Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 2328–2331, April 1983
Immunology

Linkage of β_2-microglobulin and ly-m11 by molecular cloning and DNA-mediated gene transfer

(genetic mapping/histocompatibility antigen/minor histocompatibility locus)

DAVID H. MARGULIES*, JANE R. PARNES*†, NANCY A. JOHNSON‡, AND J. G. SEIDMAN‡

*Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20205; and ‡Department of Genetics, Harvard Medical School, Boston, Massachusetts 02115

Communicated by William E. Paul, December 28, 1982

ABSTRACT **β_2-Microglobulin (β_2m) is expressed on the cell surface after introduction of a β_2m^b (C57BL/6N) genomic clone into thymidine kinase-deficient mouse L cells by cotransformation using the calcium phosphate precipitate method. Stable transformant cell lines were identified that express the β_2m^b allele, as determined by reaction of the cells with appropriate monoclonal antibodies and by two-dimensional gel electrophoresis of endogenously labeled immunoprecipitates of cell extracts. These β_2m^b transformants now express ly-m11.2, as detected by an indirect radioimmunoassay. A plasmid subclone of the β_2m^b gene that contains an 8.4-kilobase insert, after introduction into mouse L cells, similarly directs the synthesis of both the β_2m^b and the ly-m11.2 antigens. Thus, the β_2m^b and ly-m11.2 determinants most likely represent sites on the same protein structure.**

Establishment of linkage between distinct markers in the mammalian genome continues to be a focus of modern genetics at the organismic, cellular, and molecular levels (for review, see ref. 1). Recently, we have witnessed the unification of molecular biological and classical genetic methods in the mapping of polymorphic restriction endonuclease fragments in both mouse (2–4) and man (5, 6). Somatic cell hybridization, *in situ* hybridization with molecular probes, and chromosome and DNA-mediated gene transfer methods offer alternatives to the classical approaches of analysis of pedigrees and recombinant inbred strains. In the past year, several groups of workers have demonstrated the effectiveness of using DNA-mediated gene transfer of cloned cell surface antigen genes and analysis of the subsequently expressed products in assigning known serological specificities to particular genes (7–10). In an attempt to apply the methods of molecular cloning, DNA-mediated gene transfer, and characterization of transformant cells to genetic mapping, we have sought to determine the linkage between the β_2-microglobulin (β_2m) gene and the locus that encodes the ly-m11 antigenic determinant.

β_2m is an 11,800-dalton cell surface polypeptide that serves as the light chain, in noncovalent association, of class I antigens of the major histocompatibility complex (11–13). In mouse, two alleles have been identified that differ by an amino acid substitution—alanine for aspartic acid at position 85 (14–17). Genetic studies have established the linkage of β_2m to the minor histocompatibility locus, *H-3*, of mouse chromosome 2 (18–20). In the course of preparing monoclonal antibodies to particular sets of lymphoid cells, an antigenic determinant, ly-m11, was identified on virtually all cell types (21). Analysis of recombinant inbred mouse strains linked this phenotype to *H-3* as well. In addition, the locus encoding another cell surface marker, ly-4, has been mapped to *H-3* (22). The possibility that H-3, ly-m11, ly-4, and β_2m are antigenic sites on the same protein has been suggested (18–22). An immune response gene, *Ir-2*, is also closely linked to *H-3* (23). A precise understanding of the genetic structure of this region would help to define the relationships among these markers. A prerequisite for using DNA-mediated gene transfer methods in this type of analysis is that the cloned gene, when introduced into mouse L cells, be functional. Many genes that are expressed only in highly differentiated cells are not expressed normally after introduction into L cells (1). Fortunately, cell surface antigens, particularly the class I molecules, are found on L cells, and their genetically distinguishable counterparts are found in their natural cellular environment when their genes are introduced by standard methods (7–10). We anticipated, then, that β_2m would be similarly expressed. As an initial step in the fine structure mapping of these linked genes, we have asked whether a cloned gene that encodes β_2m can also direct synthesis of the ly-m11 determinant.

MATERIALS AND METHODS

Genomic Clones. The identification and characterization of a β_2m gene derived from a C57BL/6N bacteriophage library (Ch4β2-C57) has been described (24). An 8.4-kilobase (kb) *Xho* I fragment containing the four exons of the β_2m gene was subcloned into plasmid pKC7 (25). Fortuitously, the 3′ *Xho* I site in this subclone is not found in the genomic DNA and results from a deletion that occurred in the bacteriophage clone. Detailed restriction mapping of this subclone reveals no other dissimilarities from the parental bacteriophage clone.

Southern Blots. DNA was isolated from tissue culture cells, digested with the restriction endonuclease *Bgl* I (New England BioLabs), and subjected to electrophoresis in a 0.8% agarose gel as described (4). After denaturation and neutralization, DNA was transferred to nitrocellulose paper by the method of Southern (26), baked at reduced pressure at 80°C, and hybridized to the nick-translated *Sac/Kpn* β_2m probe described elsewhere (24). The probe was purified from disulfide-crosslinked polyacrylamide gels (27) and nick-translated to a specific activity of 2×10^8 cpm per μg. After hybridization for 20 hr at 40°C, filters were washed as described, dried, and autoradiographed using XAR-5 film (Kodak) at −80°C with intensifying screens for 24 hr.

DNA-Mediated Gene Transfer. Thymidine kinase-deficient DAP-3 cells (1×10^6) were exposed to a calcium phosphate precipitate containing 200 ng of pBRTK, 5 μg of Ch4β2-C57, and 30 μg of DAP-3 genomic DNA (as carrier) as described (8, 10, 28–30). Stable transformants were selected in hypoxanthine/aminopterin/thymidine medium and, after 2 wk in culture, clones were isolated as described (9, 11). Transformation with

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: β_2m, β_2-microglobulin; kb, kilobase(s).

† Present address: Dept. of Medicine, Stanford Univ., Stanford, CA.

the 8.4-kilobase subclone was carried out by a similar procedure using a calcium phosphate precipitate containing 20 μg of L cell carrier DNA, 100 ng of pBRTK, and 4 μg of the plasmid subclone.

Antisera. Rabbit anti-mouse β_2m was the gift of E. Appella (National Cancer Institute). Anti-ly-m11.2 monoclonal S19.8 was provided by U. Hammerling (Memorial Sloan–Kettering Cancer Center, New York). This is the product of a hybridoma obtained by fusion to SJL spleen cells from a mouse immunized with B10.S spleen (21). The anti-β_2m monoclonal antibody (mc-β2M-B:clone 23) given to us by F. W. Shen (Memorial Sloan–Kettering Cancer Center) was also derived from a fusion to SJL anti-B10.S spleen cells (31).

Indirect Radioimmunoassay. We added 1×10^5 L cells, transformants, or peritoneal exudate cells (from C57BL/6N mice treated 3 days earlier with 1 ml of intraperitoneal thioglycollate broth) to the wells of a 96-well microtiter dish (Corning) in growth medium containing 5% fetal calf serum as described (32). The next day cells were washed and treated with either the anti-β_2m monoclonal antibody or with the anti-ly-m11.2 monoclonal antibody. Both monoclonal antibodies were used as 1:1,000 ascites fluid dilution in growth medium. After 60 min of incubation at 4°C as described (32), cells were washed and then incubated with ^{125}I-labeled goat anti-mouse immunoglobulin (New England Nuclear) in excess antibody for 1 hr, washed again, and solubilized in 1% Nonidet P-40. The extracts were assayed in a γ-spectrometer. Data presented are means of triplicate determinations that varied by less than 10%. No correction for background was made.

Incubation of Cells with Radioactive Amino Acids, Immunoprecipitation, and Two-Dimensional Gel Electrophoresis. L cells, transformant clones, or EL4 (C57BL/6) cells were labeled for 4 hr in RPMI 1640 medium lacking methionine (GIBCO Select-Amine) supplemented with 2 mM glutamine/1% nonessential amino acids/5% fetal calf serum and containing [^{35}S]-methionine at 175 μCi/ml (Amersham; specific activity, 1,390 Ci/mmol; 1 Ci = 37 GBq) as described (10). Cell extracts were prepared by lysis with 1% Nonidet P-40, cleared with normal rabbit serum and protein A-sepharose (Pharmacia), and subjected to immunoprecipitation with rabbit anti-mouse β_2m antiserum. Immunoprecipitates bound to protein A-Sepharose were solubilized in O'Farrell's lysis buffer (33) and analyzed by two-dimensional nonequilibrium pH gradient gel electrophoresis (34) under conditions suggested by Sawicki *et al.* (35) for β_2m. The second dimension was a 12.5% polyacrylamide gel containing 0.1% $NaDodSO_4$ as described by Maizel (36). In all cases 10^6 cpm of trichloroacetic acid-precipitable material was subjected to immunoprecipitation, and 20% of the final precipitate was applied to the first dimension of the gel. Gels were treated for fluorography (37), dried, and exposed to Kodak XAR-5 film with intensifying screens at −80°C for 3 days.

RESULTS AND DISCUSSION

Recently, we identified a full-length bacteriophage library genomic clone, Ch4β2-C57, derived from a C57BL/6N library, containing the DNA sequences that encode the BL/6 allele of β_2m (24). (The relevant restriction map and probe are shown in Fig. 1*B*.) The insert in this genomic library clone is about 18.5 kb long, with the bulk of the β_2m gene from the 5′ leader sequence through to the poly(A) addition site extending over 6.0 kb. Approximately 4.0 kb flank the insert in the 5′ direction and 8.5 kb, in the 3′ direction.

To test whether all the DNA sequences necessary for normal expression of β_2m are contained in this genomic clone, the C57BL/6N β_2m^b gene was introduced into mouse L cells (β_2m^a) by cotransformation with the herpesvirus thymidine kinase gene, using the calcium phosphate precipitate method (9, 28, 29). Three transformant cloned cell lines were isolated and grown to mass culture for further study. To visualize the integrated gene and to allow estimation of the number of β_2m^b gene copies introduced into these cells, the DNA was extracted and analyzed by the Southern blotting technique with appropriate enzymatic digestion and hybridization to a specific β_2m DNA probe (Fig. 1). [The allelic difference that distinguishes β_2m^a from β_2m^b occurs at position 85 in the amino acid sequence and has been determined at the nucleic acid level to represent a single base substitution of GCC (Ala) for GAC (Asp) (24). The β_2m^b allele

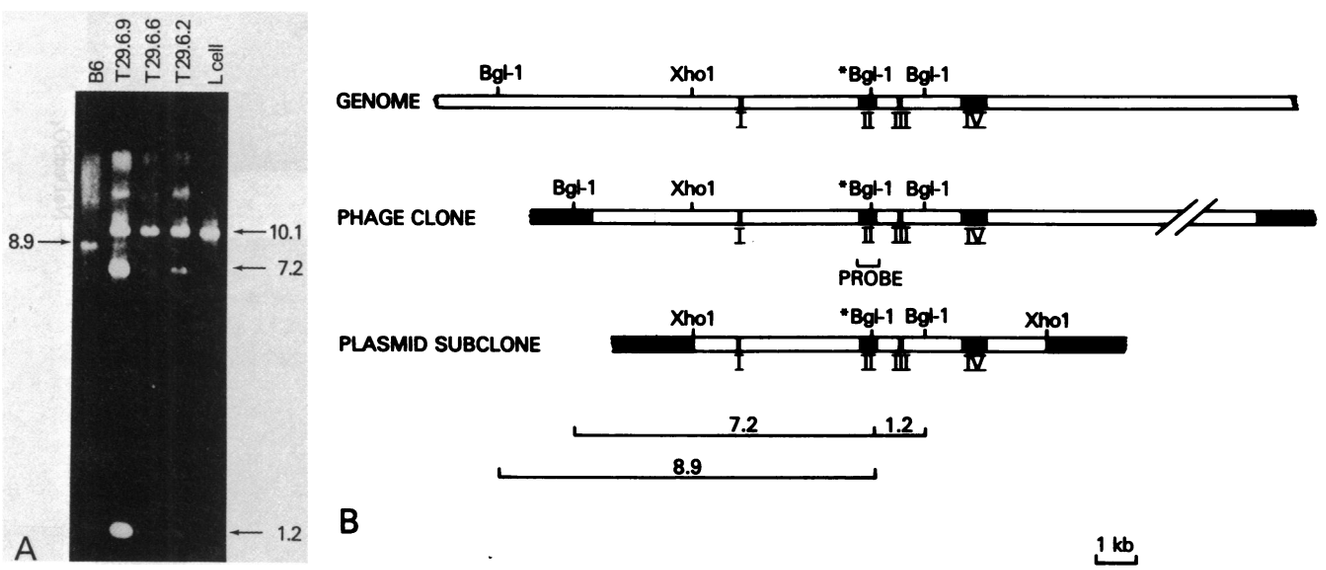

FIG. 1. (*A*) β_2m^b genes introduced into L cells by DNA-mediated gene transfer. After isolation of transformant clones, DNA was extracted and analyzed by Southern blotting. Indicated sizes were interpolated from a comigrated sample of λ DNA digested with *Eco*RI. (*B*) Restriction map of the C57BL/6N β_2m genomic clone. A detailed description of the isolation of this genomic clone from a C57BL/6N genomic library constructed in Charon 4A is given elsewhere (24). Filled in areas represent phage arms. Coding blocks of the gene are indicated by roman numerals, as reported in ref. 24. A map of the corresponding segment in the C57BL/6N genome is given for comparison. *, *Bgl* I restriction site that reflects the β_2m polymorphism; this site is present in β_2m^b and absent in β_2m^a strains.

has a unique *Bgl* I restriction site involving this codon.] The indicated probe spans the *Bgl* I restriction site. Thus, the L cell has a single 10.1-kb *Bgl* I fragment that hybridizes to the probe. C57BL/6N liver DNA has an 8.9-kb fragment and a 1.2-kb fragment. The three transformant clones, T29.6.2, T29.6.6, and T29.6.9, each have the L-cell-derived 10.1-kb fragment as well as the 1.2- and 7.2-kb fragments, both derived from the bacteriophage library clone. Fig. 1*A* thus shows that, in the three transformant clones analyzed, the β_2m^b gene has been introduced in varying copy number. Densitometric scans of these genomic blots, with appropriate standardization of the intensity differences to the putative two-copy endogenous L-cell fragment (10.1 kb), allow quantitative estimation of the number of cloned genes introduced into these cells. Clones T29.6.2 and T29.6.6 have incorporated 2 or 3 copies of the β_2m gene, while clone T29.6.9 appears to have incorporated about 10 copies.

To determine whether the newly introduced β_2m gene(s) was functional at the level of the synthesis of β_2m protein, transformant clones were screened by indirect immunofluorescence using a monoclonal anti-β_2m^b antibody (31). Preliminary data showed that, indeed, the transformants were expressing the introduced gene. To confirm this finding and establish the protein nature of the newly expressed gene product, parental L-cell and transformant clones were endogenously labeled with [^{35}S]methionine, cell extracts were prepared, and, after immunoprecipitation with a rabbit anti-mouse β_2m antiserum, subjected to two-dimensional gel electrophoresis. As shown in Fig. 2 *A* and *B*, both parental cell types [EL4 (β_2m^b) and L cell (β_2m^a)] actively synthesize a 12-kilodalton immunoprecipitable basic protein. A mixture of the two extracts (Fig. 2*D*) reveals that the two products can easily be distinguished in this two-dimensional gel system. The immunoprecipitate of transformant clone T29.6.9 separates into two distinct spots, which are indistinguishable from those seen in the mixture of EL4 (β_2m^b) and L-cell (β_2m^a) immunoprecipitates. An estimate of the cellular content of β_2m and the relative amounts of the two allelic products can be made from densitometric scans of the autoradiograms of the two-dimensional gels. Clones T29.6.2 and T29.6.6 contain about the same amount of β_2m^b as β_2m^a (data not shown). Transformant T29.6.9, however, contains 1.5- to 2.0-fold the amount of β_2m^b as β_2m^a. Thus, although T29.6.9 seems to have about 10 copies of the β_2m^b gene, it has at most 2-fold the expressed product as cellular content. This may reflect shedding, secretion, or intracellular degradation of unassembled chains. Alternatively, only some of the newly introduced β_2m^b genes may be functional. More detailed biosynthetic and transcriptional studies will be required to resolve this discrepancy.

Having determined that the C57BL/6N allele (β_2m^b) was introduced into the transformant L cells and that at least some copies were expressed at the translational level, we then sought to determine whether the ly-m11 antigenic specificity was detectable on the surface of these transformants. We found that the reactivity of the transformants with both an anti-ly-m11.2 monoclonal antibody and an anti-β_2m^b monoclonal antibody followed the same pattern (Table 1, experiment 1). Thus, the L

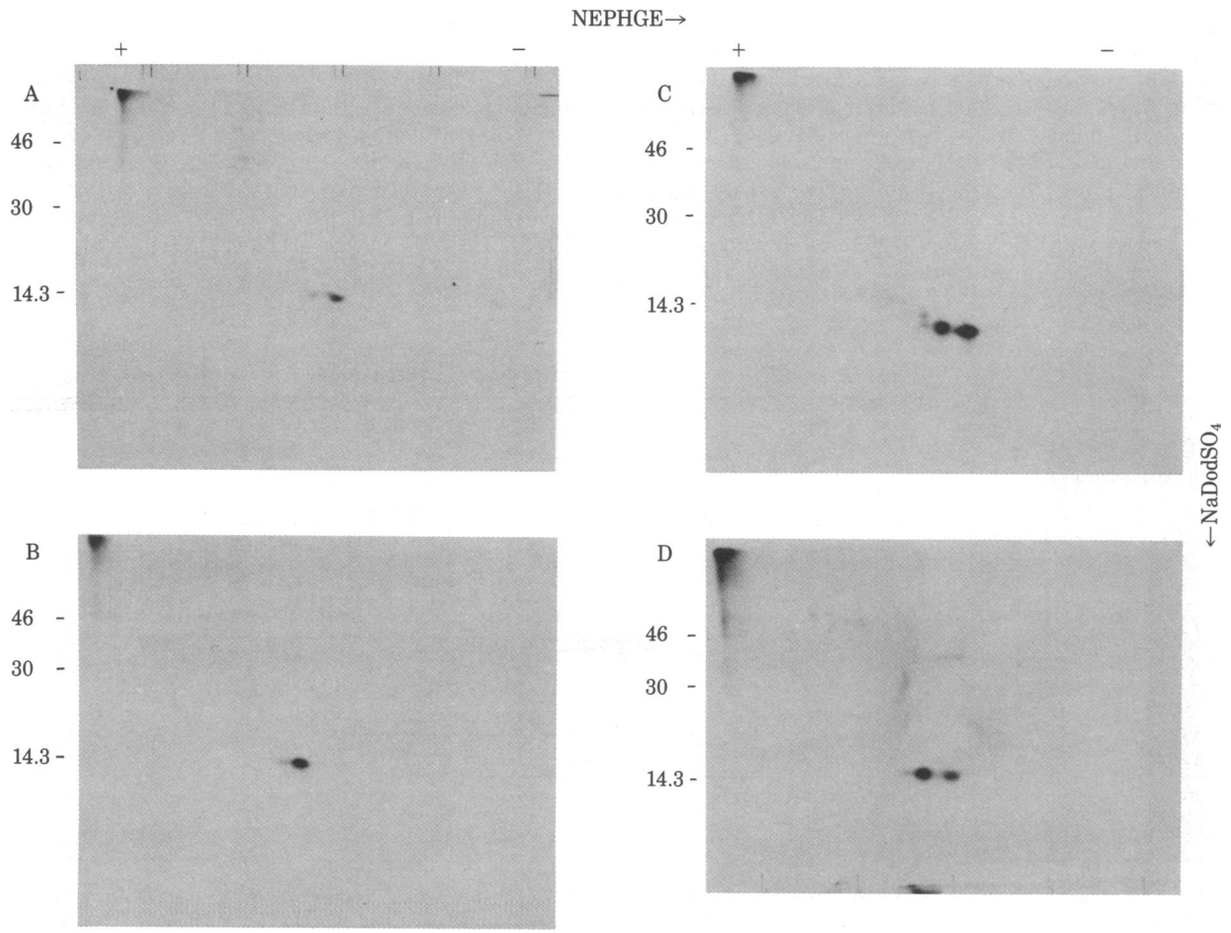

FIG. 2. Two-dimensional gel electrophoretic analysis of β_2m from L cells and various transformants. (*A*) EL4. (*B*) L cells. (*C*) T29.6.9. (*D*) EL4/L cell mixture. Horizontal dimension, nonequilibrium pH gradient electrophoresis (NEPHGE) (left to right); vertical dimension, $NaDodSO_4$ gel electrophoresis (top to bottom). Numbers on the left are molecular mass in kilodaltons.

Table 1. Indirect radioimmunoassay of L cells and various transformants

	Anti-β_2m^b	Anti-ly-m11.2	No antibody
	Experiment 1		
L cells	273	327	229
T29.6.2	3,784	3,178	198
T29.6.6	3,480	2,347	180
T29.6.9	5,282	4,381	285
C57BL/6N	2,412	2,351	982
	Experiment 2		
L cells	995	946	791
T40.9	6,211	5,135	577
T29.6.9	17,405	14,507	560

Values presented are cpm of ^{125}I.

cells do not react significantly either with the anti-β_2m^b reagent or the anti-ly-m11.2 reagent. However, all three transformant clones (T29.6.2, T29.6.6, and T29.6.9) that have incorporated the Ch4β2-C57 genomic clone now express detectable levels of both the β_2m^b specificity and the ly-m11.2 specificity. To refine the linkage of expression of β_2m^b and *ly-m11.2*, we have subcloned an 8.4-kb *Xho* I fragment that contains the entire coding block of the gene, flanked by about 1 kb to the 5′ side and 1.4 kb to the 3′ (Fig. 1*B*). This subclone was introduced into mouse L cells by DNA-mediated gene transfer. The pool of transformed cells that survived 3 wk of selection in hypoxanthine/aminopterin/thymidine medium, T40.9, was then assayed for the expression of β_2m^b and *ly-m11.2* (Table 1, experiment 2). It is apparent that this pool of transformants now expresses both determinants. The quantitative differences may reflect the mixed nature of this population.

This demonstration of linkage of β_2m and the *ly-m11.2* antigenic determinant can be interpreted in several ways. It refines the linkage analysis as determined by classical genetics using recombination frequency and recombinant inbred strains, which was capable only of determining the linkage of the two markers to within 1 centimorgan of each other (14) (an estimated 2×10^6 base pairs of DNA). Now, that linkage has been narrowed to within the same 8,400-base-pair insert that is found in the genomic subclone. A detailed protein-chemical characterization of the structure that defines ly-m11.2 will be helpful in confirming its identity with that of β_2m. Preliminary results (N. Tada and S. G. Nathenson, personal communication) show that the partial amino-terminal sequence of ly-m11.2-precipitated material is consistent with that of β_2m. Our preliminary results show that only the β_2m^b product (as identified on two-dimensional gels) is precipitated by either the anti-β_2m monoclonal antibody or the anti-ly-m11.2 monoclonal antibody from transformant T29.6.9 (unpublished data). Thus, the sites recognized by the anti-β_2m^b and anti-ly-m11.2 monoclonal antibodies are likely to be due to the same amino acid substitution.

The cell surface expression of β_2m and *ly-m11.2* in these transformant cell lines is of additional interest as we attempt to explain the biological role of this molecule. Does it merely serve to stabilize the conformation of the class I molecules in the cell membrane to allow appropriate H-2-restricted recognition or can β_2m serve independently as a restricting element or transplantation antigen? These transformant cell lines should prove useful in the continued evaluation of the relationship of other H-3-linked markers to β_2m.

We are grateful to Drs. U. Hammerling, F.-W. Shen, and E. Appella for gifts of antisera and monoclonal antibodies and to Drs. W. M. Kuehl, G. A. Evans, R. N. Germain, and D. Gasser for helpful discussions. J.G.S. is supported by grant AI18437 from the National Institute of Allergy and Infectious Diseases and Grant JFRA66 from the American Cancer Society.

1. Ruddle, F. H. (1981) *Nature (London)* **294,** 115–120.
2. Cami, B., Bregegere, F., Abastado, J. P. & Kourilsky, P. (1981) *Nature (London)* **291,** 673–675.
3. Pease, L. R., Nathenson, S. G. & Leinwand, L. A. (1982) *Nature (London)* **298,** 382–384.
4. Margulies, D. H., Evans, G. A., Flaherty, L. & Seidman, J. G. (1982) *Nature (London)* **295,** 168–170.
5. Orr, H. T., Bach, F. H., Pleough, H. L., Strominger, J. L., Kavathas, P. & DeMars, R. (1982) *Nature (London)* **296,** 454–456.
6. Botstein, D., White, R., Skolnick, M. & Davis, R. (1980) *Am. J. Hum. Genet.* **32,** 314–331.
7. Goodenow, R. S., McMillan, M., Orn, A., Nicolson, M., Davidson, N., Frelinger, J. A. & Hood, L. (1982) *Science* **215,** 677.
8. Evans, G. A., Margulies, D. H., Camaerini-Otero, R. D., Ozato, K. & Seidman, J. G. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 1994–1998.
9. Mellor, A. L., Golden, L., Weiss, E., Bullman, H., Hurst, J., Simpson, E., James, R. F. L., Townsend, A. R. M., Taylor, P. M., Schmidt, W., Ferluga, J., Leben, L., Santamaria, M., Atfield, G., Festenstein, H. & Flavell, R. A. (1982) *Nature (London)* **298,** 529–535.
10. Margulies, D. H., Evans, G. A., Ozato, K., Camerini-Otero, R. D., Tanaka, K., Appella, E. & Seidman, J. G. (1983) *J. Immunol.* **130,** 463–470.
11. Cresswell, P., Turner, M. J. & Strominger, J. L. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 1603–1607.
12. Grey, H. M., Kubo, R. T., Colon, S. M., Poulik, M. D., Cresswell, P., Springer, T., Turner, M. & Strominger, J. L. (1973) *J. Exp. Med.* **138,** 1608–1615.
13. Vitetta, E. S., Uhr, J. W. & Boyse, E. A. (1975) *J. Immunol.* **114,** 252–257.
14. Michaelson, J., Rothenberg, E. & Boyse, E. A. (1980) *Immunogenetics* **11,** 93–95.
15. Goding, J. W. & Walker, I. D. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 7395–7399.
16. Gates, F. T., III, Coligan, J. E. & Kindt, T. J. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 554–558.
17. Robinson, P. J., Graf, L. & Sege, K. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 1167–1170.
18. Michaelson, J. (1981) *Immunogenetics* **13,** 167–171.
19. Robinson, P. J., Lundin, L., Sege, K., Graf, L., Wigzell, H. & Peterson, P. A. (1981) *Immunogenetics* **14,** 449–452.
20. Goding, J. W. (1981) *J. Immunol.* **126,** 1644–1646.
21. Tada, N., Kimura, S., Hatzfeld, A. & Hammerling, U. (1980) *Immunogenetics* **11,** 441–444.
22. Snell, G. D., Cherry, M., McKenzie, I. F. C. & Bailey, D. W. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 1108–1111.
23. Gasser, D. L. (1969) *J. Immunol.* **103,** 66–70.
24. Parnes, J. R. & Seidman, J. G. (1982) *Cell* **29,** 661–669.
25. Rao, R. N. & Rogers, S. G. (1979) *Gene* **7,** 79–85.
26. Southern, E. M. (1975) *J. Mol. Biol.* **98,** 503–517.
27. Hansen, J. N., Pheiffer, B. H. & Boehert, J. A. (1980) *Anal. Biochem.* **105,** 192–201.
28. Graham, F. L. & Van der Erb, A. J. (1973) *Virology* **52,** 456–467.
29. Wigler, M., Pellicer, A., Silverstein, S., Axel, R., Urlaub, G. & Chasin, L. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 1373–1376.
30. Szybalski, W., Szybalska, E. H. & Ragni, G. (1962) *Natl. Cancer Inst. Monogr.* **7,** 75–80.
31. Chorney, M., Shen, F.-W., Michaelson, J. & Boyse, E. A. (1982) *Immunogenetics* **16,** 91–93.
32. Evans, G. A., Margulies, D. H., Shykind, B. J., Seidman, J. G. & Ozato, K. (1982) *Nature (London)* **300,** 755–757.
33. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250,** 4007–4015.
34. O'Farrell, P. Z., Goodman, H. M. & O'Farrell, P. H. (1977) *Cell* **12,** 1133–1142.
35. Sawicki, J. A., Magnuson, T. & Epstein, C. J. (1981) *Nature (London)* **294,** 450–452.
36. Maizel, J. V. (1971) in *Methods in Virology,* eds. Maramorosch, K. & Koprowski, H. (Academic, New York), Vol. 5, in press.
37. Laskey, R. A. & Mills, D. (1975) *Eur. J. Biochem.* **56,** 335–340.