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OXIDANT-INDUCED RESTRICTION POLYMORPHISM MAPS TO KINASE REGION OF C-*ABL* ONCOGENE

C.J. SCHMEICHEL and S.A. WEITZMAN*

Section of Hematology/Oncology, Department of Medicine, Northwestern University Medical School, Chicago, Illinois 60611, USA

Reactive oxygen species generated by activated human phagoctyes can cause a variety of genetic injuries and produce malignant transformation in target cells. We previously reported that DNA extracted from phagocyte-transformed 10T1/2 mouse fibroblasts contained Msp I-dependent restriction fragment length polymorphisms in the c-*abl* oncogene. The data suggested that the oxidant-induced RFLP resulted from an alteration in the methylation pattern in c-*abl*. We have now mapped one of these RFLP to a specific 'CCGG' tetramer found within the tyrosine kinase region of the gene. The polymorphic 'CCGG' site has been localized to the intron between exon 2 and 3a. Restriction analysis indicates that a repetitive sequence exists within this intron and that the RFLP is associated with this repeat.

KEY WORDS: Oxidant, phagocyte, methylation c-*abl*, tyrosine kinase, oxygen radical.

INTRODUCTION

Reactive oxidants generated by human phagocytes have been implicated in the development of several malignancies associated with chronic inflammation, including colon and bladder cancer.[1-5] Oxidants, both *in vitro* and *in vivo*, induce heritable injury to cellular DNA after which cell transformation or tumor progression are observed.[6-10] These findings suggest that the role of oxidants in carcinogenesis may involve activation of oncogenes or inactivation of anti-oncogenes important in the initiation or promotion stages of malignancy. In our laboratory we have shown that human neutrophil-derived oxidants transform C3H10T1/2 mouse fibroblasts *in vitro*.[11] The DNA from the individual cell lines transformed were positive in the NIH3T3 cell transformation focus-forming assay indicating that a stable modification of the DNA in the C3H10T1/2 cells had occurred.[12,13] Extensive screening of the DNA from the transformed cell lines revealed that Msp I restriction fragment length polymorphisms (RFLP) were present in the c-*abl* gene, but not in c-*myc*, Ki-*ras*, or v-*mos*.[14] Initial hybridization studies revealed that the polymorphisms were localized to the region of c-*abl* containing exons common to each of the four *abl* messages previously described.[14,15] As part of studies to determine the potential significance of these RFLP in oxidant-induced cell transformation, MspI sites resulting in c-*abl* polymorphisms were mapped.

*To whom correspondence should be addressed.

METHODS

Southern blot analysis was performed by standard methodologies.[16] Briefly, fifteen μg of DNA was digested for 3 hours at 37° C with 10 U/μg of restriction enzyme (purchased from Bethesda Research Laboratories, Gaithersburg, MD). DNA was electrophoresed through 0.8 to 1.0% agarose gels for 16 to 20 hours at 1.5 v/cm in 89 mM tris-borate, 89 mM boric acid, and 2 mM EDTA. DNA was transferred by capillarity to Hybond nylon membrane (Amersham, Arlington Heights, Il) according to manufacturers directions. Blots were UV fixed for 5 min at 300 nM, prehybridized for 3 to 5 hours and hybridized 20 to 24 hours at 62 to 68° C in Hoeffer hybridization chambers (Hoeffer, San Francisco, CA). Hybridization buffers included 6X SSC, 5X Denhardt's solution, and 5 mM EDTA with 0.1 mg/ml herring sperm DNA. Oligomer hybridizations were performed at 48 to 55° C for varying times and blots were washed with buffers of increasing stringency as previously described.[17,18] DNA probes were labeled with ^{32}P by the random primer method.[19] After hybridization filters were washed and subjected to autoradiography at -70° C. DNA probes were prepared from c-*abl* Type I cDNA[14] and c-*abl* genomic inserts cloned into PUC 13 plasmid. The plasmids were generously supplied by Andre Bernards (Type I), and George Daley and Michael Paskind (genomic), of the Whitehead Institute, Massachusetts Institute of Technology.

RESULTS

The murine c-*abl* gene encodes a cytoplasmic tyrosine kinase. Two major and two minor RNA messages each having variable 5′ ends have been described.[15] The mRNA

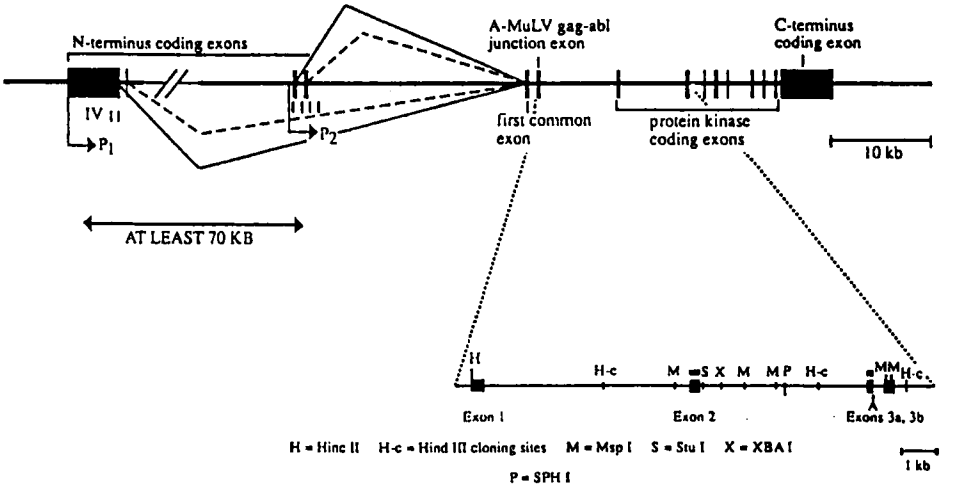


FIGURE 1 Schematic map of the mouse *cabl* gene showing the 3′ common exons encoding the tyrosine kinase region and the four alternative 5′ exons responsible for the four unique c-*abl* mRNA. P1 and P2 designate promoters which initiate the major mRNA transcripts. Restriction mapping of the region between exons 1 and 3b are shown to scale in a blow up of the kinase region. Proposed sites for the restriction endonuclease Hinc II (H), Msp I (M), Stu I (S), Xba I (X), Sph I (P) and Sma I (A) are indicated. H-c are the Hind III cloning sites used to clone the genomic sequences discussed. The mapping of the Stu I and the Xba sites, and the sequencing of exon 3a and 3b were performed by M. Paskind and G. Daley (personal communication).

species arise from splicing of alternate 5′ exons to a common set of 3′ exons (20) (Figure 1, adapted from the c-*abl* map provided by Andre Bernards). The 3′ exons encode the tyrosine kinase portion of the c-*abl* protein. The Msp I polymorphisms observed in c-*abl* in the DNA from cells transformed by neutrophil oxidants were originally detected with v-*abl*. V-*abl* consists of sequences spanning only the common exon and those exons encoding the tyrosine kinase activity of the protein.[21] The same polymorphisms were observed when c-*abl* Type I DNA was used as a probe in place of v-*abl* (data not shown). The Type I cDNA is derived from the major mouse c-*abl* mRNA and contains the 5′ variable exons together with the 3′ common exons found in v-*abl*[15,20] (personal communication, Andre Bernards). This finding confirmed localization of the Msp I RFLP to regions of c-*abl* 3′ to the variable 5′ exons.

Digestion of the Type I cDNA with the restriction endonuclease Sma I generated a 1 kb probe derived from the 5′ end of the Type I cDNA to the Sma I site designated in Figure 1. Hybridizations with this probe detected each of the RFLP described (Figure 2). RFLP were not detected using cDNA probes mapping 5′ to the Hinc II site (see map Figure 1, data not shown). Further localization of two of the RFLP was achieved with a 0.25 kb cDNA probe which spans the 3′ 0.17 kb of exon 2 and all 90 base pairs of exon 3a (Figure 1). The 0.25 kb probe detected 4 bands in the Msp I digested DNA isolated from untransformed 10T1/2 cells including a 3 kb, a 2.3 kb, a 1.8 kb and a 1.4 kb band (Figure 3A, lane 1). Each of the 4 bands were also

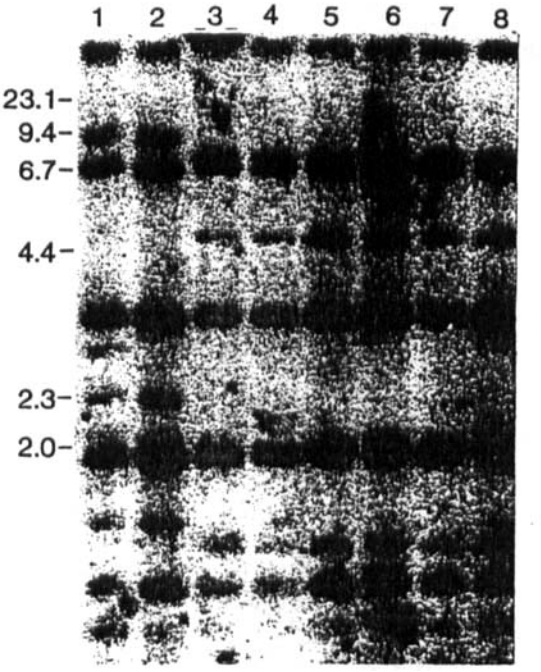
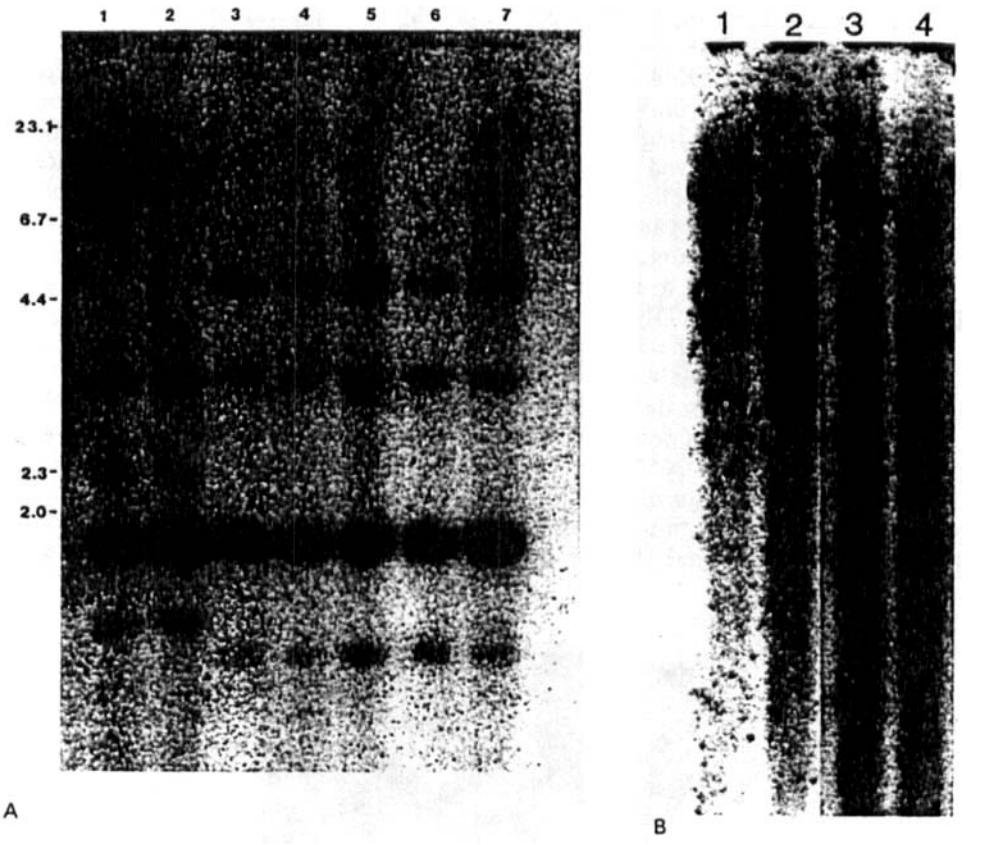


FIGURE 2 Restriction fragment length polymorphisms in Msp I sites of the c-*abl* gene in oxidant transformed cells. DNA samples digested with Msp I were analyzed in Southern blots. Lanes contain DNA from the following: 1, untransformed 10T1/2 cells; 2, 10T1/2 cells transformed chemically with 3-methylcholanthrene; 3–8, cells transformed with phagocyte-generated oxidants. The blot was hybridized with the cDNA extending from the 5′ end of the Type I cDNA to the Sma I site designated in Figure 1.



detectable in DNA from normal C3H mouse spleen.[14] The 3 kb and the 1.8 kb bands are constant in DNA from both control and transformed C3H10T1/2 cells, whereas the 2.3 and 1.4 kb bands are unique to the control DNA. In contrast, DNA from the phagocyte-transformed cells exhibit unique 4.8 and 1.2 kb bands. RFLP were not detected when hybridizations were performed with cDNA probes mapping immediatly 3′ or 5′ to the 0.25 kb cDNA probe (data not shown).

Our previous studies demonstrated that the RFLP detected in c-*abl* were specific for Msp I indicating that the Msp I recognition site, 'CCGG' was altered.[14] Analysis of the c-*abl* sequence within and proximal to the region mapped by the 0.25 kb probe revealed that no 'CCGG' sites were present in exon 1, exon 2 or exon 3a.[21,22] The intron between exon 3a and 3b also did not contain 'CCGG' sites (unpublished observation, M. Paskind and G. Daley). The cDNA 'CCGG' sites nearest to the region containing the Msp I RFLP were 20 and 100 base pairs downstrean in exon 3b. Two twenty base pair synthetic polydeoxynucleotide oligomers were constructed, each surrounding and containing one of the two exon 3b 'CCGG' sites at the center of the oligomer, based on known sequence information. Strict oligomer hybridization conditions of Southern blots prepared with Msp I-digested DNA from control and transformed cells revealed that the 5′ 'CCGG' site in exon 3b was not polymorphic (Figure 3B). Hybridizations directed at the downstream 'CCGG' site gave similar

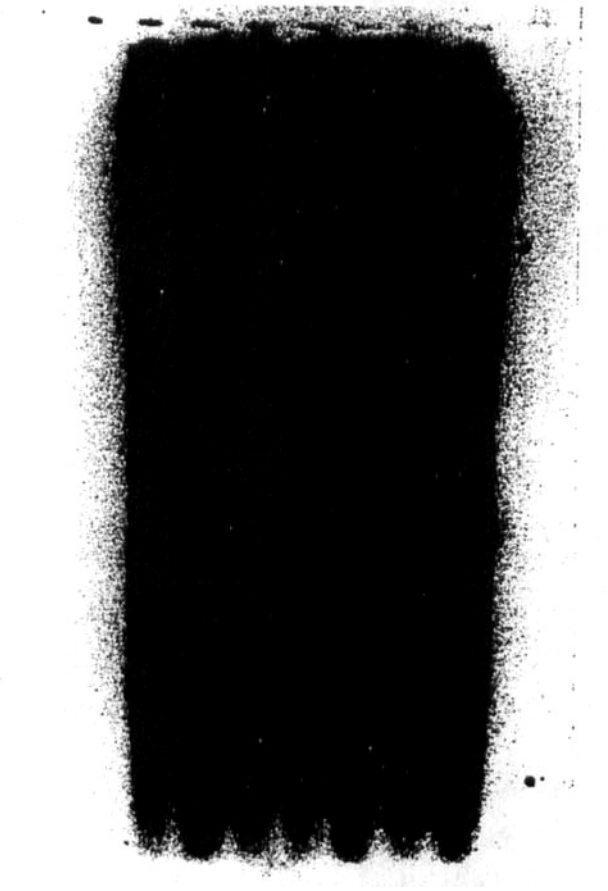


FIGURE 3 Localization of the restriction fragment length polymorphisms in Msp I sites of the *cabl* gene in oxidant transformed cells. DNA samples digested with Msp I were analyzed in Southern blots. Lanes contain DNA from the following: 1, untransformed 10T1/2 cells; 2, 10T1/2 cells transformed chemically with 3-methylcholanthrene; other lanes, cells transformed with phagocyte-generated oxidants. Hybridizations were performed with the 0.25 kb c-*abl* cDNA (A), the twenty base oligomer directed to the 5' 'CCGG' site in exon 3b (B), and the Xba I to Hind III genomic clone (C).

results (data not shown). These findings indicated that the RFLP detected by the 0.25 kb cDNA probe must result from 'CCGG' sites contained within the intron between exons 2 and 3a.

A genomic probe containing the intron sequences between the Xba I and Hind III sites (Figure 1) was hybridized to the southern blot originally probed with the 0.25 kb cDNA. An intense hybridization signal was observed along the entire length of each lane containing the individual control and experimental samples (Figure 3C). Repeat hybridizations of fresh Southern blots with the same and digested pieces of the Xba I to Hind III probe yielded identical results (data not shown). The type of hybridization signal observed is typical of probe hybridizations to repetitive sequences contained throughout the genome.[23,24] Hybridizations with genomic probes mapping 3' to the Sph I site (Figure 1) were negative for RFLP (data not shown).

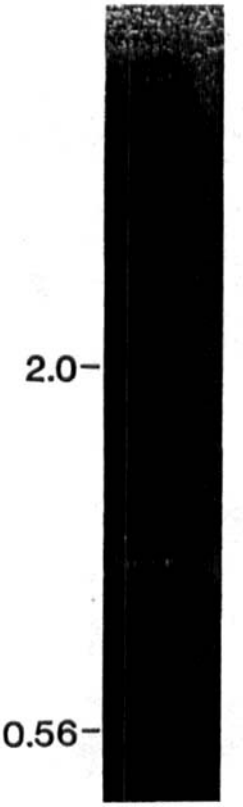


FIGURE 4 Defining the Msp I sites between the Xba I and the Hind III sites. The DNA segment of the genomic clone extending from the Xba I site to the Hind III site (Figure 1) was digested with Msp I and run into a 1% agarose mini-gel containing 0.5 ug/ml of ethidium bromide. Molecular size markers are indicated.

Msp I digestion of the genomic DNA segment mapping from the Xba I site to the 3' Hind III site demonstrated the presence of two 'CCGG' sites (Figure 4). Restriction mapping of this region placed the Msp I sites ~0.610 and ~1.2 kb 3' to the Xba I site (Figure 1). Msp I sites were not detectable between the Sph I and the 3' Hind III sites (data not shown). A single Msp I site was localized to 0.7 kb 5' to the Stu I site (Figure 1B). The absence of detectable RFLP after hybridization with probes mapping 3' to the Sph I site suggests strongly that the major RFLP detectable in c-*abl* in the DNA from oxidant-transformed cells results from an alteration in the Msp I site 0.61 kb 3' to the Xba I site.

DISCUSSION

C3H10T1/2 cells transformed by human neutrophil-derived oxidants exhibit several Msp I dependent polymorphisms in c-*abl*.[14] We have localized the major RFLP to the tyrosine kinase region of the *abl* gene. Further our data demonstrate that the RFLP

exist in the intron between exon 2 and exon 3a and that no RFLP exist in the tyrosine kinase coding region from the common exon through exon 'B'. Restriction mapping revealed that two 'CCGG' sites exist in the intron between exon 2 and 3a; one site ~0.61 kb and the second ~1.2 kb 3′ to the Xba I site downstream of exon 2. Our data suggest that the 5′ 'CCGG' site within this segment is polymorphic as hybridizations with DNA mapping downstream of the 3′ 'CCGG' site did not detect RFLP, whereas DNA hybridizing upstream of the 5′ 'CCGG' site did detect RFLP (data not shown).

Our previous studies demonstrated that the polymorphisms were restricted to alterations only in Msp I recognition sites.[14] Msp I polymorphisms could theoretically arise from point mutation, duplications, rearrangements, deletions, or from methylation of the 5′ cytosine of 'CCGG' tetramers. Absence of observable polymorphisms with other restriction enzymes effectively rules out rearrangement, duplications or deletions. The polymorphisms we have described appear homozygous suggesting that the RFLP do not result from point mutation but instead result from Msp I sensitivity to methylation at the 5′ cytosine. However, Msp I polymorphisms generated by sensitivity to 5′ cytosine methylation are infrequent. One can expect that most 'CCGG' sites in genomic sequences will be available for Msp I digestion since the majority of methylations occur at the inner cytosine residue. Therefore, assuming that each Msp I site downstream of the 5′ Hind III site is digested by Msp I, hybridization with the 0.25 kb cDNA probes will detect only the 2.3 and the 1.4 kb bands unique to the DNA from the untransformed C3H10T1/2 cells. We have not yet identified the regions which give rise to the 3 and 1.8 kb bands common to DNA from both control and transformed cells. We are currently sequencing the genomic region between the Xba I and Sph I sites to verify the predicted number and location of Msp I sites. This information should help identify the mechanism by which the observed RFLP are generated.

While methylation of DNA has long been associated with regulation of DNA transcription, the functional result of the specific methylation changes noted here are unknown.[25] It is therefore of particular interest that the RFLP in c-*abl* are associated with a region of repetitive sequences, as transcriptional regulatory sequences have been identified within and associated with repeats in rat insulin 1 and SV40 genes.[26–28] Therefore, this modification in the c-*abl* sequence may alter the timing in the cell cycle, the rate, or the magnitude of tyrosine kinase message transcribed.

Preliminary findings from our laboratory demonstrate that DNA from the oxidant-transformed C3H10T1/2 cells exhibit homozygous Msp I-dependent polymorphisms in the Genes c-*yes*, c-*src*, and possibly c-*fyn*.[29–31] These genes, like c-*abl*, encode cytoplasmic tyrosine kinases and, similar to c-*abl*, the polymorphisms have been localized to the conserved region of the genes encoding tyrosine kinase activity. Our data, therefore, suggests that the neutrophil-derived oxidants may have induced specific concerted modifications in the methylation pattern of a family of genes.

Acknowledgements

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