Differential Expression of Type-Specific *c-abl* mRNAs in Mouse Tissues and Cell Lines

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The mammalian *c-abl* proto-oncogene produces mRNAs with 5' heterogeneity from two distinct promoters and the alternative splicing of variable 5' exons. By using quantitative RNase protection assays, the relative abundance of two major *c-abl* mRNAs, type I and type IV, in several mouse tissues and cell lines has been determined. Our results demonstrate that the level of type IV *c-abl* mRNA is rather constant, whereas that of the type I mRNA varies over a 10-fold range in different tissues and cell types. This finding has interesting implications for the function of the two *c-abl* proteins.

c-abl is an evolutionarily conserved proto-oncogene (6). In mammalian cells, two *c-abl* mRNAs have been detected by Northern (RNA) blots (12, 16). The mouse *c-abl* RNAs of 6.5 and 5.3 kilobases (kb) are found in a variety of tissues and cell types throughout the mouse life cycle (12, 16). cDNA clones of the two *c-abl* mRNAs have been prepared from mouse and human cells (1, 15; J. Y. J. Wang, unpublished data). Nucleotide sequences of the cDNA clones have revealed that the two *c-abl* mRNAs differ at their 5' ends. The 5' heterogeneity is generated by the alternative splicing of 5' variable exons, transcribed from two distinct promoters, to a set of common exons (2, 15, 17; Fig. 1). The 5' exon of the longer *c-abl* mRNA, 6.5 kb in mouse (type IV [1]) or 7 kb in human (type Ib [15]), is transcribed from a promoter at least 90 or 175 kb upstream from the mouse or human *c-abl* common exons (3). The shorter *c-abl* mRNA, 5.3 kb in mouse (type I [1]) or 6 kb in human (type Ia [15]), is transcribed from a promoter approximately 17 or 19 kb upstream from the common exons (2, 15; Fig. 1). Although the lengths of the mouse and human *c-abl* RNAs differ, their nucleotide sequences are 98% homologous in these variable exons (15). In addition to the two major types of *c-abl* cDNA clones, two minor *c-abl* cDNA species (types II and III) were isolated by Ben-Neriah et al. (1). Sequences corresponding to the type II and III exons were not found in the human genome (2, 15). The type II cDNA clone contains a repetitive sequence and could be generated by splicing errors, since corresponding mRNA species could not be detected in cells (1, 2). The type III 5' exon is located immediately downstream from the type I exon in the mouse genome (Fig. 1), and the type III *c-abl* mRNA was reported to be 1 to 5% of the total *c-abl* RNAs in mouse cell line 70Z/3 (1, 2).

Because the 5' variable *c-abl* exons contain protein-coding sequences, the mouse or human *c-abl* gene encodes at least two proteins with different N-terminal amino acids (1, 15). The type IV *c-abl* protein contains an N-terminal glycine, a possible site for myristylation (14). It has been proposed that the N-terminal variability may dictate the subcellular localization of the two *c-abl* proteins, the type I soluble and the type IV membrane bound (1). As previously reported, levels of the two *c-abl* mRNAs in tissues and cell lines varied when detected on Northern blots (16). Because the different *c-abl* mRNAs encode dissimilar proteins, accurate determination

of their relative abundance may provide insight into the biological function of the two *c-abl* proteins. By using quantitative RNase protection assays, we have determined the relative concentrations of the *c-abl* mRNAs in several mouse tissues and cell lines. Our results show that the expression of the type-specific *c-abl* mRNAs is differentially regulated in normal tissues.

Cell lines and tissues. Total cellular RNA was purified as previously described (5) from brain, liver, spleen, kidney, heart, skeletal muscle, and thymus of 5- to 6-week-old or 16- to 17-week-old BALB/c mice and from the following cell lines: NIH 3T3 fibroblasts; SCRF-60A, a lymphoid fibrosarcoma cell line of New Zealand black mouse (10); 70Z/3, a pre-B-lymphoma cell line (13); S49T, a BALB/c T-cell lymphoma line (8); and S194, a BALB/c myeloma immunoglobulin A producer line (9).

Antisense RNA probes. Types I and IV *c-abl* cDNA clones were prepared from RNA of SCRF-60A cells (Wang, unpublished data). Restriction fragments from cDNA clones were subcloned in the antisense orientation into Riboprobe SP64 and SP65 plasmids (Promega Biotec). Splice junction-spanning antisense probes were derived from cDNA fragments which contained the first 290 base pairs of a common-exon sequence and a given 5' variable-exon sequence (Fig. 2A; also see Fig. 4A). Exon-specific probes contained a given unique 5' exon sequence or the first 283 base pairs of the common-exon sequence. Antisense RNA probes were prepared with SP6 RNA polymerase and α -³²P-labeled rCTP by the method of Melton et al. (11).

Relative abundance of type I and IV *c-abl* mRNAs. Quantitation of type I or IV *c-abl* RNA was achieved by RNase protection assays with antisense RNA probes which spanned the splice junction (Fig. 2A). A completely hybridized probe would lose 16 nucleotides (nt) of polylinker sequence after RNase digestion to yield either a full-length protected fragment of 396 nt for the type I mRNA or a fragment of 352 nt for the type IV mRNA (Fig. 2A). If a junction-spanning probe hybridized to a noncorresponding *c-abl* RNA, only the common-exon sequence of 290 nt would be protected from RNase digestion (Fig. 2A). Typical results of these RNase protection assays are shown in Fig. 2B. On these gels, the intensity of a full-length type I fragment (396 nt) represents the amount of type I RNA; that of the common-exon fragment (290 nt) represents the amount of other types of *c-abl* RNA in a given test RNA sample. The faint bands above the full-length protected fragments are

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FIG. 1. Schematic representation of the 5' region of the *c-abl* gene in mice and humans. The figure is not drawn to scale. P, Promoter; the symbols are self-explanatory. For details, see references 2, 3, and 15.

residual undigested probes which are longer by 16 nt. Different amounts of either total tissue or cell RNA were used in these assays to standardize the amount of *c-abl* RNAs present in the hybridization reactions (Fig. 2B). Variations in the amount of test RNA sample used in the assays did not alter the experimental results; i.e., the same

FIG. 2. RNase protection assays for types I and IV *c-abl* mRNA. (A) Types I and IV cDNA clones, junction-spanning probes, and the lengths of protected fragments. *Eco*RI (R1) to *Hinc*II (H) fragments of *c-abl* types I and IV cDNAs were cloned into SP64. A *Pst*I site (P) is 7 nt down from the 5' splice site of the first common exon. Junction-spanning antisense RNA probes containing a small amount of plasmid polylinker (L) were transcribed by SP6 RNA polymerase and yielded full-length protected fragments of 396 nt (396N) for type I, 352 nt for type IV, and 290 nt when only the common-exon region was protected. (B) Examples of RNase protection assays. Total cellular RNA from brain (38 μ g), liver (48 μ g), spleen (17 μ g), and the cell lines 70Z/3 (7.5 μ g) and S194 (14.7 μ g) were coprecipitated with gel-purified antisense RNA probes at a 1,000-fold molar excess of probe to total *c-abl* RNAs and hybridized in 80% formamide overnight at 45°C (11, 18). Unhybridized probes were digested with a combination of RNase A (80 μ g/ml) and RNase T₁ (20 μ g/ml), and the protected probes were precipitated with carrier tRNA (20 μ g) and run on 9 M urea-6% polyacrylamide gels. A small amount of background probe which escaped RNase digestion can be seen above the protected fragments in two of the lanes: brain, type IV (368 nt), and 70Z/3, type I (412 nt).

FIG. 3. Junction-spanning or variable-exon probes gave similar assay results. (A) Type I cDNA clone and the lengths of the various protected fragments. A type I junction-spanning probe of 412 nt yielded either a full-length protected fragment of 396 nt or a common-exon protected fragment of 290 nt. A common-exon probe (299 nt) yielded a protected fragment of 283 nt (283N) because cloning with *Pst*I deletes the first 7 nt of the common exons. Type I variable-exon probe (142 nt) yielded a protected fragment of 126 nt. See Fig. 2 legend for explanation of abbreviations. (B) RNase protection assay with 20 μ g of RNA from SCRF-60A cells hybridized to variable-exon probe (lane 1), common-exon probe (lane 2), or junction-spanning probe (lane 3). The three probes were of equal specific activity, and a 1,000 molar excess of each probe was used.

relative concentration of a given type of *c-abl* mRNA was obtained in liver cells whether 20 or 50 μ g of liver RNA was added to the hybridizations. This was because the probe was consistently added at a 1,000-fold molar excess over the total *c-abl* RNAs during hybridizations.

In assay with junction-spanning probes, the relative abundance of a given type of *c-abl* mRNA is determined by the intensities of the full-length and common-exon protected fragments, after correction for the number of cytidines in each fragment, according to the equation (full length/full length + common) $\times$ 100%. To verify that a junction-spanning probe could give an accurate determination of the relative abundance of a type-specific RNA, a set of exon-specific probes was tested in the protection assay (Fig. 3A). To directly measure the abundance of a given exon sequence, an antisense probe corresponding only to that exon was prepared (Fig. 3A). With an identical 1,000-fold molar excess of probe to *c-abl* RNAs, the relative abundance of the type I mRNA could also be determined by the ratio of the intensity of a type I exon protected fragment (126 nt [Fig. 3B, lane 1]) to that of a common-exon protected fragment (283 nt [Fig. 3B, lane 2]). Experiments showed that the same value was obtained whether the assay was done with a probe spanning the splice junction (Fig. 3B, lane 3) or with a 5' variable-region probe and a common-region probe (Fig. 3B, lanes 1 and 2). This result also showed that all of the type I exon sequences were in the correctly spliced form and that the junction-spanning probe did provide a valid assay of the relative abundance of type-specific *c-abl* RNA.

TABLE 1. Relative abundance of types I and IV *c-abl* mRNAs

Tissue or cell line	Mean abundance (%) $\pm$ SD ^a of:	
	Type I	Type IV
Tissues		
Liver	26 $\pm$ 4	46 $\pm$ 5
Brain	23 $\pm$ 4	47 $\pm$ 6
Kidney	28 $\pm$ 4	47 $\pm$ 3
Heart	53 $\pm$ 7	31 $\pm$ 4
Muscle	52 $\pm$ 1	25 $\pm$ 2
Spleen	45 $\pm$ 5	30 $\pm$ 1
Thymus	46 $\pm$ 3	28 $\pm$ 4
Cell lines		
S194	23 $\pm$ 5	57 $\pm$ 7
SCRF-60A	41 $\pm$ 4	29 $\pm$ 4
S49T	34 $\pm$ 3	43 $\pm$ 5
NIH 3T3	59 $\pm$ 3	24 $\pm$ 3
70Z/3	61 $\pm$ 3	21 $\pm$ 2

^a Values obtained from at least four independent experiments. The intensities of the full-length and the common-exon protected fragments were obtained by densitometry and corrected by the number of cytidines present in each fragment; relative abundance was calculated as shown in the text.

All tissues and cell lines tested contained types I and IV *c-abl* RNAs, but their relative abundance varied (Fig. 2B). The quantitative results are summarized in Table 1. Interestingly, the seven tissues can be grouped into three categories with respect to the relative abundance of the *c-abl* mRNAs (Table 1). In brain, liver, and kidney, type I RNA is half as abundant as type IV RNA, whereas it is twice as abundant in heart and skeletal muscle (Table 1). In lymphoid tissues such as spleen and thymus, type I RNA is about 1.6 times more abundant than type IV RNA (Table 1). The ages of the mice did not have an effect on the relative abundance; the same results were obtained with 5- to 6-week-old or 16- to 17-week-old tissues (spleen, liver, and brain). The tissue-specific expression pattern of the *c-abl* mRNA types was reflected in some cell lines. NIH 3T3 fibroblasts had 2.5 times more type I than type IV mRNA, a result similar to that found with muscles; and a lymphoblastoid thymoma cell line (SCRF-60A) contained 1.4 times more type I than type IV RNA, a result similar to that obtained with normal thymus (Table 1). It appears that less-differentiated cells contained more type I RNA, e.g., pre-B-cell line 70Z/3 and NIH 3T3 embryo fibroblasts, whereas mature B-cell line S194 or T-cell line S49T contained more type IV mRNA (Table 1).

Expression of type III exon sequence. In all the RNA samples tested, types I and IV mRNAs accounted for 70 to 84% of the total *c-abl* RNAs (Table 1). The other 16 to 30% could be made up by the two minor *c-abl* mRNAs (types II and III) reported by Ben-Neriah et al. (1). The type II variable exon contained a repetitive sequence, and its corresponding mRNA was not detected, but the type III mRNA was found at a very low level in 70Z/3 cells (1, 2). To measure the level of the type III *c-abl* RNA in mouse tissues, two antisense probes were prepared from the type III cDNA clone (Fig. 4A). The type III junction-spanning probe (576 nt), when hybridized to total tissue or cell RNA, did not yield any detectable full-length protected fragment (560 nt), albeit a prominent common-exon-protected fragment of 290 nt was found on gels (Fig. 4B, lanes 2 and 4). A fainter band (270 nt) was also detected in assays with the type III junction-spanning probe, and that length correlated with protection by an unspliced type III exon. The 270-nt pro-

FIG. 4. RNase protection assay of type III *c-abl* mRNA. (A) Type III cDNA clone, antisense probes, and lengths of the protected fragments. Type III junction-spanning probe of 576 nt (576N) yielded either a full-length protected fragment of 560 nt or a common-exon protected fragment of 290 nt (290N). Variable-exon probe (286 nt) yielded a protected fragment of 270 nt. See Fig. 2 legend for explanation of abbreviations. (B) RNase protection assays with type III probes. 70Z/3 RNA (15 μ g; lanes 1 and 2) or mouse brain RNA (76 μ g; lanes 3 and 4) was hybridized to either a type III variable-exon probe (lanes 1 and 3) or a junction-spanning probe (lanes 2 and 4) in the RNase protection assay. An undigested variable-exon probe of 286 nt is found in lanes 1 and 2, and a small amount of undigested junction-spanning probe of 576 nt is found in lanes 2 and 4.

ected fragment was shown to represent type III exon by using a type III variable-region-specific probe (Fig. 4B, lanes 3 and 5). The persistence of the undigested probe (286 nt) was most likely due to a large molar excess of the probe added, since the abundance of the type III exon sequence in the test RNA samples was not previously known. The type III-specific protection of the 270-nt fragment was not observed when tRNA or no RNA was added to the hybridization reactions. The unspliced type III exon was found to be present at 3 to 8% of the abundance of the *c-abl* common-exon sequences in all tissues and cell lines tested. The level of the mature type III mRNA was at least five times lower and was not detectable in our assays. The type III exon has been mapped to be contiguous with the type I exon in the mouse genome; thus, it is included in the first intron of the type I or the type IV *c-abl* RNA (Fig. 1 [2]). The finding of a type III exon sequence in total RNA samples suggests either that the first *c-abl* intron persists in cells or that the majority of the type III exon is spliced onto undefined exons in the *c-abl* locus.

Since the type III *c-abl* RNA did not make up the 16 to 30% of *c-abl* RNAs unaccounted for by the types I and IV RNAs in our assays, it is a formal possibility that yet other types of *c-abl* RNAs exist in cells and tissues. The lack of corresponding cDNA clones implies that other types of RNAs do not exist in significant abundance. Several other possibilities might explain the observed discrepancy between the sum of types I and IV RNAs and the total *c-abl* RNAs measured by the common-exon probe. The discrep-

TABLE 2. Estimated concentration of total and type-specific *c-abl* mRNAs

Tissue or cell line	No. of copies of <i>c-abl</i> mRNA/cell ^a		
	Total ^b	Type I ^c	Type IV ^c
Tissues			
Liver	20	5	10
Brain	30	7	14
Kidney	40	11	19
Heart	50	26	16
Muscle	60	31	15
Spleen	80	36	24
Thymus	110	51	30
Cell lines			
S194	50	12	28
SCRF-60A	60	25	20
S49T	80	27	34
NIH 3T3	150	92	32
70Z/3	150	89	36

^a Calculations were based on the assumption that every cell in a given tissue contained 3×10^5 poly(A) RNAs and that every cell expressed *c-abl* mRNA.

^b NIH 3T3 and 70Z/3 cells contained the highest amount of total *c-abl* RNA, 0.04 to 0.07% of total poly(A) RNA (16), which was approximately 150 copies per cell. For the other tissues and cell lines, the copy numbers of the total *c-abl* RNA were calculated relative to the levels in NIH 3T3 and 70Z/3 cells (e.g., 8 μ g of 70Z/3 RNA gave the same amount of protection to a probe as 25 μ g of S194 RNA); therefore, S194 contained approximately 50 copies of total *c-abl* RNA per cell.

^c Calculated for relative abundance as shown in Table 1, footnote *a*.

ancy could be due to experimental conditions that favored the detection of the shorter common-exon-protected fragment over the longer full-length protected fragment, e.g., less nonspecific degradation of the shorter fragment during RNase treatment. It was also possible that the 5' ends of the *c-abl* RNAs were preferentially degraded during RNA preparation. Alternatively, 16 to 30% of the common-exon sequences may have been incorrectly spliced to nonspecific sequences because of the large first intron (>90 kb) of the type IV primary transcript. Several of the *c-abl* 5' cDNA clones we have isolated from our cDNA library had 5' exons which did not contain open reading frames that were in frame with the common-exon sequences (Wang, unpublished data). One of these pseudo 5' exons was found to be located in the same genomic DNA fragment as the type IV exon, whereas another was found to share a 10-nucleotide sequence identical to the type IV exon at the splice junction. These observations showed a positional or structural relationship between the pseudo exons and the type IV exon. Although we could not exclude the possibility that these pseudo-exon-containing cDNAs were generated by template switching during reverse transcription, their isolation indicates that *c-abl* RNAs with random 5' exons may exist in cells. It is likely that the long-distance splicing event is error prone and that the pseudo-exon-containing RNAs, which resulted from mistakes in the splicing reaction, may account for the 16 to 30% loss of the common-exon sequences.

Per-cell copy number of *c-abl* mRNAs. An interesting correlation is noted in our quantitation results; in samples in which the total *c-abl* RNA level was higher, there was relatively more type I mRNA. With knowledge of the levels of the total *c-abl* RNA (16) and the relative abundance of the type-specific mRNAs (Table 1), the concentrations of the type I or type IV *c-abl* RNA in each tissue and cell line can be calculated (Table 2). Among the five cell lines tested, type

IV *c-abl* mRNA was calculated to be 20 to 36 copies per cell, whereas type I mRNA varied from 12 to 92 copies per cell (Table 2). For example, the 70Z/3 and S194 cells contained similar levels of type IV mRNA (36 versus 28 copies per cell), but 70Z/3 cells contained 7.4-fold more type I RNA than S194 cells (89 versus 12 copies per cell). The calculations for the tissues were based on the assumption that *c-abl* RNAs were expressed in every cell of a given tissue. The nonlymphoid tissues, i.e., liver, brain, kidney, cardiac, and skeletal muscle, contain similar levels of type IV mRNA (15 ± 3 copies per cell) but a sixfold variation of type I mRNA (5 to 31 copies per cell). The lymphoid tissues, thymus and spleen, contain levels of type IV mRNA 2-fold higher than those in nonlymphoid tissues; their type I mRNA levels, however, are about 7- to 10-fold higher than that found in liver or brain. These results show that the level of type IV *c-abl* RNA is rather constant in a variety of cell types, whereas the level of type I mRNA is modulated in a tissue-specific manner.

Regulation of levels of *c-abl* RNAs. Because the two *c-abl* RNAs are transcribed from separate promoters (Fig. 1), differential regulation of transcription initiation can achieve a different relative abundance of these two RNAs. As the two 5' exons compete for the same splice acceptor site of the first common exon (Fig. 1), regulation of the efficiency of splicing could also bring about different relative concentrations of these two RNAs. By using nuclear run-on transcription assays, the rate of transcription from the type I promoter was found to be the same in two B-lineage cell lines: S194, which contained 12 copies of type I RNA per cell, and 70Z/3, which contained 89 copies of type I RNA per cell (data not shown). This observation suggests that the regulation of the type I mRNA is not at the level of transcription in these two B-lineage cell lines. However, it does not rule out the possibility of tissue-specific transcription of type I mRNA in other cell types. Evidence in other systems has shown that alternative splicing may be regulated by *trans*-acting factors (4). The alternative splicing of the two *c-abl* 5' exons could also be regulated by *trans*-acting factors whose activities differ according to tissue or cell-type specificity. Alternatively, differential regulation of the stability of the type I and IV *c-abl* RNAs could also account for the variations we observed.

The finding that type IV *c-abl* mRNA is present at a constant level in all tissues and cell types has been corroborated by finding similar levels of type IV *c-abl* protein in S194 and 70Z/3 cells when antibodies specific for the amino acids of the type IV 5' exon were used (A. J. Stumf, A. O. Morla, and J. Y. J. Wang, unpublished data). Measurement of the total *c-abl* protein levels with antibodies for the common-exon sequences showed, however, that 70Z/3 cells had a level of *c-abl* protein approximately threefold higher than that in S194 cells, correlating well with the total *c-abl* RNA levels in these two cell lines (Table 2). The ubiquitous and constant expression of the *c-abl* type IV protein suggests that it may serve a function common to all cells. The *Drosophila abl* homolog encodes only one protein with an N-terminal glycine like the mammalian type IV (type Ib) protein (7). Perhaps the cellular function of the *c-abl* type IV protein has been conserved through evolution. The cell-type-specific regulation of type I *c-abl* RNA indicates that the type I protein may have a more restricted function. The types I and IV *c-abl* proteins share 1,097 identical amino acids, which contain a tyrosine kinase domain. Our results suggest that a slight N-terminal variation may confer different cellular functions on one tyrosine kinase.

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