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The c-*cbl* Proto-Oncogene Is Preferentially Expressed in Thymus and Testis Tissue and Encodes a Nuclear Protein

WALLACE Y. LANGDON,[1,2]* CRAIG D. HYLAND,[2] RAELENE J. GRUMONT,[2] AND HERBERT C. MORSE III[3]

Division of Human Immunology, Institute of Medical and Veterinary Science, Adelaide, South Australia 5000,[1] and The Walter and Eliza Hall Institute of Medical Research, PO Royal Melbourne Hospital, Victoria 3050,[2] Australia, and Laboratory of Immunopathology, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892[3]*

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Cas NS-1 is an acutely transforming murine retrovirus that induces early B-lineage lymphomas and occasional myeloid leukemias. The transforming sequence of this virus, v-*cbl*, shows no homology to known oncogenes but has some similarities to the yeast transcriptional factor GCN4. In this study we used a v-*cbl* probe to analyze mRNAs from a wide range of murine and human hemopoietic tumor cell lines and detected an 11-kilobase mRNA in all lineages. In normal mouse tissues the expression of c-*cbl* was highest in testis and thymus tissues, the predominant species in testis tissue being a 3.5-kilobase mRNA. The v-*cbl* oncogene was inserted into a bacterial expression vector to produce protein for the immunization of rabbits. Affinity-purified v-*cbl* antibodies identified abundant levels of $p100^{gag\text{-}cbl}$ in Cas NS-1-transformed fibroblasts and lower levels of a 135-kilodalton protein ($p135^{c\text{-}cbl}$) in both normal and transformed cells. Subcellular fractionation showed that $p100^{gag\text{-}cbl}$ and $p135^{c\text{-}cbl}$ are both located in the nucleus and retained following 420 mM salt extraction. These results indicate that the translational product of a c-*cbl* is a 135-kilodalton nuclear protein.

The v-*cbl* oncogene was recently identified in the acutely transforming Cas NS-1 retrovirus (10). This novel viral oncogene has no homology to previously described oncogenes but does have some similarities to the DNA-binding domain and transcriptional activation region of the yeast protein GCN4 (10). The tumors induced in mice by Cas NS-1 are predominantly pre-B-cell lymphomas (80%), with pro-B-cell and occasional myeloid tumors contributing the remainder (9; W. Langdon and H. Morse III, unpublished results). Probes prepared from v-*cbl* have identified its cellular homolog in murine and human DNA (10), and chromosome mapping has detected c-*cbl* sequences on mouse chromosome 9 closely linked to *Thy-1* and c-*ets-1* (16). The homologous region in humans is located on the long arm of chromosome 11, in a region which is disrupted by translocations in some acute lymphocytic leukemias (ALLs) and acute non-lymphocytic leukemias (ANLLs) (22). Interestingly, the phenotypes of the ALLs involving translocations in this 11q23 region show similarities to the v-*cbl*-induced pro-B-cell lymphomas in that they coexpress myeloid and early B-lineage surface markers (9, 10, 22).

This report describes the initial characterization of c-*cbl* mRNA that was performed by studying its level and pattern of expression in normal tissues and hemopoietic cell lines. We also describe the production of *cbl*-specific antibodies and the identification of a 135-kilodalton nuclear protein, which we propose is the translational product of c-*cbl*.

MATERIALS AND METHODS

Hemopoietic cell lines. A description of the murine hemopoietic cell lines has been reported previously (6). The majority of the human hemopoietic tumor lines were originally obtained from the American Type Culture Collection, Rockville, Md., and are described in the *ATCC Catalogue* (4). The origin and establishment of BALL-1, a B-cell ALL line, has previously been reported (5), and JM cells, isolated from a patient with a T-cell lymphoma, were provided by A. Boyd, Walter and Eliza Hall Institute of Medical Research.

RNA analysis. Poly(A)$^+$ RNA was prepared as previously described (12). Samples of 4 μg were electrophoresed through 1% agarose gels containing 6% formaldehyde in 1× MOPS (morpholinepropanesulfonic acid) buffer (12). After transfer to nitrocellulose (19), the RNA was hybridized overnight to random-primed probes at 42°C in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate–1× Denhardt solution–50 μg of denatured salmon sperm DNA per ml–10% dextran sulfate. Stringent washes were performed at 60°C in 0.2× SSC–0.1% sodium dodecyl sulfate, and the membranes were exposed to Kodak XAR-5 film at −70°C. The DNA probes were a 418-base-pair *Eco*RI-*Pst*I fragment from v-*cbl* (10) and a 1.1-kilobase (kb) fragment from pRGAPDH (14). GAPDH was used as a control to determine the relative amounts of mRNA in each lane. Gels were also stained with ethidium bromide before and after transfer to determine the levels of mRNA in each lane and to monitor its transfer.

Purification of recombinant v-*cbl* protein. A 1,334-bp *Nci*I-*Xba*I fragment was isolated from the Cas NS-1 proviral clone (10), and the termini were blunt ended by treatment with the Klenow (large) fragment of *Escherichia coli* DNA polymerase I. This fragment, which contains 1,126 base pairs of v-*cbl* sequence and 208 base pairs of untranslated *pol* sequence (10), was then ligated into the *Sma*I site of the expression vector pGEX-2T (17) (see Fig. 4). The plasmid was transformed into *E. coli* JM109 (21), and induction of the *tac* promoter with isopropyl-β-D-thiogalactopyranoside (IPTG) resulted in the synthesis of a 65-kilodalton glutathione-*S*-transferase–v-*cbl* fusion protein (see Fig. 4, lane GEX-cbl). Large-scale production and purification of GEX-*cbl* protein with glutathione agarose beads was carried out exactly as described by Smith and Johnson (17). To purify *cbl* protein, the agarose beads were incubated with 160 ng of human thrombin (Sigma Chemical Co.) per ml in 150 mM NaCl–2.5 mM $CaCl_2$ for 3 h at 37°C, resulting in the release

* Corresponding author.

of a 38-kilodalton *cbl* protein into the supernatant and the retention of glutathione-*S*-transferase on the beads.

Purification of v-*cbl* antibodies. Rabbits were immunized subcutaneously with 400 μg of GEX-*cbl* protein in Freund complete adjuvant and then immunized twice with 200 μg of thrombin-cleaved *cbl* protein in Freund incomplete adjuvant. The antisera recognized both the fusion and thrombin-cleaved *cbl* protein (data not shown). *cbl*-specific antibodies were purified by passing the antisera through a column containing recombinant *cbl* protein bound to cyanogen bromide-activated Sepharose 4B (Pharmacia, Inc.). Bound antibodies were eluted with 0.1 M glycine hydrochloride–0.1 M NaCl (pH 2.6) and immediately neutralized with 1 M Tris hydrochloride (pH 8.0).

Subcellular fractionation and immunoblotting. Protein extracts were prepared from 10^8 normal and Cas NS-1-transformed NIH 3T3 cells by using the cell lysis procedures and salt extraction buffers described by Dignam et al. (2). The subcellular fractions comprised a cytoplasmic extract, a 420 mM NaCl nuclear extract, and a residual nuclei fraction. The residual nuclei were treated with 50 μg of DNase I per ml for 20 min at 25°C before lysis with 2% sodium dodecyl sulfate. All three fractions were adjusted to a final volume of 0.5 ml. Aliquots of 10 μl from each fraction were separated on 12.5% denaturing polyacrylamide gels (8) and transferred to nitrocellulose membranes (20). The membranes were blocked for 1 h in PBS (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4 [pH 7.3]) containing 5% powdered milk (Blotto) and then incubated with 20 μg of affinity pure *cbl* antibody in 20 ml of Blotto for 3 h at 25°C. Membranes were washed and then incubated for 2 h at 25°C with 15 μCi of ^{125}I-labeled protein A (Amersham Corp.). Unbound protein A was removed by three washes in PBS, and the membranes were exposed to Kodak XAR-5 film at −70°C.

RESULTS

c-*cbl* expression in hemopoietic tumors. Poly(A)$^+$ RNA was isolated from a range of murine and human hemopoietic tumor cell lines and analyzed by Northern (RNA) hybridization for the presence of c-*cbl* transcripts. The v-*cbl* probe identified an 11-kb c-*cbl* mRNA in murine pre-B, B, plasmacytoma, T, erythroid, myeloid, macrophage, and mast cell tumor lines (Fig. 1). The B-cell tumor WEHI-231 showed two additional transcripts of 12.2 and 8.2 kb that were expressed at levels equivalent to the 11-kb mRNA. A 3.5-kb transcript was also expressed in a number of lineages, although it was less abundant than the 11-kb mRNA.

Human myeloid, T-cell and B-cell tumor lines were also found to express an 11-kb c-*cbl* mRNA of equivalent size to that of the murine transcript (Fig. 2). A 3.5-kb c-*cbl* mRNA was not detected in the human cells, although a high level of an additional 4-kb mRNA was observed in BALL-1 cells. In these experiments, mRNA from the murine pre-B cell line 70Z was used to determine whether human c-*cbl* mRNA was of equal size to mouse c-*cbl* mRNA. In addition to the 11-kb mRNA, 70Z expressed high levels of a 3.5-kb transcript (Fig. 2).

c-*cbl* mRNA levels are highest in testis and thymus tissues. To examine c-*cbl* expression in normal cells, we prepared poly(A)$^+$ RNA from adult mouse tissues. High levels of c-*cbl* mRNA were found in testis and thymus tissues, and low to very low levels were found in spleen, lung, brain, and heart tissues (Fig. 3). A striking feature of this survey was the presence of a highly abundant 3.5-kb transcript in testis tissue, together with relatively lower levels of 11- and 5.6-kb

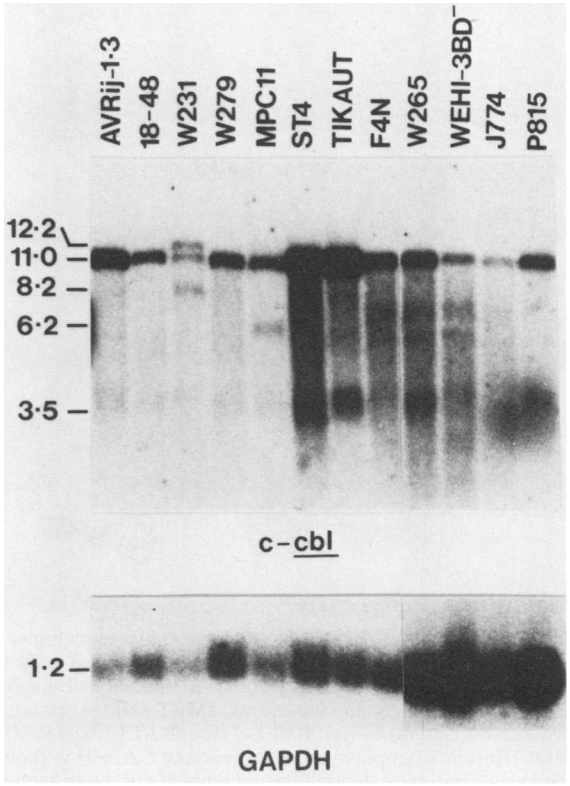

FIG. 1. Expression of c-*cbl* in murine hemopoietic tumor cell lines. The cell lines used were AVRij-1.3 and 18-48 (pre-B-cell lymphomas), WEHI-231 (W231) and WEHI-279 (W279) (B-cell lymphomas), MPC11 (plasmacytoma), ST4 and Tikaut (T cell lymphomas), F4N (erythroleukemia), WEHI-265 (W265) and WEHI-3BD$^-$ (myeloid tumors), J774 (macrophage tumor), and P815 (mast cell tumor). Expression of GAPDH is shown to provide an indication of the relative amounts of mRNA each lane.

c-*cbl* mRNAs. The reverse pattern of expression was seen in thymus tissue, where the 11-kb c-*cbl* mRNA was the dominant species. Smaller transcripts than those found in somatic cells have been observed in testis tissue examined with other proto-oncogenes such as *mos*, *abl*, *pim-1*, and N-*ras* (reviewed in reference 15). Indeed, the pattern of c-*cbl* expression seen in testis and thymus tissues shows a remarkable similarity to that of the *abl*, *pim-1*, and N-*ras* oncogenes (11, 13, 18).

c-*cbl* encodes a 135-kilodalton nuclear protein. A v-*cbl*-encoded protein of 376 amino acids was synthesized in *E. coli* by using the inducible expression vector pGEX-2T (17) (Fig. 4). This construct contained all but 14 N-terminal amino acids of v-*cbl*. Induction resulted in the synthesis of a 65-kilodalton glutathione-*S*-transferase–v-*cbl* fusion protein (Fig. 4, lane GEX-cbl), which was used for the primary immunization of rabbits. *cbl* protein purified by thrombin cleavage was used for subsequent immunizations and for the affinity purification of *cbl* antibodies. To examine the recognition of v- and c-*cbl* proteins in mammalian cells by these antibodies, we prepared extracts from normal and Cas NS-1-transformed NIH 3T3 cells. Northern hybridization of mRNA from NIH 3T3 cells had earlier confirmed the presence of an 11-kb c-*cbl* transcript (data not shown). A 3.5-kb c-*cbl* mRNA was not detected in the NIH 3T3 cells used in these experiments. The extracts were examined by immunoblotting and consisted of a cytoplasmic fraction (Fig. 5,

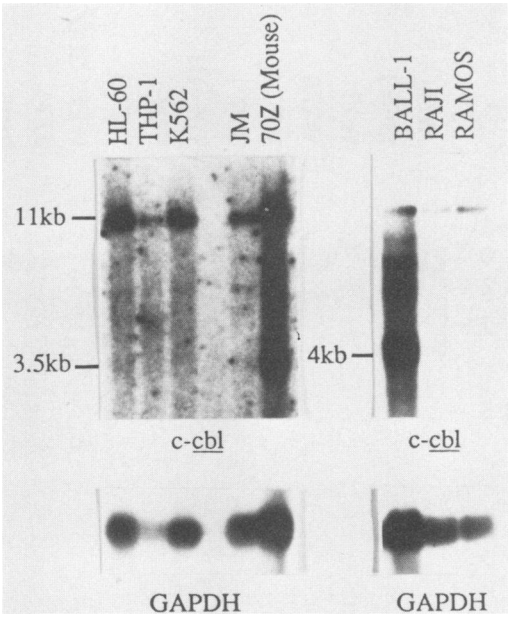

FIG. 2. Expression of c-*cbl* in human hemopoietic tumor cell lines and the murine 70Z pre-B cell line. The cell lines used were HL-60 (promyelocytic leukemia), THP-1 (monocyte leukemia), K562 (chronic myelogenous leukemia), JM (T-cell leukemia), 70Z (mouse pre-B-cell lymphoma), BALL-1 (B-cell ALL), and RAJI and RAMOS (Burkitt's lymphomas). Expression of GAPDH is shown to provide an indication of the relative amounts of mRNA in each lane.

lanes 1 and 2), a 420 mM salt-extracted nuclear fraction (lanes 3 and 4), and a residual nuclear fraction (lanes 5 and 6). The antibodies identified high levels of $p100^{gag\text{-}cbl}$ in the residual nuclear fraction of transformed NIH 3T3 cells and lower levels of an 80-kilodalton protein and a 135-kilodalton protein, p135 (lane 6). The residual nuclei from normal NIH 3T3 cells had equivalent levels of p135 to the levels in transformed NIH 3T3 cells but no 80-kilodalton protein or $p100^{gag\text{-}cbl}$ (lane 5). This suggests that the 80-kilodalton protein is a cleavage product of $p100^{gag\text{-}cbl}$. The nuclear fractions prepared by 420 mM salt extraction showed no evidence of p135 (lanes 3 and 4), and transformed cells had very low levels of $p100^{gag\text{-}cbl}$ (lane 4). No viral or endogenous *cbl* protein was evident in the cytoplasmic fractions (lanes 1 and 2). These findings indicate that p135 is most

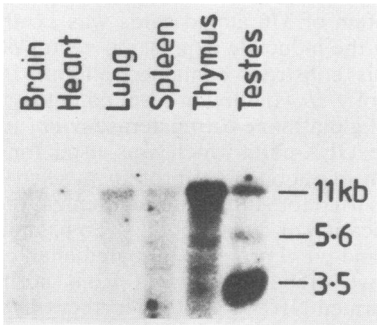

FIG. 3. Expression of c-*cbl* in adult mouse tissues. Equal amounts of mRNA from the tissues indicated above each lane were analyzed by Northern hybridization with a v-*cbl* probe. mRNA levels were monitored by ethidium bromide staining before and after transfer.

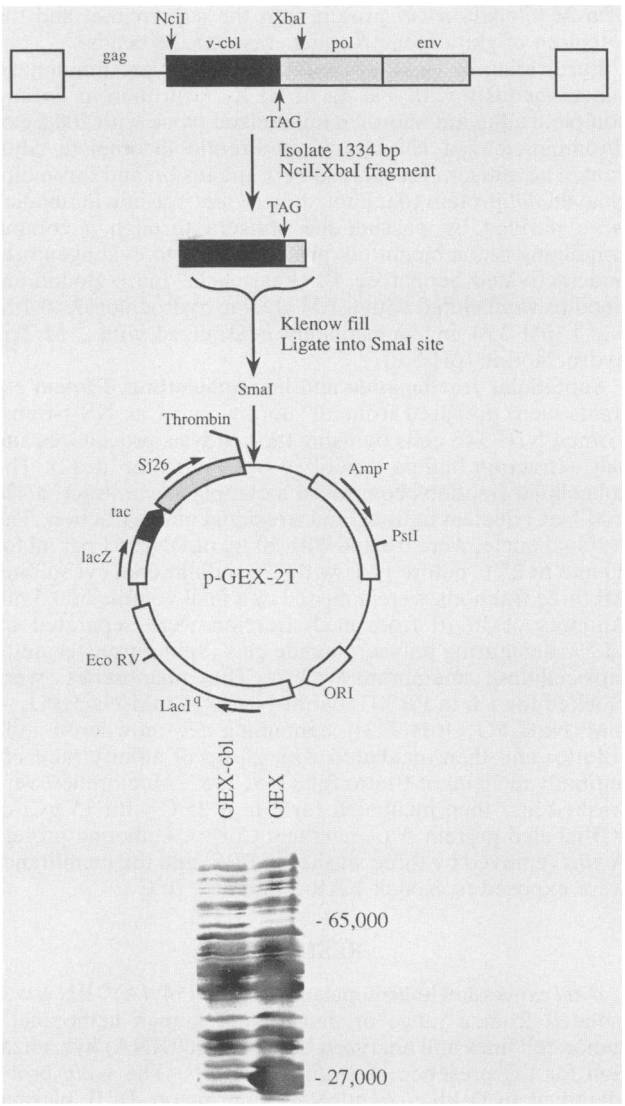

FIG. 4. Construction of the pGEX–v-*cbl* expression vector. The insertion of v-*cbl* into pGEX-2T resulted in the synthesis of a 65-kilodalton glutathione-*S*-transferase–v-*cbl* fusion protein (GEX-cbl). Sj26 is the 26-kilodalton glutathione-*S*-transferase protein of *Schistosoma japonicum*. The *pol* and *env* boxes of Cas NS-1 represent endogenously derived retroviral sequences. Purification of *cbl* protein was facilitated by the thrombin cleavage site present in pGEX-2T (17).

probably the translated product of the c-*cbl* proto-oncogene and that its location is nuclear. Furthermore, the c- and v-*cbl*-encoded proteins are tightly associated with the nucleus, since they were not extracted by salt concentrations that remove most nonhistone proteins (2). The results also suggest that the sequences responsible for the nuclear location of $p135^{c\text{-}cbl}$ are also present in v-*cbl*, which encodes 43 kilodaltons of the 100-kilodalton *gag-cbl* fusion protein (10).

DISCUSSION

In this study we have identified the transcriptional and translational products of the c-*cbl* proto-oncogene. The predominant transcriptional product in a range of murine and

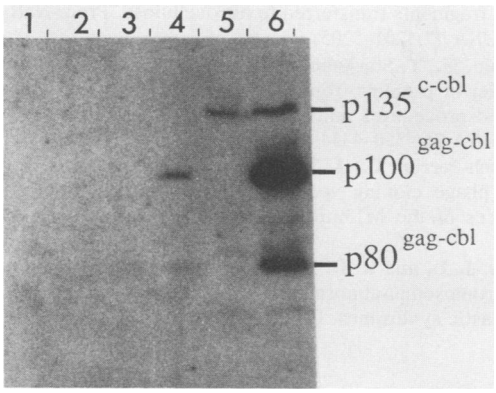

FIG. 5. Localization of v- and c-*cbl* proteins in the residual nuclear fraction of normal and Cas NS-1-transformed NIH 3T3 cells. The fractions in each lane are as follows: 1, cytoplasmic from NIH 3T3 cells; 2, cytoplasmic from Cas NS-1-transformed NIH 3T3 cells; 3, 420 mM NaCl nuclear extract from normal NIH 3T3 cells; 4, 420 mM NaCl nuclear extract from Cas NS-1-transformed NIH 3T3 cells; 5, residual nuclei from normal NIH 3T3 cells; 6, residual nuclei from Cas NS-1-transformed NIH 3T3 cells. Immunoblotting was performed as described in Materials and Methods.

human hemopoietic tumor cell lines was found to be an 11-kb c-*cbl* mRNA. A less abundant, 3.5-kb transcript was also expressed in some of the murine tumor lines. Of the 20 hemopoietic cell lines, 3 also contained c-*cbl* mRNAs of different sizes that were expressed at levels equivalent to or elevated above that of the 11-kb mRNA. Interestingly, the three tumor cell lines were all derived from B-lineage lymphomas. This raises the possibility that c-*cbl* was involved in the development of these tumors; this possibility is supported by the fact that approximately 90% of v-*cbl*-induced tumors of mice are B lineage.

For normal mouse cells, the highest levels of c-*cbl* mRNA were found in testis and thymus tissues. In testis tissue, a 3.5-kb c-*cbl* mRNA was present at markedly higher levels than the 11-kb mRNA, suggesting that the expression of this smaller transcript may have a role in c-*cbl* regulation. It is also possible that the abundant mRNA of testis tissue is the same mRNA seen at low levels in some tumor cell lines and at high levels in the murine pre-B-cell line 70Z. To date, the expression of nine other proto-oncogenes in testis tissue has been reported (reviewed in reference 15), with *fos* being the only nuclear oncogene (1, 13). The implications of germ cell proto-oncogene expression and the function of the smaller transcripts are not well understood; however, it will be important to determine whether the products of these transcripts are functional after fertilization and, if so, whether they are involved in early embryonic development.

The translational product of c-*cbl* appears to be a 135-kilodalton nuclear protein, $p135^{c\text{-}cbl}$. This finding was determined by the subcellular fractionation of NIH 3T3 cells and immunoblotting with affinity-purified antibodies to a v-*cbl* protein produced in *E. coli*. These procedures identified a single protein species of 135 kilodaltons in the residual nuclei fraction. Abundant levels of the v-*cbl*-encoded $p100^{gag\text{-}cbl}$ were detected in addition to $p135^{c\text{-}cbl}$ in the residual nuclear fraction of Cas NS-1-transformed NIH 3T3 cells. The nuclear location of the v- and c-*cbl* proteins is consistent with our observation of some sequence similarity between v-*cbl* and the yeast transcriptional factor GCN4 (10). However, the retention of *cbl* protein in the nucleus following 420 mM salt extraction indicates a tighter association with the nucleus than was described for known transcriptional factors (2). It remains to be determined, however, whether *cbl* functions as a transcriptional factor. The tight association of the *cbl* protein with the nucleus also distinguishes it from the *myc* and *myb* nuclear proteins, which are extracted with salt concentrations as low as 200 mM (3, 7). A more detailed analysis is therefore required to fully characterize the association of *cbl* with the nucleus and with other nuclear proteins. These studies may provide clues to the elucidation of its function.

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