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Genetics

Cloning of Ly-5 cDNA

(cell surface phenotypes/isoforms/hematopoiesis/congeneic mice)

F.-W. SHEN*, Y. SAGA*, G. LITMAN*, G. FREEMAN†, J.-S. TUNG*, H. CANTOR†, AND E. A. BOYSE*

*Memorial Sloan–Kettering Cancer Center, New York, NY 10021; and †Dana–Farber Cancer Institute, Boston, MA 02115

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ABSTRACT **A notable feature of Ly-5, among immunogenetic systems that identify glycoproteins of the cell surface and define the surface phenotype of cells according to their lineage, is that the *Ly-5* locus specifies a range of molecular isoforms that distinguish cells of different stages and branches of hematopoietic development. The composition of the *Ly-5* locus is of much interest in regard to how these isoforms are constructed and differentially regulated according to cell lineage. We describe here a cDNA clone, pLy-5-68, that identifies *Ly-5*. The *Ly-5* specificity of the pLy-5-68 clone was first indicated by a restriction fragment length polymorphism (RFLP), which in Southern blotting distinguishes genomic DNA of C57BL/6 (B6) mice (*Ly-5^a*) from that of B6-*Ly-5^b* congeneic mice whose genome is the same as B6 except for the segment of chromosome 1 that bears *Ly-5^b*. For the following reasons it is unlikely that pLy-5-68 represents a gene linked to *Ly-5* that was carried over with *Ly-5^b* during serial backcrossing to make the B6-*Ly-5^b* congeneic strain. In all mouse strains tested, the serological Ly-5 allotype (Ly-5.1 vs. Ly-5.2) accorded with the RFLP pattern. Cells of the ST/bJ mouse strain have unique Ly-5 serological reactions and ST/bJ DNA gives a unique (third) RFLP pattern (*Ly-5^c*) with pLy-5-68. All Ly-5$^+$ cell types reacted positively with pLy-5-68 in RNA transfer blotting, and all Ly-5$^-$ cell types tested did not. The difference in size of mRNA reactive with pLy-5-68 in cells expressing the 200-kDa Ly-5 isoform as compared with cells expressing the 220-kDa Ly-5 isoform corresponded with the difference in size of the protein components of those isoforms.**

The *Ly-5* locus of the mouse (1, 2) specifies a set of different glycoprotein isoforms, each of which is expressed on the surface of cells of a particular lineage within the hematopoietic compartment of development (3). Of five NaDodSO$_4$/PAGE bands derived from Ly-5 precipitated from cells of different hematopoietic lineages by Ly-5 antiserum or monoclonal antibody (3–5), two have been studied biochemically in some detail. These represent a 200-kDa isoform expressed by T cells and a 220-kDa isoform expressed by B cells. The size of the respective protein components of these two isoforms, which appear to be highly related to one another in two-dimensional peptide mapping, is 160 kDa and 190 kDa, and the *Ly-5* locus, on chromosome 1 (unpublished data), is the site of the protein structural gene or genes for at least these two isoforms (6). The cDNA probe described here should help to elucidate the genetic basis of Ly-5 isoform diversity.

MATERIALS AND METHODS

Preparation of Sublibraries Based on cDNA Insert Size. A T-cell cDNA library, consisting of 3.8×10^5 independent transformants, was constructed by using the pcD vector of Okayama and Berg (7) and poly(A)$^+$ mRNA of the Con-A-stimulated (22 hr) inducer T-cell clone C1.Ly1-T1 (8). Plasmid DNA was prepared by the alkaline lysis procedure (9) followed by equilibrium sedimentation in CsCl. Twelve micrograms of plasmid DNA was digested with 12 units of *Sal* I or *Cla* I endonuclease and electrophoresed in adjacent wells in a 1.3% agarose gel. DNA fragments whose sizes spanned the range 2–23 kilobases (kb) were electrophoresed in adjacent tracks. After staining with ethidium bromide, the gel was sliced into eight sections corresponding to cDNA insert sizes of 0–0.5, 0.5–1.2, 1.2–2, 2–3, 3–4, 4–5, 5–9, and 9–20 kb. DNA was extracted from each slice by the sodium iodide/glass powder method (10), recyclized with T4 DNA ligase, and used to transform *Escherichia coli*, strain MC1061. The individual transformed cultures were diluted 1:20 with LB broth containing 50 μg of ampicillin per ml and were incubated at 37°C for 2 hr with swirling. The cultures were then adjusted to 7% dimethyl sulfoxide, quick frozen, and stored at −70°C.

Preparation of Poly(A)$^+$ mRNA. Poly(A)$^+$ mRNA was prepared essentially as described by Maniatis *et al.* (11) with modification. Briefly, 1 ml of packed cells, washed with phosphate-buffered saline without Ca^{2+} and Mg^{2+} ions, was suspended in 7 ml of guanidinium isothiocyanate and homogenized (Polytron setting 7, 35 sec). The homogenate was layered onto a 3-ml cushion of 5.7 M CsCl in a Beckman SW41 ultraclear tube and centrifuged at 34,000 rpm for 20 hr at 20°C. The pellet was washed twice with 70% ethanol and resuspended in doubly distilled H_2O. Sodium acetate (3 M, pH 5.2, 0.1 vol) and absolute ethanol (2.2 vol) were added. After storage at −20°C overnight, total RNA was recovered by centrifugation. Poly(A)$^+$ mRNA was selected as described (11).

RNA Transfer Blotting. Electrophoresis of mRNA after denaturation with glyoxal and dimethylsulfoxide was carried out as described (11). The glyoxylated mRNA was transferred to GeneScreen nitrocellulose paper and hybridized as recommended (New England Nuclear).

Nick-Translation and Southern Blotting. These procedures were performed as described (12).

^{32}P-Labeled Single-Strand cDNA Synthesis and Subtractive Hybridization. These techniques were performed according to Davis *et al.* (13).

Inbred and Inbred Congeneic Mice. C57BL/6 (B6) and B6-*Ly-5^b* congeneic mice were supplied from colonies at Sloan–Kettering Institute. The latter (congeneic) strain was derived from a cross between the B6 and SJL/J strains (*Ly-5^a* × *Ly-5^b*) by 23 serial backcrosses to B6, selecting in each backcross generation for *Ly-5^b* segregants (50%) by serotyping for Ly-5.2. The expected size of a transferred chromosomal segment for 23 backcross generations is about 9 centimorgans (14). The B6-*Ly-5^b* congeneic strain has the *Pep-3^b* type of the *Ly-5^b* allele donor (SJL/J), as expected from the close linkage of *Ly-5* to *Pep-3* (2–3 centimorgans, unpublished data). Mice designated /J were obtained from

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Abbreviations: kb, kilobase(s); RFLP, restriction fragment length polymorphism.

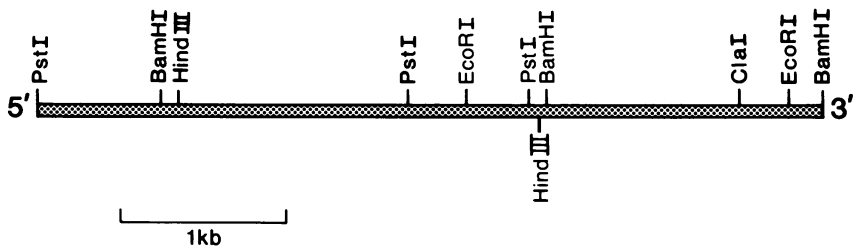

FIG. 1. Restriction map of the pLy-5-68 Ly-5.1 cDNA clone; there are no *Xho* I or *Sal* I sites.

The Jackson Laboratory. Mice not so designated were supplied from colonies at Sloan–Kettering Institute.

Cell Lines. I.29 is a spontaneous early surface Ig^+ B-cell ascites leukemia and ISL-57 is a T-cell ascites leukemia, both of the I/St strain; I.29 and ISL-57 cells were obtained from ascites fluid of I/St passage mice. BALB/c Meth A ascites sarcoma cells were obtained from ascites fluid of BALB/c passage mice.

DNA Sequence. Nucleotide sequences were determined by the method of Sanger *et al.* (15) on fragments subcloned in M13 mp10 and mp11 (16). Subcloning utilized both blunt-end and forced cloning strategies and the sequences were confirmed on both strands.

RESULTS AND DISCUSSION

Cloning Strategy. The size of Ly-5 protein of the 200-kDa isoform of T cells is about 160 kDa (6), corresponding to >4.5 kb. The T-cell cDNA library was therefore fractionated by size to give a sublibrary with inserts of >3 kb.

^{32}P-labeled single-stranded cDNA of the I.29 line of B cells was subtractively hybridized with excess mRNA of ascites sarcoma cells (Meth A), which are phenotypically Ly-5 negative, to provide a probe for screening the sublibrary. Of ≈20,000 colonies screened, 365 were thus selected. Each of the 365 clones was tested by hybridization with ^{32}P-labeled single-stranded cDNA of (*i*) ISL-57 T leukemia cells ($Ly\text{-}5^+$), (*ii*) I.29 B leukemia cells ($Ly\text{-}5^+$), and (*iii*) Meth A sarcoma cells ($Ly\text{-}5^-$); 70 clones gave the reactions appropriate to Ly-5 (*i*, positive; *ii*, positive; *iii*, negative).

These 70 clones were divided into 12 sets, and plasmid DNA was prepared from each set. Each of the 12 preparations, labeled with ^{32}P by nick-translation, was allowed to react in Southern blotting with genomic DNA (digested with various restriction enzymes) of B6 mice (*Ly-5*^{a}) and of B6-*Ly-5*^{b} congeneic mice. One of the 12 sets recognized a restriction fragment length polymorphism (RFLP) distinguishing DNA of B6 mice from DNA of B6-*Ly-5*^{b} congeneic mice. This set was further subdivided and the responsible clone, called pLy-5-68, thus was isolated.

Characterization of Clone pLy-5-68. This clone has an insert of about 4.7 kb. A restriction map is given in Fig. 1 and a partial 5′ end nucleotide sequence is given in Fig. 2. The evidence that pLy-5-68 represents *Ly-5* follows.

Southern blotting of genomic DNA. Fig. 3 shows that Southern blots of DNA from liver of B6 (*Ly-5*^{a}) and congeneic B6-*Ly-5*^{b} mice, allowed to react with pLy-5-68, are distinguished by a RFLP identified by *Bam*HI and *Bgl* II. This RFLP signifies that pLy-5-68 represents *Ly-5* or else represents a flanking gene (in the SJL/J allele-donor segment of chromosome 1 carried by B6-*Ly-5*^{b} congeneic mice) that exhibits the same pattern of selective expression in the three cell lines that were used in clone selection (see above). The latter possibility (that pLy-5-68 represents a gene linked to *Ly-5*) is rendered less likely by the fact that the strain distribution of the RFLP accorded with Ly-5 serological phenotype in all mouse strains tested by Southern blotting with pLy-5-68 (A, BALB/c, and B6, which are *Ly-5*^{a} compared with SJL/J and STS/A, which are *Ly-5*^{b}). The special case of ST/bJ is discussed separately below.

Southern blotting of DNA of Ly-5 nonexpressor cells (liver) compared with DNA of Ly-5 expressor cells showed no evidence of *Ly-5* gene rearrangement.

The special case of the ST/bJ mouse strain. Before we obtained the pLy-5-68 clone we had already found that ST/bJ gives unique reactions in serological and biochemical tests of Ly-5 (unpublished). Thus, (*i*) some Ly-5.2 antisera and all Ly-5.1 antisera reacted with ST/bJ cells and all such reactions could be traced to *Ly-5* or its vicinity because B6 cells removed all activity from Ly-5.1 antisera and B6-*Ly-5*^{b}

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                                                30                                  60
TTT GGA TTT GCC CTT CTG GAC ACA GAA GTC TTT GTC ACA GGG CAA ACA CCT ACA CCC AGT
phe gly phe ala leu leu asp thr glu val phe val thr gly gln thr pro thr pro ser

                                                90                                 120
GAT GGT GCC AGC CTC ACA ACT CTT ACA CCA TCC ACT CTG GGC CTT GCA AGC ACT GAC CCT
asp gly ala ser leu thr thr leu thr pro ser thr leu gly leu ala ser thr asp pro

                                               150                                 180
CCA AGC ACA ACC ATA GCT ACC ACA ACG AAG CAA ACA TGT GCT GCC ATG TTT GGG AAC ATT
pro ser thr thr ile ala thr thr thr lys gln thr cys ala ala met phe gly asn ile

                                               210                                 240
ACT GTG AAT TAC ACC TAT GAA TCT AGT AAT CAG ACT TTT AAG GCA GAC CTC AAA GAT GTC
thr val asn tyr thr tyr glu ser ser asn gln thr phe lys ala asp leu lys asp val

                                               270                                 300
CAA AAT GCT AAG TGT GGA AAT GAG GAT TGT GAA AAC GTG TTA AAT AAT CTA GAA GAA TGC
gln asn ala lys cys gly asn glu asp cys glu asn val leu asn asn leu glu glu cys

                                               330                 345
TCA CAG ATA AAA AAC ATC AGT GTG TCT AAT GAC TCA TGT GCT CCA
ser gln ile lys asn ile ser val ser asn asp ser cys ala pro
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FIG. 2. Partial 5′ end nucleotide sequence and deduced amino acid sequence for the pLy-5-68 cDNA clone.

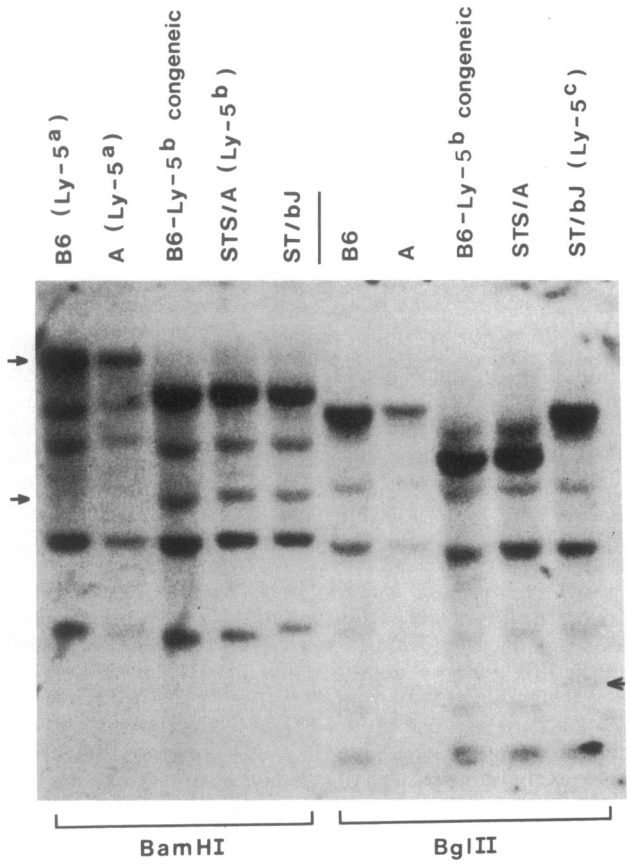

FIG. 3. Southern blots of liver DNA with the pLy-5-68 cDNA probe, showing the RFLP that distinguishes *Ly-5*^{a} mouse strains from congeneic and unrelated *Ly-5*^{b} strains (arrows, left) and also the unique RFLP pattern of ST/bJ mice (arrow, right), which give exceptional Ly-5 serological reactions. With ST/bJ cells, endonucleases *Bgl* II, *Eco*RI, and *Hin*dIII gave the exceptional RFLP banding pattern, whereas *Pst* I and *Pvu* II gave the same banding pattern as *Ly-5*^{a} cells and *Bam*HI gave the same pattern as *Ly-5*^{b} cells. All six endonucleases distinguish *Ly-5*^{a} from *Ly-5*^{b} cells.

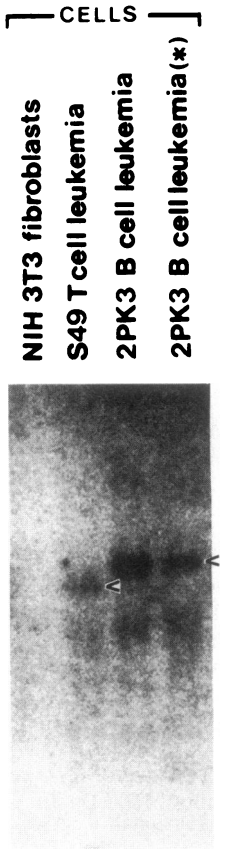

FIG. 4. Illustration of a size difference (≈4.7 kb vs. ≈5.0 kb) between mRNA reactive with pLy-5-68 in T cells (200-kDa isoform of Ly-5) as compared with B cells (220-kDa isoform of Ly-5) in RNA transfer blotting [5 μg of poly(A)$^+$ mRNA on 1% agarose gel]. (*) Indicates cytoplasmic mRNA prepared specially for maximal exclusion of nuclear RNA. See Table 1 for further cell types tested so far.

congeneic cells removed all activity from all Ly-5.2 antisera; (*ii*) Ly-5.1 and Ly-5.2 antisera precipitated from ST/bJ thymocytes the characteristic isoform of 200 kDa and from ST/bJ spleen cells the characteristic isoforms of 200 kDa, 205 kDa, and 220 kDa. These exceptional findings with ST/bJ, which are not explicable in terms of genetic contamination, have been explored for potential relevance to the structure of the *Ly-5* locus; we cite them here only for their use in validating pLy-5-68 as a probe for *Ly-5*. In that respect, the fact that ST/bJ DNA has the unique (third) RFLP pattern (Fig. 3) as well as unique serological reactions strongly implies that pLy-5-68 represents *Ly-5* and not a flanking gene. The *Ly-5* genotype of ST/bJ mice should now be termed *Ly-5*^{c}.

RNA transfer blotting. If pLy-5-68 represents a *bona fide Ly-5* gene, then RNA transfer blotting is expected to reveal a corresponding mRNA in Ly-5 expressor cells that is absent in Ly-5 nonexpressor cell types. Also, RNA transfer blotting of different Ly-5 expressor cell types might show differences in the size of pLy-5-68-reactive mRNA corresponding to differences in size of the proteins of the isoforms expressed. Both of these criteria have been met by pLy-5-68. First, all Ly-5$^+$ cell types tested were positive in RNA transfer blotting, and all Ly-5$^-$ tissue tested (liver, kidney, brain, sarcoma cells, fibroblasts) were negative (Fig. 4 and Table 1). Second, the estimated size of pLy-5-68-reactive mRNA of T cells, which express the 200-kDa Ly-5 isoform, is ≈4.7 kb, and that of B cells expressing the 220-kDa isoform is ≈5.0 kb, corresponding to the size of protein in those isoforms—namely, 160 kDa and 190 kDa (6).

Relation to cDNA Clones for T200 of the Rat. T200 of the mouse is believed to represent the same molecular system as Ly-5. Thus, it is thought that T200 antibody (rat anti-mouse) recognizes a monomorphic Ly-5 epitope, whereas Ly-5 alloantibody (mouse anti-mouse) recognizes a polymorphic Ly-5 epitope showing allelic variation (17, 18). On grounds of tissue representation and molecular isomorphism, T200 of the rat, recognized by mouse anti-rat antibody, is believed to be the rat homologue of mouse T200 and hence of Ly-5. Thomas *et al.* (19) synthesized an oligonucleotide probe based on the protein sequence of T200 of the rat and used this to isolate rat cDNA clones. The sequence given for pLy-5-68

Table 1. Summary of reactions of the pLy-5-68 clone in RNA transfer blotting of mRNA of Ly-5$^-$ cells and Ly-5$^+$ cells expressing the 200-kDa or 220-kDa isoform

Positive		
≈4.7 kb*	≈5.0 kb*	Negative
Thymocytes	Lymph node cells	Liver, kidney, brain,
Lymph node cells	Spleen cells	NIH 3T3 cells,
Spleen cells	B-cell leukemias I.29	BALB/c Meth A
T-cell leukemias EL4 (C57BL), ISL-57 (I/St), S49 (B6), and R1 (C58)	(I/St) and 2PK3 (BALB/c)	sarcoma

*Estimated size of pLy-5-68-reactive mRNA of T cells (≈4.7 kb) and B cells (≈5.0 kb).

in Fig. 2 shows no significant correspondence with the sequence given by these authors, but this may be due simply to lack of overlap; with regard to the restriction maps there is significant correspondence at the 3′ ends (*Hin*dIII, *Bam*HI, *Eco*RI sites), representing cytoplasmic domains, but none at the 5′ ends, representing cellular-exterior domains where epitopes for the Ly-5 alloantigens and monomorphic T200 antigen, recognized serologically on intact cells, should reside. Thus, it remains to be seen whether we are dealing with two molecules of a related Ly-5 T200 family.

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1. Komuro, K., Itakura, K., Boyse, E. A. & John, M. (1975) *Immunogenetics* **1**, 452–456.
2. Scheid, M. P. & Triglia, D. (1979) *Immunogenetics* **9**, 423–433.
3. Michaelson, J., Scheid, M. P. & Boyse, E. A. (1979) *Immunogenetics* **9**, 193–197.
4. Tung, J.-S., Scheid, M. P., Pierotti, M. A., Hämmerling, U. & Boyse, E. A. (1981) *Immunogenetics* **14**, 101–106.
5. Tung, J.-S., Scheid, M. P. & Palladino, M. A. (1983) *Immunogenetics* **17**, 649–654.
6. Tung, J.-S., Deere, M. C. & Boyse, E. A. (1984) *Immunogenetics* **19**, 149–154.
7. Okayama, H. & Berg, P. (1983) *Mol. Cell. Biol.* **3**, 280–288.
8. Clayberger, C., DeKruyff, R. H., Aisenberg, J. & Cantor, H. (1983) *J. Exp. Med.* **157**, 1906–1919.
9. Birnboim, H. C. (1983) *Methods Enzymol.* **100**, 243–255.
10. Vogelstein, B. & Gillespie, D. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 615–619.
11. Maniatis, T., Sambrook, J. & Fritsch, E. F. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
12. Shen, F. W., Chaganti, R. S. K., Doucette, L. A., Litman, G. W., Steinmetz, M., Hood, L. & Boyse, E. A. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6447–6450.
13. Davis, M. M., Cohen, D. I., Nielsen, E. A., DeFranco, A. L. & Paul, W. E. (1982) in *UCLA Symposia*, eds. Vitetta, E. & Fox, C. F. (Academic, New York), Vol. 24, p. 215.
14. Flaherty, L. (1981) in *The Mouse in Biomedical Research*, eds. Foster, H. L., Small, J. D. & Fox, J. G. (Academic, New York), Vol. 1, p. 215.
15. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
16. Norrander, J., Kempe, T. & Messing, J. (1983) *Gene* **26**, 101–106.
17. Scheid, M. P., Landreth, K. S., Tung, J.-S. & Kincade, P. W. (1983) *Immunol. Res.* **69**, 141–159.
18. Omary, M. B., Trowbridge, I. S. & Scheid, M. P. (1980) *J. Exp. Med.* **151**, 1311–1316.
19. Thomas, M. L., Barclay, A. N., Gagnon, J. & Williams, A. F. (1985) *Cell* **41**, 83–93.