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Germ-Line Transmission of a c-*abl* Mutation Produced by Targeted Gene Disruption in ES Cells

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A substitution mutation has been introduced into the c-*abl* locus of murine embryonic stem cells by homologous recombination between exogenously added DNA and the endogenous gene, and these cells have been used to generate chimeric mice. It is shown that the c-*abl* mutation was transmitted to progeny by several male chimeras. This work demonstrates the feasibility of germ-line transmission of a mutation introduced into a nonselectable autosomal gene by homologous recombination.

THE INTRODUCTION OF MUTATIONS into the germ line of an organism is one of the most powerful genetic methods for determining the functions of a specific gene product (*1*). Recent advances in the detection of rare homologous recombination events have facilitated the modification of defined chromosomal loci in mammalian cell lines (*2–8*). The use of these techniques in combination with cultured embryonic stem (ES) cells should now allow the replacement of normal cellular genes in the mouse germ line by mutant alleles with defined sequence alterations (*9*). ES cells are pluripotent cells derived from preimplantation mouse embryos (*10*), which can be propagated in culture and subsequently reintroduced into mouse blastocysts by microinjection to form chimeric mice. Such chimeras, if constructed with euploid ES cells, have high rates of transmission of the ES cell component in the germ line (*11*). To date, however, only mutations at the X-linked locus encoding the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT), for which there are genetic selections, have been successfully transferred into the mouse germ line by this strategy (*12, 13*).

We are interested in the function of v-*abl*, the oncogene carried by the Abelson murine leukemia virus (A-MuLV), and its cellular homolog c-*abl* (*14*). A-MuLV causes the rapid induction of lymphosarcoma in susceptible mice and can transform both fibroblasts and lymphocytes in culture (*15, 16*). The human c-*abl* has been implicated in at least two forms of cancer, chronic myelogenous leukemia and acute lymphocytic leukemia, where the gene is activated by chromosomal translocation (*17*). Although much is known about the oncogenic potential of both v-*abl* and c-*abl*, little is known about the function of the normal gene in development or in the life of the adult organism. The c-*abl* gene is transcribed in most tissues to give rise to at least two major mRNAs found in approximately equal abundance (*18*). Postmeiotic spermatids have been shown to express very high levels of a distinctive c-*abl* mRNA (*19, 20*) that is truncated in the 3′ untranslated region by polyadenylation at a novel site (*21*). The roles of the c-*abl* protein (Abl) in these cells and in the rest of the organism remain obscure, although its membership in the class of tyrosine-specific kinases suggests that it may be involved in signal transduction. The generation of defined mutations in c-*abl* in the mammalian germ line could provide insights into the function of the gene product.

We have used homologous recombination to introduce a substitution mutation into the c-*abl* locus of mouse ES cells. We chose to introduce mutations affecting only the COOH-terminal third of the Abl protein, downstream from the tyrosine kinase domain and nuclear targeting sequences (*22*). Since c-*abl* is expressed ubiquitously, we were concerned that introduction of a null mutation would have severe deleterious effects on the development of the mouse, or might even be lethal, at a very early stage. Mutations limited to this region might not represent null mutations and might generate a less severe phenotype. Deletions affecting the COOH-terminus of v-*abl* have shown that this domain is not needed for tyrosine kinase activity or for the transformation of fibroblasts but is important for transformation of lymphocytes. These deletion mutants also exhibit a reduction in the toxicity associated with high-level expression of the wild-type viral oncogene in certain cell lines (*23*). The tissue specificity of the effects of these mutations in A-MuLV suggested that we might obtain informative tissue-specific phenotypes from similar mutations in c-*abl*.

To select for the rare homologous recombination of DNA with the endogenous c-*abl* locus, we designed a DNA construct, pAbXR1, in which a promoterless neomycin-resistance gene (*neo*^{r}) is fused to c-*abl* genomic sequences (*24*); this DNA would confer resistance to the drug G418 only after certain recombination events. Expression of *neo* in the construct could be activated either when a nonhomologous integration event places the sequence next to an arbitrary cellular promoter, or, alternatively, when homologous recombination inserts

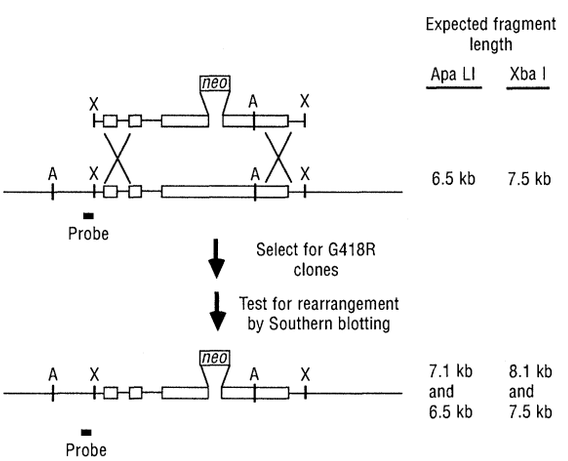

Fig. 1. Scheme for the replacement of the normal c-*abl* gene with a c-*abl–neo* fusion by homologous recombination. A linear DNA containing *neo* embedded in c-*abl* sequences, but devoid of signals for transcription and translation, is introduced by electroporation (*27*). A double crossover in the flanking c-*abl* sequences replaces the normal gene with the fusion gene and activates expression of the *neo*. After digestion of DNA from drug-resistant clones with Apa LI and Xba I, hybridization with the flanking probe EX (*28*) detects DNA fragments of novel sizes from the mutant allele, as well as fragments of the normal size from the unaltered allele. A, Apa LI; X, Xba I.

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the DNA into the target locus (*4, 5*). A requirement of the procedure is that the c-*abl* locus be expressed in the target cell line; we have shown by Northern (RNA) blots that the CCE ES cell line used in this study (*25*) does indeed express the two normal c-*abl* mRNAs (*26*). The plasmid containing the c-*abl*–*neo* fusion was digested with Xba I, the DNAs were introduced into CCE ES cells by electroporation, and the cells were plated into medium containing G418 (*27*). Drug-resistant colonies were isolated, and DNA preparations from these cell lines were examined for rearrangements by Southern (DNA) blotting.

Genomic DNAs from G418^r clones were digested with Apa LI, separated by electrophoresis, transferred to nitrocellulose, and hybridized with a labeled probe (EX) homologous to a region of c-*abl* outside the introduced DNA fragment (*28*). Integration of the *neo* sequences by homologous recombination should convert one copy of the 6.5-kb Apa LI wild-type fragment detected by this probe to a novel 7.1-kb fragment (Fig. 1). We identified seven independent (single copy) homologous integration events out of 239 colonies screened, giving a frequency of 1 in 34 G418^r clones. Examination of the genomic DNA from five of the clones by digestion with other restriction enzymes demonstrated that, in four of the five, the alteration of restriction pattern was as expected for a simple substitution of the c-*abl* region by the added DNA (Fig. 2). Details of the generation of these lines will be presented elsewhere (*29*).

The CCE cell line used for these experiments was originally derived from a single XY blastocyst of the 129/Sv//Ev strain (*25*). The strain is homozygous at the black (*b*) and agouti (*a*) loci. Additionally, the strain is homozygous for the GPI-1^c allele, encoding a rare electrophoretic variant of the glucose phosphate–isomerase enzyme. This line was chosen because it has been shown reproducibly to colonize the germ line of male chimeras with high efficiency (*13, 25*). For the generation of chimeras, host blastocysts were obtained from CD-1, MF1, and C57Bl/6 mouse strains. The CD-1 and MF1 outbred strains are both albino (homozygous for the *c* allele), whereas the C57Bl/6 inbred strain is black, nonagouti (*BB aa*). Thus, chimeric mice could be scored by the presence of pigmented coat hair in the CD-1 and MF1 genetic background, and by the presence of agouti hair in the C57Bl/6 background.

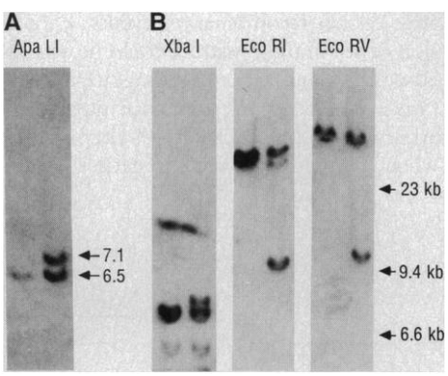

Fig. 2. Southern blot analysis of genomic DNA from individual G418^r cell lines. DNA preparations from various clones were digested and analyzed by Southern blotting. (**A**) DNAs were digested with Apa LI and probed with the Abelson-specific EX probe (*28*). Left lane, a nonhomologous transformant; right lane, clone 2b1. The bands derived from the parental c-*abl* allele (6.5 kb) and from the rearranged allele (7.1 kb) are indicated by arrows. (**B**) DNAs were digested with the indicated enzyme and hybridized with nick-translated pAb3sub3 probe (*14*). Left lanes, CCE control line; right lanes, clone 2b1.

Table 1. Rate of generation of overt chimeras with the 2b1 cell line.

Genotype of host blastocyst	Blastocysts transferred	Progeny born (percent of blastocysts transferred)	Overt chimeras (percent of progeny born)	Phenotypic males (percent of overt chimeras)
CD-1	156	49 (31)	20 (41)	15 (75)
MF1	189	80 (42)	42 (52.5)	27 (64)
C57Bl/6	331	106* (32)	29 (32)*	21 (71)

*Of these progeny, 91 survived to an age to permit scoring for coat color chimerism.

Six clonally derived cell lines, each containing one disrupted allele of c-*abl*, were initially tested for their ability to generate chimeric mice (*30*). For each line, approximately 10 to 15 cells were injected into 3.5-day-old blastocysts derived from CD-1 matings. These were then transferred into pseudopregnant females and allowed to develop to term. A single cell line, termed 2b1, that gave the best rate and extent of chimerism in the resulting live-born animals was chosen for more extensive analysis. A G-banding analysis of metaphase chromosomes showed that 2b1 was predominantly euploid XY (25/32 spreads scored; 78%). It was also noted on separate occasions that a consistently higher proportion of cells than normal (approximately 20%) were tetraploid. When the line was karyotyped after ten passage generations (approximately 35 doublings) after transformation, a significant proportion (10%) of the cells had acquired a specific chromosomal translocation (6:14 fusion).

Data on the rate of chimera formation by the 2b1 line, in combination with host blastocysts from different strains, are presented in Table 1. The overall frequency of formation of chimeric animals was approximately equal in all backgrounds (32 to 52% of live-born animals examined). The degree of ES cell colonization to the coat was markedly influenced by the genetic background of the host blastocyst (Fig. 3, A to C). We consistently found that the ES cells contributed very extensively in combination with C57Bl/6 blastocysts. In the CD-1 background coat pigmentation was less pronounced. This poor contribution in CD-1 mice could not be attributed to the use of outbred albino recipients; injections of 2b1 cells into blastocysts of MF1 mice, another outbred albino strain, yielded better chimeras, intermediate between the CD-1 and C57Bl/6 chimeras (Fig. 3C). We conclude that the genetic background provided by the CD-1 outbred strain does not favor the incorporation of 129-derived ES cells.

Since the CCE cell line was derived from a male and is of XY genotype, one indicator of good chimera formation is a distortion of the normal ratio of male to female animals among the chimeras born. This is because of the high localized incorporation of Y-bearing cells into the genital ridge, which induces the development of a male reproductive system. There was only slight sex distortion among chimeras derived from the 2b1 line in any strain background.

To screen for germ-line transmission, we caged phenotypically male chimeras with tester females. The CD-1– and MF1-based chimeras were mated to albino females, whereas the C57Bl/6-based animals were mated to nonagouti [genotype (C57Bl/6 × DBA/2) F_1] females. From these test matings, the litters were inspected for progeny carrying ES cell–derived agouti pigmentation. Glucose phosphate isomerase analysis of peripheral blood samples was used to verify that the agouti progeny were derived from the CCE cells (*25*).

Of the first 17 male C57Bl/6-based chimeric mice that were proven to be fertile, we have obtained six which have produced agouti progeny. These six chimeras have to date sired a total of 17 agouti offspring in a total of 13 litters (Table 2; examples shown in Fig. 3). In contrast, none of the 11 CD-1–based male chimeras that have been tested has produced any pigmented offspring among the 80 to 180 offspring produced per chimera (Table 2). Similarly, none of the MF1-based chimeras of the first nine tested has yet produced pigmented offspring in the

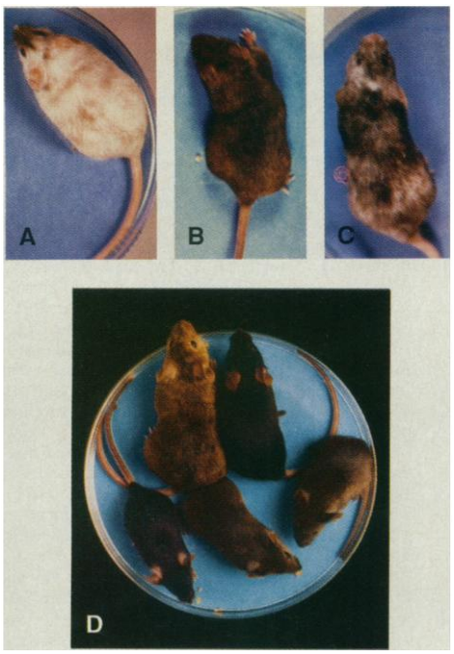

Fig. 3. Examples of chimeric mice and progeny. (**A** to **C**) Chimeras derived with clone 2b1 injected into host blastocysts of various genetic backgrounds. (A) CD-1. (B) C57Bl/6. (C) MF1. (**D**) Members of a family in which the c-*abl–neo* allele has been transmitted in the germ line. The adult brown mouse is male chimera C, and the adult black mouse is a tester female (C57Bl/6 × DBA)F_1. Two of their offspring shown are agouti, indicating transmission of the ES cell markers. Southern blot analysis revealed that one of these two inherited the c-*abl–neo* allele (Fig. 4).

first two litters.

Southern analysis of DNA from the first ten agouti progeny of the chimeric males designated "C," "J," and "O" has demonstrated that six carry the c-*abl* mutation originally present in the 2b1 cell line (Fig. 4). Thus, we have successfully introduced the original mutant allele into the mouse germ line; this allele has been named c-abl^{m1}. These mice, heterozygous for the mutant c-*abl* allele, are phenotypically normal at 10 weeks of age. None have shown the appearance of palpable tumors or lymphomas.

Our use of promoterless selectable markers as a means of selecting for rare homologous recombinants has several advantages. In the case of c-*abl*, we found that 1 in 34 drug-resistant clones had undergone a homologous recombination event. This frequency is sufficiently high to allow the direct and rapid screening of DNAs from cell clones by Southern blotting. If we assume an approximately 100-fold enrichment with this technique, the ratio of unselected homologous to nonhomologous integration events is approximately 1/3400, comparable to frequencies observed at other endogenous chromosomal loci (*2, 6, 7, 31, 32*) and at foreign, exogenously added sequences (*3–5*). The various efficiencies of homologous recombination at different target loci could depend on the level of expression of the target gene, but other factors may be important (*2, 6*).

Another advantage to the method of selection we have used is that it only relies on a single selection in the antibiotic G418, and does not require subsequent selection or screening steps. Previous studies have demonstrated that G418 selection alone does not abolish the ability of ES cells to generate germ-line chimeras (*25, 33*). We feel that it is important that the procedure minimizes the number of passages required before cells are used to generate chimeras. Even with a single selection scheme, there is considerable variability in the quality of independent clones. The culture history and growth characteristics of the ES cells are likely to be major factors in good chimera formation. The 2b1 cell line consistently gave chimeras with an extremely high contribution both to somatic and germ cell lineages. Other mutant clones tested, however, produced significantly fewer and poorer chimeras. It is unclear whether these differences between ES clones are due to exposure to the selective drug, or to heterogeneity in the starting ES cell population that is resolved in the cloning to yield cell lines with a spectrum of different potentials to differentiate. In either case, it may be important to test several independent clones for good chimera formation.

Although the selection for activation of the drug-resistance marker is a powerful method of enrichment for mutant cells, an obvious limitation to the strategy is that it is likely to be applicable only to genes expressed in ES cells. Other selection methods that have been recently described, such as "positive-negative selection" (*6, 34*) and the use of polymerase chain reaction for screening (*7*), may permit targeting of any gene sequence for homologous recombination, whether or not it is expressed. There may be unknown difficulties, however, with the generation of chimeras with cells derived from some of these procedures. Multiple drug selections may further compromise the ability of the cells to colonize the embryo; similarly, the extended passage of the cells required while the targeted cells are being retrieved from large pools may adversely affect the pluripotency of the cells.

When we used the mutant ES cell clones to generate chimeric mice, we found that the use of recipient blastocysts from different mouse strains had a significant effect on the degree of contribution to the chimeras. In particular, when CCE cells and clones derived from them were introduced into blastocysts derived from C57Bl/6 mice, many chimeras were obtained that showed a greater than 95% contribution to the coat by the ES cells (*35*). In contrast, chimeras generated in the CD-1 background had much lower levels of contribution to their coat hairs. We are scoring contributions to two different tissues in these different mouse strains (*36*). In the CD-1 albino outbred mice, chimerism is scored by the presence of pigmented coat hair and eyes resulting from ES contribution to melanocytes, derived from neural crest. In the C57Bl/6 chimeras, chimerism is scored by the presence of agouti coat hairs resulting from colonization of the hair follicle, derived from both mesoderm and ectoderm. The different scoring method, however, does not account for the difference between the two strains since, in control experiments, GPI analysis of the internal organs of chimeras generated from the parental CCE cells indicated a strong correlation between overt chimerism and ES cell colonization of the somatic tissues (*35*). We

Table 2. Breeding data from fertile male chimeras.

Mouse strain used as host blastocyst	Male chimera	ES cell–derived progeny/progeny sired
CD-1 (series 1)	3	0/159
	4	0/83
	5	0/156
	6	0/176
	7	0/80
	9	0/152
	12	0/168
	16	0/126
	17	0/178
	19	0/43
	20.12	0/88
MF1 (series 2)	101	0/26
	103	0/26
	105	0/14
	107	0/41
	108	0/23
	109	0/39
	110	0/33
	114	0/34
	116	0/24
C57Bl/6 (series 3)	C	2/6
	E	0/28
	F	0/52
	J	9/79
	K	0/78
	O	1/3
	Q	0/20
	R	0/20
	S	0/6
	U	0/16
	V	2/6
	Y	0/6
	Z	1/9
	AA	2/6
	BB	0/10
	CC	0/13
	EE	0/10

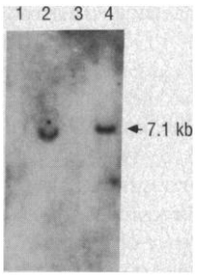

Fig. 4. Southern blot analysis of DNAs from two of the progeny of chimera J. DNAs were isolated from tail biopsies of two agouti-progeny mice (*41*) and from control cell lines, digested with Apa LI, and analyzed on Southern blots hybridized with a pSV2neo probe. Lane 1, CCE control line; lane 2, clone 2b1, containing the mutant allele; lane 3, mouse J2.1; lane 4, mouse J2.2.

therefore believe that the genotypes of the recipient blastocysts can profoundly influence the overall incorporation of ES cells in the developing conceptus. Similar conclusions were reached in an analysis of aggregation chimeras made between the 129 and C57Bl/6 strains; the 129 component was found to predominate in all somatic tissues (*37*).

The differences among strains in the relative efficiency of ES cell contribution to somatic tissues is also reflected in efficiency of contribution to the germ line. For example, although large numbers of germ-line chimeras have been obtained with 129-derived ES cells in an MF1 background (*11, 25*) and in a C57Bl/6 background (*33*), we and others have only been able to generate control germ-line chimeras in the CD-1 background at a very reduced efficiency compared to other strains (*35, 38*). The dependence on host strain may be enhanced when genetically modified ES clones are used; the choice of host can be the crucial factor in obtaining successful germ-line transmission. This conclusion is supported by our test-breeding experiments reported here: whereas none of the 11 chimeras generated in the CD-1 background and none of 9 in the MF1 background have transmitted ES cell markers to their offspring, 6 of the first 17 chimeras tested in the C57Bl/6 background have produced agouti offspring.

The c-*abl* gene has been shown to express a haploid germ cell–specific message, and the protein product of the gene has been demonstrated to be present in cells at late stages of spermatogenesis (*20, 39*). However, we have observed normal rates of transmission of the mutant allele in the passage of the gene through the germ line of chimeras. It should be possible to introduce more severe, null mutations that will abolish the kinase activity of c-*abl*, as well as mutations that specifically prevent formation of one or the other of the alternate forms of c-*abl*. In mice and humans, c-*abl* mRNA species with at least two and perhaps four alternative 5′ first exons have been detected (*40*). Since these exons encode alternate NH_2-termini of the protein, it would be of interest to eliminate each of the two most frequently used first exons and to determine what roles the different c-*abl* proteins have in the mouse.

These experiments demonstrate that a mutation can be introduced into an endogenous nonselectable gene in ES cells by homologous recombination and that the mutation can be transmitted through the germ line of the resultant chimeric mice. The results suggest that it will be possible to generate mutations in mice at any locus defined by a cloned DNA sequence. Using these techniques, we have introduced a mutation that abolishes the COOH-terminal portion of the c-*abl* gene product. Mice heterozygous for the mutation show no growth or developmental defects, demonstrating that this allele does not act in a dominant fashion. Analysis of homozygous c-abl^{m1}/c-abl^{m1} mice should ultimately reveal much about the function of the normal gene.

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CD-1 (Charles River), and MF1 (Harlan Sprague-Dawley) females were used as the source of blastocysts. (C57Bl/6 × CBA) F_1, (C57Bl/6 × DBA) F_1 (Charles River), or CD-1 females were used for foster mothers. Cells for injection were thawed and maintained in culture for no more than six additional passages. Approximately 12 to 15 cells were injected into blastocysts collected 3.5 days post coitus, as previously described (7) [A. Bradley, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, Ed. (IRL Press, Oxford, 1987), pp. 113–151]. Microinjected blastocysts were introduced into the uterine horns of pseudopregnant mice 2.5 days post coitus. Chimerism was scored by coat and eye pigmentation in the CD-1 and MF1 albino background and by the presence of agouti coat color in the C57Bl/6 background.

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Thymotaxin, a Chemotactic Protein, Is Identical to β_2-Microglobulin

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Thymotaxin, an 11-kilodalton protein chemotactic for rat bone marrow hematopoietic precursors, was purified from media conditioned by a rat thymic epithelial cell line. The NH_2-terminal sequence of thymotaxin was identical to that of rat β_2-microglobulin (β2m). Antibodies to β2m removed thymotaxin activity from the fraction containing the 11-kilodalton protein. Chemotactic activity was observed with rat plasma β2m, human β2m, and mouse recombinant β2m, further supporting the identity of thymotaxin with β2m. The directional migration, as opposed to random movement, of the cells was also confirmed. The only rat bone marrow cells that migrated toward β2m were Thy1$^+$ immature lymphoid cells devoid of T cell, B cell, and myeloid cell differentiation markers.

CHEMOTAXIS IS THE DIRECTIONAL migration of cells along soluble gradients of chemical substances. In mammals, several cell types are able to migrate in a chemotactic manner, especially cells from the hematopoietic system, for example, polymorphonuclear cells (*1*), monocytes, and mature T lymphocytes (*2*). It has been suggested (*3*) that chemotaxis is involved in directing the migration of hematopoietic precursors from their site of emergence, the bone marrow, to the thymus. Indeed, we showed that avian thymic peptides attract T cell precursors from quail bone marrow in vitro (*4*). In vivo, migration of hematopoietic precursors into the thymus is a prerequisite for T cell differentiation, which requires the influence of the thymic epithelium and thymic accessory cells (*5*). We also showed that secretion products of rat thymic epithelial cells induce the migration of the hematopoietic precursors from rat (*6*) and mouse (*7*) bone marrow. This chemotactic activity was due to an 11-kD protein called thymotaxin (*6*). In Boyden migration chambers, thymotaxin selectively attracted immature lymphoid cells, devoid of mature T and B cell differentiation markers. These nonreplicating cells failed to grow in methylcellulose when stimulated with growth factors. The selected population did acquire T cell differentiation markers and synthesized T cell receptor α and β chain transcripts on coculture with embryonic thymic tissue (*8*); thus, this population contained T cell precursors. We now report that thymotaxin is biochemically and functionally identical to rat β_2-microglobulin (β2m).

Thymotaxin, secreted in a serum-free medium conditioned by IT-45 R1 rat thymic epithelial cell line (*9*), was purified by gel filtration or reversed-phase high-performance liquid chromatography (HPLC). Column fractions were tested for their ability to induce the migration of rat bone marrow cells in a Boyden chamber assay (*10*). Bone marrow cells were partially depleted of erythroid and myeloid cells and enriched in low-density hematopoietic precursors by centrifugation on a 28% bovine serum albumin (BSA) gradient (*6*). In both gel filtration and reversed-phase HPLC, a cell migration activity was associated with the same 11-kD peptide when analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1, A and B). The active reversed-phase fraction was electroblotted onto a polyvinyl membrane and sequenced directly from membrane strips in a gas phase microsequencer (*11, 12*). The 11 NH_2-terminal amino acids were IQKTPQIQVYS, identical to those of rat β2m (*13*). Since the amino acid sequence of bovine β2m can easily be distinguished from that of rat β2m in this region, we confirmed that thymotaxin was a product of thymic epithelial cells and not a contaminant from the fetal calf serum used in the culture medium.

The biological activity of thymotaxin produced by IT-45 R1 cells was then further investigated. The thymotaxin fraction from reversed-phase HPLC (Fig. 1B, lane c) showed a peak of maximal activity at $10^{-11}M$ and another slightly lower peak at $3 \times 10^{-9}M$ (Fig. 2A). The activity found at $10^{-11}M$ was completely removed by passage over an affinity column prepared with rabbit polyclonal antibodies to mouse β2m (anti-β2m), which cross-react with rat β2m (Fig. 2A). The activity found at $3 \times 10^{-9}M$, which was not retained on the anti-β2m affinity column, seemed to be due to a second peptide of 8 kD, which comigrated with thymotaxin on reversed-phase HPLC (Fig. 1B, lane c) and which could be removed by running the reversed-phase fraction on SDS-PAGE and electroeluting the

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