Numbers and Ratios of Visual Pigment Genes for Normal Red-Green Color Vision

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Red-green color vision is based on middle-wavelength– and long-wavelength–sensitive visual pigments encoded by an array of genes on the X chromosome. The numbers and ratios of genes in this cluster were reexamined in men with normal color vision by means of newly refined methods. These methods revealed that many men had more pigment genes on the X chromosome than had previously been suggested and that many had more than one long-wave pigment gene. These discoveries challenge accepted ideas that are the foundation for theories of normal and anomalous color vision.

In humans, genes lying in a head-to-tail tandem array on the X chromosome encode long-wavelength– and middle-wavelength–sensitive visual pigments that serve red-green color vision. Men with normal color vision are proposed to have typically a single long-wave gene followed by one, two, or three middle-wave genes (*1*). This model is based on results from densitometric analysis of Southern (DNA) hybridization blots. Recent technological advances offer new methods of estimating the numbers and ratios of genes that remedy limitations inherent in quantitative Southern analysis. We used these methods to reexamine the X-linked pigment gene arrays of men with normal color vision. The results give a very different picture of the arrays than is given by the currently favored model (*1*).

We developed an assay to count the number of visual pigment genes on the X chromosome. A 183–base pair (bp) DNA fragment that lies about 50 bp upstream of the first exon of each gene (Fig. 1A) was amplified by polymerase chain reaction (PCR). One Sma I restriction site within the amplified region is present in all genes in the array except the one farthest upstream. Amplified gene segments were end labeled with ^{32}P and cleaved with endonuclease Sma I, and the products were electrophoretically separated (Fig. 1B). Three bands were observed: a 137-bp fragment from the first gene in the array, a 25-bp fragment from all except the first gene, and a 46-bp fragment from every gene in the array. The number of pigment genes per X chromosome was estimated from the relative amounts of radioactivity measured in the bands by means of PhosphorImage analysis (*2*).

First, three red-green color-blind men (dichromats) were analyzed. Previous studies have indicated that each has one X-linked pigment gene (*3*, *4*). The 25-bp Sma I fragment, present only in the genes downstream of the first one, was absent in these men (for example, see lane 1 in Fig. 1B). Heteroduplex bands seen in individuals with two or more X-linked pigment genes (Fig. 1B) were also absent. These results provide additional evidence that each of the dichromats has one X-linked pigment gene. Because they have a known gene number (one), these men serve as a reference against which results from men with normal color vision can be compared and validated. Each man has a single copy of the 137-bp fragment and each has one 46-bp fragment for every X-linked pigment gene. Thus, the ratio of the intensity of the 46-bp band to that of the unique 137-bp band yields the relative number of genes in the cluster. The number of X-linked pigment genes was determined for each of 27 men with normal color vision (Table 1). Even in this relatively small sample, the number of pigment genes per X chromosome ranged from two to nine.

A second experiment was designed to determine the ratio of long- to middle-wave genes. Amino acid substitutions encoded by

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Table 1. Estimated numbers and ratios of long- and middle-wave genes. Number of X-linked genes (N) and the middle- to long-wave ratio (M/L) are calculated as described in Fig. 1. The values are the average of triplicate experiments for each participant. Mean and standard error of the mean (SEM) are reported to one decimal place. SEMs of 0.0 are <0.05. The number of long-wave genes is calculated as N/(M/L + 1); the number of middle-wave genes is calculated as N/(L/M + 1) and rounded to the nearest whole number. Normal color vision was assayed previously by Rayleigh color matching (*11*). Participants adjusted the ratio of red to green light in a mixture to exactly match the appearance of a monochromatic yellow standard light. The proportion of red light in a mixture with green [R/(R + G)] that each man chose is given. These are the energies of the lights multiplied by constants so that the average R/(R + G) setting for men with normal color vision equals 0.50.

Participant number	Number of X-linked pigment genes (mean ± SEM)	Ratio of middle- to long-wave genes (mean ± SEM)	Number of long-wave genes	Number of middle-wave genes	Color match R/(R + G)
		Men with more than one long-wave gene			
07	9.0 ± 1.3	3.8 ± 0.3	2	7	0.47
09	7.7 ± 1.2	3.2 ± 0.4	2	6	0.48
23	6.5 ± 1.0	0.5 ± 0.0	4	2	0.46
14	6.7 ± 0.7	2.1 ± 0.1	2	5	0.53
25	6.2 ± 0.6	2.1 ± 0.1	2	4	0.52
06	6.0 ± 0.6	2.5 ± 0.1	2	4	0.53
22	5.6 ± 1.0	2.0 ± 0.2	2	4	0.53
20	4.8 ± 0.4	1.9 ± 0.1	2	3	0.44
04	4.5 ± 0.8	1.0 ± 0.1	2	2	0.47
26	5.7 ± 0.4	0.5 ± 0.1	4	2	0.48
08	3.3 ± 0.2	0.9 ± 0.0	2	2	0.54
19	3.3 ± 0.3	0.9 ± 0.1	2	2	0.54
		Men with only one long-wave gene			
17	5.4 ± 0.5	3.3 ± 0.0	1	4	0.43
02	4.4 ± 0.3	2.0 ± 0.1	1	3	0.48
05	4.0 ± 0.3	2.0 ± 0.0	1	3	0.44
18	3.7 ± 0.4	1.8 ± 0.1	1	2	0.44
03	3.6 ± 0.4	1.9 ± 0.0	1	2	0.58
11	3.5 ± 0.1	2.1 ± 0.1	1	2	0.53
24	3.4 ± 0.4	1.8 ± 0.1	1	2	0.45
15	3.3 ± 0.5	1.9 ± 0.1	1	2	0.43
01	3.0 ± 0.0	2.1 ± 0.0	1	2	0.44
12	2.9 ± 0.3	1.2 ± 0.1	1	2	0.48
27	2.7 ± 0.4	1.8 ± 0.1	1	2	0.59
21	2.2 ± 0.1	1.0 ± 0.0	1	1	0.57
10	2.2 ± 0.1	0.9 ± 0.1	1	1	0.54
13	2.2 ± 0.1	0.9 ± 0.1	1	1	0.49
28	2.1 ± 0.0	1.0 ± 0.0	1	1	0.60
		Color-blind men with only one X-linked pigment gene			
1P	1.0 ± 0.0	– –	0	1	–
2P	1.0 ± 0.0	– –	0	1	–
1D	1.0 ± 0.0	– –	1	0	–

exon 5 of the pigment genes produce the majority of the spectral difference between middle- and long-wave pigments (*3–5*). Other amino acid differences encoded by exons 2, 3, and 4 also produce spectral shifts; however, these are relatively small and are responsible for producing subtypes of pigments within the middle- and long-wave classes (*5*). Thus, the most valid estimates of the ratio of middle- to long-wave genes will rely on methods that make use of the exon 5–encoded differences that functionally distinguish long- from middle-wave pigments. A Rsa I restriction site polymorphism in exon 5 was identified earlier (*1*). This polymorphism results from nucleotide differences that specify one of the two closely spaced, spectrally active amino acid substitutions that differentiate long-wave–from middle-wave–sensitive pigment classes (*4*). We exploited this polymorphism to separate long- from middle-wave genes as follows. An ~300-bp fragment containing exon 5 from all X-linked pigment genes was amplified (Fig. 1A), end labeled, and digested with restriction endonuclease Rsa I, and the products were electrophoretically separated (Fig. 1C). Two bands were from long-wave gene fragments cut by Rsa I, and one was from middle-wave gene fragments that were not cut (Fig. 1C). The radioactivity in each band was quantitated and the middle- to long-wave gene ratio determined (Table 1). The validity of this measure was checked by examination of men who were found in the first experiment to have two X-linked pigment genes. Normal men with only two X-linked pigment genes must have a middle- to long-wave gene ratio of 1:1, because their color vision requires one long-wave and one middle-wave gene. The observed values were consistently close to one (participants 10, 13, 21, and 28). Taken together, the estimates of gene number and of middle- to long-wave gene ratio allowed the number of long-wave genes in each man to be calculated (Table 1). Men with normal color vision had as many as four long-wave genes and nearly half had at least two long-wave genes.

Our methods were not subject to the hybridization and quantitation artifacts inherent in the Southern blot procedure. Regions corresponding to the PCR primers and the labeled ends of the amplified fragments were identical for all photopigment genes, and DNA was fixed in the gel and never transferred.

Nathans and colleagues used the ratio of restriction fragments A_g/A_r (Fig. 1A) to estimate gene number with the assumption that fragment A_r was always present in a single copy (*1*). Homologous recombination has occurred among these genes with high frequency. Fragment A_r is longer than A_g because of an insert about a third of the way

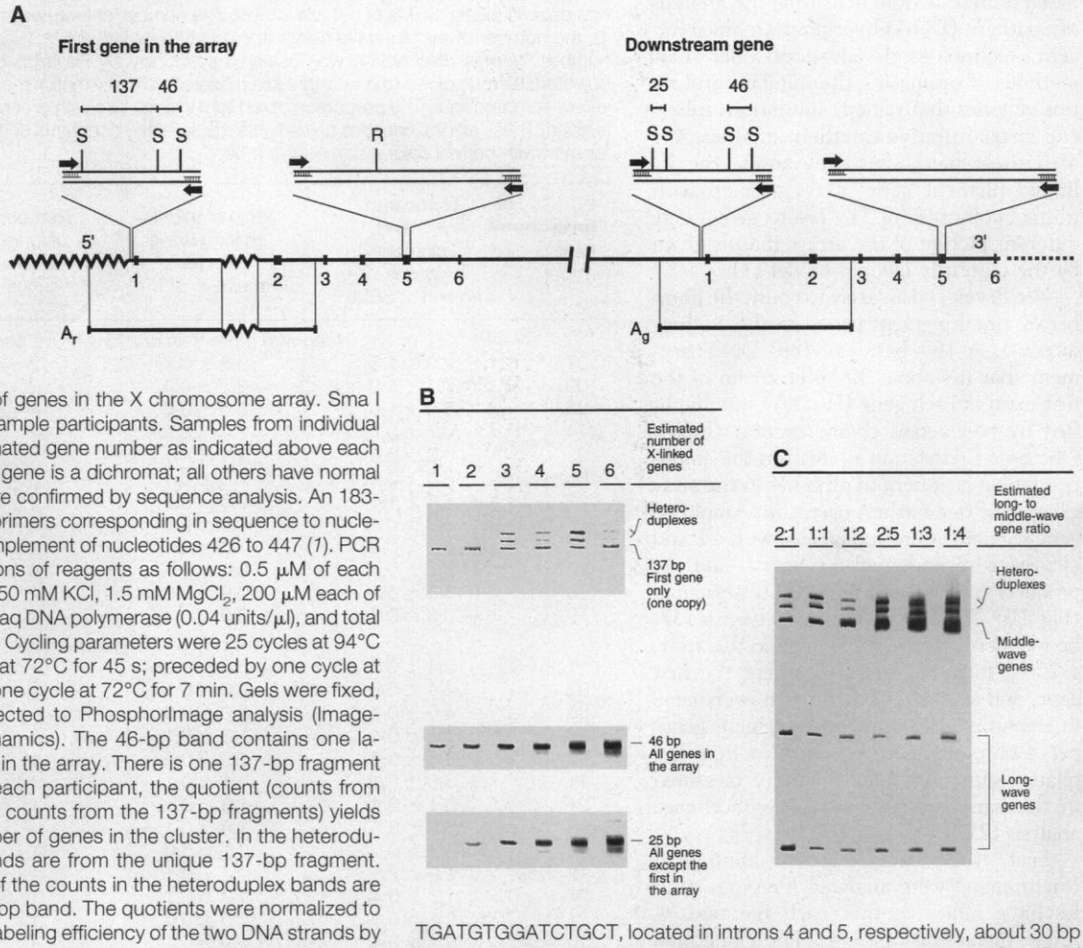

Fig. 1. (**A**) Restriction map of the 5′-most gene and of one downstream gene from the pigment gene array on the X chromosome. In detail, above the array, are the 183-bp and 300-bp fragments that were amplified and used to determine the number and ratio of long- and middle-wave genes. Sma I (S) and Rsa I (R) sites are indicated. Below the array are the extents of restriction fragments A_g and A_r used in Southern blot analysis. (**B**) Analysis of the number of genes in the X chromosome array. Sma I fragments visualized from six example participants. Samples from individual participants are ordered by estimated gene number as indicated above each lane. The leftmost man with one gene is a dichromat; all others have normal color vision. Heteroduplexes were confirmed by sequence analysis. An 183-bp fragment was amplified with primers corresponding in sequence to nucleotides 264 to 283 and to the complement of nucleotides 426 to 447 (*1*). PCR reactions contained concentrations of reagents as follows: 0.5 μM of each primer, 50 mM tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each of dGTP, dATP, dCTP, and dTTP, Taq DNA polymerase (0.04 units/μl), and total human genomic DNA (2.5 ng/μl). Cycling parameters were 25 cycles at 94°C for 1 min, at 59°C for 45 s, and at 72°C for 45 s; preceded by one cycle at 94°C for 5 min and followed by one cycle at 72°C for 7 min. Gels were fixed, dried on filter paper, and subjected to PhosphorImage analysis (ImageQuant Software, Molecular Dynamics). The 46-bp band contains one labeled fragment from each gene in the array. There is one 137-bp fragment per pigment gene cluster. For each participant, the quotient (counts from the 46-bp fragments divided by counts from the 137-bp fragments) yields an estimate of the relative number of genes in the cluster. In the heteroduplexes, half of the labeled strands are from the unique 137-bp fragment. Thus, in the quotient, one-half of the counts in the heteroduplex bands are added to the counts in the 137-bp band. The quotients were normalized to correct for the difference in the labeling efficiency of the two DNA strands by means of a multiplication factor that brought the average estimate of gene number in dichromatic men to a value of 1.0. (**C**) Analysis of the ratio of middle- to long-wave genes in men with normal color vision. Rsa I fragments were visualized from six example participants. Samples from individual participants are ordered by estimated gene ratio as indicated above each lane. Primers for amplification of the 300-bp fragment containing exon 5 were 5′-TCCAACCCCCGACTCACTATC and 5′-ACGGTATTTTGATGTGGATCTGCT, located in introns 4 and 5, respectively, about 30 bp from the intronexon junction. PCR reactions and cycling were as described in (B), except that the 59°C step was done at 60°C. One strand of each heteroduplex is from long-wave genes and the other is from middle-wave genes. Thus, half the counts in the heteroduplexes (H/2) are added to the counts in the uncut band (U), and the other half are added to the sum of the counts in the two cut bands (C). The final quotient: (U + H/2)/(C +H /2) yields an estimate of the ratio of middle- to long-wave genes in the cluster.

into the gene. The probability of a crossover is proportional to the length of homologous DNA. Thus, it is likely that crossovers have occurred within the first third of the gene, thereby duplicating long-wave gene sequences including fragment A_r. For individuals with more than one copy of A_r, the analysis assuming a single copy of A_r would have substantially underestimated the number of genes. In contrast, the Sma I restriction site polymorphism used here occurs very near the 5′ edge of the pigment gene array. As upstream sequences flanking the first gene are not repeated within the array, it is unlikely that our marker for the first gene would have been duplicated by homologous recombination. If, in any participant, our marker for the first gene was duplicated in a downstream gene, it would lead to an underestimate of the number of pigment genes per X chromosome. Our estimates indicate that the number of pigment genes per X chromosome is often much higher than was previously reported. However, our sample included men who demonstrably had a single X-linked pigment gene, and we identified men with as few as two and three genes. The fact that the new method yields estimates of small as well as large gene numbers demonstrates that it does not systematically overestimate the number of pigment genes.

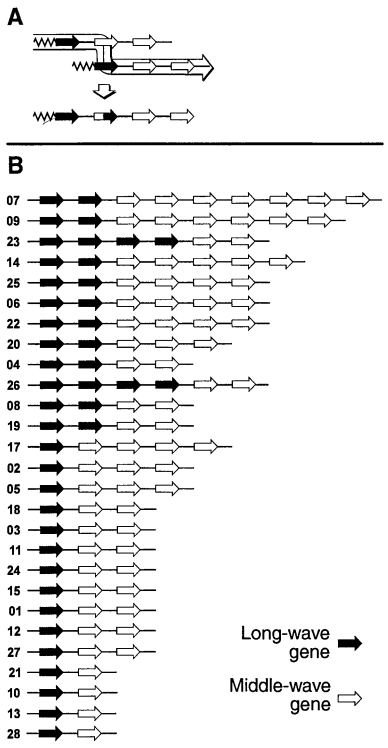

Fig. 2. (**A**) Proposed unequal intragenic recombination that would produce multiple long-wave genes in men with normal color vision. If the two parental chromosomes misalign by one gene as shown, the duplicated long-wave gene will occupy a position adjacent to the unaltered long-wave gene. Misalignments that would place the duplicate in more 3′ positions may be less likely. (**B**) Proposed gene arrangements of 27 men with normal color vision. Genes are identified as long-wave from sequences in exon 5; those that occupy positions downstream of the first gene contain 5′ sequences that were believed earlier to be exclusive to middle-wave genes in men with normal color vision, as illustrated in (A).

The experiments reported here were carried out in triplicate to establish the reliability of the method. On average, the 95% confidence interval for the counts of gene number is ±23%. Thus, for example, an estimate of four genes would have a 95% confidence interval of 3.08 to 4.92; it is unlikely that such a person has as few as three or as many as five genes. The reliability of the measure of middle- to long-wave gene ratio is even higher (the average 95% confidence interval is ± 12%).

Finally, to directly compare results from the old and new methods, we determined the A_g/A_r ratios for our participants using quantitative Southern blot analysis. The two methods often yield very different estimates of gene number. For example, Southern blot analysis of participant 14 gave an A_g/A_r ratio of 2.3. Earlier interpretations would have suggested that he has three X-linked pigment genes. In contrast, we estimated that he has seven genes—two long-wave and five middle-wave genes (Table 1). If we discard the assumption that fragment A_r is always present in a single copy and allowed participant 14 to have two copies of A_r, one for each of his long-wave genes, then the stoichiometry of the A fragments (2.3) from Southern blot analysis is consistent with seven genes (A_g/A_r = 5/2 = 2.5). Other participants had even more long-wave genes. For example, participant 26 has an A_g/A_r ratio of 2.3. The standard interpretation of this would be that he has one long- and two middle-wave genes. However, we estimated a middle- to long-wave gene ratio of 0.50 (the 95% confidence interval is 0.37 to 0.63). Table 1 shows that he was estimated to have six X-linked pigment genes—four long-wave and two middle-wave genes. Duplicate long-wave genes with fragment A_r and long-wave genes with fragment A_g represent two important products of recombination that have been overlooked when interpreting Southern blot data. Participant 26 appeared to have both; thus, the number of genes was underestimated and the ratio of middle- to long-wave genes was overestimated by the A_g/A_r ratio.

Middle-wave genes are proposed to have been duplicated by homologous recombination. The extra long-wave genes probably arose by the same mechanism. There is a difference, however. Middle-wave genes within the array can be duplicated by intergenic crossovers. The first position in the array is usually occupied by a long-wave gene (6). Long-wave genes in the first position can only be duplicated by intragenic crossovers (Fig. 2A). The duplicate long-wave genes will thus contain sequences previously identified as middle-wave (*1*, *7*). Nonetheless, they will encode a pigment with a long-wave spectrum, provided they have the parent long-wave exon 5. This explains the presence of A_g in some long-wave genes. Duplication of long-wave genes by crossovers downstream of the A_r insert could produce polymorphisms in the coding sequences of the long-wave genes within an individual. These were not detected in earlier studies (*8*) that used single-strand conformation polymorphism analysis (SSCP). This is not inconsistent with our result, because duplication of the long-wave genes may have occurred by crossovers upstream of locations examined by SSCP. However, sequences previously assumed to be exclusive to middle-wave genes appear to occur in genes that encode long-wave pigments in men with normal color vision; this unforeseen complication makes interpretation of previous results more difficult. Polymorphisms in the long-wave genes could profitably be reexamined in light of the new results presented here.

It was long assumed that people with normal color vision all have three stereotyped cone pigment genes in common. Early molecular studies revealed a surprise—there is variability in the number of pigment genes that underlie red-green color vision. The present results demonstrate that individual differences in the pigment genes are much larger than have been appreciated. These men with normal color vision had as few as two and as many as nine X-linked pigment genes. Moreover, many men had multiple long-wave genes—often two, and as many as four.

It is interesting that many men have multiple long-wave genes in the light of recent findings that there are at least two normal variants of the long-wave pigment (*8*–*10*). Individual differences in long-wave pigments are associated with differences in normal color-matching behavior (*8*, *10*). If participants are asked to mix a red and a green light to exactly match the appearance of a monochromatic yellow light, a person who has inherited one of the long-wave variants will choose a different red/green ratio as matching the yellow than will another person who has inherited a different long-wave variant. However, if some men with multiple long-wave genes have and express both variants, they might be expected to make more intermediate color matches. Color matches by the participants in this study were measured previously (*11*). Matches of participants with one long-wave

gene can now be compared with those of men with multiple long-wave genes. The absolute difference from the mean (mean = 0.50) was determined for each R/(R + G) value given in Table 1. The absolute deviation from the mean color match was, on average, larger for the men with only one long-wave gene (0.056) than for those with multiple long-wave genes (0.032). This difference is unlikely to have arisen by chance (Mann-Whitney U test = 42.5; P = 0.02). The fact that individuals with multiple long-wave genes tend to make more intermediate color matches hints that some may be expressing more than one long-wave pigment. If that is true, in total they would express at least four different cone pigments: two long-wave, a middle-wave, and a short-wave pigment. This would seem counter to the Young-Helmholtz theory that has held sway for more than two centuries, in which the presence of three pigments is proposed to explain human trichromatic color vision. In any case, we can now seek to understand how normal color vision phenotypes can arise from such an enormous variety of genotypes and to reexamine how the normal genotypes might have been altered to result in color vision defects.

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Peptide Specificity in the Recognition of MHC Class I by Natural Killer Cell Clones

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Recognition by natural killer (NK) cells of major histocompatibility complex (MHC) class I molecules on target cells inhibits NK-mediated lysis. Here, inhibition of NK clones by HLA-B*2705 molecules mutated at single amino acids in the peptide binding site varied among HLA-B*2705–specific NK clones. In addition, a subset of such NK clones was inhibited by only one of several self peptides loaded onto HLA-B*2705 molecules expressed in peptide transporter–deficient cells, showing that recognition was peptide-specific. These data demonstrate that specific self peptides, complexed with MHC class I, provide protection from NK-mediated lysis.

Natural killer cells are a distinct lymphocyte population that controls the early phase of infection by various pathogens such as viruses, intracellular bacteria, protozoa, and fungi (*1*). Natural killer cells can eliminate infected cells by direct cell lysis, but they also influence subsequent T cell responses by secreting potent mediators of inflammation (*1*). Class I molecules of the MHC expressed on target cells play an important role in the specificity of recognition by NK cells (*2–5*). Engagement of NK receptors for class I molecules turns off the lytic machinery and lymphokine production (*3, 5–7*). In humans, several NK molecules are involved in the recognition of human leukocyte antigen (HLA)–C (*6*) and HLA-B (*8*) alleles. However, the nature of the specific interaction between NK receptors and target class I molecules remains obscure. For example, it is still not known whether NK cells recognize a conventional trimolecular class I complex of heavy chain, β_2-microglobulin, and peptide or other, less stable conformations of class I (*9*). Here, the well-defined HLA-B*2705 allele was used as a tool to test how NK cells recognize class I molecules.

To isolate NK clones specific for HLA-B*2705, we generated a large panel of clones (*10*) from two individuals and screened the clones for their ability to lyse the B cell line C1R, which expresses HLA-Cw4 as the only serologically detectable allele (*11*), and its transfected derivative C1R-B*2705 (*12*). As expected from previous work (*5, 13*), B*2705 molecules on C1R cells provided protection from NK lysis by several, but not all, NK clones. The specificity of 10 randomly chosen B*2705-specific NK clones was assessed further with two other C1R transfectants (*14*) that express equivalent surface amounts of B*0801 or B*1402 molecules (Fig. 1A). Seven clones (including 2wA-08 and 2wA-29) recognized only B*2705, whereas three clones (such as 10wA-16) also recognized B*0801. Protection by several HLA-B alleles has been reported with other NK clones (*5*), perhaps the result of expression of several receptors on a single NK clone, or cross-reactivity of NK receptors with related class I alleles.

To show that B*2705 molecules were directly involved in recognition by the NK clones, we used monoclonal antibody (mAb) A6/136 (anti–class I) (*15*) to interfere with class I–mediated protection. At saturating conditions, A6/136 inhibited the protection from lysis—that is, it restored lysis—by all six NK clones tested (three are shown in Fig. 1B). A control isotype-matched immunoglobulin M (IgM) mAb had no effect. The IgM mAb B27.2M (anti–HLA-B27) (*15*) restored lysis by only one out of the six clones tested (2wA-08 in Fig. 1B), suggesting that NK clones can recognize B*2705 molecules in different ways.

Functional heterogeneity among B*2705-specific NK clones was shown by use of B*2705 molecules mutated at single amino acid residues in the peptide binding site (Fig. 2). Surface expression of mutants E45T, L95I, and D116F (*16*) transfected in C1R cells was comparable to that of wild-type B*2705 molecules, except for mutant E45T, whose expression was lower (Fig.

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