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dissertation entitled

CONTROL OF CD5 EXPRESSION ON
MURINE B-LINEAGE CELLS

presented by

Timothy Raymond Weichert

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Microbiology

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By

Timothy Raymond Weichert

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology

1994

ABSTRACT

CONTROL OF CD5 EXPRESSION
ON MURINE B-LINEAGE CELLS

By

Timothy Raymond Weichert

The CD5 surface antigen is expressed on all T-cells and on a subset of B-cells found mainly in serousal cavities of adult mice. It has previously been shown that adult murine bone marrow does not contain progenitors for $CD5^+$ B-cells, prompting some investigators to classify $CD5^+$ B-lymphocytes as a unique lineage of B-cells. However, we have found that the v-Ha-*ras* and v-*src* oncogenes, but not v-*myc*, can induce the expression of CD5 on two bone marrow-derived pre-B-cell lines not previously expressing this marker, and that this up-regulation correlates with increased steady-state levels of CD5 mRNA. This suggests that the CD5 phenotype is available to $CD5^-$ B-lineage cells and may alter the concept that CD5-expressing B-cells represent a unique, self-renewing subset of B-lymphocytes.

The lymphoid-specific expression of CD5, along with its up-regulation by transforming oncogenes, suggests that interesting mechanisms control the expression of CD5. To identify these mechanisms, we have cloned and sequenced over 1100 base pairs of the murine CD5 5′-flanking region, isolated from an NIH 3T3 fibroblast genomic library. The CD5 5′-flanking region is capable of a lymphoid-specific pattern of promoter activity that reflects *in vivo* CD5 expression. The

CD5 promoter lacks a consensus TATA box and initiates transcription from multiple sites around an initiator (INR)-like element. These characteristics may place the CD5 promoter in the same class as the mb-1 and B29 lymphoid-specific TATA-less promoters.

This Dissertation is Dedicated to My Beloved Wife

Michelle Anderson

and

To My Parents

Donald and Lois Weichert

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INTRODUCTION

Our initial involvement with the CD5 surface antigen occurred during the 'standard' phenotypic analysis of four pre-B-cell populations independently derived by v-Ha-ras infection of high density murine bone marrow cultures (R1-4; Schwartz et al., 1986). Each population expressed CD5. A few years earlier, an elegant experiment by Hayakawa, et al. (1985) had established $CD5^+$ B-cells as a unique lineage of B-cells. They found that bone marrow could not replenish the $CD5^+$ B-cell population in lethally-irradiated mice, suggesting that the progenitors of $CD5^+$ B-cells are not found in bone marrow. Yet, we possessed four transformed bone marrow-derived pre-B-cell populations that expressed CD5. A literature review demonstrated that other labs had also reported frequent establishment of $CD5^+$ pre-B-cell lines from bone marrow utilizing transforming oncogenes (Davidson et al., 1984; Holmes et al., 1986). A closer study of the literature, along with a survey of transformed cell lines in our lab, yielded a surprising result. Every pre-B-cell population established with v-Ha-ras expressed CD5. To reconcile the transformation data with the lineage model, one would have to conclude that minor, contaminating populations of $CD5^+$ B-lineage cells in the bone marrow are preferentially

susceptible to transformation by the v-Ha-ras oncogene during each independent infection. This seemed unlikely. Alternatively, the targets of the virus may include $CD5^-$ B-cells. If this was occuring, it was formally possible that the v-Ha-ras oncogene was inducing the expression of CD5 on previously $CD5^-$ cells. This has turned out to be the case.

Chapter 1 of this thesis will detail the more popular theories of $CD5^+$ B-cell lineage descent, as well as, the current status of the field. Chapter 2 details the cloning of the murine CD5 5'-flanking region and demonstrates that it is capable of a pattern of tissue-specific expression reflective of in vivo CD5 expression. Chapter 3 will demonstrate that the v-Ha-ras oncogene is capable of inducing the expression of CD5 on pre-B-cell lines. In the summary section, the status of the CD5 field will be viewed from the perspective of our findings.

CHAPTER 1

LITERATURE REVIEW

The assignment of unique lineage status to CD5-expressing B-lymphocytes has proven to be one of the most divisive controversies in immunology in the last ten years. Several unifying theories addressing the origin of $CD5^+$ B-cells have been advanced, each depending upon either the co-expression of ill-defined surface antigens or strict anatomical compartmentalization of lymphoid subsets to make its case. This chapter will summarize the characteristics of CD5 expressing B-cells, with a review of the more popular theories of their lineage descent, and will ultimately present data suggesting that the CD5 phenotype is available to a range of B-lymphocytes and is not a lineage-restricted phenomenon.

1. The CD5 Surface Antigen

1.1 Molecular Aspects of CD5

The CD5 (Ly-1, Leu-1, Lyt-1) surface antigen is a 67 kDa MW monomeric, membrane-associated glycoprotein, expressed on all T-lymphocytes and a subset of B-lymphocytes (Huang et al, 1987; Jones et al, 1986). CD5 was originally defined by the monoclonal antibody (mAb) OKT-1, which was produced from a hybridoma generated by the fusion of myeloma cells with spleen cells from mice immunized with human T-cells (Reinherz et al, 1979). Eventually, monoclonal antibodies were used to purify the CD5 protein allowing the N-terminal amino acids to be sequenced. Degenerate oligonucleotides to this sequence were

synthesized and used to probe a human T-cell cDNA library which yielded the human cDNA clone (Jones et al., 1986). The human cDNA was used to probe a murine T-cell cDNA library and the murine CD5 cDNA was isolated (Huang et al., 1987).

The murine cDNA is 2.2 kilobases (Kb) in length and has an open reading frame of 1482 bases coding for a protein of 494 amino acids (Huang et al., 1987). The full-length cDNA includes a 3' untranslated region of 457 base pairs (bp) and a 5' region upstream of the terminal methionine codon of only 6 bp. The first 23 amino acids have leader sequence homology, while residues 378 to 391 have the strong hydrophobic profile of a transmembrane sequence. Altogether, the CD5 protein consists of an N-terminal region of 347 amino acids, a 30 residue transmembrane region and a 94 residue carboxy-terminal domain. Initially, the protein was predicted to have folding homology to the hinge region of immunoglobulin (Ig) molecules, but recent work has shown greater identity to the cysteine-rich scavenger receptor family of polyanion-binding membrane-associated proteins (Freeman et al., 1990).

1.2 Function of the CD5 Molecule

In the first ten years following it's discovery, the number of functions attributed to the CD5 molecule was directly proportional to the number of investigators addressing the question. Alternate hypotheses have included CD5 as an activation antigen, a tumour neo-antigen and a marker defining a sub-lineage of B-lymphocytes (for review,

see Kipps, 1989). Until recently, very few investigators have offered rigorous analyses addressing the involvement of the CD5 molecule itself in the physiology of lymphocytes.

Van de Velde et al. (1991), using biotin-labelled CD5 protein, identified a B-cell-specific surface antigen, CD72 (Lyb-2), as a ligand for CD5. This may implicate CD5 in T-cell/B-cell interactions as well as B-cell/B-cell interactions.

The carboxy-terminal cytoplasmic tail of the CD5 coding region contains many predicted sites for protein kinase C (PKC)-mediated serine/threonine phosphorylation as well as sequences homologous to the tyrosine autophosphorylation sites of the src, yes and fgr oncoproteins (Huang et al., 1987; Chatila and Geha, 1988). Chatila and Geha (1988) have shown that activation of PKC with phorbol esters does indeed hyperphosphorylate CD5, implicating it in the transduction of extra-cellular signals (also Berridge, 1984).

The use of anti-CD5 mAb's as substitute ligands has shown that CD5 can transduce a proliferation signal in T-cells, possibly by up-regulating IL-2 and IL-2 receptors (Ledbetter et al., 1985; Ceuppens and Baroja, 1986; Spertini et al., 1991), and increase the mobilization of extra-cellular calcium in T-cells (June et al., 1987). Since these activities have been observed only in $TCR^+/CD3^+/CD5^+$ T-cells, and since activation of the TCR/CD3 complex induces T-cell activation and increased calcium flux, it was postulated that CD5 signal transduction may be integral in the TCR/CD3-mediated

proliferation response. This idea is supported by the work of Burgess et al. (1992), which showed that anti-CD5 mAbs could co-precipitate a CD5/TCR/CD3 complex that includes the protein tyrosine kinases $p56^{lck}$ and $p59^{fyn}$, which may be responsible for phosphorylating CD5 (also Osman et al., 1992). While no parallels have been found in B-cell-associated CD5, the recent placement of CD5 in the scavenger receptor family of receptors may implicate CD5 in the binding of modified proteins (i.e. acetylated lipoproteins) (Freeman et al., 1990).

2. Characteristics of B-lymphocyte Subsets Expressing CD5

2.1 CD5 as a Marker for a Distinct Lineage of B-lymphocytes

As stated previously, CD5 is a pan-T-cell marker and is expressed on a minority of B-cells. In normal animals, the distribution of $CD5^+$ B-cells appears to be compartmentalized. About thirty percent of all lymphocytes in the murine and human peritoneal, pleural and pericardial cavities are $CD5^+$ B-lymphocytes, with small populations found in murine spleen, human lymph node and human tonsil (Kipps, 1989; Kroemer et al., 1993). $CD5^+$ B-cells are not found in adult bone marrow or the peripheral circulation except in certain autoimmune diseases and chronic lymphocytic leukemia (CLL). This intriguing distribution in normal animals and apparent breakdown of compartmentalization in disease states that involve immune dysregulation, have led to the hypothesis that $CD5^+$ B-lymphocytes may be a lineage distinct from peripherally located $CD5^-$ B-cells.

The above hypothesis was apparently supported by a now-classic experiment by Hayakawa et al. (1985). In their report, the authors used adult murine bone marrow cells to reconstitute the immune system of lethally-irradiated mice and subsequently could not detect $CD5^+$ B-cells in the recipients. However, if peritoneal cells were used in the reconstitution, donor $CD5^+$ B-cells could eventually be found to repopulate the peritoneal cavity. Herzenberg et al. (1986), have interpreted this to mean that $CD5^+$ B-cells can only be replenished from $CD5^+IgM^+$ precursors and not from $CD5^-IgM^-$ pro/pre-B-cells found in adult murine bone marrow. A related experiment utilized a strain of mice whose B-cells expressed either surface IgH^A or IgH^B heavy chain alleles. The authors then injected the mice with anti-IgH^B mAb to deplete this set of mature B-cells. Even after a year, FACS (fluorescence-activated cell sorting) analysis could not detect IgH^BCD5^+ B-cells in any compartment. This suggests that while bone marrow could replenish IgH^B B-cells from $sIgM^-$ precursors, it could not renew the $CD5^+$ B-cells because their precursors reside outside the marrow as IgM^+CD5^+ cells (Lalor et al., 1989). Further permutations of these adoptive transfer experiments have found that fetal liver contains precursors for both $CD5^+$ and $CD5^-$ cells but fetal omentum can only replenish $CD5^+$ cells (for review, see Kroemer et al., 1993). In contrast to the above, Iacomini and Imanishi-Kari (1992) have repeated the bone marrow adoptive transfer experiments of Hayakawa et al. (1985) and found that $CD5^+$ B-cells could be repopulated in lethally-irradiated mice

from donor bone marrow cells. This discrepant finding of $CD5^+$ precursors in adult bone marrow has not been satisfactorily explained at this time.

2.2 Surface Markers of the $CD5^+$ B-cell

Upon examination of the populations that reconstitute $CD5^+$ B-cells in adoptive transfer experiments, a pattern of commonly expressed surface antigens has emerged. The phenotype of $CD5^+$ B-lymphocytes is generally accepted to be $CD5^+IgM^{high}IgD^{low}Mac\text{-}1^+CD45^{low}FcE\text{-}R^-IL\text{-}5R^+$ which is opposite of 'normal' B-cells (Stall et al., 1992; Waldschmidt et al., 1992; Kroemer et al., 1993). During these adoptive transfer experiments, some investigators noted that donor cells from compartments that reconstitute $CD5^+$ B-cells also reconstitute B-cells with the above surface phenotype that were $CD5^-$ (Stall et al., 1992). This population, termed the CD5 'sister ' population, only differs in that CD5 is poorly expressed or absent. This prompted a group of senior CD5 B-cell investigators to reclassify all B-cells as either B1-lymphocytes ($CD5^+$ B-cells [B-1a] and the $CD5^-$ 'sister' population [B-1b]) or B2-lymphocytes (all other B-cells) (personal account of the conference "CD5 B-cells in Development and Disease", Palm Beach Gardens, Florida, June 3-6, 1991). This nomenclature will not be heavily relied upon in this paper.

2.3 CD5-Expressing B-cells Have Monocytoid Features

$CD5^+$ B-cells tend to be adherent and occupy serousal cavities, much like macrophages (Herzenberg and Stall, 1989; Hayakawa et al., 1986). These B-cells express the classical macrophage markers CD11/MAC1 (C3R), CD11b, CD14, and CD15 as well as the B-specific antigens CD19, CD20, CD21 and CD72 (Kipps, 1989; Herzenberg et al., 1987).

Certain $CD5^+$ myeloid lines have rearranged Ig genes suggesting that they either differentiated from pre-B-cell precursors or have superimposed developmental programs (Kemp et al., 1980). Likewise, all pre-B-cell lines that can differentiate into macrophage lines have been found to be $CD5^+$ in cases where this marker has been examined (Bretz et al., 1992; Holmes et al., 1986; Davidson et al., 1988). The establishment of $CD5^+$ pre-B-cell lines with myeloid characteristics was interpreted by some to mean that monocytic features are a distinct trait of the unique CD5 lineage (Herzenberg and Stall, 1989).

2.4 CD5 B-cells in Disease

2.4.1 Autoimmunity, Autoantibodies and Natural Antibodies

Flow cytometric studies have detected increased levels of $CD5^+$ B-cells in the circulation of patients with rheumatoid arthritis and primary Sjogren's syndrome (Hardy et al., 1987; Toniguchi et al., 1987; Lydyard et al., 1987; Dauphine et al., 1988). $CD5^+$ B-cells are also found in increased numbers in systemic lupus erythematosis (SLE)-prone NZBxNZW mice (Shirai

et al., 1991). These autoimmune diseases are characterized by pathological tissue destruction thought to be mediated by autoreactive antibodies. The CD5$^+$ B-cell was implicated as a source of this autoantibody when sorted populations of this cell type, taken from rheumatoid arthritis patients, were found to secrete rheumatoid factor (an anti-IgG IgM) (Hardy et al., 1987). Subsequently, CD5$^+$ B-cells were found to secrete IgM-class antibodies to single-stranded DNA, bromelain-treated erythrocytes, thymocytes, thyroglobulin, with some having cross-reactivity to bacterial coat proteins and herpes simplex glycoproteins (Calvanico, 1993; Kipps, 1989; Mayer et al., 1990; Shirai et al., 1991).

The basis for the CD5$^+$ B-cell's Ig repertoire being limited to production of autoantibody lies in it's restricted use of Ig variable region genes (Mercolino et al., 1986; Pennel et al., 1988). In support of this, Reininger et al. (1987), described three clonally unrelated, CD5$^+$, anti-erythrocyte antibody-producing hybridomas, generated from NZB mice, that utilized identical variable heavy chain Ig genes and nearly identical variable kappa light chain Ig genes. This work was supported by that of Forster et al. (1988), which describes the generation of seventeen CD5$^+$ hybridomas with similar specificities. Surprisingly, the CD5$^+$ CH series of lymphomas (isolated from aging mice hyper-immunized with sheep erythrocytes) also rearranged the identical IgH variable regions as the autoantibody-producing hybridomas previously mentioned (Kofler, 1988; Winter et al., 1985). These studies

suggest that in vitro methods can be used to study CD5$^+$ B-cell variable chain usage. Moreover, all the sequence analyses available indicate that the Ig genes that show restricted use in hybridomas have not significantly diverged from the germline (for review, see Kipps, 1989). By all accounts, selection of variable region genes in CD5$^+$ B-cells is probably not a random event and has prompted some authors to include it as another identifying characteristic of the CD5$^+$ B-cell lineage (Kantor and Herzenberg, 1993).

CD5$^+$ B-cell-produced autoantibodies generally have low binding affinities and multiple antigenic specificities and are not the monoreactive, highly specific, IgG class antibodies thought to be needed to convey autoimmune tissue destruction. Evidence against CD5$^+$ B-cells producing the autoantibodies actually responsible for autoimmune pathology began to mount when it became apparent that the development of clinically significant autoimmune disease in NZBxNZW mice correlated with an isotype shift to IgG anti-DNA antibodies produced by CD5$^-$ cells (Shirai et al., 1991). It is not clear if this was due to expansion of anti-DNA specific CD5$^-$ B-cells or class-switching of the IgM anti-DNA CD5$^+$ cells with subsequent CD5 down-regulation.

When it became apparent that CD5$^+$ B-cells may be responsible for the production of autoreactive low-affinity IgM and not pathologic high-affinity IgG autoantibody, questions arose as to the physiological significance of these self-directed antibodies. With data demonstrating that CD5$^+$

B-cells populate the fetal mouse peritoneal cavity early in ontogeny, Kipps (1989) suggested that self-directed, low-affinity antibody could "establish a primordial network of idiotypic and anti-idiotypic interactions" to stimulate clonal expansion of certain B-cell subsets in the sterile fetal environment (also Jerne, 1984; 1985). In the adult, $CD5^+$ B-cells could produce autoantibody to form immune complexes or regulate post-thymic maturation of the T-cell antigen repertoire (Martinez et al., 1986; Kipps, 1989). The persistence of this autoantibody production from the neonatal animal into the adult may also form the basis for 'natural' immunity (humoural). These 'natural' autoantibodies may take part in removal of damaged tissue, block cross-reactive epitopes shared between host and invading pathogens, or provide a pool of non-specific antibodies that rapidly react to microbial invasion while the process of class-switching and antibody affinity-maturation is occuring (Logtenberg, 1990). While the concept of 'natural' autoantibody production as the specialized immune function of $CD5^+$ B-cells is an attractive hypothesis, Kasaian et al. (1992), have reported that Epstein-Barr virus (EBV)-transformed $CD5^-$ B-lymphocytes can be induced to express 'natural' antibodies to ssDNA, insulin, phosphorylcholine and tetanus toxoid. This suggests that $CD5^+$ B-cells do not have a unique immune function and somewhat detracts from their status as a separate B-lymphocyte lineage.

2.4.2 CD5 B-cells in Chronic Lymphocytic Leukemia and Neoplastic Transformation

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia and is characterized by invasion of blood, bone marrow, lymph node and spleen by small B-cells (95% of cases) or T-cells (5% of cases) (Deissorth et al., 1992). In B-CLL, the neoplastic cells are clonal in nature and invariably express CD5 on their surface (Freedman, 1990). These cells also express surface IgM and other markers reflective of B-1 lymphocytes (i.e. $CD5^{+}IgM^{high}IgD^{low}Mac\text{-}1^{+}CD45^{low}FcE\text{-}R^{-}IL\text{-}5R^{+}$) rather than B-2 cells (Freedman, 1990). Kipps (1988) found that about 25% of CLL's express autoantibody to determinants that autoantibodies from murine $CD5^{+}$ B-cells also identify. Thus, B-CLL is thought to be the neoplastic counterpart of naturally occuring 'B1a'-cells. Not coincidentally, there is a large body of literature dealing with the expression of CD5 on transformed B-lineage cells.

The first report of CD5 expression on B-cells actually involved spontaneously arising B-lymphomas from New Zealand and B-10 mice (Lanier, et al, 1981). Soon after, Braun (1983) reported a simple, long term splenocyte culture system that yielded oligoclonal outgrowths of immortalized $CD5^{+}$ B-cells. Davidson et al. (1984) described a series of ten spontaneously occuring or Moloney murine leukemia virus (MoMuLV)-induced $CD5^{+}$ B-lineage lymphomas that spanned the stages of B-cell differentiation. Palacios et al. (1987) were able to devise a culture system that allowed the establishment of $CD5^{+}$ pro-B

and B-lymphocytes from the bone marrow of four week old Balb/c mice.

Braun et al. (1986) subsequently found that the c-^{myc} proto-oncogene is over-expressed in the CD5^{+} lymphoblasts from splenic cultures. This was followed by anecdotal reports from other investigators that also noted a preponderance of CD5^{+} B and pre-B-lymphocytes in culture systems that relied upon transforming oncogenes for establishment of B-lineage lines (Kipps, 1989; R. Schwartz, unpublished observations). Holmes et al. (1986) authored the seminal paper addressing the utilization of oncogene-expressing retroviruses to generate CD5^{+} B-cell lines from murine bone marrow cultures. They infected bone marrow (also fetal liver cells) from 2 to 3 week old NFS/N mice with Abelson murine leukemia virus (A-MuLV; v-abl) or replication-defective retroviral vectors containing the v-src, v-Ha-ras, K-ras or v-fes oncogenes. Cell lines were established in liquid culture and then analyzed for CD5 expression via FACS analysis. It was found that v-abl and v-src infected lines did not express or weakly expressed CD5, while lines infected with v-Ha-ras vectors invariably expressed this marker. The lines were predominantly pre-B-cells regardless of tissue of origin. This result was supported by Tidmarsh et al. (1989) who found zero out of eleven cell lines generated by infection of bone marrow with A-MuLV expressed CD5. Another group found that while 4 of 4 cell lines generated by v-Ha-ras infection of murine bone marrow expressed CD5, 2 lines generated by v-Ha-ras/v-myc co-

infection did not (Schwartz et al., 1986, and unpublished observations).

3. The Role of Oncogenes in B-cell Transformation

From the above, it appears that the $CD5^+$ B-cell may be a frequent target for certain transforming oncogenes (and, presumably, their cellular homologs). Therefore, a brief overview of the cellular targets, molecular biology, and transforming characteristics of the aforementioned oncogenes is in order.

Sawyers et al. (1991) define leukemia as "...the uncontrolled proliferation or expansion of hematopoietic cells that do not retain the capacity to differentiate normally to mature blood cells." Implicit in this definition is that the genetic events that cause leukemia, such as chromosomal abberations and proto-oncogene activation, must dysregulate both growth and differentiation. The support for this idea (summarized below) has come from studies indicating that, in general, oncogenes having hematopoietic neoplastic activity confer either growth factor independence, arrest of differentiation, or both. Moreover, the phenotype of the arrested, transformed cell is thought to reflect the stage of differentiation at which the normal cellular homolog of the oncogene is active (for review, see Sawyer et al., 1991).

3.1 The abl Oncogene

The oncogene carried in the Abelson murine leukemia virus (v-abl) has intrinsic tyrosine kinase activity, which is undetectable in it's cellular homolog c-abl (Witte et al., 1980; Ponticelli et al., 1982). c-abl is closely related by homology to the c-src family of tyrosine kinases, whose normal cellular function is thought to entail signal transduction at the cytoplasmic surface of the cell membrane. In t(9⁻,22) chromosomal translocations (Philadelphia chromosome) of human chronic myelogenous leukemia (CML), mutant forms of c-abl are able to display tyrosine kinase activity. Appearence of this abl/bcr fusion product in CML is correlated with polyclonal expansion of differentiated B-lymphoid and myeloid subsets that may later evolve into a blast crisis of immature progenitors (Sawyer et al., 1991). It is at the blast stage that CML resembles murine Abelson disease in that the aggressive lymphoid tumors can be characterized as lymphoid or myeloid progenitors (Witte, 1983). To determine the lymphoid target of the v-abl oncogene, Whitlock et al. (1983) developed an in vitro culture system that supports the growth of murine bone marrow-derived early B-progenitors (perhaps even stem cells), pre-B cells and B-cells. They then infected clonal populations of B and pre-B-cells (derived by limiting dilution) with v-abl and found that immature cell subsets could still undergo differentiation to mature forms (analyzed by subsequent Ig light chain gene rearrangement). This data, combined with the fact that the Philadelphia chromosomal

fusion is seen in all lymphoid subsets in CML, suggests that the v-abl protein and the bcr/abl fusion protein target a very early bone marrow progenitor, perhaps the stem cell. An extension of this is that v-abl dysregulates growth restraint but not differentiation.

3.2 The src Family of Tyrosine Kinases

Other tyrosine kinases that can transform pre-B-cells include v-fes and v-src (Holmes et al., 1986). As members of the v-src family of tyrosine kinases, they are intimately associated with the inner leaf of the cell membrane via myristic acid residues. They are endowed with a cytosolic domain that is capable of down-regulating it's intrinsic kinase activity by auto-phosphorylation of a carboxy-terminal tyrosine. The transforming activity associated with v-src is attributable to a missense mutation that replaces the carboxy-terminal tyrosine with another amino acid, resulting in constitutive kinase activity. Probable implications of constitutive activity are chronic activation of downstream messengers such as phosphatidyl inositol 3-kinase and c-ras/GAP (for review see Cantley et al., 1991).

Recent work in the area of B-cell activation has implicated the src family of tyrosine kinases in signal transduction via the B-cell receptors sIgM and sIgD. Yaminishi et al. (1991) have shown that c-lyn physically associates with sIgM, while Burkhardt et al. (1991) used immunoprecipitation techniques to demonstrate interaction

between sIgD and c-blk, c-lyn, and c-fyn. Mizuguchi et al. (1992) demonstrated that activating the B-cell by anti-IgM cross-linking results in tyrosine phosphorylation, while Lin and Justement (1992) suggested that this signal is transduced through the sIg-asssociated proteins mb-1 and B-29.

Like v-abl transformations, the tumors derived from bone marrow transformed with retroviruses expressing v-src are of short latency (one to two weeks). In contrast, while v-abl transformants may differentiate further into B-cells, cell lines derived by infection with v-fes or v-src are of a stable pre-B or pre-B/myeloid phenotype (Pierce and Aaronson, 1983; Pierce et al., 1984).

3.3 The myc Oncogene

The c-myc proto-oncogene product is a 49,000 MW protein localized to the nucleus and is expressed in a myriad of cell types (for review, see Bishop, 1991). It's role in hematopoietic neoplasia is best characterized by it's involvement in Burkitt's lymphoma, a human B-cell lymphoma of the peri-mandibular region and endemic to Africa.

c-myc's involvement in Burkitt's lymphoma is thought to begin after the clonal (or oligoclonal) outgrowth of Epstein-Barr virus (EBV)-infected pro-B or pre-B-lymphocytes (Magrath, 1990). It is at this stage, where there is high recombinational activity at the Ig heavy chain (pro-B) or light chain (pre-B) loci, that rearrangement of the c-myc gene to an Ig promoter may occur (t[8; 14, 22 or 2] translocation),

resulting in c-myc overexpression. While it is not clear what c-myc's contribution to the development of the lymphoma is, it apparently does not affect early B-progenitors' ability to further differentiate, as evidenced by 1) pre-B lines derived from lymphomas of c-myc transgenic mice can further rearrange their Ig light chain loci (Adams et al., 1985) and 2) the observation that infection of pristane-treated mice with a recombinant retrovirus expressing c-myc leads to a high incidence of differentiated Ig-secreting plasmacytomas in mice (Sawyers et al., 1991; Magrath, 1990). Apparently, to achieve the fully transformed phenotype in myc overexpressing cell lines, it is necessary that another genetic event must occur that affects normal differentiation, such as coexpression with oncogenes such as v-Ha-ras (Schwartz and Witte, 1988). The above data suggests a role for myc in cell proliferation.

While many proto-oncogenes require some genetic mutation for transforming activity (i.e. c-abl is not oncogenic without a 5'-deletion and subsequent fusion to the bcr locus that unmasks tyrosine kinase activity; c-ras apparently requires specific point mutations that constitutively activate GTPase activity [for review see Bishop, 1991]), it is clear that simple over-expression of c-myc, via juxtaposition to an Ig promoter and within a temporal framework, is sufficient for transforming-activity in EBV-immortalized cells (for review, see Magrath, 1990). Therefore, the transforming mechanism of myc should reflect the normal function of c-myc.

Kelly et al. (1983) found that stimulators of the proliferative response in B-lymphocytes (LPS, concanavalin A) and NIH/3T3 fibroblasts (PDGF) induce the expression of c-myc. Coughlin et al. (1985) have shown that c-myc RNA is induced by EGF in human fibroblasts and that this signal is transduced through PKC. Other functions, circumstantially ascribed to myc, are repression of the non-overexpressed c-myc allele (Magrath, 1990) and entry into the cell cycle from a resting state (Luscher and Eisenman, 1990). This data, coupled with myc's nuclear localization, sequence specific DNA binding activity (Blackwell et al., 1990) and ability to hererodimerize with max (Blackwood and Eisenman, 1990) (reminiscent of the fos/jun (AP-1) transcriptional activator complex), suggests that c-myc is at the convergence of many proliferation/signal transduction pathways.

myc's seeming involvement in proliferation but not differentiation has a notable exception. Murine erythroleukemia cells (MEL cells) can be derived from splenic lymphocytes after whole mouse infection with Friend erythroleukemia virus. These immature erythroblasts can almost fully differentiate (as assayed by induction of hemoglobin synthesis) into erythrocytes by incubation with dimethyl sulfoxide (DMSO). Coppola and Cole (1986) transfected an LTR/c-myc construct into MEL cells and found that its expression was correlated with inhibition of DMSO-induced differentiation.

3.4 The ras Oncogene

The ras family of oncogenes have extensive homology to G proteins, molecules found on the cytoplasmic surface of the cell membrane and thought to be involved in the inward transduction of extra-cellular signals. The current model for the activity of G proteins is that they usually exist in the inactive state, characterized by the binding of GDP, associated with their upstream signalling molecule (probably a receptor). When a stimulus is received from upstream, GDP is exchanged for GTP, allowing the G protein to interact with a downstream cytosolic effector molecule and resulting in further propagation of the signal. Once this interaction is accomplished, the GTP is hydrolyzed to GDP and the G protein returns to the resting state. It is though that ras oncogenes represent chronically-activated G proteins that continually propagate growth or differentiation signals downstream. This chronic activation is conferred by point mutations in the c-ras gene allowing the production of oncogenic ras proteins. The ras oncogenes carried by the murine transforming viruses v-K-ras and v-Haras have Ser^{12} and Thr^{59} or Arg^{12} and Thr^{59} replacing Gly^{12} and Ala^{59}, respectively, presumably leading to chronic activation of these proteins (for review, see Barbacid, 1987).

Recent work utilizing the cytoplasmic injection of biotinylated anti-c-ras antibodies into B-cells, shows that after crosslinking surface IgM, c-ras co-caps with these

molecules. This suggests that c-ras is involved in the B-cell receptor signal transduction pathway (Graziadei et al., 1990).

c-ras protein clearly has proliferative effects on NIH/3T3 fibroblasts. Microinjection of anti-c-ras antibodies into the cytoplasm of these cells blocks serum stimulated G_0-to-S phase entry but has no effect in cycling cells (Mulcahy et al., 1985). Also, cells transformed with growth factor receptors such as v-fms, cannot enter S phase if microinjected with anti-c-ras antibodies (Smith et al., 1986). Overall, however, it does not appear that over-expression of ras is sufficient in itself to fully transform cells.

Primary rat embryo cells transfected with the v-Ha-ras oncogene may pass through a few cell divisions, but become established as cell lines at a reduced frequency compared to those also containing exogenously introduced c-myc (Barbacid, 1987). This suggests that another genetic transforming event is needed for the fully transformed phenotype. This correlates with work done with murine pre-B-cells which suggests that while bone marrow cultures infected with v-Ha-ras do establish cell lines in vitro, they require v-myc co-expression for the fully transformed phenotype (Schwartz et al., 1986).

ras's involvement in the differentiation programs of lymphoid cells is fascinating in that lymphoid lineages can be transformed by v-Ha-ras but may or may not be blocked to differentiation. The predominant outgrowth of high-density bone marrow cultures infected with v-Ha-ras are pre-B-cells

that do not further differentiate in tissue culture. These transformants proceed to clonality, have a high growth rate and form tumors at a very low rate upon challenge in syngeneic animals (Schwartz et al., 1986). However, these transformants do not take on the fully-transformed, tumor-forming phenotype unless coexpressed with a nuclear oncogene such as v-myc (Schwartz et al., 1986).

Examples of cell types that are transformed but not blocked to differentiation are: 1) Erythroid progenitors from spleens of phenylhydrazine-treated mice infected with ki-ras are able to undergo colony formation and differentiate into hemoglobin synthesizing cells (Hawkins and Scolnick, 1981), 2) Spleen cells infected with v-Ha-ras produce clonal lines of mast cells that maintain the requirement of IL-3 for growth (Rein et al., 1985), and 3) Infection of murine bone marrow cultures with v-Ha-ras yields two types of colonies in soft agar: immature B-lymphoid progenitors and cells of the myeloid phenotype (Pierce and Aaronson, 1985). While the characteristically-myeloid colonies could not be established into cell lines, the more blastic-appearing monocytes could. When the latter were treated with TPA, these blastic cells acquired myeloid markers and their cloning efficiency in soft agar dropped. It is not clear whether these myeloblasts were derived from B-lymphoid progenitors (so-called lineage infidelity) or represented the true monocyte lineage. However, Bretz et al. (1992) have found that clonal pre-B-cell lines derived from v-Ha-ras-transformed high density bone

marrow cultures can further differentiate into functional macrophages after syngeneic tumor challenge.

4. Possible Origins of CD5$^+$ B-lymphocytes

With the knowledge that CD5$^+$ B-lineage cells are undetectable (by FACS analysis) in the bone marrow of mice (see section 2.1), the following question presents itself: Are the oncogenic viruses (particularly v-Ha-ras) that give rise to a preponderance of CD5$^+$ B-lineage cell lines upon infection of murine bone marrow (see section 2.4.2 above), specifically targeting a rare population of CD5$^+$ 'B-1' cells and conferring a growth advantage via transformation, or are the viral targets more diverse (including CD5$^-$ 'B-2' cells) with the transforming event actually inducing the expression of CD5? A body of evidence does exist that suggests the latter may be possible.

4.1 The Modulation of CD5 Expression

Evidence that CD5 expression is not a static phenomenon was first introduced by Miller and Gralow (1984) who were able to induce CD5 expression on purified populations of human peripheral CD5$^-$ B-cells after in vitro treatment with PMA, an activator of protein kinase C (PKC). Since PMA has been extensively used to induce activation or differentiation of B-cells, CD5 was initially considered a marker for activation. However, further evidence has shown that most CD5$^+$ B-cells in vivo are in G_0 or G_1 and not activated (for review see Kipps,

1989). Additional evidence that CD5 antigen levels are modulated was supplied by Jyonouchi et al. (1990) who found that IL-4 down-regulated the CD5 levels on the 70z/3 cells (a murine pre-B-cell line) while LPS up-regulated CD5 mRNA levels three to four-fold. Brooks et al. (1992) showed that CD5 transcript levels are modulated as BCL_1-3B3 cells (a clonal murine B-cell line) traverse the cell cycle.

However, the most intriguing experiment demonstrating CD5 modulation was offered by Ying-zi et al. (1991). This group used FACS to sort against the typical $CD5^+$ B-cell and collected a population of $CD5^-sIgM^+IgD^{high}CD23^{high}CD45^{high}$ cells ('B-2' cells) from murine spleen. Using an anti-Mu antibody, they then cross-linked surface IgM on this population and noted an increase in the percentage of CD5-expressing cells, as well as an increase in actual cell numbers upon resorting. The authors claimed that if this increase had been due to the expansion of a small population of contaminating $CD5^+$ B-cells, a bi-modal distribution would have been evident upon FACS analysis. In their discussion, they hypothesized that there were two types of activation pathways for 'B-2'-cells, one that was LPS-inducible resulting in secreted Ig (standard B-cell activation assay) with no increase in CD5, and the other that proliferated and up-regulated CD5 expression upon anti-sIgM crosslinking (but does not produce secreted Ig). In addition, treating the CD5-inducible splenic populations with interleukin-6, yielded a surface phenotype identical to peritoneal-derived $CD5^+$ B-cells. The authors interpreted this

to mean that the CD5 antigen may be a marker for proliferation of 'B-2' cells and that $CD5^+$ B-cells may not represent a distinct lineage.

4.2 $CD5^+$ B-cells Are Made, Not Born

The main pillar of support for the unique lineage hypothesis comes from the work of Huang et al. (1986) (see section 2.1) who found that, while donor bone marrow cells were not able to reconstitute the $CD5^+$ B-cell population in lethally-irradiated mice, peritoneal-derived $CD5^+$ B-cells could. They interpreted this data to mean that $CD5^+$ B-cells could only be derived from $CD5^+$ precursors, effectively establishing the $CD5^+$ B-cell as a separate lineage. In contrast, the work of Yin-zi et al. (1991), (detailed in section 5.1) suggests that the $CD5^+$ phenotype is available to any immature (i.e. not previously activated) B-cell that receives the appropriate stimulation. While neither of these models directly addresses the literature detailing the high-frequency of establishment of $CD5^+$ B-lineage cell lines from $CD5^-$ bone marrow by infection with transforming oncogenes (section 3.2), that data is most reconcilable with the model proposed by Yin-zi et al. (1991). If, as they propose, the $CD5^+$ phenotype is available to any immature B-cell, then a signalling pathway must exist that is capable of up-regulating CD5 expression after the appropriate stimulation. Since the cellular homologs of transforming oncogenes are implicated in the transduction of intracellular signals, it is formally

possible that the targets of viruses encoding these oncogenes consist of both $CD5^+$ and $CD5^-$ B-lineage cells. Therefore, induction of CD5 expression in $CD5^-$ B-cells may be the result of oncoprotein-mediated chronic activation of signalling pathways involved in CD5 up-regulation.

While the work of Yin-zi et al. (1991) is very solid, one technical objection may be raised. The authors initially collected their $CD5^-$ B-cell pools from heterogeneous B-cell populations that presumably included $CD5^+$ B-cells. It is formally possible, although unlikely, that what they interpreted as an induction of CD5 expression on $CD5^-$ B-cells, could actually have been the expansion of a contaminating population of $CD5^+$ B-cells. While FACS analysis did not detect the bi-modal distribution one would expect from the expansion of a single population in a pool of two cell types, bi-modal peaks may have been present but obscured by background (or too close together to discriminate). The use of clonal lines of $CD5^-$ B-lineage cells would obviate this objection.

4.3 A Thesis Proposal

This thesis addresses the hypothesis that expression of the CD5 surface antigen on B-lineage cells can be induced by the introduction of transforming retroviruses and their associated oncogenes. This work relies upon $CD5^-$ clonal B-lineage cell lines to ensure that the retroviruses or their associated oncogenes are not conferring a growth advantage to

a minor contaminating population of $CD5^+$ cells, whose subsequent expansion could possibly mimic CD5 induction. Additionally, the putative CD5 promoter has been cloned in order to initiate work on a possible mechanism governing the expression of CD5.

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CHAPTER 2

Cloning and Tissue-Specific Regulation of the Murine CD5 Promoter

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ABSTRACT

A genomic clone containing 1700 base pairs of the 5'-flanking region and first exon of the murine CD5 gene was isolated by screening an NIH 3T3 fibroblast genomic library with the previously characterized murine CD5 cDNA (Huang *et al.* (1987) *PNAS* 84, 204-208). The CD5 5'-flanking region lacks a consensus TATA box but contains the reverse IgH μE2, reverse CCAAT, AP-1 and TCF-2α motifs. Transcription is initiated from multiple sites upstream of an INR-like sequence. When linked to the CAT reporter gene, the CD5 5'-flanking region was an active promoter in transient transfection assays using the EL4 (T-cell), PD36 (pre-B-cell) and M12 (B-cell) lines, but was inactive in NIH 3T3 fibroblasts. This pattern of lymphoid-specific expression reflects the pattern of *in vivo* CD5 expression. Successive 5'-to-3' deletions of the CD5 promoter/CAT reporter were transfected into T and B-cells revealing a succession of positive and negative regulatory elements until promoter activity was eliminated at position -27. This result identified sequences from -125 to +9, (which contain the INR, reverse IgH μE2 and AP-1 sites) as a minimal promoter. Isolation and characterization of the CD5 promoter represents an initial step in elucidating the control over tissue-specific expression of the murine CD5 gene.

INTRODUCTION

The murine CD5 surface antigen (also referred to as Ly-1 or Lyt-1) is a 67 kDa MW monomeric, membrane-associated glycoprotein that is structurally homologous to the cysteine-rich scavenger receptor family of polyanion-binding receptors (1,2). It is lymphoid-specific in its expression, being found on all T-lymphocytes and on a subset of B-cells found mainly in serousal cavities of adult mice. CD5 is physically associated with the TCR/CD3 complex on T-cells where it is thought to be a phosphorylation target for the protein tyrosine kinases $p56^{lck}$ and $p59^{fyn}$ during the transduction of extracellular signals (3,4). No such function has been attributed to B-cell-associated CD5, but the B-lymphocyte-specific surface antigen CD72 (Lyb-2) has been identified as a ligand for CD5 (5). The implications of CD72/CD5 binding for T-B and B-B-cell interactions have yet to be elucidated.

Some investigators consider $CD5^+$ B-cells to be a unique lineage of B-lymphocytes, the so-called B-1 cells (B-2 cells do not express CD5)(6). This idea stems from adoptive transfer experiments that showed that peritoneal-derived B-lymphocytes were capable of reconstituting $CD5^+$ B-cells in lethally irradiated mice, while bone marrow-derived $CD5^-IgM^-$ B-2 progenitors could not (7). However, other groups have found that CD5 expression on B-cells may not be the static phenomenon predicted by a lineage model and that levels of CD5 expression may be regulated. It has been found that anti-immunoglobulin (Ig)-crosslinking of surface IgM on $CD5^-$ B-

cells induces the expression of CD5, and acts as a mitogen (8). Additionally, CD5 expressed on clonal murine B-cell lines has been found to be down-regulated by lipopolysaccharide (LPS) and modulated as the cell traverses the cell cycle (9,10). CD5 expressed on human peritoneal B-cells has been found to be up-regulated in the presence of PMA (11). In either case, the lymphoid specificity of CD5 expression, together with its restriction to a B-cell subset and possible regulation in response to signal transduction events, suggests complex regulatory mechanisms may govern the expression of CD5.

To elucidate the mechanisms regulating the expression of the murine CD5 gene, we have cloned the murine CD5 promoter from an NIH 3T3 fibroblast genomic library. The CD5 5'-flanking region lacks a TATA box and transcription of the CD5 message is initiated from multiple sites flanking an INR-like sequence. The CD5 promoter is capable of a pattern of tissue-specific expression reflective of <u>in vivo</u> CD5 expression and is governed by both positive and negative regulatory elements. The promoter sequence contains consensus TCF-2α and IgH μE2 motifs, both of which have been shown to be involved in the regulation of tissue-specific expression from other lymphoid-specific promoters (12,13). Deletion of promoter sequences from -127 to -25 (which contain AP-1, SV40 core enhancer and the initiator [INR] motifs) results in loss of promoter activity. This region will be useful in elucidating regulatory mechanisms governing expression of the CD5 gene.

MATERIALS AND METHODS

Cell Culture and Transfection Assays - Murine EL4 T-cell (ATCC TIB 39), M12 B-cell (14) and PD36 pre-B-cell (12) lines were maintained in Rosewell Park Memorial Institute (RPMI) medium 1640 supplemented with 10% fetal calf serum (FCS), 50μM 2-mercaptoethanol (2ME) and 25μg/mL each of penicillin and streptomycin (penn/strep). NIH 3T3 murine fibroblast cells were maintained in Dulbecco's Modified Eagle's Media (DMEM) supplemented with 10% calf serum and pen/strep as above. The lymphoid lines were transfected by electroporation (Biorad Gene Pulser, Biorad, Inc., Hercules, CA) utilizing parameters of 280 volts and 960 μF capacitance conducted across a cuvette of 0.4 cm electrode gap (Biorad). Pulse times varied between 25 and 35 msec. Each cuvette contained 0.5 ml RPMI media/10% FCS (without 2ME or penn/strep) with 10^7 cells, 4.4 μg pGL2 luciferase reporter plasmid (Stratagene, Inc., La Jolla, CA) as an internal control (Fig. 4d) and 10.0 μg of the appropriate CAT reporter construct (Figure 4a). Electroporated cells were transferred from cuvettes to 6 cm tissue culture plates in 5.0 ml complete medium. NIH 3T3 cells were transfected using quantities of plasmid identical to the lymphoid lines but utilizing the calcium phosphate coprecipitation method (15).

After 24 hours, transfected cells were washed in ice-cold phosphate-buffered saline and divided into a 1/5th portion for luciferase assays and a 4/5th portion for CAT assays. The cells analyzed for luciferase assays were lysed in 50.0 μl

cell culture lysis buffer (Promega, Inc., Madison, WI) and 20.0 μl used for luminometry in a Turner TD-20e luminometer (Turner Designs, Sunnyvale, CA) utilizing 100.0 μl luciferin assay buffer (Promega). Light unit readings were collected for 15 seconds (after a delay time of 30 seconds) and the ratio of each sample value to the lowest sample value determined (normalized luciferase ratios). Normalized luciferase ratios were used to determine the volume of cell extract used in the CAT reaction (16). Cell extracts for CAT assays were prepared by freeze-thawing cells in 0.25 M Tris-HCl for 3 cycles, then heating to 65°C for 20 mins. CAT reactions were carried out utilizing 1.0 μl of 50.0 mCi/mmol [^{14}C]chloramphenicol, 25.0 μg of n-butyryl Coenzyme A, with cell extract and 0.25 M Tris-HCl in a total volume of 125.0 μl, and incubated at 37°C for 10 hours. Conversion of [^{14}C]chloramphenicol to butyrylated forms was directly determined from TLC plates on an Ambis Radioanalytic Imaging System (Ambis, San Diego, CA).

Cloning Strategy and Sequencing - An NIH 3T3 fibroblast genomic library, generated by *Bam*HI partial digest (15 kb average insert size) and cloned into the *Xho*I sites of the Lambda Fix II vector (Stratagene), was probed with the *Nco*I-*Eco*RV restriction fragment of the murine CD5 cDNA clone (Fig. 1a)(17). 10^5 plaques were screened resulting in the isolation of six hybridizing clones. Two of these clones were analysed, with one (LambdaFixIIcl.41-CD5) ultimately yielding the 5'-

Figure 1. *Cloning Strategy and Partial Restriction Map of the CD5 5'-Flanking Region.* **A.** Partial restiction map of the CD5 cDNA (17). The *Nco*I-*Eco*RV restriction fragment was used as a probe to isolate LambdaFixIIcl.41-CD5 from an NIH 3T3 fibroblast genomic library. Also, the 263 bp *Bam*HI-*Bst*EII fragment of the CD5 cDNA was ligated in the antisense orientation into pSP64 to create the pSP640-263 in vitro transcription vector (panel E). **B.** Partial restriction map of LambdaFixIIcl.41-CD5 genomic clone. This clone was digested with *Sac*I and fragments ligated into the corresponding site in SK-pBluescript (Stratagene) (panel C). **C.** Restriction map of pBlu.41. Nylon transfer-membrane colony lifts of pBluescript transformants were probed with the *Nco*I-*Eco*RV fragment of the murine CD5 cDNA (panel **A**) to identify pBlu.41. Arrow below panel represents direction of double-stranded sequencing using a primer complementary to the pBluescript vector. **D.** Restriction map of M13mp19proCD5. LambdaFixIIcl.41-CD5 (panel B) was digested with *Nco*I, yielding a 2.0 kbp fragment. This fragment was digested with *Sph*1 and directionally subcloned into the *Sph*1 and *Pst*I sites of M13mp19 to create M13mp19proCD5. Arrows below figure represent direction of single-stranded sequencing using an M13mp19-specific primer and a second primer complementary to insert sequences. **E.** Restriction map of the in vitro transcription vector pSP640-263pro. The *Nco*I-*Stu*I restriction fragment of M13mp19proCD5 was subcloned into the *Nco*I and *Sma*I sites of pSP640-263 (panel A).

Abbreviations: A, *Ava*I; Acc, *Acc*I; B, *Bam*HI; dB, destroyed *Bam*HI site; Bx, *Bst*XI; B2, *Bst*EII; E1, *Eco*RI; E5, *Eco*RV; H, *Hin*cII; N, *Nco*I; S, *Sac*I; Sa, *Sau*96I; Sph, *Sph*I; St, *Stu*I; V, vector sequences; M13, M13mp19 vector sequences; Blu, pBluescript vector sequences.

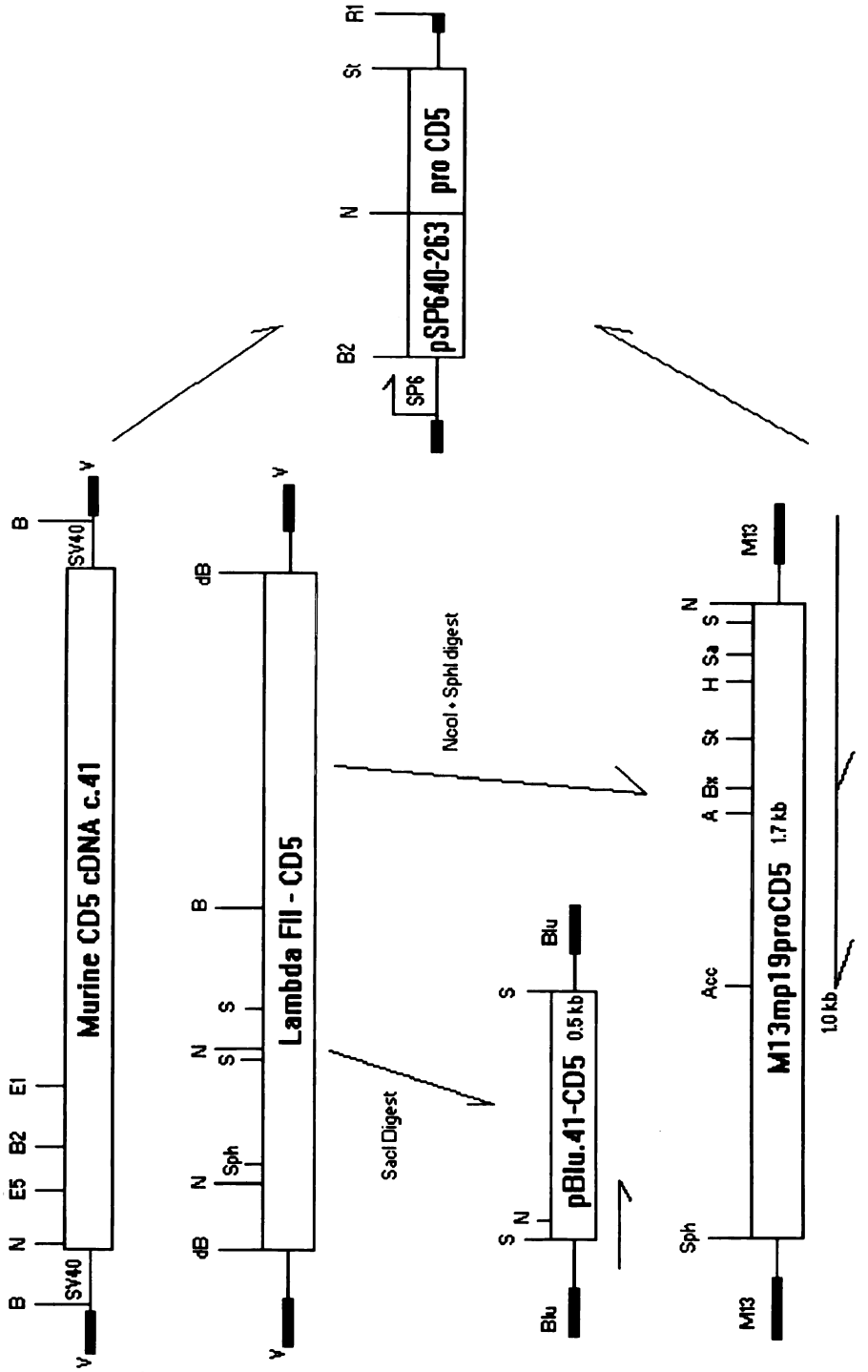

Figure 1. *Cloning Strategy and Partial Restriction Map of the CD5 5'-Flanking Region*

flanking region (Fig. 1b). Restriction fragments from a *Sac*I digest of LambdaFixIIcl.41-CD5 were subcloned into the SK-pBluescript plasmid and rehybridized with the *Nco*1-*Eco*RV cDNA probe to select a positive clone (pBlu.41) with a 500 bp insert (Fig. 1c). Double-stranded, dideoxy-sequencing (Sequenase version 2.0; USB Inc., Cleveland, OH) utilizing the pBluescript reverse primer (5'-AACAGCTATGACCATG-3'; Stratagene)(18) determined that this fragment terminated 29 bp upstream of the coding region. The 500 bp fragment was then used as a probe to identify a genomic clone containing a 2.0 kb *Nco*I genomic clone fragment, which was made blunt-ended with Klenow fragment of DNA polymerase I (Klenow) and digested with *Sph*I. This yielded a 1.7 kb fragment that was directionally subcloned into the *Pst*I (blunt-ended with T4 DNA polymerase) and *Sph*I sites of M13mp19, creating M13mp19pro (Fig. 1d). This effectively regenerated the 3' *Nco*I site of the genomic clone. Approximately 1.0 kb of this insert was sequenced utilizing the M13 forward sequencing primer (5'-TGACCGGCAGCAAAATG-3')(USB) and an upstream primer (5'-CCTGCTCACTCCTGATG-3') complementary to the insert sequence at -496 to -512.

Plasmid Constructs - To create the <u>in vitro</u> transcription vector pSP64CD5 for RNAse protection assays, the murine CD5 cDNA was digested from the MD-10 vector (17) by *Bam*HI (Fig. 1a), cloned into the corresponding site in the pSP64 vector (Promega) and then antisense orientation was verified by

multiple restriction enzyme digestions. This construct, pSP64CD5, was then digested with *Bst*EII (restriction site found at +263 of the CD5 cDNA) and *Hinc*II (this site is found in the pSP64 polylinker between the SP6 promoter and the CD5 insert). The resulting restriction fragments were subjected to low-melting-point-agarose (LMP) gel electrophoresis and the band containing the vector sequences and the first 263 base pairs (bp) of the CD5 cDNA was isolated from the agarose. The vector was made blunt-ended with Klenow and the vector recircularized with T4 DNA ligase, creating pSP640-263. pSP640-263 was then digested with *Nco*I and *Sma*I (the *Sma*I site is found in the pSP64 polylinker upstream of the cDNA insert) and the *Nco*I-*Stu*I 5'-flanking region fragment (Fig. 1d) ligated into it to generate pSp640-263pro (Figure 1e).

The 1700 bp *Nco*I (blunt-ended with Klenow)-*Sph*I fragment from M13mp19CD5 (Fig. 1d) was directionally subcloned into the *Pst*I (made blunt-ended by T4 DNA polymerase) and *Sph*I sites of the pCAT-Basic reporter vector (Promega)(Fig. 4a and 4b). This vector was digested with *Nco*I and *Hin*dIII and the resulting 1700 bp fragment subcloned into the corresponding sites in the pCAT-Enhancer vector (Promega) (Fig. 4c). The -125 deletion constructs were created by directly ligating a purified *Sau*96I-*Nco*I (both blunt-ended with Klenow) fragment of the 1700 bp M13mp19CD5 insert into the *Pst*I (made blunt-ended with T4 DNA polymerase)-*Hin*dIII (made blunt-ended with Klenow) sites of pCAT-Basic and pCAT-Enhancer vectors. Correct orientation was verified by multiple restriction

enzyme digestions. Sequential 5′-to-3′ deletions of promoter/reporter constructs were accomplished by utilizing the *Hin*dIII site of the reporter vector and the appropriate internal restriction sites (Fig. 4a an 4d).

RNA Isolation and Characterization - Cytoplasmic RNA was isolated from EL4 and NIH 3T3 cells by an SDS/urea method (19) and analysed by RNAse protection assay (20). Riboprobes were synthesized in vitro in the presence of 10 units of SP6 (New England Biolabs (NEB), Beverly, MA), 1x SP6 transcription buffer (NEB), 6.0 μM of 400 Ci/mmol [α-^{32}P]CTP, 150.0 μM each of unlabeled ATP, UTP and GTP, 25.0 μM unlabeled CTP and 1.0 ug linearized vector (Fig. 1e). Probes were hybridized with 180.0 μg cytoplasmic RNA in 80% formamide, 40.0 mM PIPES pH 6.7, 0.4 M NaCl and 1.0 mM EDTA overnight at 50° C. Riboprobe-cytoplasmic RNA hybrids were digested in 10.0 mM Tris-HCl, pH 7.5, 5.0 mM EDTA, 300.0 mM NaCl, 2.0 μg/ml RNAse T1 and 40.0 μg/ml RNAse A at 30° C for 30 minutes. Protected RNA species were electrophoresed in a 0.4 cm-thick 5.0% polyacrylamide/urea sequencing gel (19).

RESULTS

Sequence of the CD5 5′-Flanking Region and First Exon - Sequence analysis of pBlu.41 (Figure 2) reveals that an intron begins at +58 bp downstream of the terminal nucleotide reported as the 5′-end of the murine CD5 cDNA (17). Our data shows a single nucleotide discrepancy in this 58 bp coding

Figure 2. *Sequence of the CD5 5'-Flanking Region.* The sequence from -27 to +87 was derived from pBlu.41 and the sequence from +9 to -1119 was derived from M13mp19proCD5. Numbers preceded by '-' at the right margin refer to bp upstream of a transcriptional initiation site at +1. Location of known enhancer motifs are highlighted by overlining or underlining. The N-terminal methionine of the coding sequence is identified as 'met'. A guanine nucleotide not matching the reported cDNA sequence (17) is identified by a solid box.

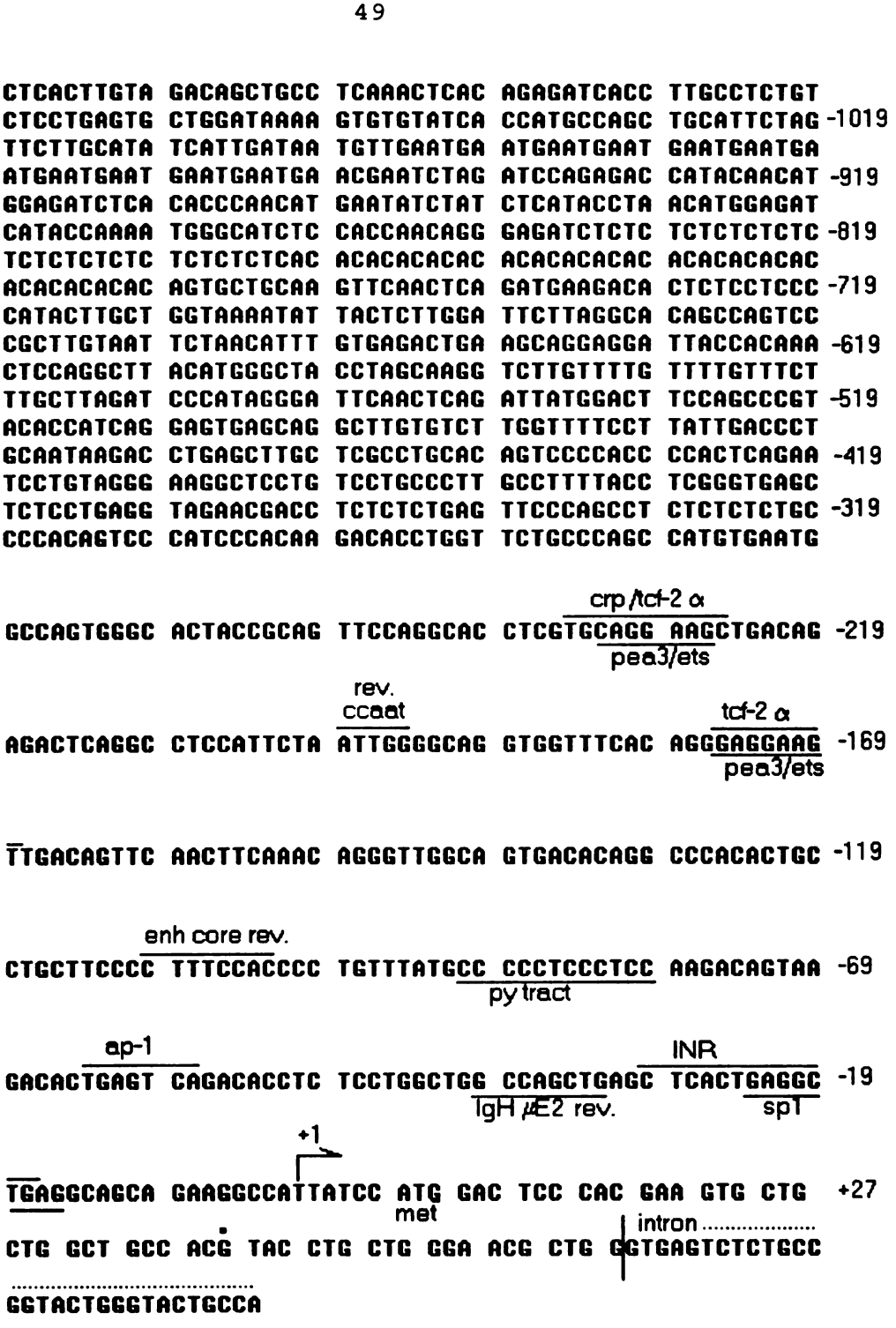

FIGURE 2. *Sequence Analysis of the CD5 5'-Flanking Region*

region when compared to the reported cDNA sequence. Originally reported as adenine (17), we find a guanine nucleotide at base +39. While this A to G alteration may represent a polymorphism between the C57Bl/6 strain used to isolate the CD5 cDNA and the Swiss mouse strain (NIH 3T3) used to clone the 5'-flanking region, the RNAse protection data (Figure 3) in this area does not display truncated RNA species, suggesting that the base is indeed guanine. In either case, the A-to-G change is silent, with both resulting triplets coding for threonine.

The 5' End of the CD5 Message Maps to the 5'-Flanking Region - To determine if the 5'-end of the CD5 transcript actually mapped to our 5'-flanking region genomic clone and to determine the transcriptional start site of the mRNA, a hybrid gene was constructed in the pSP64 vector (Figure 1e). The *Stu*I-*Nco*I fragment of the CD5 5'-flanking region was directionally cloned into the pSp640-263 vector already containing the terminal 263 bp fragment of the CD5 cDNA. This vector, pSp640-263pro, maintains the fidelity of the *Nco*I coding region-junction area, without additions or deletions, and produces a riboprobe (run-off the SP6 promoter) complementary to the 5'-end of the CD5 coding region that extends into the flanking region.

Figure 3 shows an RNase protection assay of EL4 T-cell cytoplasmic RNA compared to fibroblast RNA ($CD5^-$ cell type) using the riboprobes generated from the pSp640-263pro vector.

FIGURE 3. *The 5'-Termini of CD5 mRNA Map to the CD5 5'-Flanking Region.* RNAse protection analysis of CD5 mRNA. Lane 1, NIH 3T3 fibroblast RNA probed with probe P. Lane 2, EL4 RNA probed with probe P'. Lane 3, EL4 RNA probed with probe P. Lane M, single-stranded RNA ladder (BRL, Gaithersburg, MD) end-labelled with T4 polynucleotide kinase and [γ-^{32}P]UTP. Size of marker bands is given in nucleotides on right border of figure. Lane P, probe P. This probe was generated by in vitro transcription utilizing the *Eco*RI-linearized pSP640-263proCD5 vector (Figure 1e). Full-length probe P is 516 bases, of which 471 are CD5-specific (263 bases complementary to CD5 cDNA and 208 bases complementary to the CD5 5'-flanking region) while 45 bases are encoded by the pSP64 polylinker. Lane P', probe P'. This probe was generated by in vitro transcription reaction utilizing the *Nco*I-linearized pSP640-263proCD5 vector (Figure 1e). Full-length probe P' is 290 bases, of which 263 bases are complementary to the CD5 cDNA sequence and 27 bases are pSP64 polylinker sequence. Arrowheads without tails at the left border of the figure identify multiple protected species of EL4 RNA in lane 3. The arrowhead with a tail at the left border identifies a single protected species of EL4 RNA in lane 2.

FIGURE 3. *The 5'-Termini of CD5 mRNA Map to the CD5 5'-Flanking Region*

EL4 RNA (Figure 3, lane 3) displays protected species of heterogeneous length terminating in the putative promoter region of pSp640-263proCD5. No protected species are evident with fibroblast RNA (Figure 3, lane 1). pSp640-263 control protections, utilizing probe P' (complementary to the CD5 coding region only) displays a single protected band terminating at the 5'-*Nco*I site of the coding region, while most protected RNA species using probe P (which includes the 5'-flanking region) terminate up to 100 bases beyond this point. This suggests that CD5 transcription initiates from multiple sites in the 5'-flanking region.

The reported cDNA sequence reveals only 6 nucleotides upstream of the ATG coding for the N-terminal methionine of the leader sequence (17). Due to the apparent heterogeneity of start sites, the cDNA sequence may have been derived from an mRNA species initiated at a site close to the coding sequence. Therefore, downstream nucleotide numbering will commence with the 5'-terminal thymidine (T) of the published cDNA sequence (17). S1 nuclease protections utilizing both synthetic oligonucleotide probes and single and double-stranded restriction fragments, as well as primer extension analyses with multiple oligos, were unable to map the 5'-termini of CD5 transcripts with any finer detail, possibly due to secondary structure of the flanking region.

The CD5 5'-Flanking Region Lacks a Consensus TATA Box - The 5'-flanking region of CD5 lacks a consensus TATA box (Figure

2). Two general types of TATA-less lymphoid-specific promoters have been described. The first, represented by the TdT promoter, uses the initiator (INR) sequence, 5'-CTCANTNGGAGAC-3', to initiate transcription from a single site in the INR sequence. The group represented by Ig variable region promoters expressed in pre-B-cells, and the mb-1 and B-29 genes, also contains this INR sequence but initiates transcription from multiple sites upstream of the INR (21,22). The 5'-flanking region of CD5 has an INR-like sequence (5'-CTCACTGAGCTG-3'; underlined bases match the INR sequence) at -29, overlying a putative SP1 binding site (Figure 2). In the mb-1 promoter, SP1 is thought to act as a tethering factor for TFIID and the transcription complex (21). Our clone probably belongs to the mb-1 class of TATA-less promoters although the multiple initiation sites of CD5 appear to range further upstream than those described for this type of promoter.

The CD5 5'-Flanking Region is Capable of Tissue-Specific Promoter Activity - CD5 mRNA is expressed in a lymphoid-specific fashion in mice and humans (17). To investigate whether our putative promoter could initiate transcription in a lymphoid-specific manner at levels reflective of those found *in vivo* in T-cells and B-cells (T-cell steady-state mRNA levels are consistently greater than B-lineage levels; FACS analysis of surface CD5 protein also reflects this difference), we utilized transient transfection techniques to introduce the -1700 pCAT-Basic reporter vector construct

FIGURE 4. *Restriction Map of the CD5 5'-Flanking Region Deletion Constructs.* **A.** The -1700 bp CD5 5'-flanking region fragment was subjected to serial 5'-to-3' deletions with *Acc*I (-1107), *Ava*I (-374), *Bst*XI (-275), *Stu*I (-208), *Hinc*II (-165), *Sau*96I (-125) and *Sac*I (-27). Numbering of deletion constructs corresponds to the restriction site used to generate them. The maps of -275 through -27 have been expanded to show greater detail. Location of known enhancer motifs are identified above each construct beginning with -275. **B.** Restriction map of pCAT-Bacic vector (Promega). **C.** Restriction map of pCAT-Enhancer vector (Promega). **D.** Restriction map of the pGL2-Luciferase control vector (Promega).

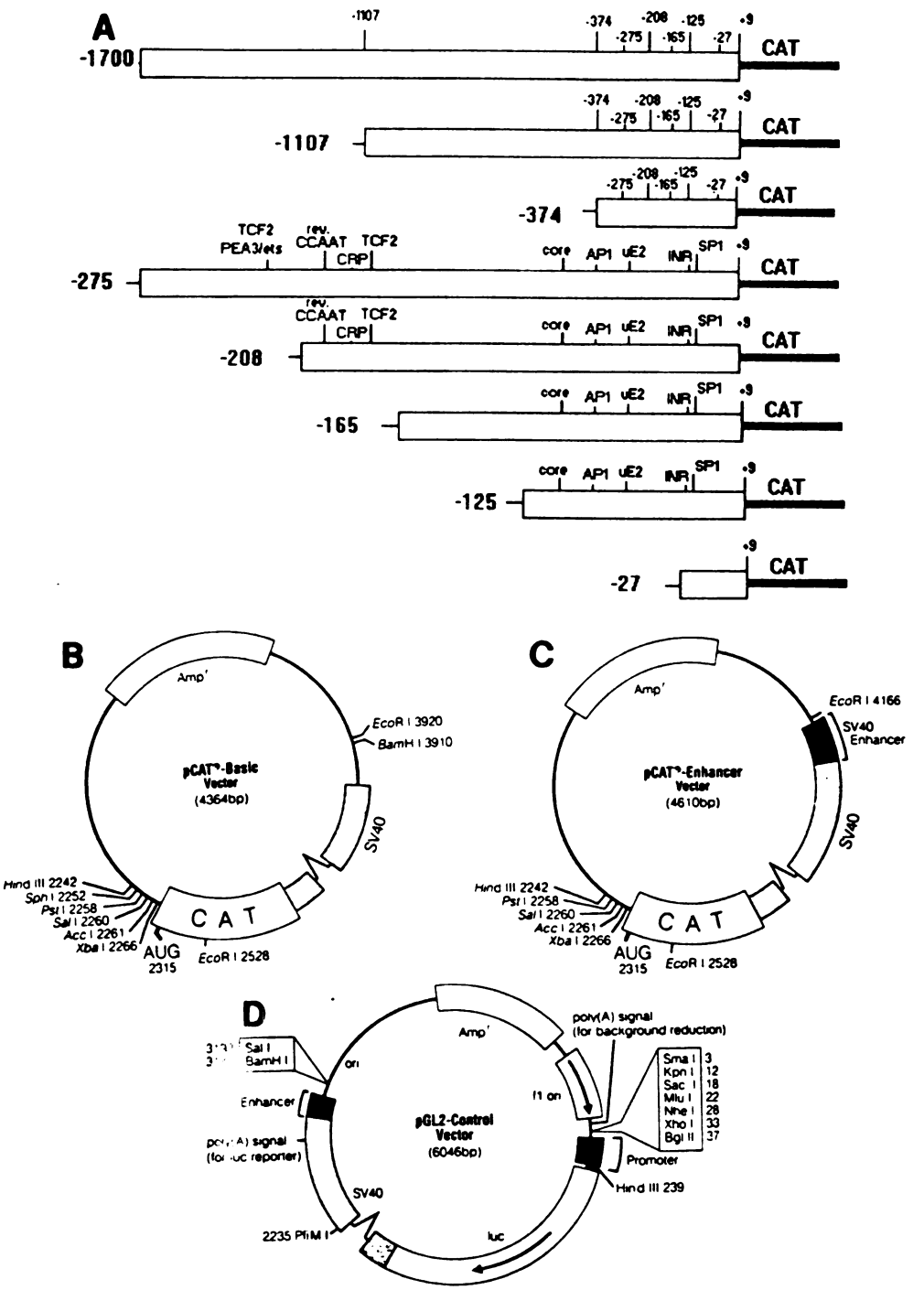

FIGURE 4. *Restriction Map of the CD5 5'-Flanking Region Deletion Constructs*

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(Figure 4a) into EL4 (T-cell), M12 (B-cell), PD36 (pre-B-cell) and NIH 3T3 (fibroblast) cell lines. Figure 5 shows that promoter/CAT activity in these cell types is indeed reflective of relative steady state mRNA levels found in these cell types. EL4 T-cells displayed the greatest activity, followed by PD36 pre-B-cells (0.8x the value of EL4) and M12 B-cells (0.5x the value of EL4), with NIH 3T3 fibroblasts displaying negligible CAT activity. CAT activity from the pCAT-Basic vector alone was at background levels, while expression from the vector containing the SV40 promoter and enhancer regions (a positive control for expression) showed the greatest activity in any given line. If the orientation of the CD5 promoter is reversed in the pCAT-Basic vector, expression decreases 10-fold (Figure 6).

Sequences Flanking -27 to -125 are Capable of Acting as a Minimal Lymphoid-Specific Promoter - Figure 4a shows the structure of progressive 5'-to-3' CD5 promoter truncations constructed in the -1700 pCAT-Basic and -1700 pCAT-Enhancer vectors. When these constructs were transiently transfected into EL4 cells, it was found that the vector containing the -27 to +9 fragment was incapable of expression in either plasmid, while the -125 to -27 fragment contains sequences sufficient for promotion (Figure 7). Putative enhancer elements located in this fragment (Figure 4a) that are capable of transcriptional activation in other systems, include an AP-1 site at -63 (23), a reverse IgH chain μE2 enhancer motif at

FIGURE 5. *Tissue-Specific Expression of the CD5 Promoter.* Cell lines were transfected as described in Materials and Methods. PD36 cells are poorly transfectable, with the pCAT-Basic vector transfectants displaying only 1.5 light units of activity. All cell extracts volumes were normalized to this value for CAT reactions. After chromatography, TLC plates (PE SIL G/UV; Whatman, Inc., Hillsboro, OR) were sprayed with surface autoradiography enhancer (EN^3Hance; NEN, Boston, MA) and exposed for 48 hours. Numbers immediately below figure represent the ratio of [^{14}C]chloramphenicol converted by a given transfectant to [^{14}C]chloramphenicol converted by the EL4 -1700 transfectant. Figures are representative of at least two replications per cell line.
Abbreviations: -1700, -1700 pCAT-Basic vector (Figure 4a); V, pCAT-Basic vector; '+', pCAT-positive control vector (contains the SV40 promoter and enhancer regions; Promega).

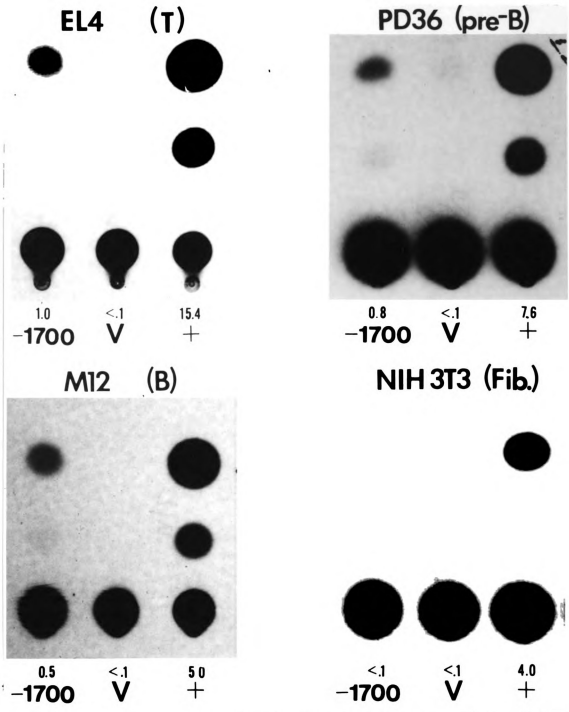

FIGURE 5. *Tissue-Specific Expression of the CD5 Promoter*

FIGURE 6. *Maximal CD5 Promoter Activity is Orientation-Dependent.* The -1700 promoter fragment (Figure 4) was cloned into the pCAT-Basic vector in the reverse orientation. The -1700rev. pCAT-Basic construct was transiently transfected, in parallel with the -1700 pCAT-Basic vector, into EL4 cells. The pCAT-positive control vector had the the lowest light unit readings (2.0) and volumes of cell extracts utilized for CAT reactions were normalized to this number. Numbers immediately below the figure represent the ratio of [^{14}C]chloramphenicol converted by a given transfectant to [^{14}C]chloramphenicol converted by the -1700 pCAT-Basic transfectant. Figure represents data from one experiment.
Abbreviations: '+', pCAT-positive control vector; -1700, -1700 pCAT-Basic in the forward orientation (Figure 4); -1700rev., -1700 pCAT-Basic in the reverse orientation; V, pCAT-Basic vector only.

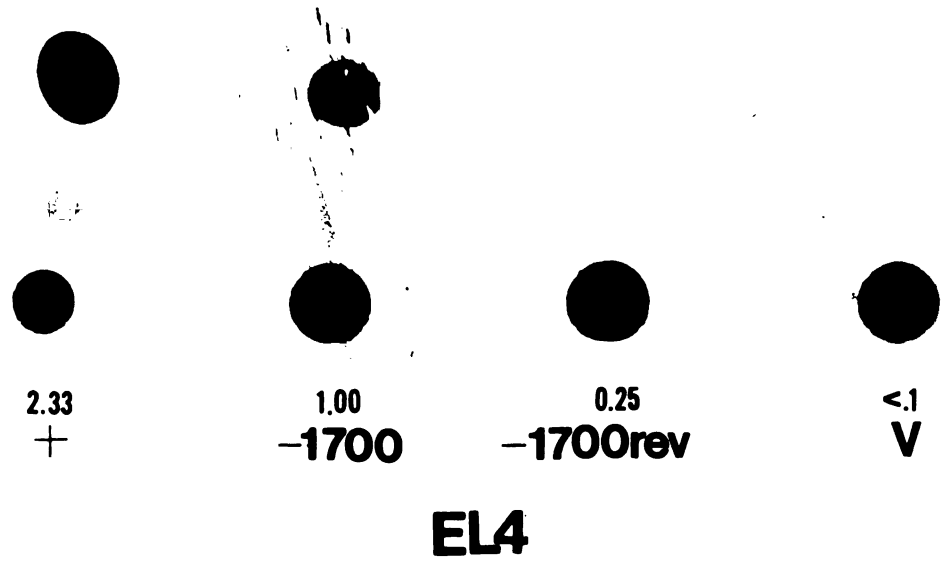

FIGURE 6. *Maximal CD5 Promoter Activity is Orientation-Dependent*

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-39 (13) and a reverse SV40 core element at -109 (24). The *Sac*I site used to generate the -27 deletion destroys a putative INR site and an overlying SP1 motif, possibly accounting for the loss of activity.

The CD5 promoter contains a reverse IgH chain enhancer element, μE2, at -39 (5'-GCCAGCTG-3'). μE2 is capable of enhancing expression from minimal promoters in both lymphoid and non-lymphoid cells (13). μE2 is found in the ENH_{iH} intron of the IgH heavy chain locus, situated between the μE5 and the μE3 enhancer motifs. Studies utilizing a CAT expression vector construct consisting of only a TATA box and the μE2, μE3 or μE5 enhancers in various combinations, have shown that μE2 is not required for tissue-specific expression of this locus (25). While μE3 alone is active in all tissue types, it's non-lymphoid expression is suppressed by μE5. Constructs consisting of μE5 and μE2 in tandem do not enhance expression in any cell type. While this work implicates μE5 as the genetic switch determining tissue-specific expression, it does not address the ability of μE2 to enhance expression in the presence of other cis-acting elements. The CD5 promoter may be a good candidate for studying the capability of μE2 to act as an enhancer outside the IgH locus.

The py tract (8-to-14 pyrimidine base pairs) of Ig enhancers is found up to 41 bp upstream of heptamer/octamer motifs and has been found to augment transcriptional enhancement from these motifs. Deletion of the py sequences have been shown to reduce promoter activity 2-3 fold in B-

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cells (26). CD5 has a 12 bp pyrimidine tract at -90 (5'-CCCCCTCCCTCC-3') which ends 40 bp upstream of the reverse μE2 motif. While the effect of py tracts on enhancement by μE motifs is not known, the CD5 promoter may provide the opportunity to study such an interaction.

Regulation of the CD5 Promoter in Lymphoid Cells is Mediated by Positive and Negative Elements - Serial 5'-deletions of the CD5 promoter region reveal a succession of positive and negative regulatory elements in both EL4 and M12 cells (Figures 7 and 8). Expression from individual promoter deletion constructs, in both EL4 and M12 cells, appear to follow similar trends. Although [^{14}C]chloramphenicol conversion values are higher with deletion constructs expressed in M12 cells, corrected values accounting for the 5-fold less extract loaded in EL4 CAT assays suggest that mutants express comparably in both cell lines.

The -275 and -125 promoter deletions in EL4 cells (Figure 7, upper panel) each display >2-fold increases in expression over the preceding construction and may represent loss of silencer elements from the promoter. The largest decrease in expression compared to a preceding construction, is found in the -165 deletion transfected into EL4 cells (14 fold decrease) and may represent loss of an important enhancer element found between positions -208 and -165 (Figure 7, upper panel). Interestingly, the -165 deletion construct also displayed a drop in expression in M12 cells (3-fold; Figure 8,

FIGURE 7. *Activity of CD5 Promoter Deletion Constructs in EL4 Cells.* The deletion constructs depicted in Figure 4a were transfected into EL4 cells with the -208 pCAT-Enhancer construct yielding the lowest light unit values (4.5). All cell extract volumes were normalized to this value for CAT reactions. Numbers along the lower border of the figure correspond to the promoter deletion construct (Figure 4a) cloned into either the pCAT-Basic vector (upper panel) or the pCAT-Enhancer vector (lower panel). Numbers along the upper border of the upper panel represent the ratio of [^{14}C]chloramphenicol converted by a given transfectant to [^{14}C]chloramphenicol converted in -1700 pCAT-Basic cell extracts and apply to the upper panel only. Data is representative of three replications of this experiment.
Abbreviations: B, pCAT-Basic vector; E, pCAT-Enhancer vector; '+', pCAT-positive control vector.

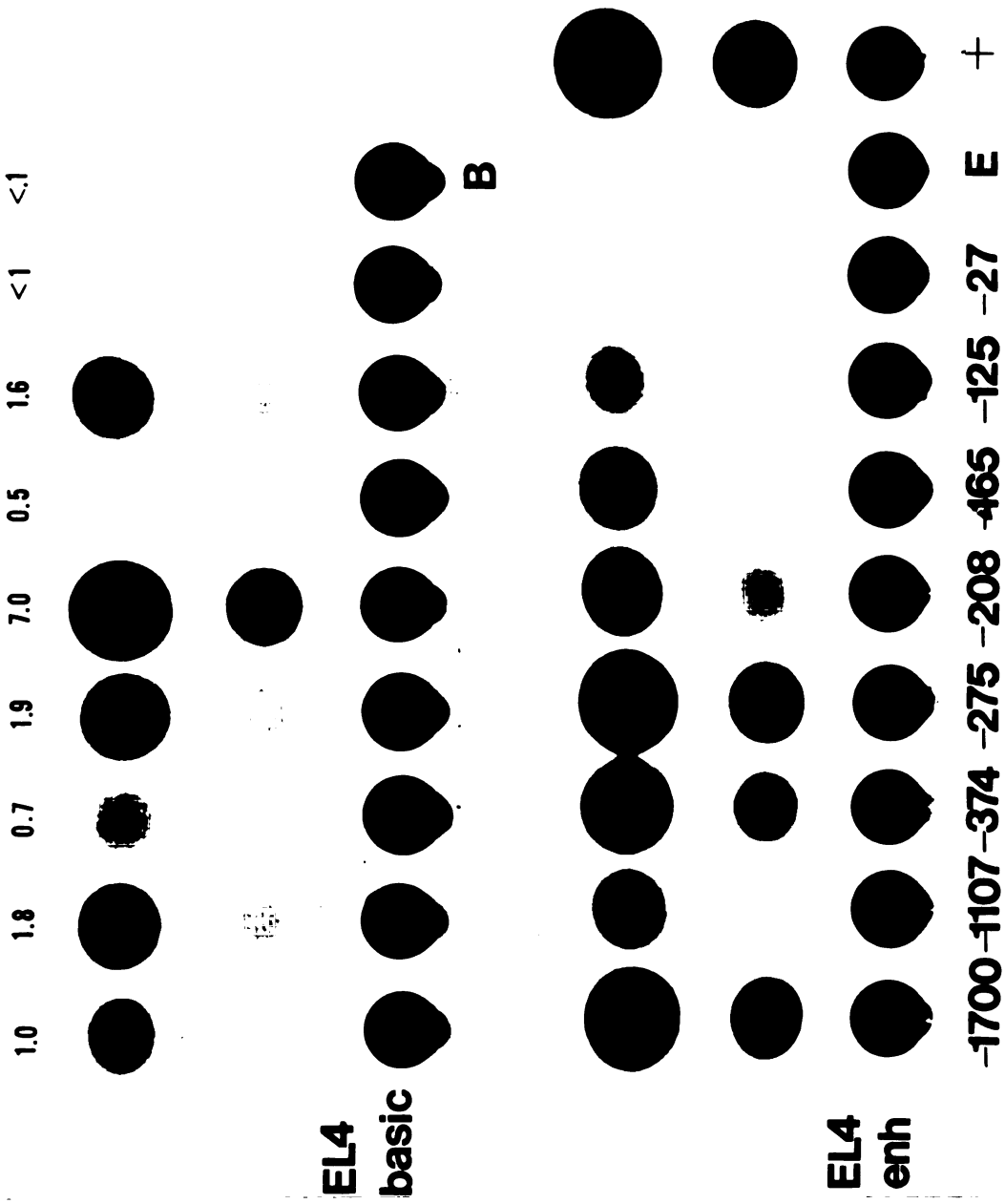
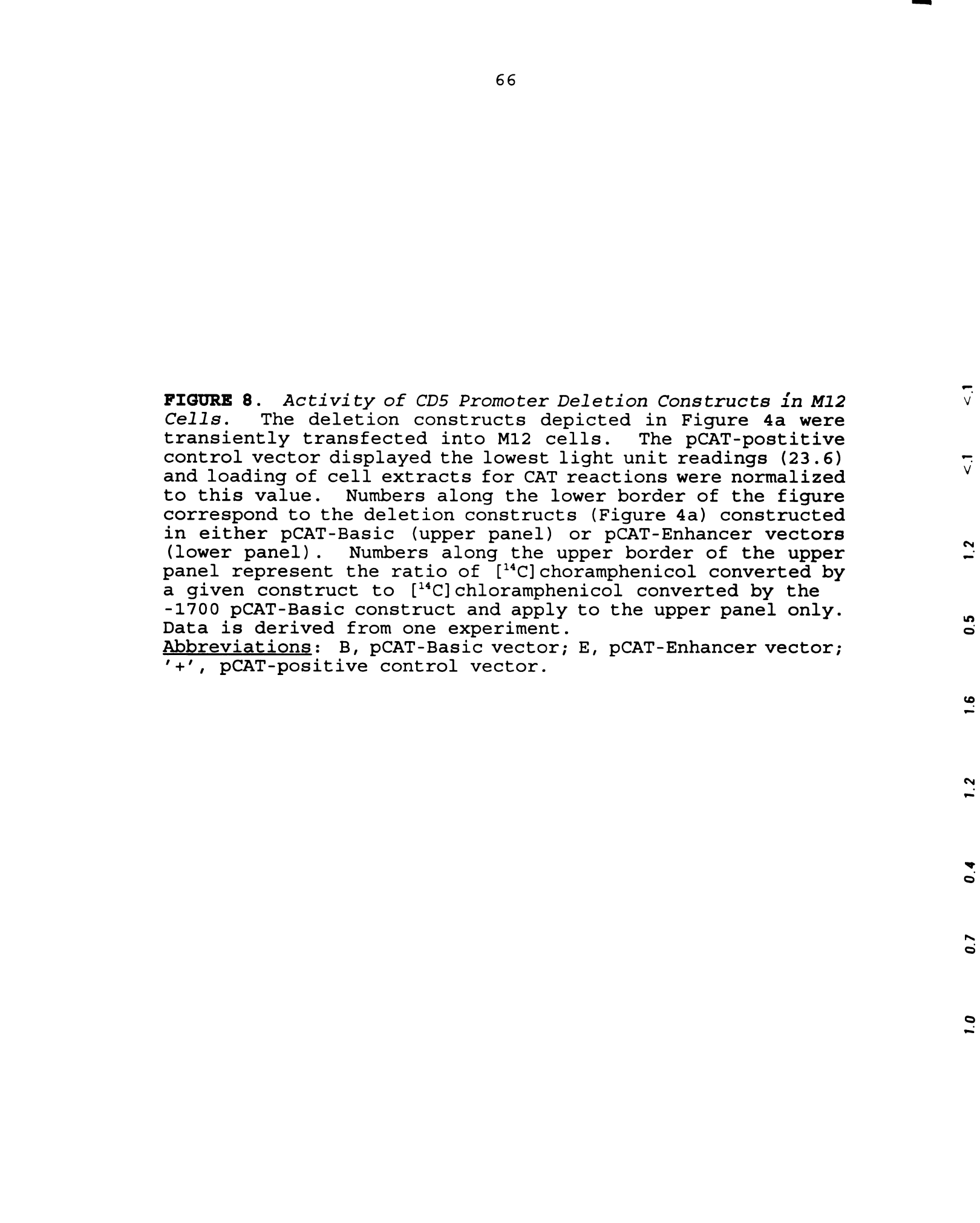


Figure 7. *Activity of CD5 Promoter Deletion Constructs in EL4 Cells.*

FIGURE 8. *Activity of CD5 Promoter Deletion Constructs in M12 Cells.* The deletion constructs depicted in Figure 4a were transiently transfected into M12 cells. The pCAT-postitive control vector displayed the lowest light unit readings (23.6) and loading of cell extracts for CAT reactions were normalized to this value. Numbers along the lower border of the figure correspond to the deletion constructs (Figure 4a) constructed in either pCAT-Basic (upper panel) or pCAT-Enhancer vectors (lower panel). Numbers along the upper border of the upper panel represent the ratio of [^{14}C]choramphenicol converted by a given construct to [^{14}C]chloramphenicol converted by the -1700 pCAT-Basic construct and apply to the upper panel only. Data is derived from one experiment.
Abbreviations: B, pCAT-Basic vector; E, pCAT-Enhancer vector; '+', pCAT-positive control vector.



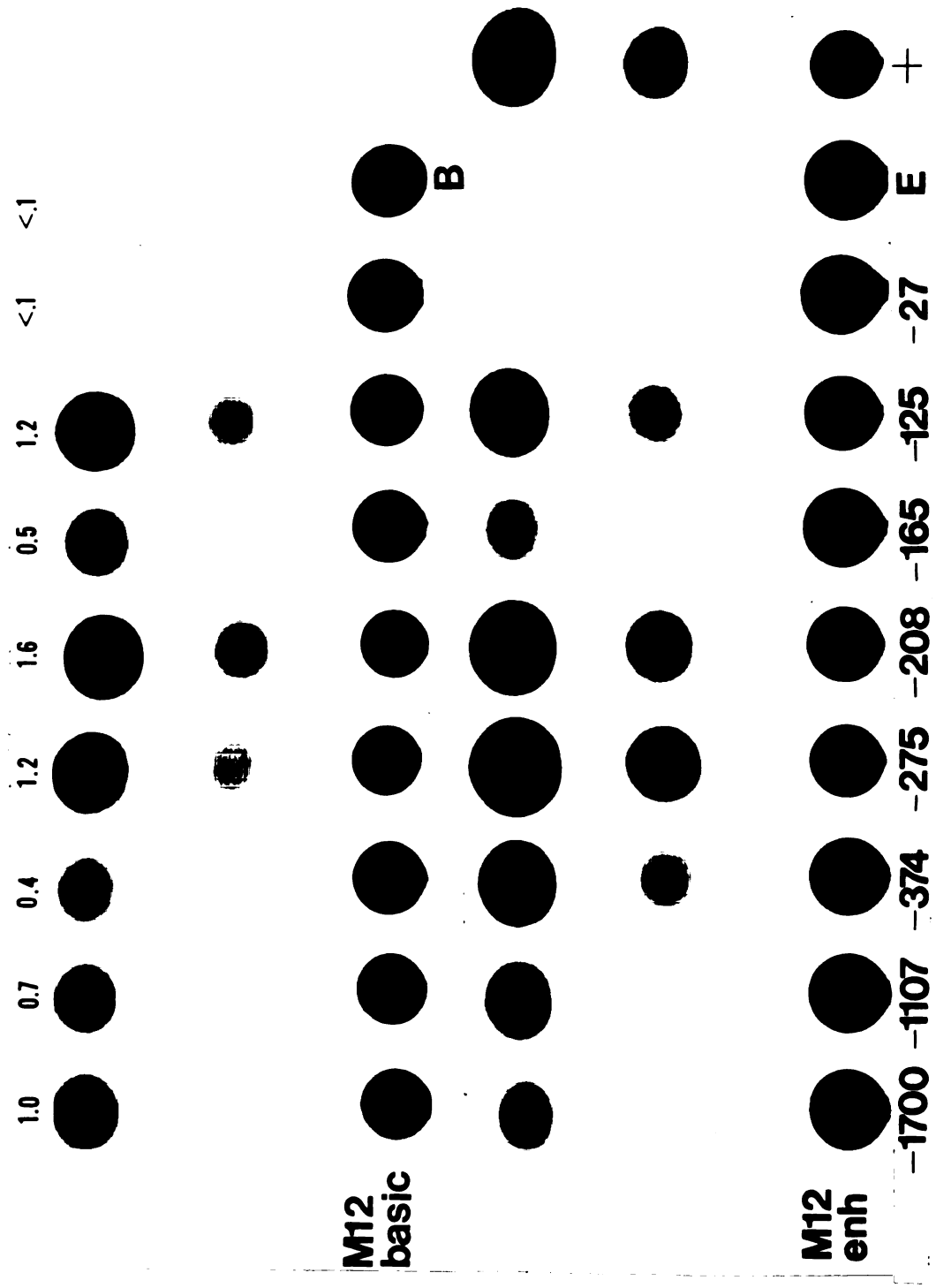


Figure 8. *Activity of CD5 Promoter Deletion Constructs in M12 Cells.*

upper panel), suggesting that T-cells and B-cells have common factors that recognize an enhancer element between positions -165 and -208. Known enhancer elements found in this region (Figure 4a) include a reverse CCAAT box (-198), thought to bind the C/EBP family of transcriptional activators (27), and a PEA3/ets motif (-174) (28), and a TCF-2α motif (13).

The CD5 promoter contains a reverse CCAAT box motif at -198. This motif has been shown to bind a diverse family of proteins capable of transcriptional enhancement including IgEBP-1 (29), C/EBP (27) and NF-IL6 (30). IgEBP-1 is found in all tissues although levels are highest in EL4 T-cells and pre-B-cells (29). NF-IL6 can be found in most tissue types (30), while C/EBP is found in liver, adipose tissue, small intestine, placenta, lung and in non-dividing tissue (27). C/EBP, and other C/EBP-related proteins (CRP's) are able to heterodimerize, possibly conferring tissue specificity or gradations of enhancement to promoters containing this motif. The CRP core motif is 5'-TT/GNNGNAAT/G-3' with further specificity conferred by flanking sequences. The CD5 promoter contains three degenerate CRP motif homologies: one in reverse orientation at -198 (which includes the reverse CCAAT box) and two in standard orientation at -177 and -234. The sites at -177 and -198 deviate one base from the consensus sequence, while the site at -234 is a perfect match. The ubiquitous expression of the C/EBP family and ability to enhance transcription from degenerate motifs makes the above sites possible candidates for binding of transcriptional

activators in the CD5 locus. The redundancy of these sites may be responsible for the lack of effect observed with the deletion that removes only the upstream -234 CRP site.

The CD5 promoter contains TCF-2α motifs at -175 and -231 (Figure 2). This domain, in the TCR-α promoter, has been shown to bind the lymphoid-specific factor LEF-1 in nuclear extracts from the 1200M T-cell line (13). This motif was capable of enhancing transcription from heterologous promoters in M12 B-cells, PD36 pre-B-cells and EL4 T-cells. Transcription was minimal in NIH 3T3 cells, suggesting that this factor is capable of conferring tissue-specific activity (13). While this motif overlaps the PEA3/ets motifs at -175 and -231, it would represent an ideal candidate for initial investigation of factors conferring tissue-specificity to the CD5 promoter. Again, the redundancy of these sites may explain the lack of effect with the deletion that removes only the upstream -231 TCF-2α site.

Sequences Upstream of -275 May Repress Expression of the CD5 Promoter in Fibroblasts - Figure 9 displays CAT expression from pCAT-Basic vector deletion constructs transfected into NIH 3T3 fibroblasts. In order to observe low levels of expression, CAT reactions were loaded with 15-fold more cell extract than used for the NIH 3T3 CAT reactions displayed in Figure 5. The deletion constructs -1700, -1107 and -374 display equal but low-level promoter activity. This activity increases 3.5-to-5-fold for deletion constructs -275 to -125.

FIGURE 9. *Activity of CD5 Promoter Deletion Constructs in NIH 3T3 Fibroblasts.* The deletion constructs in Figure 4a were transfected into NIH 3T3 fibroblasts. The -125 pCAT-Basic construct displayed the lowest light unit levels (21.0) and all cell extract volumes for CAT assays were normalized to this value. Numbers along the lower border of the figure correspond to the deletion constructs in Figure 4a. Numbers along the upper border of the figure represent the ratio of [^{14}C]chloramphenicol converted by a given cell extract to the [^{14}C]chloramphenicol converted by the -1700 pCAT-Basic cell extract. The pCAT-positive control transfection is found in the NIH 3T3 panel in Figure 5. Data is derived from one experiment.
Abbreviations: V, pCAT-Basic vector.

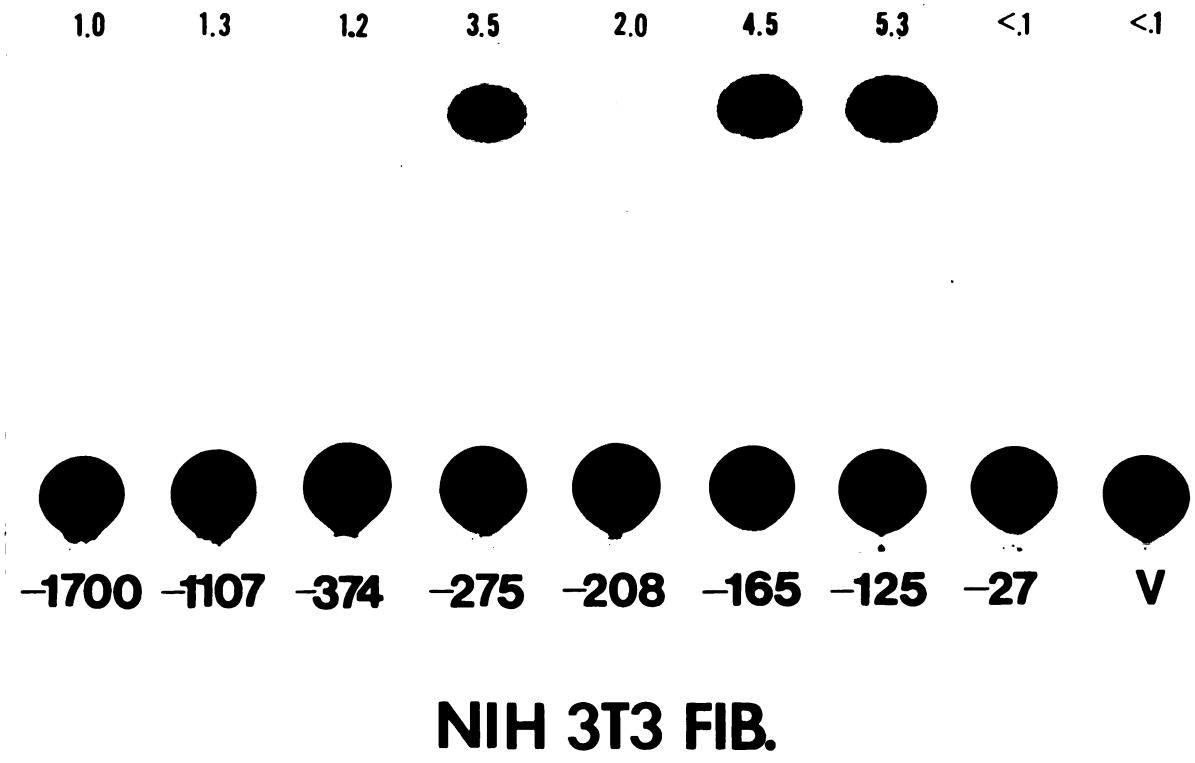


Figure 9. *Activity of CD5 Promoter Deletion Constructs in NIH 3T3 Fibroblasts.*

This pattern suggests the loss of a negative regulatory element between positions -374 and -275. The model system for the suppression of lymphoid-specific promoter activity in fibroblasts is found in the IgH locus. As previously stated, the μE5 motif represses the enhancer activity of the μE3 element in fibroblasts, but not in lymphoid cells (25). While there is no consensus μE5 motif in the CD5 promoter, the fibroblast transfection data suggests the loss of a μE5-like element between positions -374 and -275.

DISCUSSION

The promoters of the B-cell-specific genes mb-1 and B29, share many characteristics with the murine CD5 promoter. They are all surface glycoproteins expressed on B-lineage cells and as previously stated, lack TATA boxes. All initiate transcription from multiple sites in and around an INR-like sequence. The promoters of all three genes contain Ig enhancer elements that may be capable of conferring lymphoid specificity (20,21,31). The B29 promoter has the most extensive collection of Ig enhancer sites, including IgH μE4, μE3, Ig octamer/heptamer (5'-ATTTGCAT-3') motifs and a py tract (31). The octamer and μE elements have been found to confer B-cell specific activity to Ig promoters and are capable of binding proteins in a variety of cell types (23). Deletion of the py tract upstream of octamer/heptamer sites has been found to reduce promoter activity 2-3 fold in B-cells (25). While the mb-1 promoter contains a near-perfect octamer

motif, data suggests that tissue-specificity may be conferred by a different cis-acting element (32). The mb-1 promoter contains a functionally important PEA3/ets motif (5'-CAGGAAGTG-3'), that has been shown to bind the transcription fator MUF2 in PD36 and EL4 cell extracts (33). Subsequent examination has revealed that this motif can bind ets-1, ets-2 and PU.1 proteins in vitro. ets-1 is expressed in B and T-cells, PU.1 in myeloid and B-cell lineages, while ets-2 has ubiquitous tissue distribution. These factors have been found to bind the PEA3/ets motif with different affinities and could possibly influence the tissue-specific expression of genes containing this motif (34). The CD5 promoter contains PEA3/ets sites at -174 (5'-AGGAAG-3') and at -232 (5'-CAGGAAG-3'; Figure 2), and MUF2 has been shown to be present in PD36 cells (34). This could be an important enhancer motif involved in CD5 expression.

In summation, we have cloned and sequenced 1119 bp of the 5'-flanking region of the murine CD5 gene. This region can serve as a tissue-specific promoter that mirrors the in vivo expression of CD5 in lymphoid cells and lack expression in fibroblasts. A deletional analysis reveals that transcriptional regulation of CD5 is achieved through a complex array of both positive and negative regulatory elements. An IgH μE2 enhancer motif at -39 is a good candidate as a critical element for lymphoid-specific expression. The region between -374 and -275 may harbor a μE5-like element capable of repressing expression of CD5 in

fibroblasts. Additional CRP motifs at -198 and -234 PEA3/ets motifs at -174 and -232, and TCF-2α motifs at -175 and -231 are also plausible elements for involvement in lymphoid-specific expression. Further characterization of tissue-specific factors interacting with enhancer motifs found in this region of the CD5 promoter, may lead to a better understanding of control over tissue-specific expression.

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CHAPTER 3

Regulation of Murine CD5 Expression by Transforming Oncogenes

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ABSTRACT

The murine CD5 surface antigen is a frequent marker on B-lineage cell lines produced from bone marrow infected with v-H-*ras* and v-*src*. Since $CD5^+$ B-cells cannot be detected in adult murine bone marrow by FACS analyis, the viral targets of transformation are either a minor contaminating population of $CD5^+$ B-lineage cells or the transforming oncogenes are inducing the expression of CD5 on B-lineage cells not previously expressing this marker. We have found that v-H-*ras* and v-*src*, but not v-*myc*, can induce the expression of CD5 on two $CD5^-$ pre-B-cell lines established from murine bone marrow, and that this induction correlates with increased steady-state levels of CD5 mRNA. The existence of a signal transduction pathway capable of modulating the expression of CD5 may alter the concept that $CD5^+$ B-lineage cells represent a unique, self-renewing subset of B-lymphocytes.

INTRODUCTION

The murine CD5 surface antigen (also referred to as Ly-1 or Lyt-1) is a 67 KDa MW monomeric, membrane-associated glycoprotein structurally homologous to the cysteine-rich scavenger receptor family of polyanion-binding receptors (Kipps, 1989; Freeman *et al.*, 1990). CD5 is lymphoid-specific in its expression, being found on all T-lymphocytes and on a subset of B-cells found mainly in serousal cavities of adult mice. In addition, FACS (fluorescence-activated cell sorter) analysis has determined that small populations of $CD5^+$ B-cells reside in adult murine spleen and fetal liver, but not in adult bone marrow or peripheral circulation (Kipps, 1989). CD5 is physically associated with the TCR/CD3 complex on T-cells where it is thought to be a phosphorylation target for the protein tyrosine kinases $p56^{lck}$ and $p59^{fyn}$ during the transduction of extracellular signals (Burgess *et al.*, 1992; Osman *et al.*, 1992). No such function has been attributed to B-cell-associated CD5, but the B-lymphocyte-specific surface antigen CD72 (Lyb-2) has been identified as a ligand for CD5 (Van de Velde *et al.*, 1991). The implications of CD72/CD5 binding for T-B and B-B-cell interactions have yet to be elucidated.

CD5-expressing B-lineage cell lines are established at high frequency in specialized culture systems that support the growth of B-lymphocytes *in vitro*. A simple, long term splenocyte culture system has been described in which $CD5^+sIgM^+Ia^+FcR^+C3R^+$ B-cell lines are established after a 3 to

4-week crisis period (Braun, 1983). Also, a culture system that allows establishment of CD5$^+$ pro-B-cell lines from bone marrow of 4-week-old Balb/c mice has been described (Palacios *et al.*, 1987). In this system, murine bone marrow cells are first depleted of myelocytes and mature lymphocytes, and then passaged in WEHI-3 conditioned media known to contain IL-3.

CD5 expression is rare on pre-B-cell lines spontaneously arising from Whitlock-Witte high density bone marrow cultures established from bone marrow of 4-week-old Balb/c mice (R. Schwartz, unpublished observations; Whitlock *et al.*, 1983). However, if bone marrow is first infected with a transforming retrovirus expressing the v-H-*ras* oncogene and then cultured in the Witte-Whitlock system, CD5$^+$ pre-B-cell lines are the sole outgrowth (Schwartz *et al.*, 1986 and unpublished observations). Interestingly, if bone marrow is coinfected with v-H-*ras* and v-*myc*-expressing retroviruses and replated as above, the resultant pre-B-cell lines are CD5$^-$ (Schwartz *et al.*, 1986 and unpublished observations).

Another method utilizing transforming retroviruses to establish CD5$^+$ B-lineage cell lines from bone marrow at high frequency has been reported. In this system, bone marrow was harvested from 2 to 5-week-old mice and infected with replication defective retroviruses expressing either v-*src*, v-H-*ras*, v-K-*ras*, v-*fes*, or v-*abl*. After infection, the cells were suspended in soft agar. Individual colonies were picked and then expanded in 'bulk' cultures on feeder layers for 6 weeks. Analysis of these immortal lines for CD5 revealed that

less than 50% of the individual cells, in a given cell line established with v-*abl*, expressed CD5 (2 lines), 2 out of 6 lines established with v-*src* expressed CD5 and all lines established with v-*fes* (3 lines total), v-H-*ras* (5 lines total), or v-K-*ras* (1 line) expressed this marker (Pierce and Aaronson, 1982; Holmes *et al.*, 1986). This work was extended by another group who found zero out of eleven cell lines, established by Abelson murine leukemia virus (A-MuLV; v-*abl*) infection of murine bone marrow, expressed CD5 (Tidmarsh *et al.*, 1989).

$CD5^+$ B-cells are thought to represent a unique lineage of B-lymphocytes, 'B-1' cells, which are distinct from the $CD5^-$ 'B-2' cells found in adult murine bone marrow, lymph nodes and peripheral circulation (for review, see Kantor and Herzenberg, 1993; Herzenberg and Stall, 1989). The support for classification of $CD5^+$ B-cells as a unique lineage comes, in part, from adoptive transfer experiments utilizing lymphocytes derived from different immune compartments. It was found that if adult murine bone marrow was used to reconstitute the immune system of lethally-irradiated mice, $CD5^+$ B-cells could not be detected in the peritoneal cavities of the recipients. However, if peritoneal lymphocytes were used as donor cells in the reconstitution, donor $CD5^+$ B-cells could eventually be found to repopulate the peritoneal cavity (Hayakawa *et al.*, 1985). The interpretation of this experiment was that $CD5^+$ B-cells could only be replenished from $CD5^+sIgM^+$ precursors residing in the peritoneal cavity, and not $CD5^-sIgM^-$ pro/pre-B-

cells found in adult bone marrow. If this model is applied to the data demonstrating high frequency of establishment of $CD5^+$ B-lineage lines from murine bone marrow (utilizing transforming retroviruses), one may conclude that there is a small contaminating population of $CD5^+$ B-lineage cells in bone marrow that is preferentially susceptible to transformation by certain oncogenes.

A necessary extension of the lineage model is that CD5 expression is a constitutive phenomenon and not available, as a phenotype, to B-cells that do not have $CD5^+$ precursors. However, evidence has been accumulating that suggests that CD5 expression on murine B-cells may be subject to some modulation. CD5 expression on the 70z/3 murine pre-B-cell line has been found to be down-regulated by IL-4 and up-regulated 3-to-4-fold by lipopolysaccharide (LPS) (Jyonouchi *et al.*, 1990). Also, CD5 transcript levels are modulated as the murine B-cell line BCL_1-3B3 traverses the cell cycle (Brooks *et al.*, 1992). However, the most intriguing experiment demonstrating CD5 modulation was offered by Ying-zi et al. (1991). This group used FACS to sort against the 'typical' $CD5^+$ B-cell and collected a population of $CD5^-$ $sIgM^+IgD^{high}CD23^{high}CD45^{high}$ cells ('B-2' cells) from murine spleen. Using an anti-Mu antibody, they then cross-linked surface IgM on this population and noted an increase in the percentage of CD5-expressing cells, as well as an increase in actual cell numbers upon resorting. The authors claimed that if this increase had been due to the expansion of a small

population of contaminating CD5$^+$ B-cells, a bi-modal distribution would have been evident upon FACS analysis. In their discussion, they hypothesized that there were two types of activation pathways for 'B-2'-cells, one that was LPS-inducible, resulting in secreted Ig with no increase in CD5 (standard B-cell activation assay), and the other that proliferated and up-regulated CD5 expression upon anti-sIgM crosslinking (but does not produce secreted Ig). In addition, treating the CD5-inducible splenic populations with interleukin-6 yielded a surface phenotype identical to peritoneal-derived CD5$^+$ B-cells. The authors interpreted this to mean that the CD5 antigen may be a marker for proliferation of 'B-2' cells and that CD5$^+$ B-cells may not represent a distinct lineage.

What model, then, is most easily reconciled with the data demonstrating oncogene-mediated, high frequency establishment of CD5$^+$ B-lineage cell lines from murine bone marrow? If the targets of transforming retroviruses expressing v-H-*ras* are restricted to CD5$^+$ B-lineage cells, then the lineage model would be more compelling. However, if the retroviral targets are more diverse and include CD5$^-$ B-cells, then the cell line establishment data would be most reconcilable with the idea that the CD5 phenotype is available to any immature B-cell. If this were the case, it may be possible that the oncogenes expressed in these CD5$^-$ B-cells are activating a signal transduction pathway that results in the induction of CD5 expression.

While the work demonstrating CD5 up-regulation on sorted populations of splenic 'B-2' lymphocytes (Yin-zi *et al.*, 1991) is very solid, one technical objection is evident. This work relied upon the purification of 'B-2' cells, from heterogenous B-cell populations, that presumably included $CD5^+$ B-cells. It is formally possible, although unlikely, that what was interpreted as an induction of CD5 expression, could actually have been the expansion of a contaminating population of $CD5^+$ B-cells. The use of clonal cell lines of the $CD5^-$ B-lineage would obviate this objection.

In this paper we present evidence that the v-H-*ras* and v-*src* oncogenes, but not the v-*myc* oncogene, is capable of inducing CD5 expression in clonal pre-B-cell lines derived from adult murine bone marrow. Also, in an attempt to define a mechanism for this induction, we have utilized the recently characterized murine CD5 promoter (Weichert and Schwartz, manuscript in preparation) to elucidate signal transduction events converging on the CD5 gene.

RESULTS

CD5-Expressing Pre-B-Cell Lines Are Established At High Frequency by Infection of Murine Bone Marrow with a v-H-ras-Expressing Retrovirus

The establishment of four pre-B-cell lines (R1-R4) by infection of murine bone marrow with a v-H-*ras*-expressing retrovirus, and the establishment of two pre-B-cell lines (RM1 and RM2) by co-infection with retroviruses expressing v-H-*ras*

and v-*myc*, has been previously described (Schwartz *et al.*, 1986). The R1-R4 cell lines were all found to express surface CD5 at low levels (but appropriate to $CD5^+$ B-cells), while the RM1 and RM2 lines did not express CD5 (representative cell lines R3 and RM2 shown in Figure 1). Since identical bone marrow pools were utilized in each type of infection, the above data might suggest that the cellular targets of transformation are different for the v-H-*ras* virus, compared to those targeted by coinfection of H-*ras* and with v-*myc* viruses. Alternately, it is possible that the retroviral targets are identical and that all properties including CD5 expression, among the R1-R4 cell lines, were induced by the transforming oncogenes.

CD5 Expression in High Density Bone Marrow Cultures is Conferred by the v-H-ras Oncogene

The R1-R4 cell lines were established with retroviral stocks containing a replication-defective v-H-*ras* virus and the Moloney murine leukemia virus (MoMuLV) as helper. To determine the contribution of each type of virus to establishment of $CD5^+$ pre-B-cell cultures, cells were pooled from 10-week-old Witte-Whitlock high density bone marrow cultures (only plates yielding $>10^6$ non-adherent cells per ml were used), and infected with either MoMuLV alone, v-H-*ras* virus containing MoMuLV helper virus, or helper-free stocks of v-H-*ras* virus. After 4 weeks in continuous culture, the MoMuLV-infected cell lines had become moribund and were

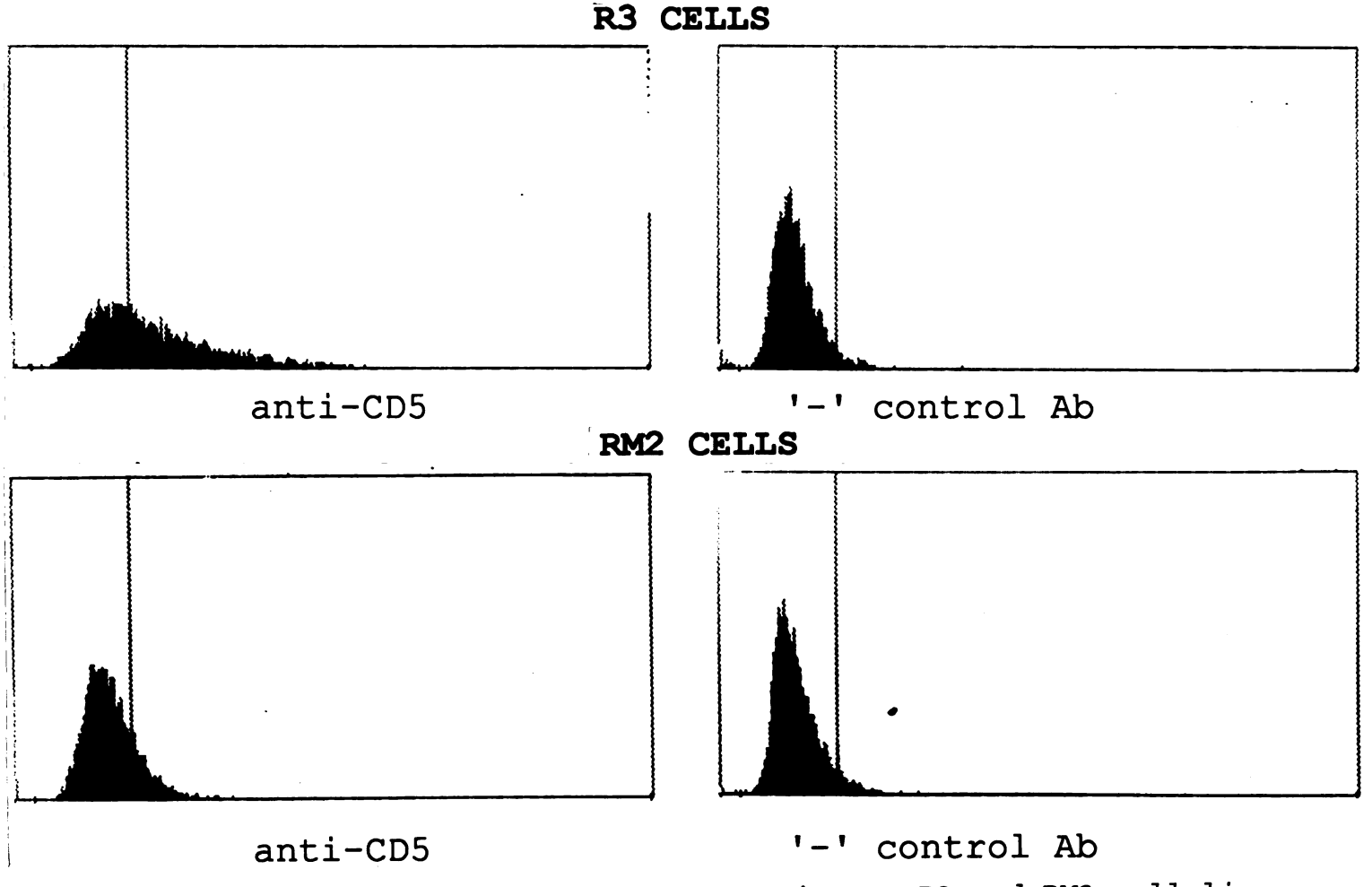

Figure 1 *FACS analysis of CD5 surface expression on R3 and RM2 cell lines*

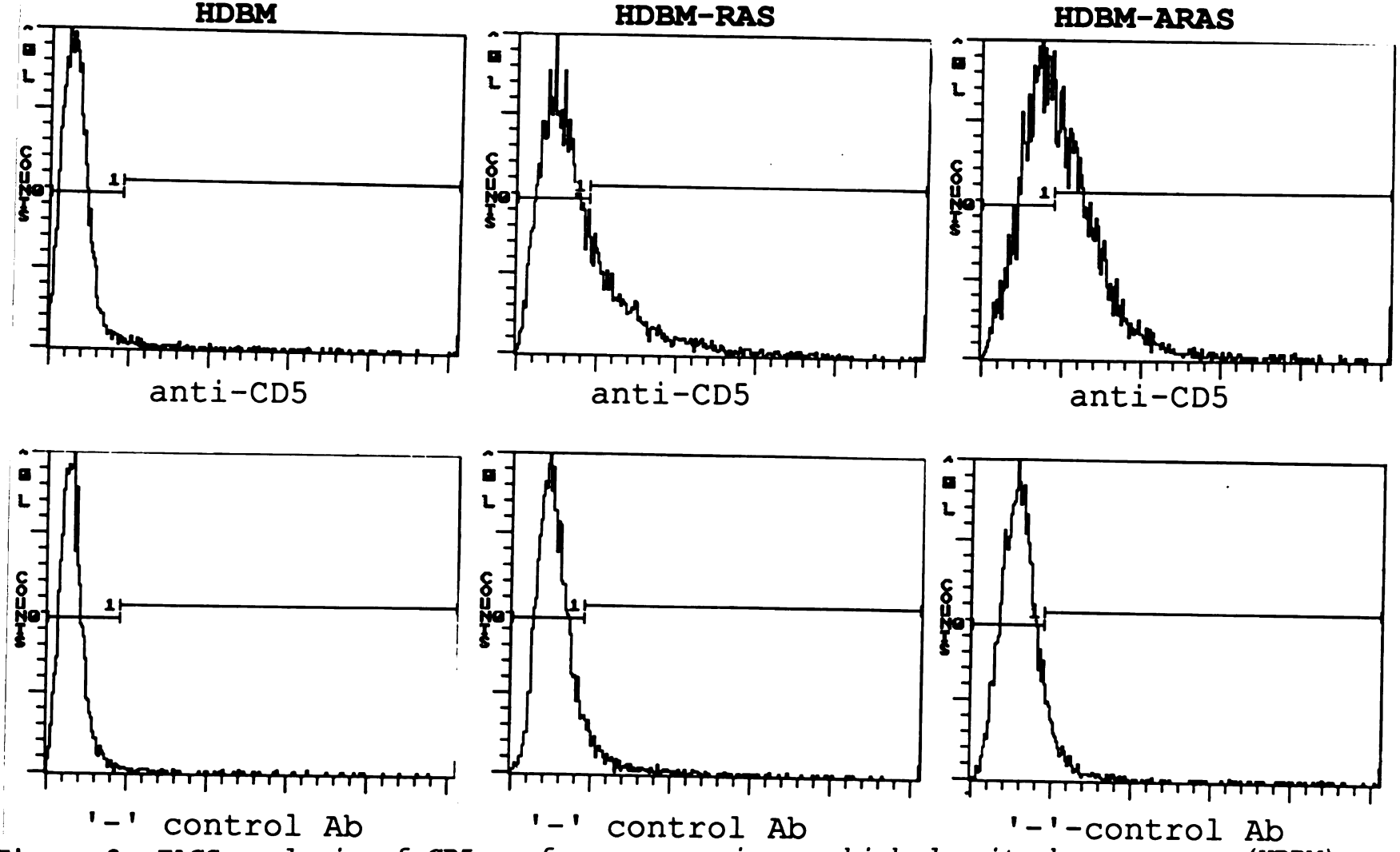

Figure 2 *FACS analysis of CD5 surface expression on high density bone marrow (HDBM) cultures infected with v-H-ras.* HDBM cultures were infected with v-H-*ras* with MoMuLV helper virus (HDBMoras), or helper-free v-H-*ras* (HDBMAras).

discarded, while the other cultures flourished. FACS analysis of the surviving cultures demonstrated that while uninfected cells were $CD5^-$, cultures established with v-H-*ras* virus, with or without MoMuLV helper virus, expressed CD5 (Figure 2). This demonstrates that the v-H-*ras* oncogene is sufficient to confer the CD5 phenotype.

v-H-ras Can Induce CD5 Expression on a Bone Marrow-Derived Clonal Pre-B-Cell Line

CD5 expression on high density bone marrow cultures may be the result of the v-H-*ras* virus conferring a growth advantage to a preferentially targeted, minor population of $CD5^+$ pre-B-cells residing in murine bone marrow. Alternately, v-H-*ras* may be inducing the expression of CD5 on $CD5^-$ pre-B-cells. To determine if v-H-*ras* could up-regulate CD5 expression, clone 3, a $CD5^-$ clonal pre-B-cell line, established from murine high density bone marrow cultures, was utilized (Whitlock *et al.*, 1983). Four cell populations (Clone 3R1-R4) were created by independently infecting Clone 3 with v-H-*ras* virus and MoMuLV helper virus. FACS analysis established that CD5 was faintly expressed on the surface of the v-H-*ras* infected cell populations (representative cell lines Clone 3 and Clone 3R2 shown in Figure 3). Northern analysis of cytoplasmic RNA from Clone 3 infectants, demonstrates >5-fold induction of steady-state levels of CD5 mRNA over the uninfected control (Figure 4). This clearly demonstrates that v-H-*ras* is capable of up-regulating CD5 expression on pre-B-cells and that this

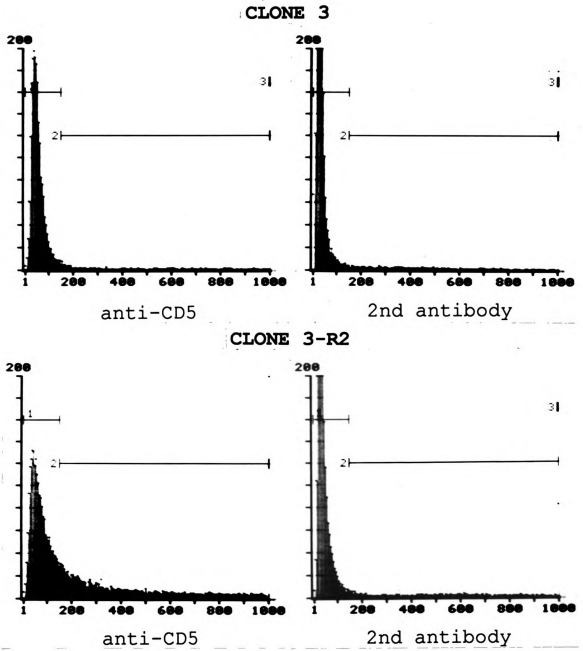

Figure 3 *FACS analysis of CD5 surface expression on Clone 3 cells infected with v-H-ras*

Figure 4 *Northern analysis of CD5 expression in Clone 3 cells infected with v-H-ras.* The polyA$^+$ equivalent of 150.0 μg cytoplasmic RNA was denatured, electrophoresed in a 1% agarose-formaldehyde gel and transferred to a nylon membrane. The positions of ethidium bromide-stained 28S and 18S rRNAs are marked. The membrane was hybridized with a CD5 probe and the position of the CD5 message (2.2 kb) is marked. The membrane was stripped and rehybridized with a β_2-μglobulin probe. Position of the β_2-μglobulin transcript (0.9 kb) is marked. Lane 1, Clone 3, uninfected; Lane 2, Clone 3R1; Lane 3, Clone 3R2; Lane 4, Clone 3R3; Lane 5, Clone 3R4.

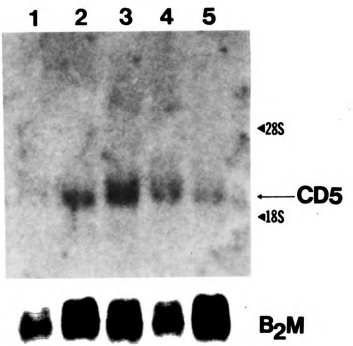

FIGURE 4 *Northern analysis of CD5 expression in Clone 3 cells infected with v-H-ras*

induction correlates with a rise in steady-state CD5 mRNA levels.

Modulation of CD5 Expression by Transforming Oncogenes

It has been reported that infection with v-*src*, and possibly the v-*abl* oncogene, as well as v-H-*ras*, can establish $CD5^+$ B-lineage cell lines from murine bone marrow (Holmes *et al.*, 1986). To examine the effect of these oncogenes on CD5 expression and to extend the observation to other $CD5^-$ B-lineage cell lines, the clonal pre-B-cell line PD36 (established from adult murine bone marrow), and the clonal B-cell line M12 (derived from a lymphoma of an adult Balb/c mouse), were utilized (Travis *et al.*, 1991; Kim *et al.*, 1979). Initially, we repeated our examination of v-H-*ras* induction of CD5 expression. Stably-transfected PD36 and M12 cell populations were established by electroporation of pSV(X)-H-*ras* or pSV(X)neo retroviral expression vectors, with subsequent selection with G418. FACS analysis demonstrated that EL4 T-cells (a positive control for CD5 staining) and PD36ras strongly expressed surface CD5 (Figure 5) while M12ras showed very faint fluorescence (Figure 6). PD36 and M12 cells (Figures 5 and 6, respectively), as well as their pSV(X)neo-transfected counterparts, were also very faint for CD5 staining. The above data demonstrates that up-regulation of CD5 expression by v-H-*ras* is a reproducible phenomenon in at least 2 independently-derived pre-B-cell lines, while no up-regulation was observed in a B-cell line.

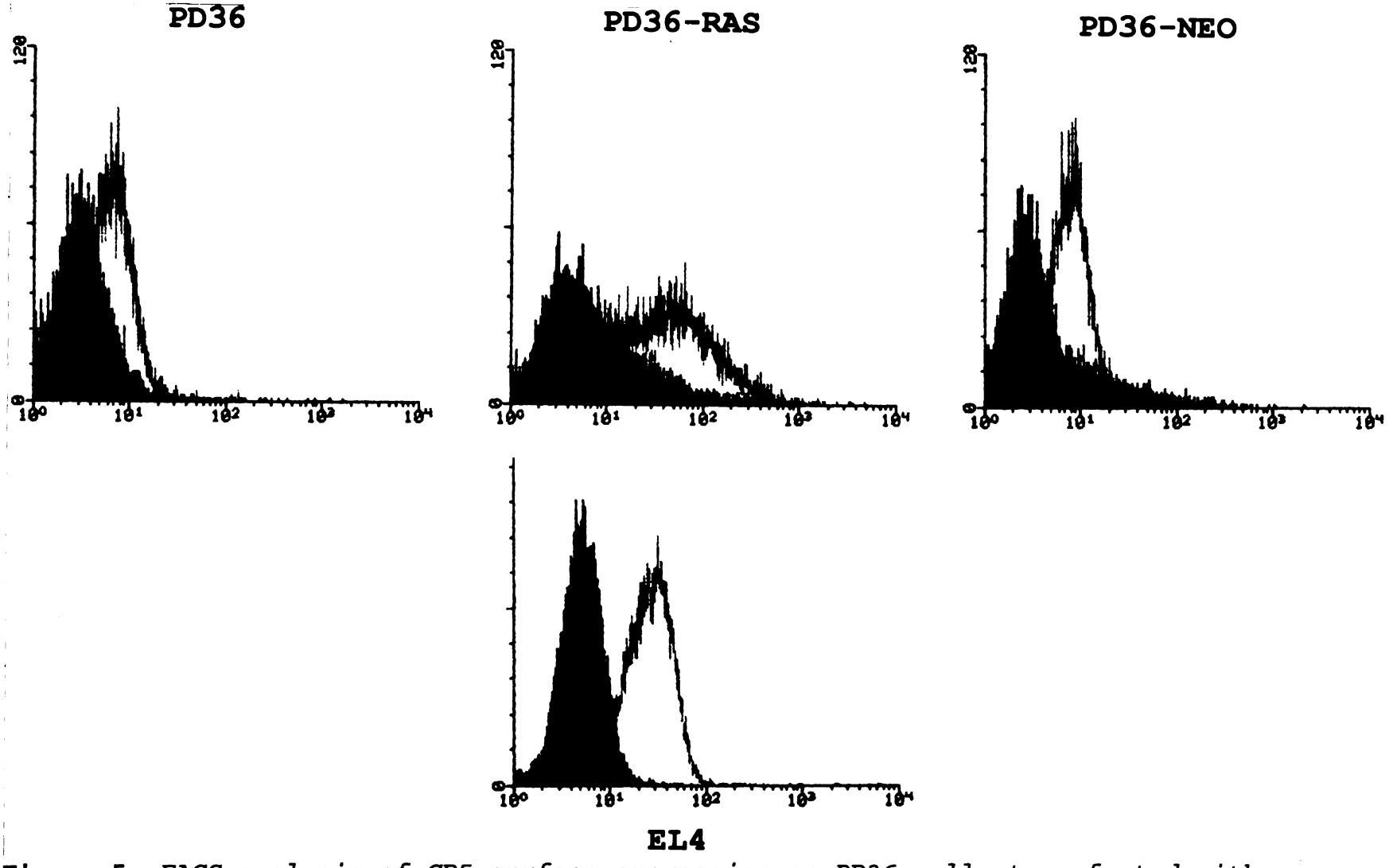

Figure 5 *FACS analysis of CD5 surface expression on PD36 cells transfected with pSV(X)-H-ras.* Shaded peaks represent staining with an isotype-matched negative-control antibody.

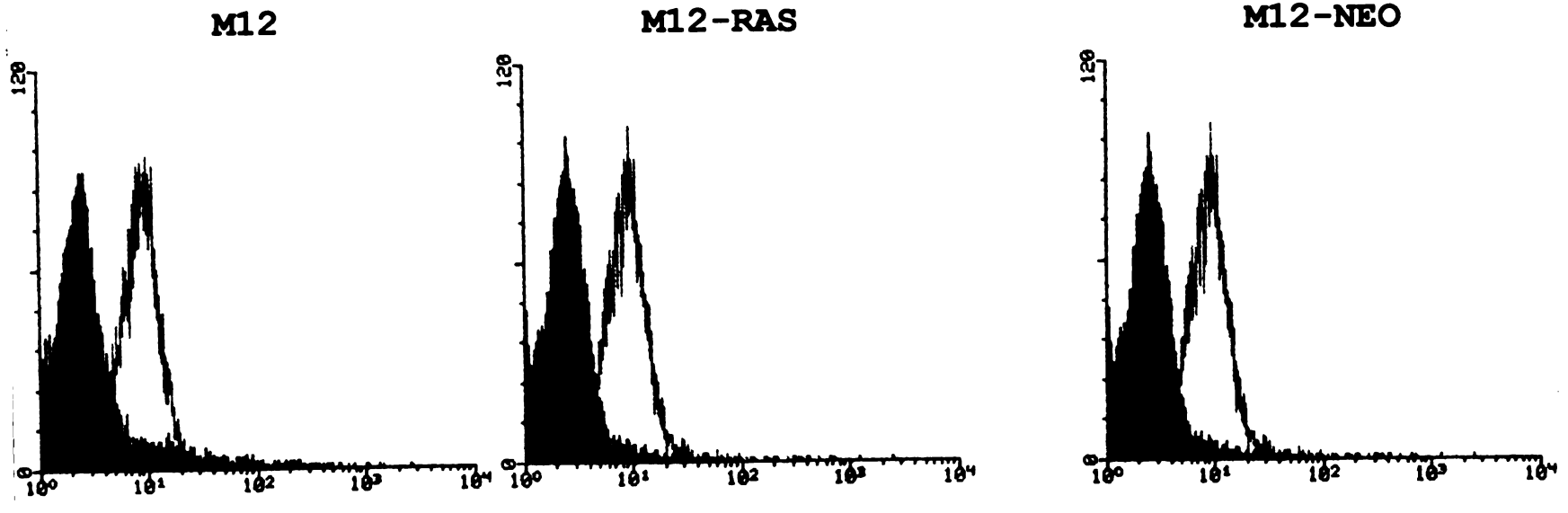

Figure 6 *FACS analysis of CD5 surface expression on M12 cells transfected with pSV(X)-H-ras.* Shaded peaks represent staining with an isotype-matched negative-control antibody.

We then extended our examination to oncogenes other than v-H-*ras* that might modulate CD5 expression. Infection of PD36 cells with v-*src*, v-*myc* and H-*ras* helper-free retroviral vectors was performed in duplicate. Infection with a G418-resistant vector served as a control. Duplicate A-MuLV (v-*abl*) infections of PD36 utilized MoMuLV helper virus, thus requiring MoMuLV-infected negative controls. Northern analysis demonstrates that the PD36 populations infected with v-H-*ras* virus each expressed 14-fold more CD5 message than uninfected PD36, and an average of 6-fold more than the populations infected with the G418-resistant control virus (Figure 7). PD36 populations infected with A-MuLV averaged 2.3-fold greater expression of CD5 than uninfected PD36, as did their MoMuLV-infected counterparts. One of the PD36 populations infected with v-*src* virus demonstrated a 7.7-fold increase in CD5 expression over PD36, while CD5 transcript levels in the other v-*src* population were not above background (data not shown). In contrast to infected populations, the PD36ras population, established by electroporation of pSV(X)-H-*ras*, displayed only 4.4-fold and 3-fold increases over PD36 and PD36neo, respectively (Figure 8). This suggests that introduction of retroviral vectors by infection, rather than electroporation, may allow more efficient expression of the oncogenes involved in CD5 modulation. PD36 populations infected with v-*myc* virus do not express CD5 transcript (Figure 7). The above data demonstrate that v-H-*ras* (all lines tested) and v-*src* (1 out of 2 lines) are capable of

Figure 7 *Northern analysis of PD36 cells infected with v-H-ras, v-myc, A-MuLV, v-src and MoMuLV.* The polyA$^+$ equivalent of 150.0 μg of cytoplasmic RNA was denatured, electrophoresed in a 1% agarose-formaldehyde gel and transferred to a nylon membrane. The positions of ethidium bromide-stained 28S and 18S rRNAs are marked. The membrane was hybridized to a CD5 probe and the position of the CD5 transcript (2.2 kb) is marked. The membrane was stripped and rehybridized with a β_2-μglobulin probe. The position of the β_2-μglobulin transcript (0.9 kb) is marked. Lanes: 1, PD36, uninfected; 2 and 3, pSV(X)neo1 and 2; 4 and 5, MoMuLV1 and 2; 6 and 7, v-H-*ras*1 and 2; 8, v-*src*1; 9 and 10, A-MLV1 and 2; 11 and 12, v-*myc*1 and 2; E, EL4.

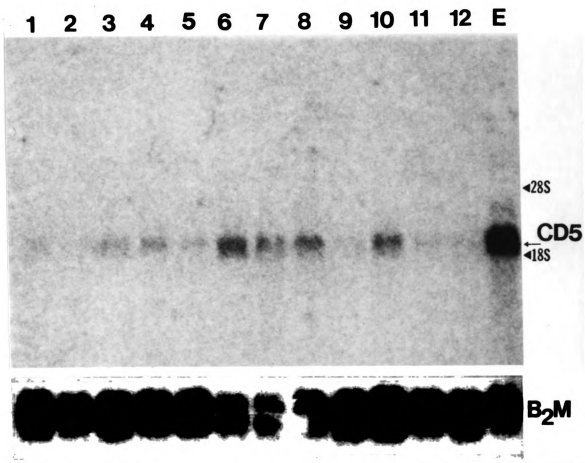

Figure 7 *Northern analysis of PD36 cells infected with v-H-ras, v-myc, A-MuLV, v-src and MoMuLV*

Figure 8 *Northern analysis of PD36 cells stably-transfected with pSV(X)-H-ras.* The polyA$^+$ RNA equivalent of 100.0 μg cytoplasmic RNA was denatured, electrophoresed in a 1% agarose-formaldehyde gel and transferred to a nylon membrane. The positions of ethidium bromide-stained 28S and 18S rRNAs are marked. The membrane was hybridized to a CD5 probe and the position of the CD5 transcript (2.2 kb) is marked. The membrane was stripped and rehybridized to a β_2-μglobulin probe. The position of the β_2-μglobulin transcript (0.9 kb) is marked. Lanes: E, EL4; 1, PD36, untransfected; 2, PD36neo; 3, PD36ras.

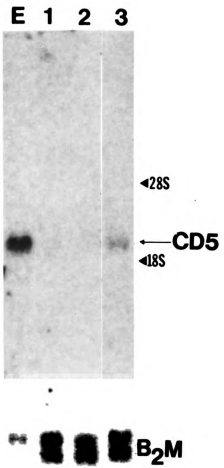

Figure 8 *Northern analysis of PD36 cells stably transfected with pSV(X)-H-ras*

up-regulating CD5 expression on PD36 pre-B-cells. Interestingly, G418-resistant virus and MoMuLV-infected negative controls demonstrated a 2.3-fold increase in CD5 expression over uninfected PD36. This could represent either a modest effect of the retroviral vector on CD5 expression or a by-product of the infective process.

The Effect of v-H-ras on Expression From the Murine CD5 Promoter

We have previously described the cloning of the murine CD5 promoter and have demonstrated its tissue-specific expression (Weichert and Schwartz, manuscript in preparation). To determine if the v-H-*ras*-mediated effects on CD5 expression occurred at the transcriptional level, the full-length CD5 promoter/CAT construct was transfected into PD36ras cells. CD5 promoter expression in PD36ras cells was not greater than control values in PD36neo cells (Figure 10).

The inability of stably integrated v-H-*ras* to transactivate the CD5 promoter in PD36 cells (Figure 9), suggests that post-transcriptional mechanisms may govern v-H-*ras*-induced up-regulation of CD5 expression. Nuclear run-on assays were unable to detect transcriptional activity at the CD5 locus (data not shown), suggesting that expression from the CD5 gene may be below the limits of detection for this assay. However, these results do not conclusively prove that v-H-*ras* is incapable of activating the CD5 promoter. It is possible that v-H-*ras*-responsive elements lie further

Figure 9 *Activity of the CD5 promoter in PD36ras cells.* The -1700 pCAT-Basic deletion construct was transiently transfected into PD36 cells stably-expressing pSV(X)-H-*ras* or pSV(X)neo. The pCAT-positive control vector transfected into PD36neo cells yielded the lowest light unit values (1.5). All cell extract volumes were normalized to this value for CAT reactions. Numbers immediately below the figure are the ratio of [^{14}C]chloramphenicol converted by a given cell extract to [^{14}C]chloramphenicol converted by cell extracts from the -1700 pCAT-Basic vector transfected into PD36neo cells. Figure is representative of one replication of this experiment.
Abbreviations: -1700, -1700 pCAT-Basic deletion construct; V, pCAT-Basic vector; '+', pCAT-positive control vector.

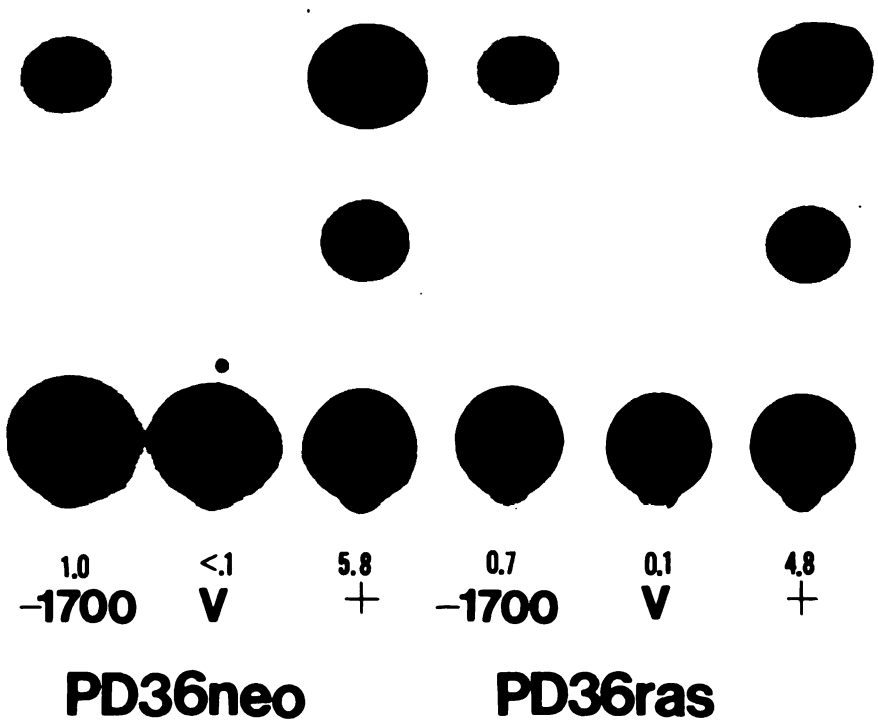

Figure 9 *Activity of the CD5 promoter in PD36ras cells*

upstream than the 1.7 kb promoter fragment we have cloned. Furthermore, v-H-*ras*-induction of CD5 expression may only occur in the context of activated chromatin.

DISCUSSION

We have demonstrated that transforming retroviruses can up-regulate the murine CD5 surface antigen on two bone marrow-derived pre-B-cell lines, PD36 (Travis *et al.*, 1991) and Clone 3 (Whitlock *et al.*, 1983). This effect is consistently seen with the v-H-*ras* oncogene and can be as high as 5-to-14-fold. The v-*src* oncogene showed similar induction in one infected population, but not in another. The v-*abl* oncogene and MoMuLV, itself, have a more modest 2-fold effect on CD5 expression. The contrasting CD5 expression in duplicate v-*src*-expressing cell populations may be the result of a threshold effect involving the level of expression of the infected oncogene. Indeed, the v-*src*-expressing population that fails to express CD5 has a much lower level of v-*src* mRNA than the $CD5^+$ population (data not shown). In any event, these results parallel previous findings of CD5 expression in v-H-*ras* transformed pre-B-cells (Holmes *et al.*, 1986), inconsistent CD5 espression in v-*src* transformed cells (Holmes *et al.*, 1986), and a lack of or weak CD5 expression in v-*abl* (Holmes *et al.*, 1986; Tidmarsh *et al.*, 1989). Most significantly, it is now clear that v-H-*ras* expression can induce CD5 expression in pre-B-cells not previously expressing this surface antigen.

Infection with MoMuLV by itself showed a modest 2-fold induction of CD5 mRNA levels. This may reflect MoMuLV acting in a separate signalling pathway than v-H-*ras*, or that v-H-*ras* is a more efficient activator of a common pathway. In any case, the effect of MoMuLV is inconsequential in comparison to the induction mediated by v-H-*ras*.

The two PD36 v-*myc* infected lines did not show up-regulation of CD5 (Figure 7). If v-*myc* suppression is dominant over v-H-*ras* induction, these findings would corroborate the data showing lack of CD5 expression on the RM1 and RM2 cell lines established by coinfection with v-H-*ras* and v-*myc* viruses (Figure 1). The question of v-*myc* dominance is currently under investigation in our lab.

The fact that v-H-*ras* consistently up-regulated CD5 expression on PD36 cells and v-*myc* consistently had no effect, suggests that a bonafide signal transduction pathway exists in pre-B-cells that converges on the CD5 gene. It has not been determined whether modulation of CD5 transcript levels occurs by increasing the rate of transcription from the CD5 locus or at a post-transcriptional level. Data showing that the CD5 promoter/CAT constructs were not any more active in PD36 cells stably-expressing v-H-*ras*, suggests a post-transcriptional mechanism. Alternatively, the CD5 promoter may need to be in the context of chromatin to be trans-activated by v-H-*ras*.

Several precedents for H-*ras* oncogene activation of cellular promoters have been detailed. The AP-1 (PEA1 in mouse) motif (5'-C/GTGACTC/A-3') has been shown to bind a

factor that is induced by the expression of an activated H-*ras* oncogene (derived from human bladder cancer) in human erythroleukemia cells, and to enhance expression from a heterologous promoter during transient transactivation assays utilizing H-*ras* constructs (Yamaguchi *et al.*, 1989). This site was also found to be activated by phorbol esters. A reverse PEA1 motif is found at -63 in the murine CD5 promoter (Weichert and Schwartz, manuscript in preparation). Phorbol esters have been shown to up-regulate CD5 expression on human T- and B-cells but not on murine lymphocytes (Miller and Gralow, 1984; Carrera *et al.*, 1989; Kipps, 1989). This suggests that PEA1 sites may display some heterogeneity in their response to phorbol esters and H-*ras*. This is apparently the case with the *ras*-responsive element (RRE) in the murine retrotransposon NVL3. The NVL3 LTR has been shown to contain two PEA1 sites activated by phorbol esters, only one of which is responsive to v-H-*ras* (Owen *et al.*, 1990). An overlapping ets binding site was subsequently defined and a *ras*-responsive factor, capable of activating transcription *in vitro* through this motif, was shown to be induced by human EJ H-*ras* (Reddy, *et al.*, 1992). While the above work was done in fibroblasts, members of the ets family of DNA binding proteins have been found in T- and B-cells (ets1) and B-/myeloid cells (ets2). The CD5 promoter contains two potential ets binding sites, but these are distal to the PEA1 site. The PEA-1 site at -63 in the CD5 promoter may be a candidate for addressing

the involvement of cis-acting promoter elements in v-H-*ras* modulation of CD5 expression.

Interesting correlations can be drawn between control of expression of the LFA-1 surface antigen and the murine CD5 gene. LFA-1 is a lymphoid-specific surface antigen involved in cellular adhesion (while CD5 has been shown to bind the B-cell specific surface antigen CD72, this function has only been postulated for CD5-expressing cells *in vivo*). The level of surface LFA-1 has been shown to be enhanced by EJ H-*ras* expression in EBV-immortalized human B-lymphoblastoid cell lines. This up-regulation was found to occur at the level of increased LFA-1 α-chain mRNA levels (Endo *et al.*, 1991). Another group found that c-*myc* expression down-regulates LFA-1 expression on EBV-immortalized B-cells by an unknown mechanism (Inghirami *et al.*, 1990). In addition, PMA up-regulates both LFA-1 and CD5 on human B-cells. IL-4 down-regulates CD5 on murine pre-B-cell lines while upregulating LFA-1 on human lymphoblastoid lines (Miller and Gralow, 1984; Jyonouchi *et al.*, 1990; Rousset *et al.*, 1989). Further insights as to the role of H-*ras* and v-*myc* in modulation of expression of LFA-1 may be applicable to murine CD5.

We have shown that the CD5 phenotype is available to pre-B-cells via v-H-*ras* expression. This is in direct conflict with the classification of CD5 B-cells as a unique lineage of B-lymphocytes on a number of points. First, PD36 populations stably-expressing v-H-*ras* are $CD5^+$ and were not derived from $CD5^+sIgM^+$ precursors. Adoptive transfer experiments showing

the inability of murine bone marrow donor cells to regenerate $CD5^+$ B-cells in lethally-irradiated mice indicates that production of $CD5^+$ pre-B-cells by bone marrow is not possible (Hayakawa, *et al.*, 1985). Second, the lineage model suggests that $CD5^+$ pre-B-cells should not exist at all in adult mice. Data on generation of $CD5^+$ B-cell lines from murine splenocyte cultures alone contradicts this (Braun, 1983). Thirdly, the consistent effects on CD5 expression by v-H-*ras* suggest that a signal transduction pathway capable of modulating CD5 levels exists in pre-B-cells. This is in contrast to the lineage model which only allows for static CD5 levels on B-cells (Kantor and Herzenberg, 1993).

As compelling as our data may seem, one must be wary of applying data derived from transformed cell lines to *in vivo* systems. It is formally possible that the pre-B-cell populations found to express CD5 after infection with the v-H-*ras* retrovirus, represent an as yet uncharacterized lineage capable of CD5 modulation. However, both peritoneal-derived $CD5^+$ B-cells and $CD5^+$ pre-B-cells derived from infection of murine bone marrow with v-H-*ras*, have been found to have myeloid characteristics (Kipps, 1989; Bretz *et al.*, 1992) suggesting that they belong to related lineages. One could also argue that the cell lines are displaying lineage promiscuity and that the overlapping phenotypes of CD5 expression on $sIgM^-$ B-cells are a property of the transforming oncogenes and may never occur *in vivo* (Greaves *et al.*, 1986). While this is a valid point, it is doubtful that the existence

cultured without feeder-layers. Virus stocks consisted of culture medium from NIH3T3 cell lines that had been cotransfected with a proviral clone of MoMuLV and p120*abl* (Rosenberg, 1982). Helper-free virus stocks consisted of culture medium from the Psi2 cell line (Mann *et al.*, 1983) that had been transfected with pSV(X)-H-*ras*, pMMCV-neo, (Schwartz *et al.*, 1986), pSV(X)neo (pZipSV(X)1 of Cepko *et al.*, 1984) or p-*src* (Nori *et al.*, 1991). Infections utilized 10^5 cells per ml culture media added to an equal volume of virus stock and Polybrene (Sigma Chemical Co., St. Louis MO, USA) to a final concentration of 8.0 μg ml^{-1}. After a 3 hour incubation at 37°C, the cells were pelleted and resuspended in culture medium. If infections utilized helper-free stocks, G418 antibiotic (Gibco, Inc., Grand Island, NY, USA) was added to a final concentration of 15.0 μg per ml for PD36 cells or 8 μg per ml for M12 cells.

Electroporation was also utilized to stably introduce pSV(X)-Ha-*ras* and pSV(X)neo vectors into PD36 and M12 cells. 10^7 cells were suspended in 0.5 ml RPMI 1640 media supplemented with 10% FCS (without 2ME or pen/strep) and 10.0 μg of the relevant vector. Electroporations were carried out utilizing a 0.4 cm electrode gap cuvette in a Bio-Rad Gene Pulser (Bio-Rad, Inc., Hercules, CA, USA) at 280 volts and 960 μF capacitance. Pulse times varied between 25 and 35 msec. Cells were transferred to 6 cm tissue culture plates in 5 ml complete media. At 24 hours, G418 was added to plates as above. Successful introduction of vectors was confirmed by

Northern blot analysis of virally encoded transcripts (data not shown).

For transiently-transfected populations, each 0.4 cm cuvette contained 0.5 ml RPMI media/10% FCS (without 2ME or pen/strep) with 10^7 cells, 4.4 μg pGL2 luciferase reporter plasmid (Stratagene, Inc., La Jolla, CA) as an internal control, 5.0 μg of the appropriate CAT reporter construct (10.0 μg of CAT reporter construct for PD36 cells stably-expressing pSV(X)-H-*ras* or pSV(X)neo; Weichert and Schwartz, manuscript in preparation) and 10.0 μg of pSV(X)-H-*ras* or pSV(X)neo (M12 cells only). Electroporation parameters were 280 volts and 960 μF capacitance. Pulse times varied between 25 and 35 msec. Electroporated cells were transferred from cuvettes to 6 cm tissue culture plates in 5.0 ml complete medium. After 24 hours, transfected cells were washed in ice-cold phosphate-buffered saline (1xPBS) and divided into a 1/5th portion for luciferase assays and a 4/5th portion for CAT assays. The cells analyzed for luciferase assays were lysed in 50.0 μl cell culture lysis buffer (Promega, Inc., Madison, WI) and 20.0 μl used for luminometry in a Turner TD-20e luminometer (Turner Designs, Sunnyvale, CA) utilizing 100.0 μl luciferin assay buffer (Promega). Light unit readings were collected for 15 seconds (after a delay time of 30 seconds) and the ratio of each sample value to the lowest sample value determined (normalized luciferase ratios). Normalized luciferase ratios were used to determine the volume of cell extract used in the CAT reaction (Gorman *et al.*,

1983). Cell extracts for CAT assays were prepared by freeze-thawing cells in 0.25 M Tris-HCl for 3 cycles, then heating to 65°C for 20 mins. CAT reactions were carried out utilizing 1.0 μl of 50.0 mCi/mmol [^{14}C]chloramphenicol, 25.0 ug of n-butyryl Coenzyme A, with cell extract and 0.25 M Tris-HCl to a volume of 125.0 μl, and incubated at 37°C for 10 hours. Conversion of [^{14}C]chloramphenicol to butyrylated forms was directly determined from TLC plates (PE SIL G/UV; Whatman, Inc., Hillsboro, OR) on an Ambis Radioanalytic Imaging System (Ambis, San Diego, CA).

Nucleic Acid Isolation and Analysis

Cytoplasmic RNA was isolated by an SDS/urea method (Schwartz *et al.*, 1986). PolyA$^+$ RNA was selected by the method of Rave *et al.* (1979). RNAs were electrophoresed through 1% agarose-formaldehyde gels (Rave *et al.*, 1979) and transferred to nylon membranes (Sambrook *et al.*, 1989). Membranes were hybridized to probes in an aqueous solution of 4xSSC, 5x Denhardt's solution, 10.0 μg ml^{-1} denatured salmon sperm DNA, 5.0% dextran-sulfate and 0.045M NaPi pH 6.8 at 65°C for 12 hours. Membranes were washed to high stringency in 0.1 x SSC and 0.1% SDS at 65°C.

Hybridization probes were prepared by random priming (USB, Inc., Cleveland OH, USA) with the incorporation of 5'-[α-^{32}P]dATP (3000 ci mmol^{-1}; ICN, INC., Costa Mesa, CA, USA). The v-H-*ras* probe was a 0.46 *Eco*RI fragment corresponding to v-H-*ras*-encoding sequences of Harvey murine sarcoma virus

(Ellis *et al.*, 1980). The v-*myc* probe was a 0.8 kb *Hin*dIII-*Bam*HI fragment corresponding to v-*myc* coding sequences of the MMCVneo vector (Wagner, *et al.*, 1985). The v-*src* probe was a 0.8 kb *Pvu*II fragment corresponding to v-*src* coding sequences (Nori *et al.*, 1991). The v-*abl* probe consisted of the entire pUCabl2.3 vector which contains a 2.3 kb fragment of a v-*abl* genomic clone construct (Rosenberg *et al.*, 1982). The CD5 probe was a 0.44 kb *Eco*RV-*Eco*RI fragment corresponding to CD5 cDNA sequences in the MD10 vector (Huang *et al.*, 1987). A 0.8 kb *Bam*HI fragment corresponding to env coding sequences of the Friend-MuLV was utilized to detect MoMuLV expression (Silver and Kozak, 1986). A 1.4 kb *Bgl*II-*Eco*RI fragment of pSV(X)neo was used to detect expression of this vector in control infections (Cepko *et al.*, 1984). The 0.55 kb *Pst*I fragment of β_2-μglobulin was used as a probe to assess RNA loading (Parnes and Seidman, 1982).

Antibodies and Cell Staining

Actively dividing cell populations that displayed greater than 99% cell viability (assayed by trypan-blue dye exclusion) were utilized for FACS analysis. 10^6 cells were collected from tissue culture plates, washed in ice-cold phosphate-buffered saline (1x PBS) and incubated with anti-CD5 antibody or an isotype-matched negative control antibody (the negative control antibody for Clone 3 cells was the FITC-conjugated second antibody) and incubated 30 minutes on ice. HDBM, PD36 and M12 cells were incubated with Fc fragment (Pharmingen, San

Diego CA) 30 minutes on ice before the initial incubation with anti-CD5 antibody. Cells were pelleted, washed in 1x PBS and resuspended in R-phycoerythrin-avidin (Pharmingen) or incubated with FITC-conjugated second antibody (Clone 3 cells only) for 30 minutes on ice. Detection of surface CD5 by FACS analysis was accomplished on an Ortho Cytofluorograph System 50 flow cytometeror or a Becton/Dickinson Vantage II flow cytometer. Rat anti-murine CD5 monoclonal antibodies were biotinylated Ly1F (R1-R4, RM1 and RM2 staining; J. Braun, personal communication), FITC-conjugated 53.7.3 (Clone 3 staining; Ledbetter and Herzenberg, 1979) or biotinylated RM-Lyt-1 (HDBM, PD36, M12 and EL4 staining; Pharmingen). Isotype-matched biotinylated negative control monoclonal antibodies utilized were L3T4 (R1-R4, RM1 and RM2 staining; J. Braun, personal communication) and RM-Lyt-2B (HDBM, PD36, M12 and EL4 staining; Pharmingen). The FITC-conjugated rabbit anti-rat IgG second antibody utilized for Clone 3 staining was MY S861 (W. Esselman, personal communication).

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SUMMARY

The ultimate goal of our work with CD5 is to more clearly define the role of $CD5^+$ B-cells in the cellular immune system. Toward this end, we have proceeded with an initial investigation into the mechanisms governing murine CD5 expression in B-cells. We have taken a molecular approach, rather than a cellular one, to address this question. The reason for this lies in the differential expression of the CD5 gene. CD5 is expressed in a lymphoid-specific manner, being found on all T-cells, but only on a fraction of B-cells. In addition, it appears that expression of CD5 can be induced on the $CD5^-$ B-cells. There are few, if any, surface antigens that are expressed in this manner. Thus, by studying the 'response' (i.e. tissue-specific expression and CD5 inducibility), we will be able to more clearly define the 'stimulus' (i.e.the immune stimuli to which $CD5^+$ B-cells are responding). Since all such stimuli must ultimately converge on the CD5 locus, we have cloned and initially characterized the CD5 promoter. We have also identified two activators of signal transduction pathways, v-Ha-ras and v-src, that can up-regulate CD5 expression.

At this point, the question to be asked is "Are $CD5^+$ B-cells a unique lineage of B-lymphocytes?" The answer is a definite 'maybe'. Our data demonstrates that the $CD5^+$ phenotype is available to pre-B-cells not previously expressing this marker. The work of Ying-zi et al. (1991) demonstrates that CD5 is up-regulated on B-cells activated

through their surface receptors. The above clearly shows that CD5 is not a lineage-restricted marker. However, neither adequately addresses the lineage status of peritoneal-derived $CD5^+$ B-cells. Peritoneal-derived $CD5^+$ B-lymphocytes do not exist in a chronic state of activation and their progenitors are not derived from adult bone marrow (Hayakawa et al., 1985). A way to unify our data with that of Ying-zi et al. (1991) and Hayakawa et al. (1991), is to suggest that there are two lineages of B-cells, one that permanently expresses CD5 and is not bone marrow-derived, and one that can modulate CD5 surface expression.

However, another model is formally possible. If CD5 expression is dependent on the stage of differentiation at which a B-lineage cell received the appropriate stimulation (a stimulus capable of inducing CD5 expression), then the existence of a single lineage of B-cells would suffice. Our data demonstrates that only pre-B-cells can be induced to express CD5 by transforming oncogenes, and that B-cells are refractive to this type of stimulation. The work of Ying-zi et al. (1991) demonstrates that sIgM cross-linking is the type of stimulation to which spleen-derived B-cells are responsive. Peritoneal-derived $CD5^+$ B-cells may have received the appropriate stimulation at a very early stage in B-cell differentiation and now permanently express CD5. Thus, the ultimate factor in determination of the CD5 phenotype is whether or not the signalling pathway that converges on the CD5 gene, and results in CD5 up-regulation, has been

activated. This is the area in which our work may have the most long-term impact. When elements that are targets for signals that converge on the CD5 promoter have been identified, the trans-acting factors that activate these elements can be identified. Identification of trans-acting factors that are integral members of receptor-linked signal transduction pathways will yield insights to the origin of extra-cellular signals and, ultimately, to the immune function of the CD5 surface antigen.

Future Directions

Two further experiments will strengthen the Northern data that demonstrates the modulation of CD5 mRNA by transforming oncogenes (Figure 7, Chapter 3). The first will firmly establish a cause-effect relationship between expression of the v-src oncogene and the up-regulation of CD5. PD36 cells infected with v-src display a 7-fold increase in steady-state levels of CD5 mRNA (Figure 7, Chapter 3). The transforming activity of v-src from this retroviral construct is temperature-dependent (Nori et al., 1991). At the permissive temperature (37° C), v-src is capable of oncogenic transformation. Changing the environment of the incubator to the non-permissive termperature (39.5° C) will prevent transformation by v-src. If the modulation of CD5 mRNA levels reflects changes in v-src expression, a direct link between expression of v-src and induction of CD5 will be established.

The second experiment will address the mechanism by which v-Ha-ras and v-myc coinfection of high density bone marrow

establishes cultures of $CD5^-$ pre-B-cells, while v-Ha-*ras* infection yields $CD5^+$ pre-B-cells (Schwartz et al., 1986). The above may represent v-*myc* suppression of v-Ha-*ras*-induced CD5 up-regulation. This hypothesis can be addressed by infecting PD36 cells that stably express v-Ha-*ras*, with v-*myc* and assessing the level of CD5 mRNA expression. Down-regulation of CD5 expression in the doubly-infected cells, will solidify data suggesting that a bonafide signal transduction pathway converges on the CD5 gene and argues against the idea that v-Ha-*ras*-induced CD5 up-regulation is a by-product of the infective process.

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