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Molecular Cloning and Serological Characterization of an Altered *c-abl* Gene Product Produced in *Ph*¹ CML Patients

A.-M. Mes-Masson, J. McLaughlin, and O. Witte

Summary

The reciprocal translocation between human chromosomes 9 and 22, termed the Philadelphia chromosome (*Ph*¹), is observed in more than 90% of patients with chronic myelogenous leukemia. This translocation fuses sequences from a variable distance 5′ to the c-*abl* locus on chromosome 9 to sequences in a breakpoint cluster region (*bcr*) on chromosome 22. The appearance of the *Ph*¹ chromosome is correlated with the production of a novel 8.7-kb RNA transcript containing both *bcr* and c-*abl* sequences as well as with a 210-kd phosphoprotein ($p210^{c\text{-}abl}$) representing non-*abl* polypeptide sequences fused to c-*abl*-derived sequences. Antibodies prepared to a number of different c-*abl* domains and to *bcr* determinants were employed to characterize the normal and altered c-*abl* gene products. By combining a variety of cDNA cloning techniques, we have isolated *bcr*/*abl* clones representing 8.7 kb of contiguous mRNA sequence.

A. Introduction

The c-*abl* gene is the normal cellular homolog of v-*abl* (p160), the transforming gene of Abelson murine leukemia virus (A-MuLV) (Witte et al. 1979; Goff et al. 1980). A-MuLV is a replication-defective, rapidly transforming retrovirus which can transform early B lymphoid and other hematopoietic cell types both in vitro and in vivo (Rosenberg et al. 1975; Rosenberg and Baltimore 1976; Whitlock and Witte 1986). The v-*abl* protein is a chimeric protein in which the amino terminal sequences of the protein are derived from the group antigen gene (*gag*) of the Moloney murine leukemia virus, while the remaining carboxy terminal sequences of the protein are derived from a large protein of the murine c-*abl* gene (Witte et al. 1979; Reddy et al. 1983; Wang et al. 1984). Several lines of evidence have shown that the tyrosine-specific kinase activity of the v-*abl* protein, encoded by c-*abl*-derived sequences, mediates the ability of A-MuLV to cause neoplastic transformation (Witte et al. 1980; Rosenberg et al. 1980; Prywes et al. 1983).

Recently, c-*abl* cDNA cloned from both human and mouse cell lines (Shtivelman et al. 1985; Ben-Neriah et al. 1986a) has been used to map the c-*abl* locus. The mature c-*abl* mRNA represents the assembly of 11 different exons whose sequences are brought together by virtue of an RNA splicing mechanism (Fig. 1). Exons 1, 2, and part of exon 3 represent 5′ c-*abl* sequences not found in v-*abl*. In addition, four different exon 1 species, which are individually spliced to the remaining exons, have been found in murine cells (exon 1/types 1–4) (Ben-Neriah et al. 1986a). Exon 1/type 1 and exon 1/type 4 are the most common forms of exon 1 found in mature murine c-*abl* mRNA. To date, exon 1/type 1 has been identified in human cells and shares extensive sequence homology with the murine form of exon 1/type 1.

Chronic myelogenous leukemia (CML) is a disease of the pluripotent stem cell which progresses through a series of stages

Molecular Biology Institute, University of California, Los Angeles, CA 90024, USA

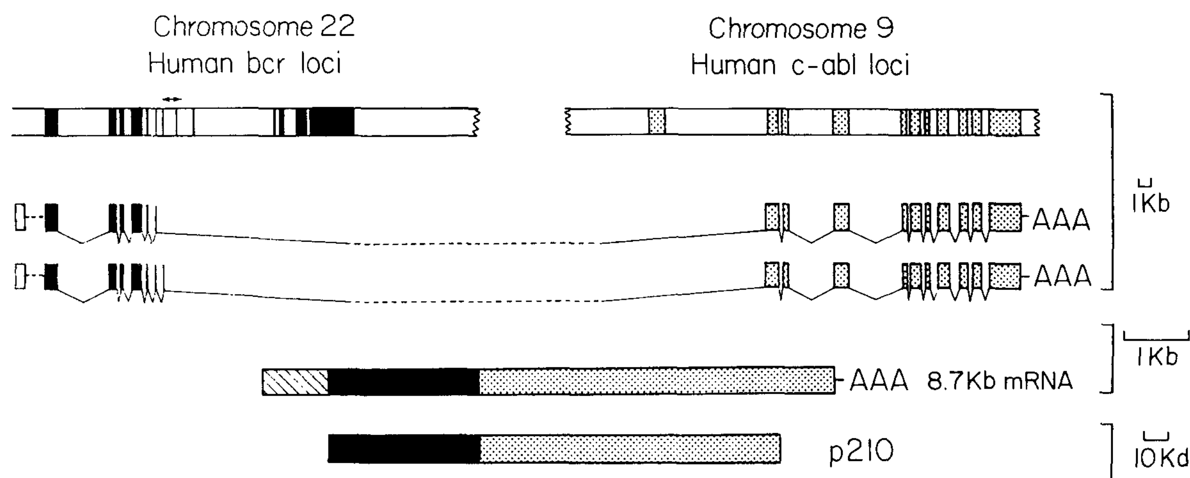

Fig. 1. Model for generating the CML-specific 8.7-kb mRNA and P210$^{c\text{-}abl}$. The *bcr* locus on chromosome 22 (Heisterkamp et al. 1985) and the c-*abl* locus on chromosome 9 (Shtivelman et al. 1985) are partners in the Ph^1 translocation whose breakpoints occur within a 5.8-kb region on chromosome 22 within the *bcr* gene (↔) and at a more variable distance 5′ to the c-*abl* gene (Groffen et al. 1984; Heisterkamp et al. 1983). The variable breakpoint and intron sequences are removed by splicing to generate the 8.7-kb mRNA. As indicated in the figure, microheterogeneity in the splicing pattern of the 8.7-kb mRNA has been observed (Shtivelman et al. 1985). cDNA cloning results demonstrated the presence of approximately 1 kb of novel sequence at the 5′ end of the 8.7-kb mRNA (*hatched box*) which does not form part of the normal *bcr* gene transcript (Mes-Masson et al. 1986). The 8.7-kb mRNA gives rise to an altered form of c-*abl*, P210$^{c\text{-}abl}$, which has a structurally altered amino terminus (Konopka et al. 1984a)

(chronic, accelerated, and blast crisis) where cells become less regulated in their growth and differentiation properties (for review see Koeffler and Golde 1981). Translocation of the c-*abl* gene from chromosome 9 to 22 (t9:22), resulting in the Philadelphia chromosome (Ph^1), occurs in over 90% of CML patients (Rowley 1973). The translocation breakpoint generally occurs within a limited region in a gene on chromosome 22 (termed the *bcr* gene) and at a variable distance 5′ to the c-*abl* gene on chromosome 9 (Heisterkamp et al. 1983; Groffen et al. 1984). Analysis of RNA expression in Ph^1-positive CML cell lines has detected a unique 8.7-kb c-*abl* mRNA (Cananni et al. 1984; Collins et al. 1984). cDNA cloning of a portion of this 8.7-kb mRNA suggests that transcription begins on chromosome 22 and continues through the junction with chromosome 9 to some point downstream of the c-*abl* gene (Shtivelman et al. 1985; Grosveld et al. 1986). RNA splicing joins exons from the *bcr* gene on chromosome 22 to the first common exon of c-*abl* (exon 2) in such a way as to preserve the reading frame of the c-*abl* gene (Fig. 1). A structurally altered c-*abl* protein (P210$^{c\text{-}abl}$), with an in vitro kinase activity similar to that of the v-*abl* protein, has been detected in a number of Ph^1-positive CML cell lines and direct clinical specimens (Konopka et al. 1984a; Konopka and Witte 1985). These results have strongly implicated c-*abl* in the pathogenesis of CML.

B. P145 and P210 Protein Structure

Large regions of the v-*abl* protein, expressed as fusion proteins in bacteria, were used as immunogens to prepare antisera specific for different regions of the v-*abl* protein (Konopka et al. 1984b). Since v-*abl*-specific antisera displayed cross-reactivity to c-*abl* and P210$^{c\text{-}abl}$, these immunological reagents were used to characterize both forms of the *abl* protein (Konopka et al. 1984a; Konopka and Witte 1985). The P210$^{c\text{-}abl}$ form has acquired new amino acids at its N-terminus, while C-terminal sequences remain indistinguishable from c-*abl*, a configuration reminiscent of the relationship between v-*abl* and c-*abl*. A similar, if not identical, protein is seen in a number of CML lines and patient samples, despite the wide range of different t9:22 breakpoints. In vivo

tyrosine phosphorylation has been observed for P210$^{c\text{-}abl}$ but not P145, although both display in vitro tyrosine activity. Although either protein can act as its own substrate in the in vitro tyrosine kinase assay, c-*abl* appears to be phosphorylated at one or two major sites, whereas P210 is phosphorylated at two to three major sites and a number of minor sites. The direct relationship between the 8.7-kb mRNA and P210 has recently been demonstrated using antiserum prepared against *bcr* determinants (Ben-Neriah et al. 1986 b). This antiserum was able to immunoprecipitate P210 and a 190-kd protein which is a candidate for the normal *bcr* protein.

C. Hybrid 8.7-kb mRNA Structure

The CML-specific 8.7-kb mRNA has been shown, by partial cDNA cloning, to be a hybrid transcript of *bcr* and c-*abl* sequences (Shtivelman et al. 1985; Grosveld et al. 1986). While a portion of the hybrid gene had been cloned, we were interested in isolating a clone which represented the entire mRNA, with special attention to clones reaching the 5′ end. To enhance recovery of *abl*-related mRNA, poly (A) selected RNA from K562 cells, a CML-derived cell line, was initially size selected over a sucrose gradient generating a ten fold enrichment of the CML-specific mRNA. Since the hybrid 8.7-kb mRNA represents a long transcript, a cDNA cloning protocol which optimized the recovery of large cDNA clones was established. Although this protocol is described in more detail elsewhere (A.-M. Mes-Masson, J. McLaughlin and O. N. Witte, in preparation), a few features stand out as being important in the recovery of large cDNA clones. These include optimizing conditions for first-strand cDNA synthesis, priming first-strand synthesis with both oligo d(T) and an internal oligonucleotide from the conserved tyrosine kinase domain of c-*abl*, and, after second-strand synthesis and the addition of EcoRI linkers, size-selecting the resulting cDNA library over a sucrose gradient before ligation to lambda arms. When this cDNA library was initially screened with a v-*abl* homologous probe, over 1000 phage plaques scored positive. In order to concentrate our efforts on clones which would provide information on upstream sequences of the hybrid mRNA, a subset (300) of the phage DNA was tested for its ability to hybridize to a number of probes, including exon 1, exon 2, and exon 3 of c-*abl*, as well as to oligonucleotides complementary to known *bcr* mRNA sequences. In this manner, clones which putatively represent the entire CML-specific 8.7-kb mRNA were isolated.

In order to establish the identity of the isolated cDNA clones, we sequenced regions corresponding to those reported to be found in *bcr*- and c-*abl*-specific sequences. Partial sequence analysis confirmed the identity of these clones. In addition, we determined the sequence at the 5′ end of various cDNA clones, which will be reported elsewhere (Mes-Masson et al. 1986). The cDNA clones were analyzed by using various portions of the cDNA clones as probes in a Northern analysis of RNA from two different CML-derived cell lines, K562 and EM2, as well as from HL60, a promyelocytic leukemia cell line. Sequences derived from the c-*abl* gene (approximately 5.7 kb at the 3′ end of the 8.7 mRNA) hybridized with the CML-specific 8.7-kb mRNA in K562 and EM2 cells, in addition to the normal 7-kb and 6-kb c-*abl* mRNA in all cell lines. Sequences from the 5′ end of the mRNA up to, but not including, the first kb of sequence were also able to hybridize with the 8.7-kb mRNA in K562 and EM2 cells, as well as to normal 4.5- and 6.7-kb *bcr* mRNA in all cell lines. A different pattern of hybridization was observed when the first Kilobases of 5′ cDNA sequences were used to probe the Northern blot. In this instance, while hybridization still occurred with the 8.7-kb mRNA in K562 and EM2 cells, the sequence failed to hybridize with either of the normal *bcr* mRNAs. However, this sequence does appear to hybridize strongly to an RNA species of approximately 4 kb in length in all three cell lines tested. The identity of this 4-kb RNA is presently being investigated. These results suggest the presence of non-*bcr* sequences at the 5′ end of the 8.7-kb mRNA (Fig. 1).

We are presently attempting to express the P210$^{c\text{-}abl}$ protein produced by the 8.7-kb mRNA in normal cell lines in order to assess

the role of P210$^{c\text{-}abl}$ in oncogenic transformation. We hope that eventually the overexpression of the P210$^{c\text{-}abl}$ protein in either eukaryotic or prokaryotic vectors will allow us to study the function of this protein in greater detail.

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