The RNA we injected is a mixture of mRNA for many different proteins. Until the mRNA responsible for the expression of the Na-H exchanger is isolated, it will be impossible to determine whether the injected RNA caused the expression of a native but previously unexpressed Na-H exchanger, or whether the injected RNA directly encoded a Na-H exchanger. If the latter explanation is correct, this would be the first example of the expression of a mammalian ion exchanger or co-transporter in a foreign system. Our approach for expressing a Na-H exchanger could have important implications for studying the properties and regulation of Na-H exchange, and suggests an approach for cloning the gene(s) for the Na-H exchanger.

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Structural organization of the *bcr* gene and its role in the Ph′ translocation

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The Philadelphia (Ph′) chromosome, an abnormal chromosome 22 (ref. 1), is one of the best-known examples of a specific human chromosomal abnormality strongly associated with one form of human leukaemia, chronic myelocytic leukaemia (CML). The finding[2] that a small region of chromosome 9 which includes the c-*abl* oncogene is translocated to chromosome 22 prompted studies to elucidate the molecular mechanisms involved in this disease. We have demonstrated previously that the chromosome 9 of one patient with CML contains a breakpoint 14 kilobases (kb) 5′ of the most 5′ v-*abl*-homologous exon[3]. These data suggest a role for c-*abl* in CML, a theory supported by the presence of an abnormally sized *abl* messenger RNA[4,5] and protein[6] in the CML cell line K562. The region involved in the translocation on chromosome 22 has also been identified: all Ph′-positive patients examined to date have a breakpoint within a 5.8-kb region, for which we have proposed the name 'breakpoint cluster region' (*bcr*)[7]. To determine whether *bcr* contains protein-encoding regions, probes from *bcr* were tested for their ability to hybridize to complementary DNA sequences. A 0.6-kb *Hin*dIII/*Bam*HI *bcr* restriction enzyme fragment proved suitable for isolating several cDNA clones from a human fibroblast cDNA library[8]. Using *bcr* cDNA sequences, we obtained data strongly suggesting the presence of a chimaeric *bcr*/*abl* mRNA in the leukaemic cells of Ph′-positive CML patients. The recent isolation of cDNA clones containing *bcr* and *abl* sequences confirms this finding[12]. Because the *bcr* part of the chimaeric mRNA could be required to induce the transforming activity of the human c-*abl* oncogene, we have now initiated studies to characterize the normal '*bcr* gene' and to determine the effect of a translocation within its coding domain. We demonstrate that as a result of the Ph′ translocation, a variable number of *bcr* exons are included in the chimaeric *bcr*/*abl* mRNA. The *bcr* gene sequences in this mRNA could be responsible for the transition of the *abl* cellular proto-oncogene into an oncogene.

The largest cDNA, V1-3, containing an insert of 2.2 kb, was characterized in detail by restriction enzyme mapping (Fig. 1*a*) and sequence analysis (Fig. 1*b*). The cDNA contains one long open reading frame, starting at the poly(G) tail at the 5′ end and continuing to nucleotide 1,770, where a stop codon is encountered. All other reading frames have many stop codons within the entire region. The long open reading frame has the coding capacity for 589 amino-acid residues, corresponding to a protein of relative molecular mass (M_r) ~65,000; at the 3′ end, a polyadenylation signal occurs at nucleotide 2,182 followed by a poly(A) tail beginning at base 2,208 indicating that the cDNA contains the complete 3′ end of the gene. Although translational start sequences are encountered at the 5′ end, it is unlikely that this cDNA contains a complete copy of the mRNA, as Northern blot hybridizations indicate the presence of *bcr* mRNAs of ~4.0 and ~6.5 kb. Computer searches of newly isolated protein sequences derived directly from proteins or deduced from cDNA nucleotide sequences frequently result in the identification of proteins with partial homology. Such information is valuable, frequently allowing the assignment of a preliminary function to an unknown protein. Therefore, the PIR FASTP program[10] was used to search for *bcr*-homologous proteins; no proteins with significant homology were found, indicating that the *bcr* protein exhibits an as yet unidentified cellular function.

To determine the orientation of the *bcr* gene on chromosome 22, 5′ and 3′ probes were prepared from the V1-3 cDNA and hybridized to cosmids[7] containing human chromosome 22 sequences. This established that the 5′ end of the *bcr* gene is towards the centromere of chromosome 22 and is retained after the Ph′ translocation; the 3′ end of the *bcr* gene lies in the direction of the telomere and is translocated to chromosome 9 in the t(9; 22) translocation. The cDNA hybridizes to restriction enzyme fragments distributed over a region of up to 45 kb of chromosome 22 DNA (Fig. 2*a*). Within this region, a minimum of 13 exons are present. To determine the exact position and number of exons within the breakpoint cluster region, all hybridizing regions in *bcr* were sequenced and compared with the V1-3 cDNA. Four relatively small exons, designated 1-4, were present within *bcr*, varying in size from 76 to 105 base pairs (bp) (Fig. 2*b*); in the cDNA, these exons correspond to nucleotides 483-836 (Fig. 1*b*). As *bcr* was defined as the area on chromosome 22 in which the Ph′ breakpoints are found, we conclude that the breakpoints occur within a gene.

Having determined the position of the exons within *bcr*, we investigated whether the breakpoints occur in exon or intron regions. For CML DNA such as that of CML patient C481, this was readily determined. We had previously demonstrated[7] a breakpoint within a 1.2-kb H/Bg *bcr* fragment in several CML DNA samples, including that of patient C481. As no coding sequences are located within this region (see Fig. 2), patients such as C481 must have a chromosomal breakpoint in the intron between the exons designated 3 and 4.

A less simple situation was encountered in the DNA of patients 0311068 and 7701C. Nonetheless, cloning of $9q^+$ breakpoint fragments from these DNAs (data not shown) and restriction enzyme analysis followed by Southern hybridization enabled us to locate the breakpoints between exons 2 and 3 (see Fig. 2). The breakpoints in the previously cloned[3,7] $9q^+$ breakpoint fragments of patients 0319129 and 02120185 were analysed by DNA sequencing. In addition, we cloned the $22q^-$ breakpoint

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Fig. 1 *bcr* cDNA V1-3. *a*, Restriction enzyme map of V1-3. The black bar indicates the open reading frame sequences, the thin line non-translated sequences. The 3′ end of the cDNA containing the poly(A) tail is depicted as A_n. Restriction enzymes include: A, *Ava*I; Ap, *Apa*I; B, *Bgl*II; Bs, *Bst*EII; H, *Hin*dIII; Hf, *Hin*fI; P, *Pst*I; PII, *Pvu*II; S, *Sau*3A. *b*, DNA sequence and translation of V1-3 sequence. Amino-acid residues are indicated with a one-letter symbol below the sequence. The exact position of exons designated 1–6 (see Fig. 2*a*) within V1-3 *bcr* cDNA is shown by the numbers above the sequence. DNA sequencing was performed using the dideoxy-chain termination method[14] on restriction enzyme fragments of the cDNA subcloned into M13 phage[15]. All regions were sequenced on both strands.

fragments of patient 0319129 (unpublished results) and of the cell line K562. DNA sequence analysis of the breakpoint regions of these fragments and that of the sequence of the corresponding chromosome 22 regions enabled us to define the point of translocation for these DNAs (see Fig. 2). None of the breakpoints analysed here could be located within an exon, indicating that in the Ph′ translocation, breakpoints occur within intron regions of *bcr*. For four of the six DNA samples analysed, the translocation would result in the transcription of a mRNA that includes *bcr* exons 1 and 2 (see Fig. 2); in two CML DNAs the third exon would be additionally incorporated in the chimaeric *bcr*/c-*abl* mRNA. Exon 3 encodes only 25 amino acids, not substantially increasing the size of the chimaeric protein.

A knowledge of the DNA sequence of the translocation junction point may provide additional information about the mechanism of chromosomal translocation. Figure 3*a* shows the sequence of the region containing the crossover point for translocation in the DNA of patient 0319129 and compares this with normal chromosome 22 and 9 DNA sequences. In 0319129 DNA, the chromosomal break has occurred in a rather 'precise' manner, leading to the generation of a $22q^-$ and $9q^+$ sequence exactly reflecting the sequence of the normal chromosome 22 and 9 DNA sequences. However, at the breakpoint, a C nucleotide is found in both the $9q^+$ and 22q sequences, whereas the chromosome 22 sequence contains a G at that position. Note that both chromosomes 9 and 22 contain limited stretches of homology near the break (Fig. 3*a* underlined); a DNA search revealed homology of this region to human *Alu* repetitive sequences.

No such similar stretches of homologous sequences between chromosomes 9 and 22 occur at the breakpoint in the DNA of patient 0212015 (Fig. 3*b*) or in that of K562 DNA (data not shown). In the $9q^+$ DNA of patient 0212015, sequences are found between the breakpoints on chromosomes 9 and 22 (Fig. 3*b*) which are not present in the sequenced region of the normal chromosomes 9 and 22. This suggests that in the $9q^+$ chromosome a secondary event has taken place; it is possible that after translocation of chromosome 22 sequences to chromosome 9, a deletion affecting chromosome 9 sequences occurred. The arrow indicating the breakpoint on chromosome 9 (see Fig. 3*b*) would then represent the point of deletion. In addition, compared with the normal chromosome 9 sequences, the chromosome 9 sequences of the $9q^+$ fragment contain 13 nucleotide changes within an 81-bp stretch. These changes may reflect differences between individuals (the control chromosome 9 sequences were isolated from a human lung carcinoma cosmid library); however, the number of nucleotide substitutions would be very high in such an event. Moreover, the chromosome 22 part of the $9q^+$ fragment contains no nucleotide changes in either intron or exon sequences, favouring the explanation of inefficient DNA repair on deletion of chromosome 9 sequences.

These results indicate that *bcr* is part of a gene oriented with its 5′ end towards the centromere of chromosome 22. In the Philadelphia translocation, a break occurs within *bcr*, and sequences from chromosome 9 which contain the human c-*abl* oncogene in all Ph′-positive cases examined[7,9] are translocated to the 3′ of the truncated *bcr* gene. The joining of *bcr* and c-*abl* sequences is highly specific for CML, as this configuration has been found in complex translocations[9] and even in the leukaemic cells of one CML patient cytogenetically lacking the Ph′ chromosome[11]. Because the orientation of c-*abl* on chromosome 9 is also centromere-5′-3′-telomere, *bcr* and c-*abl* are joined in a

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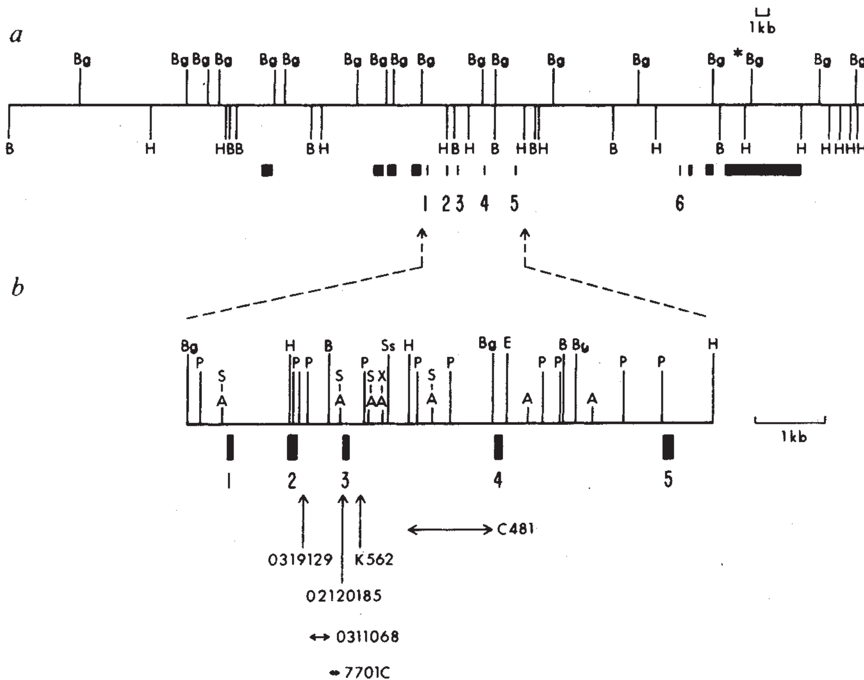

Fig. 2 Genomic organization of the *bcr* gene. *a*, Restriction enzyme map of chromosome 22 sequences encompassing *bcr*. Exons are indicated by black boxes below the restriction enzyme map. The position of the numbered exons has been determined by sequencing; all other exons were located by hybridization to the V1-3 cDNA. The asterisk indicates a polymorphic *Bgl*II restriction enzyme site. *b*, Restriction enzyme map of the breakpoint cluster region, with the exons as indicated in *a*. Below the map, the approximate positions of the breakpoints in different CML DNAs are indicated by horizontal or vertical arrows. Restriction enzymes include: A, *Ava*I; B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI, H, *Hin*dIII; P, *Pst*I; S, *Sma*I; Ss, *Sst*I; X, *Xho*I. The breakpoint of the 22q$^-$ chromosome from DNA 0319129 was cloned as a 9.5-kb *Bgl*II fragment in Charon 30 according to previously described methods[16]. 9q$^+$ fragments were isolated by cloning a 7.2-kb *Bam*HI fragment and a 7.7-kb *Eco*RI fragment into Charon 30 and λgtwes, respectively.

head-to-tail fashion on the Ph' chromosome. Although the distance between the most 5' v-*abl* homologous to v-*abl* and the physical breakpoint may vary from 14 kb (patient 0319129) to >100 kb (K562), the effect of the translocation on the expression of the *bcr* gene and c-*abl* seems to be very similar in different patients: in K562 and in Ph'-positive CML patients, abnormal RNA transcripts of ~8.5 kb are detected, which hybridize to both c-*abl* and 5' *bcr* exon probes. The molecular cloning of a chimaeric cDNA from K562 cells has provided definitive proof for the existence of chimaeric mRNA[12]. The chimaeric mRNAs must be the result of transcription initiating at the promoter of the *bcr* gene; depending on the exact location of the breakpoint, the transcript will include all 5' exons in addition to either exons 1 and 2 or exons 1, 2 and 3 of *bcr*. Recent sequence analysis of K562 *bcr*/*abl* cDNA confirms the variable presence of the third exon. In K562 we found that, as predicted, the third exon is present in the cDNA, immediately preceding c-*abl* sequences. In contrast, Southern blot analysis of the molecularly cloned 22q$^-$ genomic DNA fragment of, for example, patient 0319129 unambiguously demonstrates that in this patient exon 3 has been removed from the Ph' chromosome and will not be included in a chimaeric *bcr*/*abl* transcript. Transcription continues into the c-*abl* oncogene, including, as a minimum, the most 5' v-*abl*-homologous exon and all exons 3' of it, including the phosphotyrosine acceptor site[13]. We do not know whether the inclusion of exon 3 in the chimaeric mRNA has an effect on the progression of the disease.

Chromosomal aberrations may be generated by specific events involving recombination-prone DNA sequences. Alternatively, such recombination events could occur almost at random. In either case, a very limited number of translocations will result in gene alterations leading to the disruption of normal growth and differentiation. In the Ph' translocation, we have found that breakpoints on chromosome 9 are spread over a region of up to 100 kb. The breakpoints on chomosome 22 occur within a smaller region of around 5.0 kb. Nonetheless, no sequence homology can be found between breakpoint regions of different CML patients or coding regions of c-*abl* and *bcr* genes. Therefore, we may conclude that the processes underlying the Ph' translocation are random recombination events. Once such recombinations result in a genomic configuration that allows the transcription of chimaeric *bcr*/*abl* mRNA, malignant proliferation of specific cell types may occur. It seems highly likely that this chimaeric mRNA is translated into protein because an

Fig. 3 Breakpoint sequences of the DNAs of two CML patients. *a*, Sequence of 0319129 DNA; the sequences are in a 5'-3' orientation. Normal chromosome 9 sequences (first line) are from non-CML DNA; the 9q$^+$ and 22q$^-$ sequences (second and third lines) are from DNA of patient 0319129. Normal chromosome 22 sequences (fourth line) are from non-CML DNA. An arrow indicates the breakpoint on chromosomes 9 and 22; the nucleotide C found in both the 9q$^+$ and 22q$^-$ sequences at the breakpoint is boxed. Limited regions of homology between the normal chromosome 9 and 22 sequences are underlined. *b*, Breakpoint sequence of 02120185 DNA; normal chromosome 9 and 22 sequences (first and third lines in each set) were from non-CML DNA. The 9q$^+$ sequence on the second line contains an area boxed to indicate that it does not originate from the normal chromosomes 9 or 22 sequenced in the present experiments. Dots above the chromosome 9 sequences indicate nucleotide differences at those positions from the 9q$^+$ chromosome. The beginning of exon 3 (see Fig. 2*b*) in the 9q$^+$ and 22 sequence is indicated in the figure. Small restriction enzyme fragments containing the breakpoints were chosen for sequence analysis, based on restriction enzyme mapping data and comparison with normal chromosome 9 and 22 maps.

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abnormally sized 210,000-M_r c-*abl* protein was detected in K562 cells. In contrast to the normal c-*abl* protein, the P210 has tyrosine kinase activity[6].

It is tempting to speculate that the *bcr* moiety of the fusion protein is responsible for this effect. However, although the consequences of the Ph' translocation on a molecular level are becoming evident, it remains to be established whether this phenomenon is actually the cause or merely one of the steps that eventually result in CML.

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Cloning, sequencing and expression of cDNA for a novel subunit of acetylcholine receptor from calf muscle

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The nicotinic acetylcholine receptor (AChR) from fish electric organ has a subunit structure of $\alpha_2\beta\gamma\delta$, and this is thought to be also the case for the mammalian skeletal muscle AChR[1-3]. By cloning and sequencing the complementary or genomic DNAs, we have previously elucidated the primary structures of all four subunits of the *Torpedo californica* electroplax[4-6] and calf muscle AChR[7-10] and of the α- and γ-subunits of the human muscle AChR[7,11]; the primary structures of the γ-subunit of the *T. californica* AChR[12] and the α-subunit of the *Torpedo marmorata* AChR[13,14] have also been deduced elsewhere. We have now cloned DNA complementary to the calf muscle messenger RNA encoding a novel polypeptide (the ε-subunit) whose deduced amino-acid sequence has features characteristic of the AChR subunits and which shows higher sequence homology with the γ-subunit than with the other subunits. cDNA expression studies indicate that the calf ε-subunit, as well as the calf γ-subunit, can replace the *Torpedo* γ-subunit to form the functional receptor in combination with the *Torpedo* α-, β- and δ-subunits.

The initial cDNA clone for the calf ε-subunit was isolated by cross-hybridization with a calf γ-subunit cDNA probe in an experiment aimed at cloning an upstream cDNA sequence for the calf γ-subunit by primer extension (see Fig. 1 legend). Figure 1 shows the nucleotide sequence of the mRNA encoding the calf ε-subunit precursor, deduced from the cDNA sequence, together with the predicted primary structure of the polypeptide. We deduce that the ε-subunit precursor consists of 491 amino acids including a hydrophobic prepeptide[15] of 20 amino acids, and calculate the relative molecular masses of the ε-subunit and its precursor to be 52,568 and 54,562, respectively.

Figure 2 shows the alignment of the amino-acid sequences of the calf ε-subunit precursor and the calf, human and *T. californica* γ-subunit precursors. The mature calf ε-subunit shows 53, 53 and 57% sequence homology with the mature calf, human and *Torpedo* γ-subunits, respectively, whereas the degrees of its homology with other mature AChR subunits are as follows: calf, human and *Torpedo* α-subunit, 30% (refs 4, 7), calf β-subunit, 37% (ref. 8), *Torpedo* β-subunit, 39% (ref. 5), calf δ-subunit, 45% (ref. 10) and *Torpedo* δ-subunit, 44% (ref. 5). Homology among the mature γ-subunits is 92%, 56% and 55%, respectively, for the calf/man, calf/*Torpedo* and man/*Torpedo* pairs. Because the calf ε-subunit shows similar degrees of homology with the calf, human and *Torpedo* γ-subunits, the divergence of the ε- and γ-subunits may have occurred around the time of separation of mammals and fish. Thus, it is possible that fish have a second, undetected gene encoding a γ/ε-family subunit, which may or may not now be active.

The calf ε-subunit shares structural features common to all four subunits of the fish and mammalian AChR[4-12,14]. It contains four strongly hydrophobic segments[4-12,14] (M1-M4) and an amphipathic segment[16-18] (MA). Our expression studies using site-directed mutagenesis of the α-subunit cDNA indicate that these five putative α-helical transmembrane segments are all required for the operation of the ionic channel[18]. The hydrophilicity profile[19] and the predicted secondary structures[20] of the calf ε-subunit are also similar to those of the other AChR subunits[4-12,21]. These findings suggest that the ε-subunit is oriented across the membrane in the same manner as the four other subunits. Three cysteine residues (aligned at positions 128, 142 and 231) and one potential *N*-glycosylation site[22] (aligned at position 141) are conserved in all AChR subunits whose sequences are known[4-14]. The calf ε-subunit contains two additional potential *N*-glycosylation sites (aligned at positions 66 and 307), one of which (position 66) is assigned to the extracellular side of the membrane[4-12,14]. Because our expression studies are consistent with the presence of a disulphide bridge between the conserved cysteine residues 128 and 142 of the α-subunit[18], the corresponding cysteine residues of the ε-subunit may also form a disulphide bond.

To examine whether the ε-subunit can function as an AChR subunit, we synthesized the mRNA specific for this subunit (or the γ-subunit) *in vitro*[23,24] using the cDNA template and injected the mRNA, combined with the *Torpedo* α-, β- and δ-subunit-specific mRNAs synthesized similarly[18], into *Xenopus* oocytes. The oocytes were then tested for response to acetylcholine (ACh) applied ionophoretically by recording the intracellular potentials. All of the 61 oocytes injected with the calf ε-subunit and the three *Torpedo* mRNAs and 38 of the 56 oocytes injected with the calf γ-subunit and the three *Torpedo* mRNAs responded to ACh (Fig. 3) with mean ACh sensitivities (for the responsive oocytes) of 510 and 8 mV μC^{-1}, respectively. None of 60 control oocytes injected only with the *Torpedo* α-, β- and δ-subunit-specific mRNAs was responsive ($<\sim 0.2$ mV μC^{-1}). The mean ACh sensitivity of 70 responsive oocytes out of 72 oocytes injected with the *Torpedo* α-, β-, γ- and δ-subunit-specific mRNAs[18] was 1,300 mV μC^{-1}. The atropine-resistant and (+)tubocurarine-sensitive nature of the observed ACh responses (Fig. 3) indicates the formation of the nicotinic AChR. These results show that the calf ε-subunit, as well as the calf γ-subunit, can be functionally substituted for the *Torpedo* γ-subunit.

Blot hybridization analysis using ε-subunit cDNA probe of poly(A)$^+$ RNA from the skeletal muscle of a 4-month-old fetal calf revealed a hybridization-positive species with an estimated size of ~4,200 nucleotides (Fig. 4*A*, lane *a*). No hybridizable species was detected, however, in poly(A)$^+$ RNA from the skeletal muscle of a 7-month-old fetal calf (Fig. 4*A*, lane *b*), a 9-month-old fetal calf or a newborn calf or from adult bovine diaphragm (data not shown).

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