Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 2452–2455, April 1984
Genetics

Expression of immunoglobulin heavy chain at a high level in the absence of a proposed immunoglobulin enhancer element in *cis*

(fluorescence-activated cell sorter analysis/sodium dodecyl sulfate/polyacrylamide gel electrophoresis/Southern blot analysis/RNA dot blotting/complementation for immunoglobulin chain synthesis)

MATTHIAS R. WABL AND PETER D. BURROWS

Friedrich-Miescher-Laboratorium der Max-Planck-Gesellschaft, Spemannstrasse, 37-39, D-7400 Tübingen, Federal Republic of Germany

Communicated by N. K. Jerne, January 9, 1984

ABSTRACT **The major intron between the *J* and *C* gene segments of the immunoglobulin heavy (H) chain locus contains an enhancer-like sequence, and it has been proposed that this enhancer is necessary to achieve high levels of H chain expression. We have isolated a subclone of the lymphoid pre-B cell line 18-81 that lacks this enhancer but nevertheless produces μ chain at the level characteristic of pre-B cells. Another subclone with a larger deletion does not produce μ chain, but upon fusion with a myeloma that does not produce any immunoglobulin chain, μ chain is expressed by the homolog from the pre-B subclone. The hybridoma lacks the proposed enhancer element in *cis*; nevertheless it produces as much μ chain as other plasma cell hybridomas. Therefore, this enhancer element is not obligatory for a high level of H chain production.**

Enhancer elements are short DNA sequences that increase the transcriptional activity in *cis* of homologous or various heterologous promoters that are within a few kilobases (kb) upstream or downstream. They have been found in the genome of DNA viruses and in the long terminal repeat sequence of many retroviruses (reviewed in ref. 1). Recently, an enhancer element located within the J_H–C_μ intron has been described for the immunoglobulin heavy (H) chain genes (2–4). This enhancer element for the H chain gene was mapped to a 0.8-kb *Xba* I/*Eco*RI restriction fragment residing 3′ of J_4 in the J_H–C_μ intron (2–4). The enhancer element was identified and mapped in transfection experiments with plasmids containing either the immunoglobulin H chain gene or another gene with or without the putative enhancer sequence. By using the amount of RNA or protein expressed by these transfected genes as a measure of gene activity, it was found that the presence of the enhancer element, in either orientation, was necessary for high levels of gene expression. The purpose of the present communication is to determine whether this enhancer element is necessary for H chain expression in cells that contain a functional H chain gene, generated by the normal ontogenetic event and present at the correct position on chromosome 12.

For this study we used derivatives of the Abelson-virus-transformed mouse pre-B-cell line 18-81. This line accumulates deletions within the J_H–C_μ intron during growth *in vitro* (5, 6), allowing us to correlate the deletions in this region with loss of immunoglobulin production. The 18-81 cell line contains cells that synthesize only H chain (as well as some that synthesize no immunoglobulin chain) (7, 8). The cells of this line can switch from μ to γ2b chain synthesis *in vitro* (6). The cell line is diploid for chromosome 12. Both homologs have correctly joined *VDJ* segments. The variable (V) region alleles contain either J_2 or J_3 (5, 6); hence we name them *V2* and *V3*. Cells of the 18-81 line usually express only the *V3* allele (5, 6), because the *V2* allele contains an amber termination codon (9). However, a few cells continuously produce H chain from both homologs; that is, they also express the *V2* allele (10). The deletions in the J_H–C_μ intron occur on both alleles and vary in length and position (unpublished data). We found subclones of 18-81 that lack the enhancer element of the J_H–C_μ intron but still produce as much immunoglobulin chain as cells with the enhancer element on the expressed allele.

MATERIALS AND METHODS

Subcloning. Cell line 18-81 was subcloned by limiting dilution (0.15 cell per well) with syngeneic or xenogeneic (Wistar rat) peritoneal exudate cells as feeder cells.

Southern Blot Analysis. Southern blot analysis (11) was performed as described (6). For the gel to separate *Pst* I fragments, 1.5% agarose was used instead of the usual 0.8%. The kidney DNA was from the BALB/c mouse strain. The plasmids containing the DNA probes were generously provided by F. Blattner.

Fluorescence-Activated Cell Sorter (FACS) Analysis. Analysis with the FACS was performed as follows: First 4×10^6 cells were washed once in filtered phosphate-buffered saline, pH 7.1, containing 1% (wt/vol) bovine serum albumin and then once in filtered phosphate-buffered saline without Ca^{2+} and Mg^{2+}. After centrifugation the supernatant was removed and the cell pellet was resuspended to give a homogeneous suspension in a small volume of buffer. Ten milliliters of fixative (95 vol of ethanol and 5 vol of acetic acid, at −20°C) was added quickly by ejecting it from a syringe. After incubation for 10 min at −20°C, the cells were washed once in phosphate-buffered saline without Ca^{2+} and Mg^{2+} and then once in phosphate-buffered saline/bovine serum albumin. After centrifugation the cells were resuspended in 20 μl of buffer, 20 μl of fluorescein-coupled goat antiserum specific for μ chain (a gift from J. Kearney, Birmingham, AL) (12) was added, and the mixture was incubated for 10 min on ice. The cells were then washed three times in phosphate buffered saline/bovine serum albumin and analyzed in the FACS.

NaDodSO$_4$/Polyacrylamide Gel Electrophoresis. Cells were cultured in the presence of [^{35}S]methionine for 20 min. H chain from cell lysates was precipitated with antibodies specific for μ chain, reduced, and analyzed on NaDodSO$_4$/10% polyacrylamide gels as described (8).

Measurement of Steady-State Level of mRNA Specific for μ chain. Relative levels of μ-chain-specific mRNA were compared among the various cell lines by the technique of cytoplasmic dot hybridization (13). Briefly, cells were lysed in

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: H chain, heavy chain; V region, variable region; C region, constant region; *J*, joining segment; FACS, fluorescence-activated cell sorter; kb, kilobase(s).

0.5% Nonidet P-40 and the nuclei were removed by centrifugation. The cytoplasm was denatured and serial 1:1 dilutions were dotted onto nitrocellulose filters as described (13).

Cell Fusion. Clone A33-1 was fused with the myeloma Ag8653 (14) as described (8). The myeloma Ag8653 contributes no H or light (L) chains to the hybridomas. Clone A1 is a bromodeoxyuridine-resistant thymidine-kinase-negative subclone of 18-81. In this case, both fusion partners could be selected against in medium containing hypoxanthine, aminopterin, and thymidine.

RESULTS

The genealogy of the subclones of 18-81 that are used in this study is shown in Fig. 1. As shown by Southern blot analysis, in cells of the early 18-81 line both alleles contain the enhancer. DNA from a hybridoma, H6, derived from a fusion between an early 18-81 cell and a myeloma, Ag8653, reveals three *Eco*RI restriction fragments that hybridize with a probe covering the sequence of the proposed enhancer ("*E* probe," the 0.8-kb *Xba* I/*Eco*RI restriction fragment of the J_H–C_μ intron, Fig. 2*A*). In Fig. 2*B*, the 6.4-kb band is contributed by the myeloma, and the 2.4- and 1.8-kb bands represent the *V2* and *V3* alleles of 18-81, respectively, hence the excluded and the productive allele (5, 10).

A Subclone That Lacks the Enhancer on the Active Allele But Still Produces μ Chain. The clone A3 contains both the *V2* and *V3* alleles of the cell line 18-81. Clone A3 and subclone A35 have indistinguishable DNA arrangements as judged by Southern blot analysis using several restriction enzymes. The enzyme *Bam*HI cuts within the C_μ segment, so two bands (9.4 and 11.2 kb) of embryonic DNA hybridize with a μ cDNA probe (6). In clone A3 and subclone A35, the two J_H–C_μ alleles are represented by 5.6- and 3.5-kb *Bam*HI restriction fragments (6). Rearrangement involving J_3 would give rise to *Bam*HI fragments of the same size or larger than the 9.4-kb embryonic fragments. On the other hand, deletions in the J_H–C_μ introns of both alleles result in the smaller fragments that we observe. The 3.5-kb fragment represents the productive *V3* allele (6); it is lost in a subclone, A38, that produces no μ chain (6). Both bands also hybridize with a probe including J_3 and J_4 ("J34 probe" in Fig. 2*C*). However, the E probe hybridizes only with the 5.6-kb band that comes from the excluded allele (Fig. 2*C*). Therefore the excluded *V2* allele, but not the productive *V3* allele, contains the DNA segment where the immunoglobulin enhancer element was reported to be located (3, 4). This finding is confirmed by Southern blot analyses using the restriction ezymes *Eco*RI (Fig. 2*D*), *Bgl* I, and *Pst* I (not shown). Several other considerations lead us to conclude that the region around the *Eco*RI site of the major intron (i.e., the intron between *J* and C_μ) is deleted. The *Eco*RI and *Bgl* I sites must be deleted, because *Eco*RI or *Bgl* I digestion yields restriction fragments that contain both the J_3 and C_μ fragments (*Eco*RI, Fig. 2*D*; *Bgl* I, not shown). The first two *Xba* I sites 3′ to J_4 must be also deleted, because DNAs of different subclones of A3 that have switched to γ2b production show very different *Xba* I fragments representing the productive allele when hybridized with J34 probe, although they have the same *V3* allele (not shown).

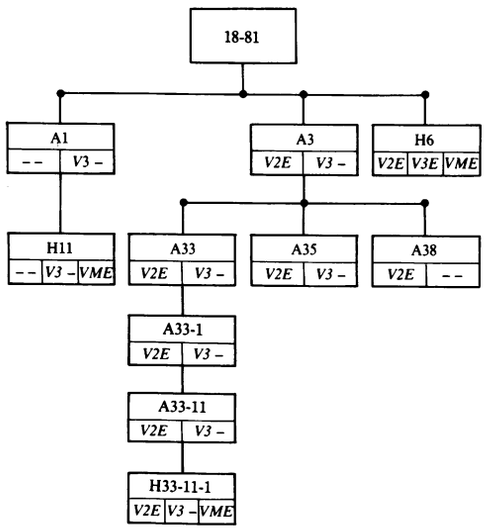

FIG. 1. Genealogy of subclones and hybridomas derived from the Abelson-virus-transformed line 18-81. Abelson-virus-transformed cells are designated "A," and their hybridomas are designated "H." The *V* region alleles are listed below the subclone designation. *V2* and *V3* are alleles from 18-81 containing J_2 and J_3, respectively; *VM* indicates the *V* region allele of myeloma Ag8653. *E* indicates the putative enhancer present on the allele.

As shown by FACS analysis using fluorescein-coupled antibodies specific for μ chain (Fig. 3*A*) and by immunoprecipitation and $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 3*B*), most of the cells of subclone A35, which lacks the enhancer element on the productive allele, produce μ chain. The steady-state level of μ chain produced in cells of clone A35 is the same as in clone A3 (not shown) and is somewhat less than in clone A33-1 (Fig. 3*A*). In subclone A33-1 the μ chain is produced from the *V2* allele (10), which contains the enhancer element (Fig. 2 *A–D*); the *V3* allele codes for a γ2b chain (6). As measured by incorporation of [^{35}S]methionine into μ chain for 20 min (data not shown), the rate of μ chain synthesis in clone A35 is one-third or one-fourth of the rate in clone A33-1. We conclude that the difference in the amount of μ chain produced by pre-B cells in the presence and absence of a known *cis* enhancer element is less than an order of magnitude.

A Subclone That Lacks the Enhancer and Does Not Produce μ Chain Can Be Activated to μ Chain Production by Fusion with a Myeloma. One could argue that the enhancer element acts in a differentiation stage-specific manner—e.g., only in plasma cells. Pre-B cells do not make large amounts of μ chain (15). In fact, the rate of μ chain synthesis in plasma cell hybridomas Sp7 and Sp6 is 10 to 20 times higher than in the clone A3 (Fig. 3*B*, lanes 7 and 8, and 3, respectively), but only 5 times higher than in clone A33-1. There is also a difference in the steady-state levels of mRNA specific for μ chain. Hybridoma Sp6 contains about 16 times more μ-specific RNA than do clones A3, A35, and A33-1 (Fig. 4, lane 8, as compared to lanes 3, 4, and 5). However, we will show in this section that the rate of H chain synthesis and the steady-state level of μ-specific RNA in plasma cells is also independent of the presence or absence of the enhancer element.

In the J_H–C_μ intron of the *V3* allele of subclone A1 there is a deletion of at least 6 kb which is larger than the deletion in clone A35. Furthermore, in subclone A1, the *V2* allele is lost completely (Fig. 2 *C* and *D*). There is no restriction fragment hybridizing with the *E* probe. Clone A1 produces neither μ chain (Fig. 3*A* and Fig. 3*B*, lane 2), nor mRNA specific for μ chain (Fig. 4, lane 2). Hybridomas were produced by fusing cells of this clone to a myeloma, Ag8653, that produces no immunoglobulin (ref. 14; no μ chain: Fig. 3*B*, lane 1), makes no μ mRNA (Fig. 4, lane 1), and contains no C_μ gene (Fig. 2*D*). Fifty hybridomas were tested, and all of them synthesized μ chain.

Hybridoma H11, as well as some other hybridomas from the above fusion, also expresses κ chain and secretes a complete Ig molecule (8). Hybridoma H11 synthesizes μ chain at the same rate as hybridoma H33-11-1 (Fig. 3*B*, lanes 5 and 6), which was derived from a subclone, A33-1, containing the enhancer element in *cis*, and at the same rate as the plasma cell hybridomas Sp7 and Sp6 (Fig. 3*B*, lanes 7 and 8, respectively). Hybridoma H11 has the same steady-state lev-

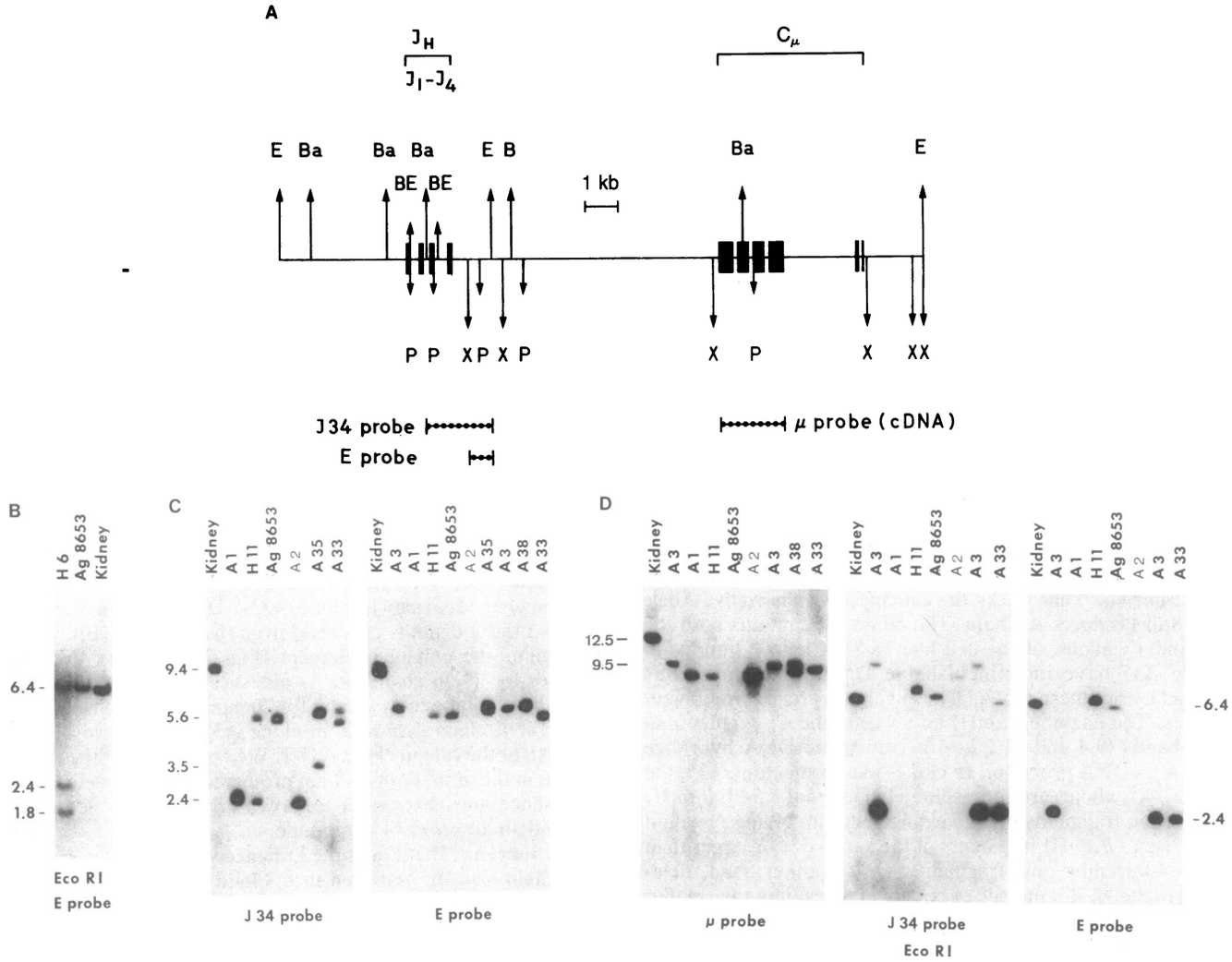

FIG. 2. Southern blot analysis to assign the presence or absence of the putative enhancer sequence to the J_H–C_μ intron alleles. Twelve micrograms of DNA per lane was applied. (*A*) Map. Ba, *Bam*HI; BE, *Bst* EII; B, *Bgl* I; E, *Eco*RI; P, *Pst* I; X, Xba I. Probes cover the regions indicated by ⊢⊣. *E* probe, *Xba* I/*Eco*RI fragment; J34 probe, *Bam*HI/*Eco*RI fragment; μ probe, cDNA. (*B–D*) Southern blots. In *D*, the 6.4-kb fragments of H11 and Ag8653 hybridizing with J34 and *E* probe seem to be of slightly different length. This is due to different DNA concentration. On numerous other gels these fragments are exactly at the same position.

el of μ-specific mRNA as the other hybridomas (Fig. 4, lanes 6, 7, and 8).

It is unlikely that there was selection for some preexisting μ-producing cells in fusion or in growth of the μ-producing hybridomas. At the time of fusion the A1 clone contained very few μ-producing cells, below 0.1%. Furthermore, cells that produce H chain without L chain should, if anything, be selected against (16–18). There is no enhancer element present in the hybridoma H11 apart from the one contributed by the myeloma (Fig. 2). Nor is there any evidence that the putative enhancer element of the myeloma was brought into *cis* position by recombination. Both the *E* probe and the J34 probe hybridize with the same restriction fragment. When digested with the enzymes *Bam*HI, *Eco*RI, *Bgl* I, and *Xba* I (for Ag8653 and H11 see Fig. 2, for H33-11-1 not shown), identical fragments are found in the hybridoma and myeloma. We conclude that the known enhancer element in the J_H–C_μ intron is not required for heightened-level H chain expression in plasma cells.

DISCUSSION

We have shown that mRNA and H chain can be produced at high levels in the absence of a well-characterized enhancer element. It is difficult to reconcile the results described here with the interpretations given by Banerji *et al.* (2), Gillies *et al.* (3), and Neuberger (4) for their transfection experiments. It is conceivable that other enhancer elements exist near the C_μ locus and would come into play in the absence of the known enhancer element. Such unknown enhancers would therefore act at greater distance but still yield the same level of H chain expression as the former enhancer. If such enhancers were 5′ of the V_H segment, each V_H segment would need its own set of enhancers. On the other hand, we can imagine that the enhancer element is required for a high level of transcription in the transfection assay rather than in "normal" cells—i.e., perhaps it is a "transfection enhancer" rather than an immunoglobulin enhancer. This would, in part, explain the results of Banerji *et al.* (2) and Neuberger (4), who performed transient expression assays. The enhancer element might also be needed for the homing of the plasmid. In the work of Gillies *et al.* (3), plasmids containing the H chain gene were presumably integrated into the cellular DNA. In this case the enhancer sequence would be needed for guided insertion into active transcription sites of the myeloma.

In our experiments we found no evidence for the need of a known enhancer element acting *cis*. However, since μ-specific mRNA production was increased 16-fold when clone

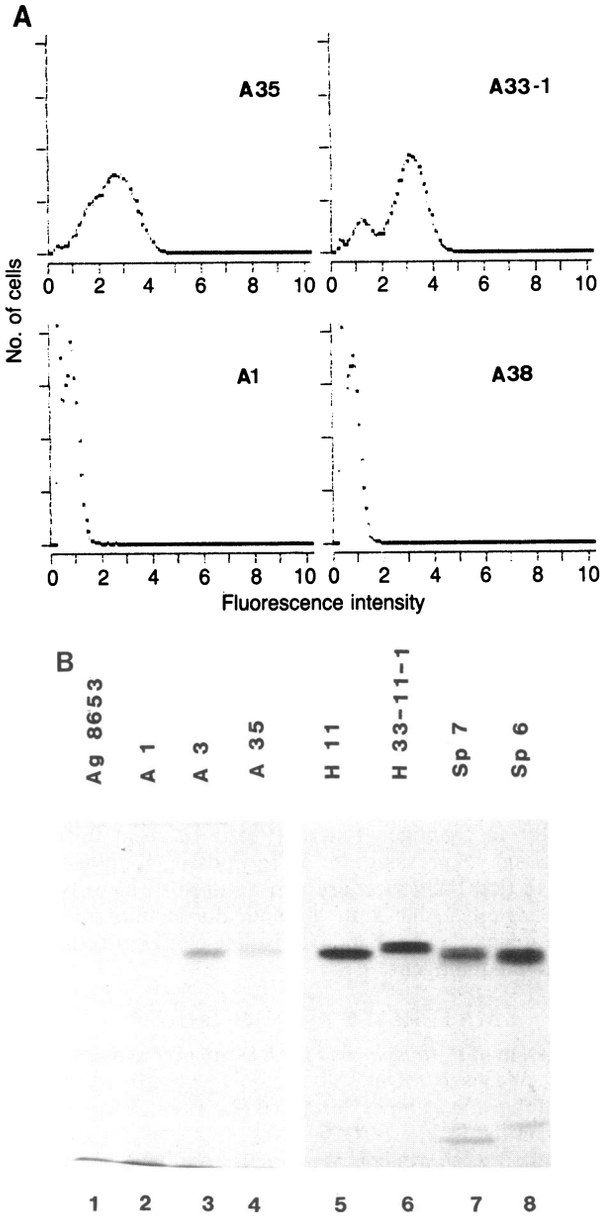

FIG. 3. (*A*) FACS analysis to determine the amount of μ chain production in clones A35, A33-1, A1, and A38. The *y*-axis has a linear scale; the *x*-axis, a logarithmic scale. (*B*) Fluorograph of NaDodSO$_4$/polyacrylamide gel electrophoresis of [^{35}S]methionine-labeled μ chains produced by Abelson-virus-transformed cells of the 18-81 cell line and by hybridomas. Lanes 1–4 were exposed 11 times longer than lanes 5–8 from the same gel. The μ chain of H33-11-1 apparently differs from the one of H11 and Sp6 and Sp7. It is derived from the usually excluded *V2* allele of 18-81.

A33-1 was fused to a myeloma, there may be *trans*-acting expression elements that enhance the steady-state mRNA production upon the transition from pre-B cells to B cells or from B cells to plasma cells. We would also like to point out that there must be an additional element in the myeloma that, after fusion, allows expression of μ chain from the gene carried by clone A1. This expression element, or "expressor," is needed specifically for the μ chain expression, because evidently the other proteins are synthesized without it. It must act *trans* and, therefore, is not an enhancer and is likely to encode a protein. Because the inability of clone A1 to synthesize mRNA for μ chain is correlated with a large deletion in the J_H–C_μ intron, we speculate that this expressor is encoded, at least partly, in the major intron of the H chain gene.

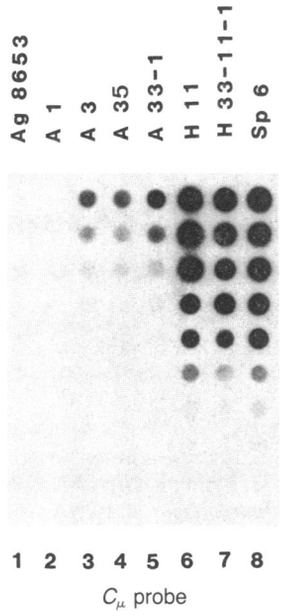

FIG. 4. Titration of steady-state level of mRNA specific for μ chain: Cytoplasmic dot hybridization. Serial 1:1 dilution steps starting with the denatured cytoplasm (13) from 4×10^5 cells (except Ag8653, which was from 2×10^5 cells). Hybridization probe: μ cDNA (Fig. 2).

We thank G. Beck-Engeser for expert technical assistance and W. Leiserson (Basel Institute for Immunology) for performing the FACS analysis. We appreciate discussions with Dr. C. Steinberg (Basel), Dr. J. Johnson (Munich), Dr. B. Hirt (Lausanne), Dr. S. Tonegawa (Cambridge), and Dr. K. M .rcu (Stony Brook).

1. Khoury, G. & Gruss, P. (1983) *Cell* **33,** 313–314.
2. Banerji, J., Olson, L. & Schaffner, W. (1983) *Cell* **33,** 729–740.
3. Gillies, S. D., Morrison, S. L., Oi, V. T. & Tonegawa, S. (1983) *Cell* **33,** 717–728.
4. Neuberger, M. S. (1983) *EMBO J.* **2,** 1373–1378
5. Alt, F. W., Rosenberg, N., Casanova, R. J., Thomas, E. & Baltimore, D. (1982) *Nature (London)* **296,** 325–331.
6. Burrows, P. D., Beck-Engeser, G. B. & Wabl, M. R. (1983) *Nature (London)* **306,** 243–246.
7. Siden, E. J., Baltimore, D., Clark, D. & Rosenberg, N. E. (1979) *Cell* **16,** 389–396.
8. Burrows, P. D., Beck, G. B. & Wabl, M. R. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 564–568.
9. Alt, F. W., Rosenberg, N., Enea, V., Siden, E. & Baltimore, D. (1982) *J. Mol. Cell Biol.* **2,** 386–400.
10. Wabl, M. R., Beck-Engeser, G. B. & Burrows, P. D. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 867–870.
11. Southern, E. M. (1975) *J. Mol. Biol.* **98,** 503–517.
12. Kearney, J. F. & Lawton, A. R. (1975) *J. Immunol.* **115,** 671–676.
13. White, B. A. & Bancroft, F. C. (1982) *J. Biol. Chem.* **257,** 8569–8572.
14. Kearney, J. F., Radbruch, A., Liesegang, B. & Rajewsky, K. (1979) *J. Immunol.* **123,** 1548–1550.
15. Raff, M. D., Megson, M., Owen, J. J. T. & Cooper, M. D. (1976) *Nature (London)* **259,** 224–226.
16. Wilde, C. D. & Milstein, C. (1980) *Eur. J. Immunol.* **10,** 462–467.
17. Coffino, P. & Scharff, M. D. (1971) *Proc. Natl. Acad. Sci. USA* **68,** 219–223.
18. Köhler, G. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 2197–2199.