Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 7616–7620, December 1983
Immunology

Evidence for extensive polymorphism of class I genes in the rat major histocompatibility complex (*RT1*)

(restriction fragment polymorphism/genetic mapping)

MELANIE PALMER*, PETER J. WETTSTEIN†‡, AND JEFFREY A. FRELINGER*

*Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, NC 27514; and †The Wistar Institute of Anatomy and Biology, 36th Street at Spruce, Philadelphia, PA 19104

Communicated by Ray D. Owen, September 6, 1983

ABSTRACT **The major histocompatibility complex of the rat (*RT1*) has been poorly characterized with respect to the number, linkage, and polymorphism of class I genes. To estimate the number of class I *RT1* genes and the relative extent of their polymorphism, we performed Southern blot analysis with liver DNA from rat strains expressing eight *RT1* haplotypes. After digestion with *Eco*RI and *Bam*HI, the DNA was separated on agarose gels, blotted onto nitrocellulose, and hybridized with mouse *H-2* cDNA probes, pH-2III and pH-2IIa. Ten to 20 *Eco*RI and 13 to 20 *Bam*HI bands hybridized with pH-2III and pH-2IIa; restriction fragment length patterns were observed to be highly polymorphic. The restriction fragments associated with different *RT1* haplotypes differed by 17–70%; this range is similar to the differences observed between mouse *H-2* haplotypes. The same restriction fragment pattern was observed in DNA from three different rat strains sharing the same *RT1* allele, confirming that the patterns were *RT1*-associated. Further, the $RT1^{l}$ and $RT1^{lv1}$ haplotypes, which differ at a single previously identified *RT1*-linked locus, were associated with *Eco*RI restriction pattern differences of 39–50%, confirming the supposition that *RT1* class I genes identified by previous serological and T-cell-mediated assays have identified only a minority of the actual number of *RT1*-linked class I genes. In summary, the results reported in this communication demonstrate that the *RT1* complex encompasses a large family of highly polymorphic class I genes similar to the *H-2* and *HL-A* complexes of mouse and man.**

The major histocompatibility complexes (MHCs) of mammals include polymorphic class I and class II genes (1), whose products regulate various aspects of the immune response. Class I genes encode approximately 45,000-dalton cell surface glycoproteins; in the case of the mouse MHC, *H-2*, approximately 30 class I sequences have been identified by gene cloning experiments (2). Among these genes are the previously identified *K*, *D*, and *L* (3) genes expressed on the majority of somatic cells and the *Qa* (4) and *Tl* genes (5) differentially expressed on lymphocyte subpopulations. Class II genes encode Ia molecules composed of two glycoprotein subunits with molecular weights of approximately 32,000 and 28,000 (6); class II molecules are primarily expressed on B cells (7) and macrophages (8).

The outstanding characteristic of class I and class II MHC genes of the two most extensively studied species, mouse and man (*HL-A*), is their high level of polymorphism. In the mouse, approximately 50 different alleles at both the *H-2K* and *H-2D* loci have been identified in inbred and wild mice (9, 10). The rat MHC, *RT1*, has been suggested to be significantly less polymorphic than the *H-2* and *HL-A* complexes. The *RT1* complex is composed of (*i*) class I genes, which are *RT1.A* (*H-2K* and *H-2D* analogs) (11, 12), *RT1.C* (*Qa* analog) (13), and *RT1.E* (14), and (*ii*) class II genes mapping to the *RT1.B* region (15, 16). Serological and T-cell mediated assays suggest that inbred and wild rats express a limited number of *RT1* haplotypes composed of class I and class II genes of limited diversity (17–19). Two alternatives may account for such presumed, limited diversity. First, *RT1* is actually not highly polymorphic, relative to other studied MHCs, due to the limited diversity of progenitors of inbred rat strains and present-day wild rats. Second, the *RT1* complex genes are, indeed, highly polymorphic and the serological and T-cell assays employed in previous studies were not sufficiently sensitive to distinguish different alloantigenic specificities.

Further, despite a recently published linkage map of the *RT1* complex (20), the relative map positions of *RT1* class I and class II genes are unknown; the reported linkage map has not ordered the *RT1.A* and *RT1.B* loci. This inadequacy is encountered, in part, because the total number of *RT1.A* and *RT1.B* genes is unknown. In addition, the low frequency of recombination within the *RT1* complex suggests that recombination analysis will yield little information on the relative map positions of *RT1*-linked genes.

It is obvious that the most direct approach to the questions of *RT1* gene polymorphism and linkage is to study the DNA itself. Mouse and human cDNA probes homologous to class I and class II genes of the rat can be employed to identify individual *RT1* sequences. This approach would take advantage of the relatively conserved nature of portions of mammalian MHC genes. The results presented in this communication constitute our initial characterization of *RT1* class I genes at the DNA level. Mouse cDNA probes homologous to relatively polymorphic exons and relatively conserved exons of H-2 genes were employed to generate restriction fragment patterns from genomic DNA extracted from rats expressing eight different *RT1* haplotypes. The results described herein strongly suggest a high level of polymorphism of *RT1* class I sequences that has not been detected previously.

MATERIALS AND METHODS

Rats. The employed rat strains and their respective *RT1* haplotypes are presented in Table 1. All rats were bred in The Wistar Institute animal facility.

DNA Extraction and Restriction Enzyme Digestion. Liver DNA was isolated from rats fasted for 48 hr according to the described technique (21). Twenty-microgram genomic DNA samples were digested for 3 hr at 37°C with 40 units of *Eco*RI or *Bam*HI (New England BioLabs). Duplicate samples containing 1 μg of λ DNA were employed to ensure complete digestion.

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.

Abbreviation: MHC, major histocompatibility complex.

‡To whom reprint requests should be addressed.

Table 1. Rat strains and respective *RT1* haplotypes

Strain	*RT*
LEW	*l*
F344	*lv1*
WF	*u*
BN	*n*
DA	*a*
BN.DA-*RT1*^{a}	*a*
F344.DA-*RT1*^{a}	*a*
AUG	*c*
BUF	*b*
MNR	*m*

cDNA Probes. The pH-2IIa and pH-2III mouse cDNA probes (22) were generously provided by M. Steinmetz (Division of Biology, California Institute of Technology, Pasadena, CA). Intact plasmids were nick-translated and used directly as probes.

Agarose Gel Electrophoresis and Hybridization. Ten-microgram samples of digested DNA were electrophoresed on 0.8% agarose gels for 500 V·hr. Electrophoresed DNA was stained with ethidium bromide and nicked with UV irradiation for 5 min. After denaturation and neutralization, DNA was transferred to nitrocellulose paper by the method of Southern (23) and baked at 80°C for 2 hr. Nitrocellulose filters were hybridized with the ^{32}P-labeled pH-2IIa or pH-2III mouse cDNA probes (1×10^6 cpm/ml) in (*i*) 50% dextran sulfate/formamide; (*ii*) 1× Denhardt's solution (24); and (*iii*) competing DNA and RNA as described (25). Hybridization was performed at 42°C for 20 hr. Hybridized filters were washed twice in 300 mM NaCl/30 mM sodium citrate/0.1% $NaDodSO_4$ at 25°C and six times in 15 mM NaCl/1.5 mM sodium citrate/0.1% $NaDodSO_4$ at 52°C. Hybridized filters were exposed to Kodak XR-5 film with a DuPont Lightning Plus intensifying screen at −70°C.

RESULTS

Two murine cDNA probes were employed to identify rat DNA sequences homologous to mouse class I *H-2* sequences. The 5′ probe, pH-2III, encompasses a 300-base-pair cDNA insert extending over *H-2* class I codons 63–160 in the relatively polymorphic exons (22). The 3′ probe, pH-2IIa, encompasses a 442-base-pair cDNA insert of codons 183–329 in the relatively conserved exons; the pH-2IIa probe was derived from cDNA clone pH-2II by removing a repetitive sequence in the 3′ untranslated region. The results of hybridization of the pH-2III probe with *Eco*RI-digested rat genomic DNA fragments are presented in Fig. 1 *Upper*. DNA samples from 11 rat strains expressing eight *RT1* haplotypes were digested with *Eco*RI and DNA samples from 6 rat strains were digested with *Bam*HI (data not shown). Twelve to 19 *Eco*RI bands and 14 to 19 *Bam*HI bands hybridized with the pH-2III probe (Tables 2 and 3). As indicated by the range in number and intensity of hybridizing restriction fragments, extensive differences in restriction pattern were associated with different *RT1* haplotypes. The percentage of differences between hybridizing restriction frag-

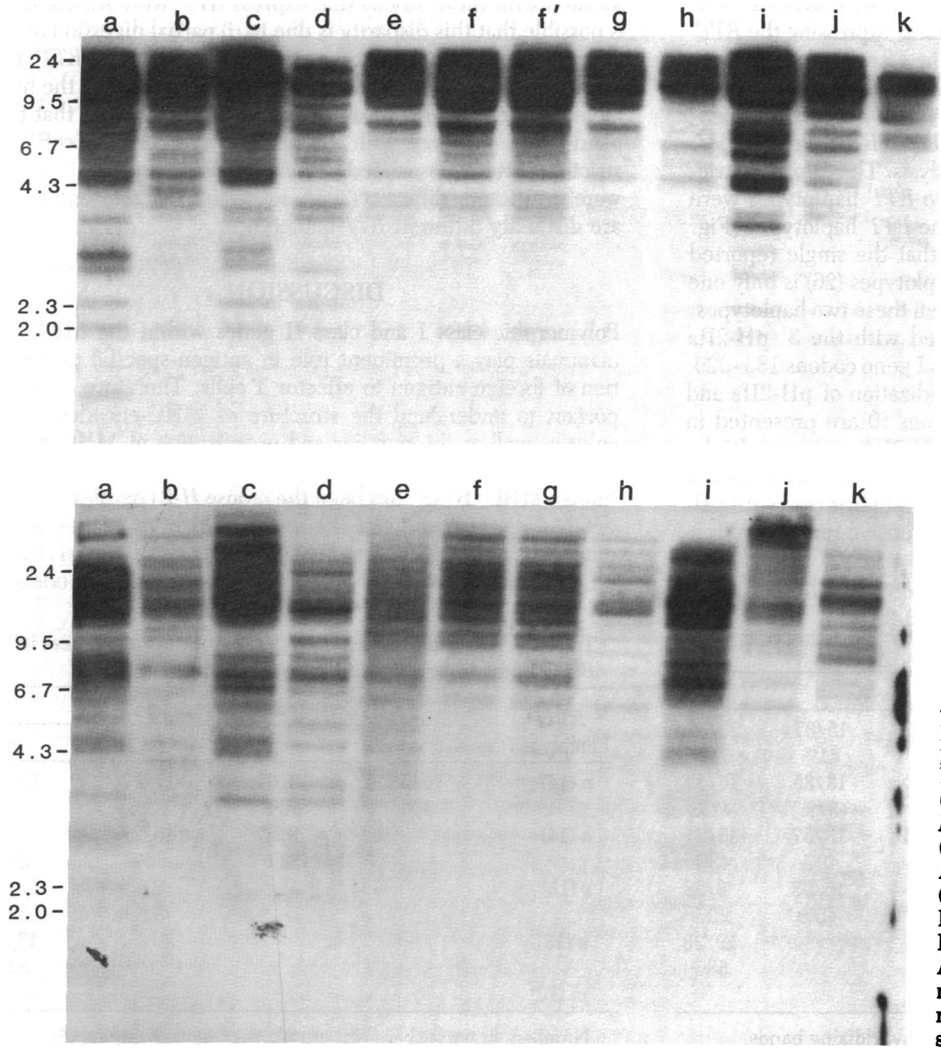

FIG. 1. Genomic Southern blots of *Eco*RI fragments hybridized with the pH-2III (*Upper*) and pH-2IIa (*Lower*) probes. Lanes in *Upper*: a, LEW (*RT1*^{l}); b, F344 (*RT1*^{lv1}); c, WF (*RT*^{u}); d, BN (*RT1*^{n}); e, DA (*RT1*^{a}); f, F344.DA-*RT1*^{a}(1) (*RT1*^{a}); f′, F344.DA-*RT1*^{a}(2) (*RT1*^{a}); g, BN.DA-*RT1*^{a} (*RT1*^{a}); h, AUG (*RT1*^{c}); i, BUF (*RT1*^{b}); j, MNR (*RT1*^{m}); k, B10.P. Lanes in *Lower*: a, LEW; b, F344; c, WF; d, BN; e, DA; f, BN.DA-*RT1*^{a}; g, F344.DA-*RT1*^{a}; h, AUG; i, BUF; j, MNR; k, B10.P. High molecular weight bands in *Upper* were resolved after a shorter (4-hr) exposure time.

Table 2. Summary of *RT1* haplotype-associated differences in *Eco*RI-derived bands hybridizing with pH-2III

RT1 haplotype	*RT1* haplotype						
	lv1	*u*	*n*	*a*	*c*	*b*	*m*
l (19)	18/36 50%	12/35 39%	13/33 39%	18/32 56%	15/31 48%	8/38 21%	17/29 59%
lv1 (17)		11/33 33%	11/31 35%	6/30 20%	13/29 45%	12/36 33%	13/27 48%
u (16)			8/30 27%	9/29 30%	10/28 36%	7/35 20%	16/26 62%
n (14)				9/27 33%	8/26 31%	9/33 27%	10/24 42%
a (13)					13/25 52%	12/32 38%	13/23 57%
c (12)						13/31 42%	6/22 27%
b (19)							15/29 52%
m (10)							

Numbers in parentheses are numbers of hybridizing bands.

Table 4. Summary of *RT1*-associated differences in *Eco*RI-derived bands hybridizing with pH-2IIa

RT1 haplotype	*RT1* haplotype						
	lv1	*u*	*n*	*a*	*c*	*b*	*m*
l (16)	14/36 39%	19/33 58%	18/34 53%	19/35 54%	18/33 55%	23/33 70%	20/30 67%
lv1 (20)		15/40 38%	18/37 49%	17/39 44%	13/37 35%	15/37 40%	20/34 59%
u (17)			11/35 31%	6/36 17%	10/34 29%	10/34 29%	15/31 48%
n (18)				11/37 30%	15/35 43%	9/35 26%	18/32 56%
a (19)					10/36 28%	12/36 33%	13/33 39%
c (17)						12/34 35%	15/31 48%
b (17)							15/31 48%
m (14)							

Numbers in parentheses are numbers of hybridizing bands.

ments associated with different *RT1* haplotypes is between 20% and 62% for *Eco*RI fragments and between 27% and 63% for *Bam*HI fragments; these percentages are presented in Tables 2 and 3, respectively.

Three strains expressing the $RT1^a$ haplotype on three different genetic backgrounds—i.e., DA, BN.DA-$RT1^a$, and F344.DA-$RT1^a$ (Fig. 1)—were included in this analysis. The restriction patterns from all three rat strains expressing the $RT1^a$ haplotype were identical, confirming the association of restriction fragment polymorphism with inheritance of *RT1* haplotypes. Two strains, LEW and F344, which express independently derived and nearly identical $RT1^l$ and $RT1^{lv1}$ haplotypes, respectively, were included in this analysis. The *Eco*RI restriction patterns associated with these two $RT1^l$ haplotypes were as different from each other as from the $RT1^l$ haplotypes (Fig. 1 *Upper*). This observation indicates that the single reported difference between these two $RT1^l$ haplotypes (26) is only one of many class I gene differences between these two haplotypes.

Similar experiments were performed with the 3′ pH-2IIa probe, which encompasses mouse class I gene codons 183–329. The genomic blots generated by hybridization of pH-2IIa and *Eco*RI fragments of genomic DNA from 10 are presented in Fig. 1 *Lower*. As in the case of the pH-2III probe, multiple, hybridizing bands were observed; 14–20 *Eco*RI fragments and 13–20 *Bam*HI fragments (data not shown) hybridized with pH-2IIa (summarized in Tables 4 and 5). Extensive restriction fragment length polymorphism was observed. The percentage of fragment length differences associated with different *RT1* haplotypes varied from 17% to 70% for *Eco*RI fragments and from 29% to 63% for *Bam*HI fragments (Tables 4 and 5). Only a single high molecular weight band distinguished BN.DA-$RT1^a$ and F344.DA-$RT1^a$ from DA; otherwise the restriction patterns associated with these strains that express $RT1^a$ were identical. It is possible that this disparity is due to (*i*) partial digestion or (*ii*) a single sequence difference between DA and BN.DA-$RT1^a$ and F344.DA-$RT1^a$. The former appears more likely given the high molecular weight of the band in question and the fact that this band is not observed with BN DNA. Further, the *Eco*RI restriction patterns associated with $RT1^l$ (LEW) and $RT1^{lv1}$ (F344) were significantly different (39%), suggesting that $RT1^l$ and $RT1^{lv1}$ are distinctly different *RT1* haplotypes.

Table 3. Summary of *RT1* haplotype-associated differences in *Bam*HI-derived bands hybridizing with pH-2III

RT1 haplotype	*RT1* haplotype				
	n	*a*	*c*	*u*	*m*
lv1 (18)	12/34 35%	15/35 43%	16/32 50%	15/37 41%	20/32 63%
n (16)		9/33 27%	10/30 33%	13/35 37%	14/30 47%
a (17)			11/31 35%	18/37 49%	15/31 48%
c (14)				15/33 45%	8/28 29%
u (19)					19/33 58%
m (14)					

Numbers in parentheses are numbers of hybridizing bands.

DISCUSSION

Polymorphic class I and class II genes within the MHCs of mammals play a prominent role in antigen-specific presentation of foreign antigen to effector T cells. Therefore, it is important to understand the structure of MHC-encoded molecules as well as the number and organization of MHC genes. The most extensively analyzed MHC of an animal model of the human MHC, *HL-A*, has been the mouse *H-2* complex. A more detailed understanding of the diversity of MHC genes among mammalian species necessitates the analysis of the MHCs of other mammals, especially those that provide useful models for

Table 5. Summary of *RT1* haplotype-associated differences in *Bam*HI-derived bands hybridizing with pH-2IIa

RT1 haplotype	*RT1* haplotype			
	a	*c*	*u*	*m*
n (14)	10/28 36%	13/27 48%	17/27 63%	12/34 35%
a (14)		9/27 33%	15/27 56%	10/34 29%
c (13)			8/26 31%	13/33 39%
u (13)				17/33 52%
m (20)				

Numbers in parentheses are numbers of hybridizing bands.

human diseases. Toward this end, we have undertaken the characterization of the rat MHC, *RT1*.

The *RT1* complex of rats is composed of class I and class II genes. The class I genes can be subdivided into: (*i*) *RT1.A* genes, which appear to be analogous to *H-2K*, *D*, and *L* in the mouse (11, 12); (*ii*) *RT1.C* genes (13), which appear to be analogous to the *Qa* genes in the mouse; and (*iii*) *RT1.E* (14). These analogies are made on the basis of tissue distribution. Further, the class II genes coding for Ia-like antigens are encoded in the *RT1.B* region (15, 16). However, the scarcity of recombinants within the *RT1* complex has hindered the quantitation of class I and II genes as well as assignment of their relative map positions within *RT1*.

Given the apparent inability of classical genetic techniques to dissect the *RT1* complex, we have initiated the characterization of *RT1* class I genes by recombinant DNA techniques. In this communication we have demonstrated at least 20 class I sequences, extrapolated from the number of bands hybridizing on Southern blots with mouse cDNA probes specific for (*i*) the second and third, relatively polymorphic, *H-2* class I exons, and (*ii*) the fourth through eighth, relatively conserved, *H-2* class I exons. This is probably a minimal estimate since intensely hybridizing bands may represent multiple copies of class I sequences. Also, higher molecular weight bands are not as well resolved and may contain multiple species. These results are consistent with those obtained for *H-2*, in which 30 class I genes have been identified by cosmid cloning (2) after the identification of approximately 13–16 restriction fragments in Southern blot analysis (27, 28). These results clearly indicate that the majority of rat class I sequences have not been identified previously by serological and biochemical techniques.

Another important observation reported in this communication is the obviously extensive polymorphism of *RT1* class I sequences. This conclusion is reached upon estimation of the differences between restriction fragment patterns associated with the eight tested *RT1* haplotypes. The percentage of differences between restriction fragment patterns associated with different *RT1* haplotypes is on the same order as that observed for *H-2* haplotypes (27, 28). Inclusion of inbred strains defining the $RT1^a$ haplotype on three different genetic backgrounds—i.e., DA, BN.DA-$RT1^a$, and F344.DA-$RT1^a$—confirmed the association of polymorphic restriction patterns with the *RT1* haplotypes of the donors.

The observed dissimilarity among restriction patterns ranged from 17% to 70%. Most interestingly, the magnitude of similarity did not correlate with previously reported *RT1* haplotype similarity, which has been based on a variety of serological and T-cell mediated assays. The level of polymorphism observed with *RT1* class I sequences is comparable to that observed by similar recombinant DNA techniques in the mouse (27, 28). Given the large number of mouse *H-2* haplotypes and the extensive polymorphism of their associated class I genes, we conclude that class I sequences in the *RT1* complex exhibit a level of polymorphism similar to that observed for *H-2*. This conclusion directly conflicts with conclusions drawn previously from serological analyses of *RT1* haplotypes of inbred and wild rats, which suggest that the *RT1* complex exhibits limited polymorphism (17–19). However, the serological analyses expectedly suffer from a lack of sensitivity required to detect more than a limited number of RT1 class I alloantigens. The documentation of extensive polymorphism in the rat *RT1* complex is extremely important in that it supports the contention that one of the main characteristics of MHC-linked loci is their high level of polymorphism within a species. Further, since we have not been able to identify the restriction fragments encoding serologically detected class I genes, it is possible that the conventional *RT1* products are similar and are encoded by one of the low-polymorphism bands. Definitive answers will come from studies of cloning and expression of rat class I sequences by DNA-mediated gene transfer.

The observations reported in this communication offer greater resolution in identifying the *RT1* haplotype origin of class I alleles associated with specific *RT1* haplotypes. In particular, the $RT1^a$ and $RT1^m$ haplotypes exhibited grossly dissimilar restriction patterns despite their apparent coinheritance of at least one *RT1.A* allele (29, 30). Of greater interest is the dissimilarity of restriction patterns associated with the $RT1^l$ and $RT1^{lv1}$ haplotypes of LEW and F344, respectively. The only reported difference between the *RT1* haplotypes of these two strains has been their expression of different alleles at the *CT* locus (26), a potential analog of mouse *Qa* loci. The important conclusion to be drawn from the observations concerning the LEW and F344 *RT1* haplotypes is that identity at *RT1.A* and *RT1.B* loci should not be extrapolated to suggest identity at all other *RT1* loci. In fact, the majority of mouse class I sequences have been mapped to the *Qa-T1* region (31), implying that even though two rat strains express identical *RT1.A* and *RT1.B* alleles they are not necessarily identical at any other class I loci.

Up to the present, identification of single genes within the rat *RT1* complex and assignment of immunological functions to these genes has been extremely difficult. Exploitation of recombinant DNA techniques will facilitate this identification by enabling one to clone *RT1* sequences and introduce them into new environments and characterize their gene products in isolation.

The authors acknowledge the excellent technical assistance of Mrs. Devorah Schiff. This research was supported by Grants AI-18972, CA-10815, and AI-20288 from the National Institutes of Health. J.A.F. is the recipient of an American Cancer Society Faculty Research Award.

1. Klein, J. (1975) *Biology of the Mouse Histocompatibility Complex* (Springer, Berlin).
2. Steinmetz, M., Moore, K. W., Frelinger, J. G., Sher, B. T., Shen, F.-W., Boyse, E. A. & Hood, L. (1981) *Cell* **25**, 683–692.
3. Goodenow, R. S., McMillan, M., Orn, A., Nicolson, M., Davidson, N., Frelinger, J. A. & Hood, L. (1982) *Science* **215**, 677–679.
4. Flaherty, L. (1976) *Immunogenetics* **3**, 533–539.
5. Flaherty, L. (1980) in *The Role of the Major Histocompatibility Complex in Immunology*, ed. Dorf, M. E. (Garland STPM, New York), pp. 33–57.
6. Uhr, J. W., Capra, J. D., Vitetta, E. & Cook, R. G. (1979) *Science* **206**, 292–297.
7. Sachs, D. H. & Cone, J. L. (1972) *J. Immunol.* **114**, 165–169.
8. Hammerling, G. J., Mauve, G., Goldberg, E. & McDevitt, H. O. (1974) *Immunogenetics* **1**, 428–437.
9. Klein, J. (1979) *Science* **203**, 516–521.
10. Gotze, D., Nadeau, J., Wakeland, E. K., Berry, R. J., Bonhomme, F., Egorov, I. K., Hjorth, J. P., Hoogstraal, H., Vives, J., Winking, H. & Klein, J. (1980) *J. Immunol.* **124**, 2675–2681.
11. Bogden, A. E. & Aptekman, P. M. (1960) *Cancer Res.* **20**, 1272–1282.
12. Gunther, E. & Stark, O. (1977) in *The Organization of the Major Histocompatibility Systems in Man and Animals*, ed. Gotze, D. (Springer, New York), pp. 207–253.
13. Stock, W. & Gunther, E. (1982) *J. Immunol.* **128**, 1923–1928.
14. Kunz, H. W., Gill, T. J. & Misra, D. N. (1982) *J. Immunol.* **128**, 402–408.
15. Gunther, E., Stark, O. & Koch, C. (1978) *Eur. J. Immunol.* **8**, 206–212.
16. Sachs, D. H., Humphrey, G. W. & Lunney, J. K. (1977) *J. Exp. Med.* **146**, 381–393.
17. Shonnard, J. W., Cramer, D. V., Poloskey, P. E., Kunz, H. W. & Gill, T. J. (1976) *Immunogenetics* **3**, 193–200.
18. Cramer, D. V., Davis, B. K., Shonnard, J. W., Stark, O. & Gill, T. J. (1978) *J. Immunol.* **120**, 179–187.
19. Shonnard, J. W., Davis, B. K., Cramer, D. V., Radka, S. F. & Gill, T. J. (1979) *J. Immunol.* **123**, 778–783.

20. Gill, T. J., Kunz, H. W., Schaid, D. J., VandeBerg, J. L. & Stolc, V. (1982) *J. Immunogenet.* **9**, 281–293.
21. Blin, N. & Stafford, D. (1976) *Nucleic Acids Res.* **3**, 2303–2308.
22. Steinmetz, M., Frelinger, J. G., Fisher, D., Hunkapiller, T., Pereira, D., Weissman, S. M., Uehara, H., Nathenson, S. & Hood, L. (1981) *Cell* **24**, 125–134.
23. Southern, E. M. (1975) *J. Mol. Biol.* **98**, 503–517.
24. Denhardt, D. T. (1966) *Biochem. Biophys. Res. Commun.* **23**, 641–646.
25. Jahn, C. L., Hutchison, C. A., III, Phillips, S. V., Weaver, S., Hagwood, N. L., Volva, C. F. & Edgell, M. E. (1980) *Cell* **21**, 159–168.
26. Marshak, A., Doherty, P. C. & Wilson, D. B. (1977) *J. Exp. Med.* **146**, 1773–1790.
27. Cami, B., Bregegere, F., Abastado, J. P. & Kourilsky, P. (1981) *Nature (London)* **291**, 673–675.
28. Pease, L. R., Nathenson, S. G. & Leinwand, L. A. (1982) *Nature (London)* **298**, 382–385.
29. Stark, O., Kunz, H. W. & Gill, T. J. (1978) *J. Immunogenet.* **5**, 261–273.
30. Gotze, D. (1978) *Immunogenetics* **7**, 491–506.
31. Wiinoto, A., Steinmetz, M. & Hood, L. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 3425–3429.