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Identification of the Cloned Gene for the Murine Transplantation Antigen H-2K^b by Hybridization with Synthetic Oligonucleotides

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The H-2K^b gene is a member of the large major histocompatibility complex class I gene family. Since many members of this family cross-hybridize with class I cDNA probes, the cloned H-2K^b gene was identified by hybridization with specific oligonucleotide probes. This clone was definitively shown to encode the H-2K^b polypeptide by partial DNA sequencing and by serological and tryptic peptide analyses of the expressed product.

The murine transplantation antigens are a group of biochemically similar proteins, collectively referred to as class I molecules, which are coded for by a multigene family on chromosome 17. These genes (K, D, and L) are located within the H-2 major histocompatibility complex and are highly polymorphic (5). Since members of the major histocompatibility complex multigene family are 80 to 90% homologous and therefore extensively cross-hybridize with class I cDNA probes, identification of an individual member within the gene family requires the demonstration of its expressed product in transformed cells or on random sequencing of each of the members. As an alternative, we used synthetic oligonucleotide probes, constructed to display greater hybridization specificity than do cDNA probes, to distinguish the H-2K^b gene from the rest of the class I gene family. By comparing published class I sequences from our (9, 11, 13–15) and other (2, 4, 8, 16, 19) laboratories, we have defined an H-2K^b-associated oligonucleotide sequence (19 bases) within the coding sequence for amino acid residues 94 through 100. A recombinant genomic DNA library was screened with class I probes, and 35 positive clones were subsequently tested with the specific nonadecanucleotide probe. Two clones hybridized with the nonadecanucleotide. Subsequently, both were shown to be identical by DNA restriction analysis. One clone (C1.4.1) was analyzed by classical techniques to demonstrate the presence of the H-2K^b gene. The expressed product of the cloned DNA was characterized with serological techniques and comparative tryptic peptide analysis. Finally, the partial DNA sequence of the gene was found to encode a protein identical to the previously published H-2K^b antigen (11). The isolation of these clones has allowed us to determine the complex DNA coding sequence for the H-2K^b antigen.

To construct specific probes, we synthesized oligonucleotides complementary to two regions of class I genes (Z.-K. Tan, S. Ikuta, T. Huang, A. Dugaiczyk, and K. Itakura, Cold Spring Harbor, Symp. Quant. Biol., in press). The first region represents the coding sequence for amino acid positions 62 through 68. In this region, the amino acid sequence of the two b haplotype transplantation antigens H-2K^b and H-2D^b are the same, but the amino acid sequences of transplantation antigens from other haplotypes differ (Table 1). The second region represents the coding sequence for amino acid residues 94 through 100. Every murine class I antigen has a different sequence in this region (Table 1), H-2K^b and H-2D^b differing by six nucleotides in the DNA sequence. For region 1, a 19-base oligonucleotide designated K^{b}D^b-19 was chemically synthesized on the basis of the H-2K^b DNA sequence (13). This oligonucleotide is complementary to the H-2K^b DNA sequence and may be expected to be complementary to H-2D^b as well (Table 1). For region 2, two 19-base oligonucleotides were synthesized, one complementary to the H-2K^b sequence (K^b-19) and the other complementary to the H-2D^b sequence (D^b-19) (Table 1).

A genomic library was prepared in the λ

TABLE 1. Sequences of oligonucleotide probes and comparison with class I sequences[a]

	Region		Sequences							No. of differences with respect to:	
										KD^b-19	D^b-19
1	Probe K^bD^b-19	3′	G C C	C T C	T G T	G T C	T T T	C G G	T 5′		
	K^b		C G G Arg	G A G Glu	A C A Thr	C A G Gln	A A A Lys	G C C Ala	A A G Lys	0	
	D^b		Arg	Glu	Thr	Gln	Lys	Ala	Lys	—[b]	
	L^d		C G G Arg	A T C Ile	A C G Thr	C A G Gln	A T C Ile	G C C Ala	A A G Lys	6	
	27.1		C G G Arg	G A G Glu	A C A Thr	C A G Gln	A T G Met	G C C Ala	A A G Lys	2	
	K^d		Glu	Gln	Thr	Gln	Arg	Val	Lys	—	
	D^d		Arg	Glu	Thr	Arg	Arg	Ala	Lys	—	
	K^k		Arg		Thr			Ala	Lys	—	
	AA positions		62						68		
2	Probe K^b-19	3′	G A T	A A G	T C C	A C T	A G A	G A C	C 5′		
	Probe D^b-19	3′	G T G	A G G	T C G	T C T	A C A	G A C	C 5′		
	K^b		A C T Thr	A T T Ile	C A G Gln	G T G Val	A T C Ile	T C T Ser	G G C Gly	0	6
	D^b		A C A Thr	C T C Leu	C A G Gln	C A G Gln	A T G Met	T C T Ser	G G C Gly	6	0
	L^d		A C A Thr	C T C Leu	C A G Gln	T G G Trp	A T G Met	T A C Tyr	G G Gly	8	4
	27.1		A C A Thr	C T C Leu	C A A Gln	T G G Trp	A T G Met	T A T Tyr	G G C Gly	8	4
	K^d		Thr	Phe	Gln	Arg	Met	Phe	Gly	—	—
	D^d		Thr	Leu	Gln	Trp	Met	Ala	Gly	—	—
	K^k		Thr				Ile	Tyr	Ile	—	—
	AA positions		94						100		

[a] Sequences of oligonucleotide probes and comparison with class I sequences. The sequence of synthetic oligonucleotide probes is shown and compared with previously published protein and DNA sequences from class I molecules (data taken from K^b [11, 13, 14], D^b [15], L^d [2, 8], 27.1 [19], K^d [4], D^d [9], and K^k [16]). Oligonucleotides were synthesized by the phosphotriester solid-phase method as described previously (Tan et al., in press).

[b] —, Data not available.

bacteriophage vector Ch4 with DNA isolated from spleens of C57BL/6Kh mice (b haplotype) as described previously (6). Recombinant phage were screened with probes prepared from class I antigen cDNA clones pH-2III and pH-2IIa (18). Phage that hybridized with the class I-specific probes were subsequently rescreened with 5′-^{32}P-labeled oligonucleotides K^bD^b-19, K^b-19, and D^b-19. The DNA of five representative class I clones (Fig. 1) was digested with *Xba*I endonuclease and analyzed by blot hybridization to illustrate the specificity of the probes pH-2III (Fig. 1A), K^bD^b-19 (Fig. 1B), and K^b-19 (Fig. 1C). All of the DNA samples hybridized with the class I antigen probe pH-2III; two of them hybridized with the K^bD^b-19 probe. It was clear that only C1.4.1 DNA hybridized with the K^b-19 probe. The D^b-19 probe did not hybridize to any of the DNA samples. Of the first 35 clones which hybridized with pH-2III, 12 hybridized with K^bD^b-19 and the remainder did not. The oligonucleotide K^b-19 hybridized to 2 of these 12 clones (designated C1.4.1 and C1.8.1), whereas D^b-19 did not hybridize to any of the clones. Region 1 (Table 1) contains sequences which are common among several but not all class I genes; region 2 contains sequences which are restricted to a smaller subset of sequences, including the H-$2K^b$ gene, and may be unique to H-$2K^b$.

Several approaches were used to determine

whether clone C1.4.1. actually contained the H-2K^b gene. DNA isolated from the purified phage was introduced into the cell line Ltk$^-$ by cotransformation with a herpesvirus thymidine kinase gene by procedure described previously (22). After 20 days of selection in hypoxanthine-aminopterin-thymidine medium, the surviving cells were tested directly for the antigenic expression of H-2K^b by an anti-mouse immunoglobulin rosette assay (1). The L cells which were cotransformed with herpes simplex virus DNA and with the genomic clone C1.4.1 expressed the H-2K^b antigen (Table 2). Absorption tests in which cotransformed cells were used as targets were performed to verify the results obtained by the rosette assay (Table 3) and confirmed that the transformed L cells express H-2K^b serological determinants. Previous reports (2, 3, 8) in which a similar analysis of transformed Ltk$^-$ cells was used yielded the unexpected result that different genes derived from the same haplotype encode indistinguishable products by their serological markers. Consequently, we have examined the H-2K^b product of the C1.4.1 transformed cell line by comparative tryptic peptide analysis.

One of the transformed cell lines (C1.4.G1) was metabolically labeled with [^{3}H]arginine.

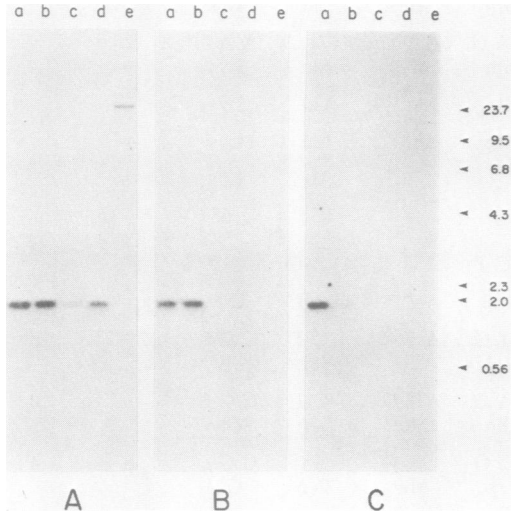

FIG. 1. Hybridization specificity of oligonucleotide probes. The DNA from five Charon 4 clones containing class I sequences were isolated from phage lysates as described previously (6). Digestion of the DNA with the endonuclease *Xba*I was performed according to the directions of the supplier (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). The resulting DNA fragments (1 μg per lane) were fractionated by electrophoresis on four identical 0.8% agarose gels in 40 mM Tris-acetate (pH 7.9)–1 mM EDTA for 16 h at 1 V/cm. The DNA fragments were transferred to nitrocellulose paper by the blotting procedure described previously (12). The nitrocellulose filters were hybridized with the nick-translated class I probe pH2III (A), 5′-[^{32}P]K^{b}D^b-19 (B), and 5′-[^{32}P]K^b-19 (C). Hybridization conditions in (A) were carried out as described previously (12). In (B) and (C) hybridization was done in 0.9 M NaCl–0.18 M Tris (pH 8.0)–0.012 M EDTA–10× Denhardt solution–0.1% sodium dodecyl sulfate with 10 ng of labeled oligonucleotide probe (specific activity, ≈10^9 cpm/μg) per ml at 55°C for 2 h. The oligonucleotides were labeled as described previously (21). The filters were washed at room temperature for 15 min with three changes of 0.9 M NaCl–0.09 M sodium citrate and again at 55°C for 1 min with the same buffer and then dried and autoradiographed. The D^b-19 probe was found not to hybridize to any of the class I clones (data not shown). Lanes contain DNA as follows: a, C1.4.1; b, A5.2.1; c, 18N.3.1; d, A9.1.1; e, C1.7.1. Lambda phage DNA digested with *Hin*dIII endonuclease was included as a DNA fragment size marker. The position and size (in kilobases) of the fragments are shown to the right.

TABLE 2. Serological analysis of transformed L cells with a rosette assay[a]

Antiserum	Antigen detected	Positive/total clones	
		Ptk + C1.4.1	Ptk alone
B6-H-2^k anti-EL4 (BALB/C × A/J) anti-HTI	H-2K^b	34/40	0/35
A/J anti-HTH (BALB/C × B6) anti-BP8	H-2D^b	0/40	0/35
	H-2K^k	20/20	35/35

[a] Cotransformed L cells, surviving 20 days of selection in hypoxanthine-aminopterin-thymidine medium, were incubated with anti-H-2 antisera. Binding of immunoglobulins to the surfaces of L cells was visualized by rosettes formed between the L cells and human erythrocytes coated with rabbit anti-mouse immunoglobulin as described previously (1). As a control, anti-H-2K^k antiserum was shown to bind to cells transformed with either the herpesvirus thymidine kinase gene (ptk) plus C1.4.1 or the herpesvirus thymidine kinase gene alone, since Ltk$^-$ cells are H-2^k haplotype.

TABLE 3. Specificity of anti-H-2^b binding as determined by absorption assay[a]

Strain	Absorption with: H-2K	Absorption with: H-2D	Absorption
B6	b	b	+
B10.A(3R)	b	d	+
B10.A(5R)	b	d	+
BALB/C	d	d	−
BALB.B	b	b	+
BALB.G	d	b	−

[a] The absorption test, in which cloned cotransformed L cells were used as targets, was performed by abosrbing B6-H-2^k anti-EL4 serum with mouse spleen cells from genetically defined inbred strains. The absorbed sera were tested with the direct binding assay (Table 2).

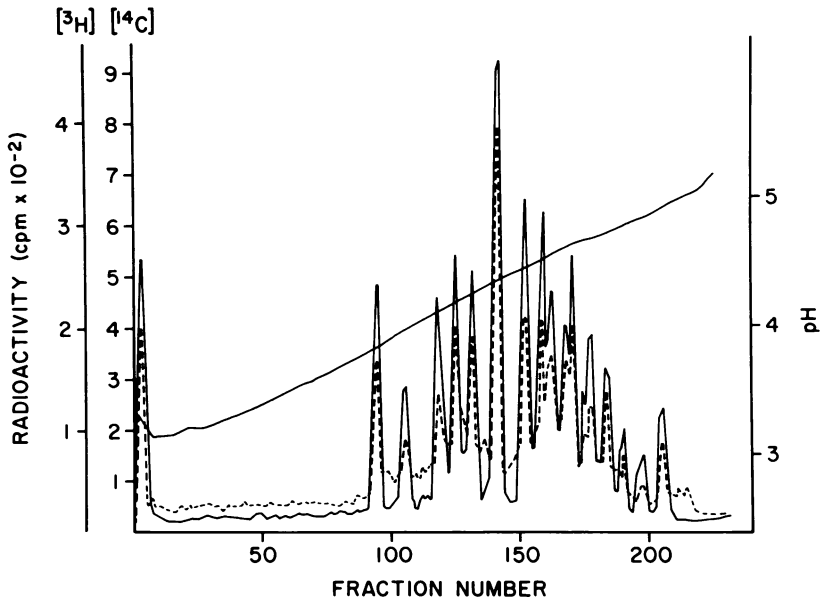

FIG. 2. Tryptic peptide comparison between H-2K^b glycoproteins from C1.4.G1 transformed L cells and C57BL/6Kh mouse spleen cells. [^{3}H]arginine-C1.4.G1 (- - -) and [^{14}C]arginine-C57BL/6Kh (——) H-2K^b glycoproteins were digested with trypsin and resolved on an ion exchange column (Spherix XX8-60-0 resin; Phoenix Co., Long Island, N.Y.) developed with a pyridine-acetate gradient as described previously (10).

Glycoproteins were isolated from the solubilized membrane fraction (10), and molecules bearing H-2K^b serological specificities were immunoprecipitated. These molecules were mixed with a similarly prepared immunoprecipitate derived from [^{14}C]arginine-radiolabeled C57BL/6Kh mouse spleen cells. A comparative tryptic peptide map of the 44,000-molecular weight glycoproteins from the transformed cell line C1.4.G1 and the spleen cells is shown in Fig. 2. There was complete concordance between the arginine-labeled peptides, thus providing biochemical evidence that the C1.4.1 clone encodes the H-2K^b gene.

Since approximately 80% of the DNA sequence is available for the coding region of the K^b gene (13, 14), we isolated the *Xba*I restriction fragment that contains the previously unsequenced portion of the gene and determined the remaining DNA sequence by dideoxy sequencing (17) directly on the isolated fragment. The K^b-19 oligonucleotide, as well as an oligonucleotide (9 bases long) complementary to K^b-19 (see Fig. 3), were used as primers (20). The deduced DNA sequences, when translated into an amino acid sequence, agreed completely with that previously determined for H-2K^b amino acids 1-91 (11). In addition, the expected intron-exon boundaries (19) are found interrupting the codons for residues 1 (Gly) and 91 (Gly). The data, together with the partial K^b sequence report earlier (13, 14), provides the complete sequence of the coding region for the H-2K^b gene. A restriction map of the *Eco*RI endonuclease fragment that encodes the K^b gene is also shown in Fig. 3.

Mellor et al. (7) have recently reported the isolation of an H-2K^b gene from a cosmid library constructed with spleen DNA from C57BL/10 mice. Since our primary interest is in the analysis of the H-2K^b mutants, the H-2K^b gene isolated in this study was from C57BL/6Kh, the strain from which the mutants arose. This gene should prove invaluable for the elucidation of the mechanism of these mutations. In our approach, hybridization to oligonucleotides is used as a criterion for gene identification as an alternative to L cell cotransformation or random DNA sequencing of cross-hybridizing members of a gene family. This technique could be applied to the identification of genes even if the genetic sequences cannot be expressed in cotransformed L cells. We have recently shown (1a) that even genes which differ by only a single nucleotide can be differentiated on the basis of hybridization to specific oligonucleotides. Therefore, this approach has broad applicability to other multigene families.

Using synthetic oligonucleotides, we identified the following three types of class I genes in this study, on the basis of hybridization behavior: (i) K^{b}D^b-19 and K^b-19 negative (ii) K^{b}D^b-19 positive and K^b-19 negative, and (iii) K^{b}D^b and K^b positive. It is possible that as yet unidentified

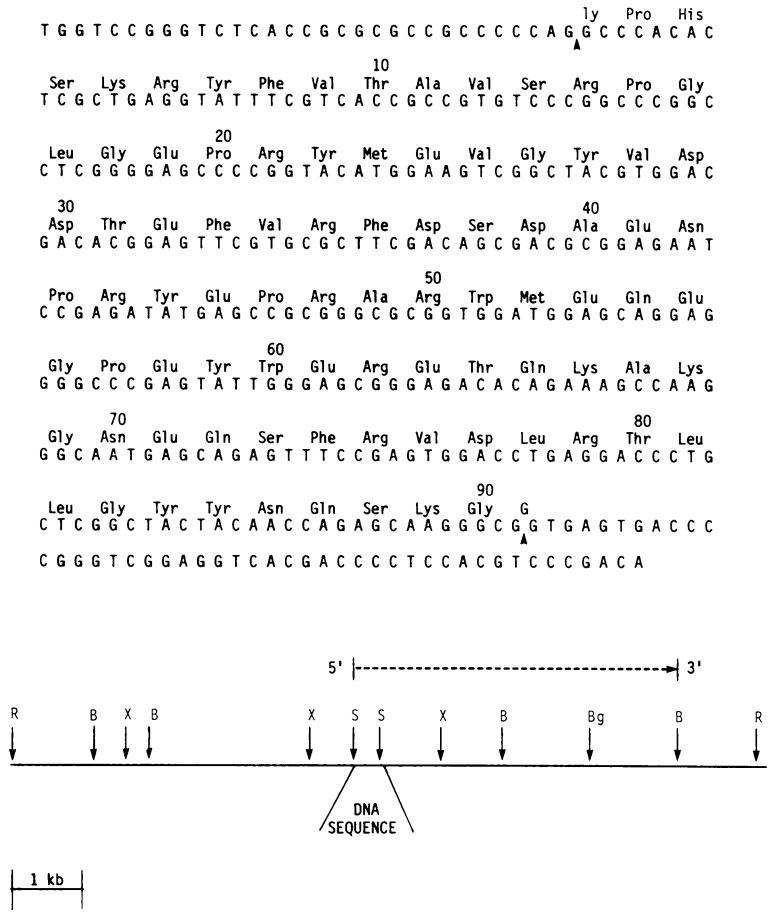

FIG. 3. Partial DNA sequence and restriction map of a 10.5-kilobase *Eco*RI subclone of C1.4.1. The 1.8-kilobase *Xba*I fragment containing the region of the gene complementary to the probes K^bD^b-19 and K^b-19 (Fig. 1) was isolated and sequenced by the dideoxysequencing method as described elsewhere (20), with K^bD^b-19 and the nonamer 5′ CGGGAGACA 3′ as sequencing primers. The DNA sequence and the deduced amino acid sequence are shown. A number of the amino acid residues is as found in the mature protein (11). Codons 1 (Gly) and 91 (Gly) are interrupted by splice sites (▲, 19). The restriction map shows the positions of *Eco*RI (R), *Bam*HI (B), *Sma*I (S), *Xba*I (X), and *Bgl*II (Bg) endonuclease sites. The region of the clone that hybridizes to the class I cDNA probes pH-2III and pH-2IIa is designated by the broken line. Transcriptional orientation was determined by DNA sequencing and blot hybridization results. The region sequenced is shown.

genes would be K^bD^b-19 negative and K^b-19 positive, showing independent assortments of these two regions in the genome. This point awaits further study. The two clones that hybridized to both K^bD^b-19 and K^b-19 probes contained the H-$2K^b$ gene.

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LITERATURE CITED

1. **Albino, A. P., K. O. Lloyd, A. N. Houghton, H. F. Oettgen, and L. Old.** 1981. Heterogeneity in surface antigen and glycoprotein expression of cell lines derived from different melanoma metastases of the same patient. Implications for the study of tumor antigens. J. Exp. Med. **154**:1764–1778.

1a. **Conner, B. J., A. A. Reyes, K. Itakura, R. L. Teplitz, C. Morin, and R. B. Wallace.** 1983. Detection of sickle cell β^s-globin allele by hybridization with synthetic oligonucleotides. Proc. Natl. Acad. Sci. U.S.A. **80**:278–282.

2. **Evans, G. A., D. H. Margulies, D. Camerini-Obero, K. Ozato, and J. G. Seidman.** 1982. Structure and expression of a mouse major histocompatibility antigen gene, H-$2L^d$. Proc. Natl. Acad. Sci. U.S.A. **79**:1994–1998.

3. **Goodenow, R. S., M. McMillan, A. Orn, M. Nicolson, N. Davidson, J. A. Frelinger, and L. Hood.** 1982. Identification of a BALB/c H-$2L^d$ gene by DNA-mediated gene transfer. Science **215**:677–679.

4. **Kimball, E. S., S. G. Nathenson, and J. E. Coligan.** 1981. Amino acid sequence of residues 1-98 of the H-2K^d murine histocompatibility alloantigen: comparison with H-2K^b and H-2D^b reveals extensive localized differences. Biochemistry **20**:3301–3308.
5. **Klein, J., and F. Figueroa.** 1981. Polymorphism of the mouse H-2 loci. Immunol. Rev. **60**:23–57.
6. **Maniatis, T., R. C. Hardison, E. Lacy, J. Lauer, C. O'Connell, D. Quon, G. K. Sim, and A. Efstratiadis.** 1978. The isolation of structural genes from libraries of eukaryotic DNA. Cell **15**:678–701.
7. **Mellor, A. L., L. Golden, E. Weiss, H. Bullman, J. Hurst, E. Simpson, R. F. L. James, A. R. M. Townsend, P. M. Taylor, W. Schmidt, J. Ferluga, L. Leben, M. Santamaria, G. Atfield, H. Festenstein, and R. A. Flavell.** 1982. Expression of murine H-2K^b histocompatibility antigen in cells transformed with cloned H-2 genes. Nature (London) **298**:529–534.
8. **Moore, K. W., B. T. Sher, Y. H. Sun, K. A. Eakle, and L. Hood.** 1982. DNA sequence of a gene encoding a BALB/c mouse L^d transplantation antigen. Science **215**:679–682.
9. **Nairn, R., S. G. Nathenson, and J. E. Coligan.** 1981. Amino acid sequence of cyanogene bromide fragment CN-C (residues 29-48) of the mouse histocompatibility antigen H-2D^d: a comparison of the amino-terminal 100 residues of H-2D^d, D^b, K^d and K^b reveals discrete areas of diversity. Biochemistry **20**:4739–4745.
10. **Nairn, R., K. Yamaga, and S. G. Nathenson.** 1980. Biochemistry of the gene products from murine MHC mutants. Annu. Rev. Genet. **14**:241–277.
11. **Nathenson, S. G., H. Uehara, B. M. Ewenstein, T. J. Kindt, and J. E. Coligan.** 1981. Primary structural analysis of the transplantation antigens of the murine H-2 major histocompatibility complex. Annu. Rev. Biochem. **50**:1025–1052.
12. **Pease, L. R., S. G. Nathenson, and L. A. Leinwand.** 1982. Mapping class I gene sequences in the major histocompatibility complex. Nature (London) **298**:382–385.
13. **Reyes, A. A., M. J. Johnson, M. Schold, H. Ito, Y. Ike, C. Morin, K. Itakura, and R. B. Wallace.** 1981. Identification of an H-2K^b-related molecule by molecular cloning. Immunogenetics **14**:383–392.
14. **Reyes, A. A., M. Schold, K. Itakura, and R. B. Wallace.** 1982. Isolation of a cDNAclone for the murine transplantation antigen H-2K^b. Proc. Natl. Acad. Sci. U.S.A. **79**:3270–3274.
15. **Reyes, A. A., M. Schold, and R. B. Wallace.** 1982. The complete amino acid sequence of the murine transplantation antigen H-2D^b as deduced by molecular cloning. Immunogenetics **16**:1–9.
16. **Rothbard, J. B., T. P. Hopp, J. M. Edelman, and B. A. Cunningham.** 1980. Structure of the heavy chain of the H-2K^k histocompatibility antigen. Proc. Natl. Acad. Sci. U.S.A. **77**:4239–4243.
17. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. **74**:5463–5467.
18. **Steinmetz, M., J. G. Frelinger, D. Fisher, T. Hunkapiller, D. Pereira, S. M. Weissman, H. Uehara, S. G. Nathenson, and L. Hood.** 1981. Three cDNA clones encoding mouse transplantation antigens: homology to immunoglobulin genes. Cell **24**:125–134.
19. **Steinmetz, M., K. W. Moore, J. G. Frelinger, B. T. Sher, F.-W. Shen, E. A. Boyse, and L. Hood.** 1981. A pseudogene homologous to mouse transplantation antigens: transplantation antigens are encoded by eight exons that correlate with protein domains. Cell **25**:683–692.
20. **Wallace, R. B., M. J. Johnson, S. V. Suggs, K. Miyoshi, R. Bhatt, and K. Itakura.** 1981. A set of synthetic oligodeoxyribonucleotide primers for DNA sequencing in the plasmid vector pBR322. Gene **16**:21–26.
21. **Wallace, R. B., J. Shaffer, R. F. Murphy, J. Bonner, T. Hirose, and K. Itakura.** 1979. Hybridization of synthetic oligodeoxyribonucleotides to ϕX174 DNA: the effect of single base pair mismatch. Nucleic Acids Res. **6**:3543–3557.
22. **Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlaub, and L. Chasin.** 1979. DNA-mediated transfer of the adenine phosphoribosyl transferase locus into mammalian cells. Proc. Natl. Acad. Sci. U.S.A. **76**:1373–1376.