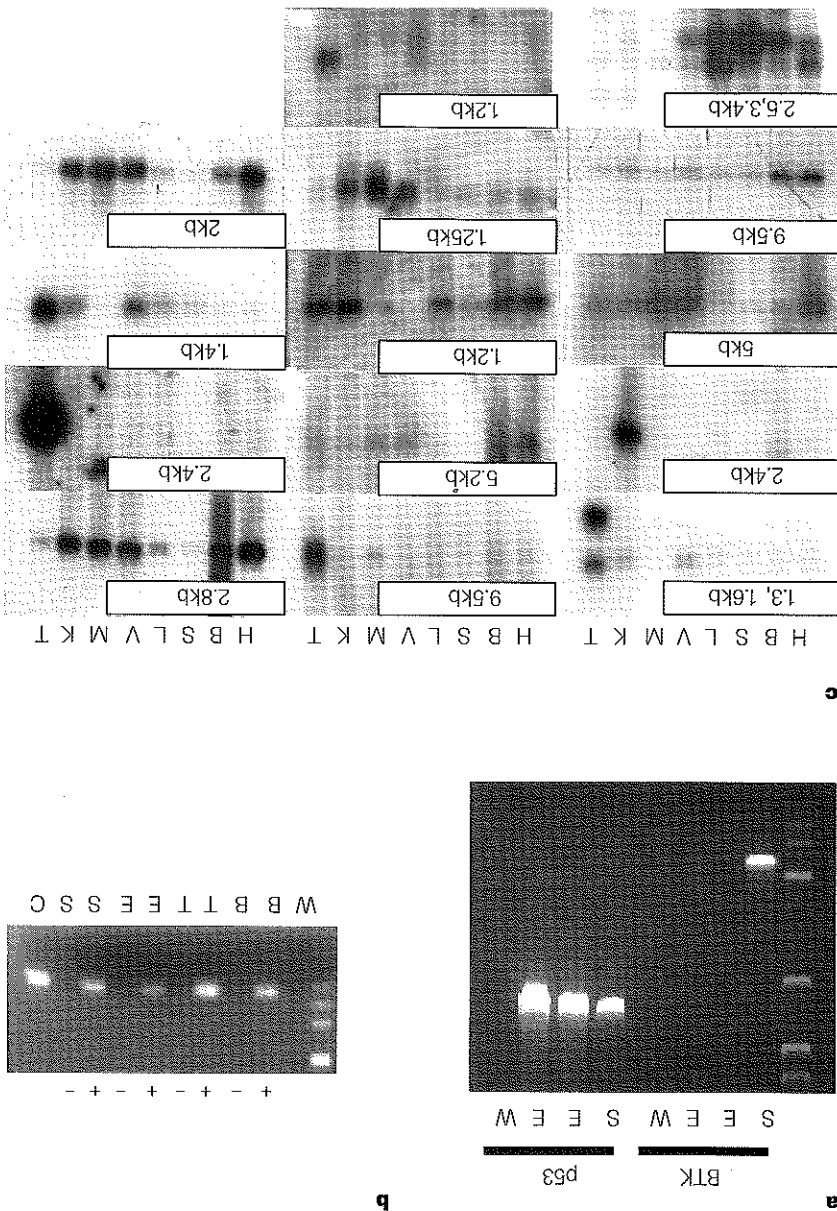


Figure 4 Expression of unknowns. **a**, First-strand cDNA was synthesized using RNA from spleen (S), two ES cell RNA samples (E) or water (W). PCR was carried out on the first strand cDNA using primers specific for *btk* or *p53* transcripts. The integrity of the ES cell RNA and reverse transcription reactions was confirmed using primers for *p53*, a gene known to be expressed in ES cells. **b**, First-strand cDNA was synthesized using RNA isolated from brain (B), testis (T), day 10.5 embryos (E) and ES cells (S). First-strand cDNA reactions were carried out in the presence (+) or absence (–) of reverse transcriptase and a water (W) and positive control (C) were included. PCR was carried out on first-strand cDNA using primers specific for 37 different unknown OSTs. **c**, Ten unknown OSTs were used as probes on multitissue northern blots made with poly(A)⁺ RNA. The blots were washed at high stringency. H, heart; B, brain; S, spleen; L, lung; V, liver; M, skeletal muscle; K, kidney; T, testis.

several reports suggest that most of the genome is accessible to retroviral integration^{15,16} with a subset of the integration events landing in 'hotspots', and with the remainder representing random integrations¹⁷. Our data support this model because most genes in Omnibank are represented by single gene trap events. Additionally, this percentage of single hit genes has remained stable as the number of OSTs has increased. To provide support that VICTR20 has a large target size and is mutagenic, a large-scale infection of ES cells was carried out, followed by selection for mutations in *Hprt* using 6-thioguanine. One 6-thioguanine-resistant colony was identified in 80,000 gene trap events and Southern blot analysis and RT-PCR confirmed it to be the result of an integration in the *Hprt* locus (data not shown). Although the gene target size for VICTR20 will become apparent as the OST numbers grow, our data suggest that the number of potential target genes is large.

We have automated the processing of mutagenized ES cells in the 96-well plate format resulting in the addition of at least 500 OSTs to the Omnibank library each week. This library represents a new type of functional genomics resource as it both contains sequence information of mutated genes and provides the ability to rapidly produce mouse mutants to analyse the function of large groups of genes. Future work will involve the production and analysis of mice from this resource and the further development of vectors and delivery systems that will maximize the number of unique gene trap events in Omnibank. □

Methods

Vectors and cell culture. PGKpuroSD, SAβgeoPGKpuroSD and SAIRESβgeoPGKpuroSD were all placed into the *Xho*I site of pGen⁺ (ref. 18) in reverse orientation. VICTR3 and VICTR20 were packaged in the retroviral producer cell line GP+E86 as described¹⁸. Electroporation and retroviral infection were carried out as previously described and ES cells were selected for 10 days in 2.75 mg ml⁻¹ puromycin (Sigma)^{18,19}. After expansion, two portions of each ES cell clone were stored in vapour-phase cryofreezers and one copy was processed to obtain sequence tags.

RNA isolation, 3' RACE and sequencing. RNA was isolated from ES cells grown on 35-mm plates using RNazol (Tel-Test). First-strand cDNA synthesis and 3' RACE PCR was carried out as previously described⁷ using Qt primer to prime cDNA synthesis and two rounds of PCR using primers Qo and P_o (5'-AAG CCC GGT GCC TGA CTA GCT AG-3'). PCR products were purified using Sephadex G-50 (DNA grade, Pharmacia) and sequenced with dye terminator cycle sequencing reactions and AmpliTaq FS DNA polymerase (Perkin Elmer). Reactions were carried out using 7 pmol of primer OBS (5'-CTG TAA AAC GAC GGC CAG TC-3') and approximately 30–120 ng of 3' RACE product. Reactions were run on an ABI Prism 377 with an XL upgrade according to the manufacturer's instructions.

RT-PCR and northern blotting. RT-PCR was carried out using 40 cycles and the reaction conditions described above for 3' RACE. Northern blots were prepared using Clontech multitissue blots and were washed at a high stringency of 65 °C in 0.2 × SSC, 0.1% SDS for 1 h.

Received 7 October 1997; accepted 10 February 1998.

- Adams, M. D. *et al.* Complementary DNA sequencing: "Expressed sequence tags" and the Human Genome Project. *Science* **252**, 1651–1656 (1991).
- Plasterk, R. H. Reverse genetics of *Caenorhabditis elegans*. *Bioessays* **14**, 629–633 (1992).
- Spradling, A. C. *et al.* Gene disruptions using P transposable elements: an integral component of the *Drosophila* genome project. *Proc. Natl Acad. Sci. USA* **92**, 10824–10830 (1995).
- Gossler, A. & Zachgo, J. in *Gene Targeting: A Practical Approach* (ed. Joyner, A. L.) 181–227 (Oxford Univ. Press, New York, 1993).
- Friedrich, G. & Soriano, P. Insertional mutagenesis by retroviruses and promoter traps in embryonic stem cells. *Meth. Enzymol.* **225**, 681–701 (1993).
- Chen, Z., Friedrich, G. A. & Soriano, P. Transcriptional enhancer factor 1 disruption by a retroviral gene trap leads to heart defects and embryonic lethality in mice. *Genes Dev.* **8**, 2293–2301 (1994).
- Zambrowicz, B. P. *et al.* Disruption of overlapping transcripts in the ROSA βgeo 26 gene trap strain leads to widespread expression of βgalactosidase in mouse embryos and hematopoietic cells. *Proc. Natl Acad. Sci. USA* **94**, 3789–3794 (1997).
- Townley, D. J., Avery, B. J., Rosen, B. & Skarnes, W. C. Rapid sequence analysis of gene trap integrations to generate a resource of insertional mutations in mice. *Genome Res.* **7**, 293–298 (1997).
- Hicks, G. G. *et al.* Functional genomics in mice by tagged sequence mutagenesis. *Nature Genet.* **16**, 338–344 (1997).
- Friedrich, G. & Soriano, P. Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice. *Genes Dev.* **5**, 1513–1523 (1991).
- Skarnes, W., Moss, J., Hurlley, S. & Beddington, R. Capturing genes encoding membrane and secreted proteins important for mouse development. *Proc. Natl Acad. Sci. USA* **92**, 6592–6596 (1995).

- Altschul, S. F., Gish, W., Miller, W., Meyers, E. W. & Lipman, D. J. Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410 (1990).
- Oeljen, J. C. *et al.* Large-scale comparative sequence analysis of the human murine Bruton's tyrosine kinase loci reveals conserved regulatory domains. *Genome Res.* **7**, 315–329 (1997).
- de Weers, M. *et al.* The Bruton's tyrosine kinase gene is expressed throughout B cell differentiation, from early precursor B cell stages preceding immunoglobulin gene rearrangement up to mature B cell stages. *Eur. J. Immunol.* **23**, 3109–3114 (1993).
- Chang, W., Hubbard, C., Friedel, C. & Ruley, E. Enrichment of insertional mutants following retrovirus gene trap selection. *Virology* **193**, 737–747 (1993).
- Withers-Ward, E. S., Kitamura, Y., Barnes, J. P. & Coffin, J. M. Distribution of targets for avian retrovirus DNA integration *in vivo*. *Genes Dev.* **8**, 1473–1487 (1994).
- Craigie, R. Hotspots and warm spots: integration specificity of retroelements. *Trends Genet.* **8**, 187–190 (1992).
- Soriano, P., Friedrich, G. & Lawinger, P. Promoter interaction in retrovirus vectors introduced into fibroblasts and embryonic stem cells. *J. Virol.* **65**, 2314–2319 (1991).
- Zhu, B., Bogue, M. A., Lim, D.-S., Hasty, P. & Roth, D. B. Ku86-deficient mice exhibit severe combined immunodeficiency and defective processing of V(D)J recombination intermediates. *Cell* **86**, 379–389 (1996).
- Hasty, P., Rivera-Perez, J., Chang, C. & Bradley, A. Target frequency and integration pattern for insertion and replacement vectors in embryonic stem cells. *Mol. Cell. Biol.* **11**, 4509–4517 (1991).

Supplementary information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from Mary Sheehan at the London editorial office of Nature.

Acknowledgements. We thank A. Bradley for ideas; M. Brandt, J. Fitzgibbon, C. Mossel, M. Newhouse, D. Potter and T. Smith for technical assistance; J. D. Wallace and A. Benjamin for tissue culture; D. Beer, L. Grocki, D. Leibham, T. Malley and S. Patel for automation and sequencing; B. Kohlhauf for computer support; and P. Hasty, R. Burgess, A. Turner and M. Nehls for discussion.

Correspondence and requests for materials should be addressed to B.P.Z. (e-mail: materials@icxgen.com). Sequences of clones given in Supplementary Information have been deposited in GenBank, accession numbers AF046239–AF046781. Lexicon will distribute the ES cell clones described here to requesting investigators for non-commercial research.

Role of Rel/NF-κB transcription factors during the outgrowth of the vertebrate limb

Yumi Kanegae*††, Ana Teresa Tavares*‡, Juan Carlos Izpisua Belmonte* & Inder M. Verma*

* The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, California 92037, USA

† These authors contributed equally to this work.

The development of the vertebrate limb serves as an amenable system for studying signaling pathways that lead to tissue patterning and proliferation¹. Limbs originate as a consequence of a differential growth of cells from the lateral plate mesoderm at specific axial levels². At the tip of the limb primordia the progress zone, a proliferating group of mesenchymal cells, induces the overlying ectoderm to differentiate into a specialized structure termed the apical ectodermal ridge. Subsequent limb outgrowth requires reciprocal signalling between the ridge and the progress zone^{3–6}. The Rel/NF-κB family of transcription factors is induced in response to several signals that lead to cell growth, differentiation, inflammatory responses, apoptosis and neoplastic transformation⁷. In unstimulated cells, NF-κB is associated in the cytoplasm with an inhibitory protein, I-κB. In response to an external signal, I-κB is phosphorylated, ubiquitinated and degraded, releasing NF-κB to enter the nucleus and activate transcription⁷. Here we show that Rel/NF-κB genes are expressed in the progress zone of the developing chick limb bud. When the activity of Rel/NF-κB proteins is blocked by infection with viral vectors that produce transdominant-negative I-κBα proteins, limb outgrowth is arrested. Our results indicate that Rel/NF-κB transcription factors play a role in vertebrate limb development.

Rel/NF-κB transcripts are first detected in the chick presumptive limb bud mesoderm at stage 15 HH⁸, coincident with the first sign of limb budding (Fig. 1a). As limb outgrowth proceeds, transcripts

† Present address: Institute of Molecular Genetics, University of Tokyo, 4-6-1 Shirokanedai, Minato-Ku, Tokyo 108-8639, Japan.