GIP would be well suited for such a function. For example, the ability of GDI-GIP to stimulate the release of CDC42Hs from membranes could serve to initiate the cycling of this GTP binding protein between different cellular compartments, and its ability to inhibit GDP dissociation as well as GTP hydrolysis would insure that CDC42Hs remains in the GDP- or GTP-bound state while in transit to these locations. Recently, the GDI protein has been shown (*25*) to be part of a cytosolic complex with the Rac GTP binding protein involved in superoxide production in neutrophils (*25–27*). This raises the question of whether the GDI protein functions both in the assembly of this complex and in the maintenance of the activation of the reduced nicotinamide adenine dinucleotide oxidase by preserving the GTP-bound state of Rac.

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29. GTPase inhibitory protein (GIP) activity was first detected in DEAE fractions enriched in GDI. Therefore, the first two purification steps were performed to obtain partially purified GDI. These fractions were obtained by homogenization of bovine cerebral tissue (70 g) in 250 ml of TED [25 mM tris-HCl (pH 8.0), 1 mM EDTA, and 1 mM dithiothreitol (DTT)] containing 0.25 mM phenylmethylsulfonyl fluoride (PMSF), leupeptin (5 μg/ml), aprotinin (5 μg/ml), and pepstatin A (2 μg/ml). The membranes were removed by centrifugation at 30,000*g* for 30 min. Ammonium sulfate was added to the supernatant to 40% saturation, stirred for 30 min, and centrifuged at 11,300*g*. The supernatant was removed, ammonium sulfate was added to 80% saturation, and the mixture was centrifuged again at 11,300*g*. The pellet, which represented the 40 to 80% ammonium sulfate fraction, was dissolved in 20 ml of TED containing 0.25 mM PMSF, leupeptin (5 μg/ml), and aprotinin (5 μg/ml). CDC42Hs-GDI activity, as assayed by the inhibition of the dissociation of labeled GDP (Fig. 2) (*3*), was present in the 40 to 80% ammonium sulfate fraction. The redissolved pellet was then dialyzed against 1 liter of TED (two changes) for 20 hours. The dialyzed sample was then applied to a DEAE Sephacel (Sigma) column (2.5 cm × 15 cm) equilibrated in TED buffer. The protein was eluted with a 400-ml gradient of 0 to 300 mM NaCl. The peak in GDI activity eluted at approximately 175 mM NaCl. GIP activity was also detected in these peak fractions. The peak in GDI activity was pooled (~60 ml) and concentrated to 10 ml. Half of the concentrate was dialyzed in a solution that contained 350 ml of bis[2-hydroxyethyl]iminotris[hydroxymethyl]methane, 2-bis[2-hydroxyethyl]-amino-2-[hydroxymethy]-1,3 propanediol (Bis-Tris) (pH 7.0), 1 mM EDTA, and 1 mM DTT (BIS-TED) supplemented with 0.1% CHAPS and 5% glycerol. This dialysate was applied to a Pharmacia fast protein liquid chromatography (FPLC)–Mono Q column (HR 5/5) equilibrated in BIS-TED containing 0.1% CHAPS. The protein was eluted with a 30-ml gradient of 0 to 500 mM NaCl. GDI and GIP activity co-eluted at approximately 175 mM NaCl. The peak in GDI-GIP activity was pooled and dialyzed against MED [20 mM Na-2-(*N*-Morpholino)ethanesulfonic acid (Na-MES) (pH 6.1), 1 mM EDTA, and 1 mM DTT] containing 0.1% CHAPS and 5% glycerol and injected onto a Pharmacia FPLC–Mono S (HR 5/5) column equilibrated in MED and 0.1% CHAPS. The protein was eluted with a 25-ml gradient of 0 to 500 mM NaCl. The peak in GDI activity and GIP activity co-eluted at 80 mM NaCl.

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Selecting T Cell Receptors with High Affinity for Self-MHC by Decreasing the Contribution of CD8

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Selective events during T cell repertoire development in the thymus include both the positive selection of cells whose receptors recognize self–major histocompatibility complex (MHC) molecules and negative selection (tolerance) of cells whose interaction with self-MHC is of high affinity. The affinity of T cell interactions with class I MHC molecules includes contributions by both the T cell receptor and the CD8 coreceptor. Therefore, by decreasing the affinity of the interaction with CD8, T cells whose receptors have relatively high affinities for self-MHC may survive negative selection. Such T cells were generated and those T cells reactive with self-MHC plus antigen also displayed low affinity for self.

The affinity of a T cell for class I MHC is attributable to binding by both the clonotypic portion of the T cell receptor (TCR), which binds allele-specific portions of the highly polymorphic α1,α2 domains of class I and its associated peptide ligand, and the CD8 coreceptor that specifically binds a nonpolymorphic region within the α3 domain of the same class I molecule (*1–3*). During maturation in the thymus it is this combined affinity that determines the fate of the developing T cell. In order for a thymocyte to develop into a functional $CD8^+$ T cell, its clonotypic receptor must recognize a self–class I MHC molecule expressed on the thymic epithelium (positive selection) (*4*). Presumably, this maximizes the probability that T cells will recognize foreign antigens presented by self–MHC molecules. However, all T cells that are potentially autoreactive by the criteria that they have sufficiently high affinity for the selecting MHC molecule to permit stimulation in the absence of foreign antigen must be eliminated before maturation (*5*). This paradox has been reconciled by proposing that thymic selection permits maturation of cells with low affinity, yet eliminates cells with no affinity or high affinity for self-MHC. Although numerous experiments using TCR transgenic lines (*4, 5*) and CD8 transgenic lines (*6*) have shown the validity of a number of predictions based on such an affinity model of thymic selection, by definition the affinity of the TCR for the syngeneic restriction molecule must be below the threshold necessary for detection of a response and, therefore, direct evidence for such affinity has been difficult to obtain (*7*).

Now we describe a situation in which a human class I molecule (HLA-A2) for which the affinity of interaction with murine CD8 is suboptimal (*8–11*), has been used to study the effect of decreased CD8 interaction on the resultant A2-restricted T cell repertoire. This is contrasted with the A2-restricted repertoire that develops in response to positive selection by the chimeric $A2/K^b$ molecule that contains a mu-

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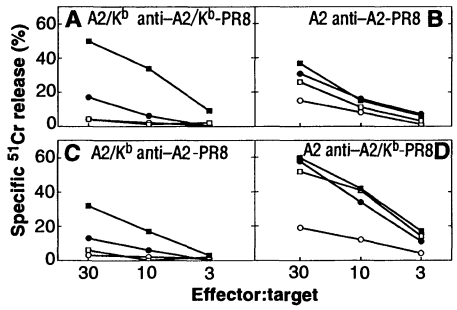

Fig. 1. Optimal stimulation of M(57–68)-specific CTLs from virus-primed A2 and A2/K^b transgenic mice required antigen presentation by A2/K^b stimulators. Responder spleen cells from A2/K^b-Tg (**A** and **C**) or A2-Tg (**B** and **D**) mice previously immunized with PR8 were stimulated with the indicated influenza virus (PR8)–infected spleen cells in vitro. The resultant effector cells were assayed for lytic activity against Jurkat (J) cells transfected with either A2 or A2/K^b or the same targets pulsed with M(57–68) peptide. Targets were as follows: J-A2 (○); J-A2/K^b (□); peptide-pulsed J-A2 (●); and peptide-pulsed J-A2/K^b (■). The derivation of the A2/K^b-Tg founder, which is the homozygous form of Tg mouse line 66, has been described (*11*). The A2-Tg founder was derived by injecting a genomic clone of A2 (*16*) into fertilized eggs obtained by crossing (C57BL/6 × SJL)F_1 mice as described (*7*). The A2-Tg line expressed the A2 transgene product on 100% of cells in peripheral blood and spleen as detected by flow cytometry (*11*). Progeny obtained by subsequent breeding of the founder with C57BL/6 were used in these experiments. All mice were homozygous for *H-2^b*. The indicated mice were primed intraperitoneally with 300 HAU hemagglutinating units of A/PR/8/34 (PR8) influenza in the form of allantoic fluid. After 3 weeks, cultures were established using the indicated irradiated, PR8-infected spleen cells as stimulators (*11*). After 6 days, effector cells were assayed for cytotoxicity against the indicated transfected lines in a 6-hour ^{51}Cr release assay (*8*). The M(57–68) peptide (*11*) was used at 100 μg/ml to pulse targets during ^{51}Cr labeling.

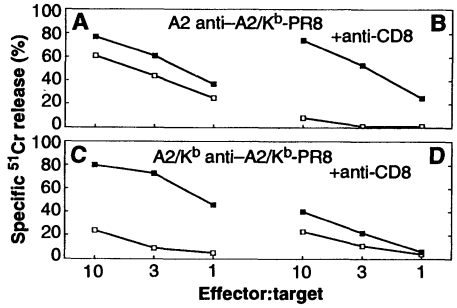

Fig. 2. Lytic activity by (M57–68)-specific effectors from A2-Tg (**A** and **B**) and A2/K^b-Tg mice (**C** and **D**) inhibited by monoclonal antibody (MAb) to CD8 (B and D). CTL lines were established as in Fig. 1 and restimulated with peptide-pulsed A2/K^b-Tg stimulators (*11*). Anti-CD8 inhibition was done as described (*8*). Targets were J-A2/K^b (□) or peptide-pulsed JA2/K^b (■). The data represent the results of a 4-hour ^{51}Cr release assay.

rine α3 domain and accordingly demonstrates increased affinity of interaction with murine CD8 (*8*, *9*, *11*). Both types of transgenic (Tg) mice expressed the transgene product on 100% of peripheral blood lymphocytes as determined by flow cytometric analysis (*8*, *11*). The average expression of the transgene product was approximately 30% lower for cells from A2-Tg mice than for A2/K^b-Tg mice; however, this difference was within the range of variation observed within each line. We have compared the ability of cytotoxic T lymphocytes (CTLs) derived from transgenic mice that express comparable amounts of A2 or A2/K^b transgene products to utilize these molecules as restriction elements during an influenza virus–specific response.

A2- and A2/K^b-Tg mice were immunized with influenza virus (A/PR/8/34) and spleen cells from the mice were tested for their response to an A2-restricted viral epitope contained within a synthetic peptide representing residues 57 to 68 of the matrix protein [M(57–68)] (*12*). As reported, A2/K^b-Tg mice could mount an A2/K^b-restricted, M(57–68)-specific response (*11*). Also as reported (*13*), an A2-restricted M(57–68)-specific response was detectable in A2-Tg mice; however, it was significantly weaker than the response by A2/K^b-Tg (compare Fig. 1, A and B). In these same cultures the D^b-restricted response to the influenza nucleoprotein (NP) was assayed using EL-4 targets pulsed with peptide NP(365–380) and was identical in both A2 and A2/K^b-Tg (78% at an effector-to-target ratio of 30:1). The magnitude of the A2-restricted response could be increased by using virus-infected A2/K^b-Tg cells as stimulators (Fig. 1D). This result suggested that the in vivo A2-restricted response may be comparable for A2 and A2/K^b mice but that optimal clonal expansion in vitro required antigen presentation by A2/K^b-bearing cells. This was supported by the small response observed when virus-infected A2-bearing cells, instead of infected A2/K^b-bearing cells, were used to stimulate an influenza-specific response using A2/K^b-Tg responders (Fig. 1C).

The responses by A2/K^b-Tg and A2-Tg mice differed in two respects. First, A2/K^b-Tg mice only weakly recognized antigen presented by A2 (Fig. 1A). Lysis was three to ten times higher when antigen was presented by targets that expressed A2/K^b, despite the greater amount of A2 on the A2 target cells than on the A2/K^b target (810 versus 500 linear fluorescence units) (*8*). Although these data were obtained with the use of a human cell line (Jurkat) transfected with either A2 or A2/K^b as targets, identical results were obtained when transfected murine were used as targets [EL4-A2/K^b and EL4-A2 (*8*)]. These findings are consistent with the ability of murine CD8 to interact with the α3 domain of A2/K^b but not A2, thereby boosting the avidity of interaction with targets expressing A2/K^b (*8–11*). It was, therefore, anticipated that lysis by A2/K^b-Tg but not A2-Tg effectors would be CD8-dependent. In support of this interpretation, antibody to CD8 had little effect on A2/K^b-restricted M(57–68)-specific lysis by effectors derived from A2-Tg mice (Fig. 2, A and B); in contrast, antibody to CD8 inhibited lysis by effectors from A2/K^b-Tg mice (Fig. 2, C and D).

A second difference between the responses by A2-Tg and A2/K^b-Tg mice was that A2-restricted effectors from A2-Tg could lyse A2/K^b-bearing targets in the absence of the M(57–68) antigen. Such recognition was dependent on the interaction of CD8 with the α3 domain of A2/K^b, because it was completely blocked by antibody to CD8 (Fig. 2B). This suggested that A2-restricted CTLs had low affinity for the A2 restriction element and that by incorporating CD8 in the interaction the avidity was boosted sufficiently to trigger cytolysis in the absence of antigen. Thymic selection usually prevents maturation of cells with such high affinity for the MHC restriction element; however, due to the weak interaction between murine CD8 and A2, it was possible that cells whose receptors had such relatively high affinities could escape negative selection.

If this interpretation were correct, then it would be anticipated that effectors from A2-Tg mice responsible for lysis of A2/K^b targets in the absence of M(57–68) were the same ones responsible for recognition of A2 plus M(57–68) (as opposed to an independently stimulated population). To test this, A2-restricted CTLs were obtained that had no prior exposure to A2/K^b and were analyzed clonally. Spleen cells from a virus-infected A2-Tg mouse were stimulated in vitro with infected A2-Tg cells, thus delaying exposure to A2/K^b-bearing cells. After 7 days, the population was restimulated with M(57–68) presented by A2/K^b-Tg stimulators. The resultant effector cell population was cloned by limiting dilution and all clones that lysed M(57–68)-pulsed, A2/K^b targets were tested for recognition of A2- and A2/K^b-bearing cells in the presence or absence of M(57–68) (Table 1). All of the clones that recognized M(57–68) presented by A2 also recognized A2/K^b in the absence of antigen. Thus, the entire repertoire of M(57–68)-specific, A2-restricted CTLs derived from A2-Tg mice

Table 1. A2-restricted M(57–68)-specific CTL clones also recognized A2/K^b. Clones were derived by limiting dilution (*17*) of an effector cell population originally obtained by stimulation with infected A2-Tg cells of A2-Tg spleen cells from an influenza-primed mouse. Stimulators used in the cloning experiment were peptide [M(57–68)]-pulsed A2/K^b-Tg spleen cells (*11*). Clones were detected using peptide-pulsed JA2/K^b targets to assay 60 μl of the contents of each culture well in a 6-hour ^{51}Cr release assay. The contents of wells exhibiting lytic activity were restimulated (*11*) with peptide-pulsed A2/K^b-Tg spleen cells and tested 6 days later for lytic activity against Jurkat (J) cells that had been transfected with A2 or A2/K^b. Data are presented for all clones that had at least 10% specific lysis of one or more targets.

Specific ^{51}Cr release (%)						
Experiment 1				Experiment 2		
J-A2	J-A2 + M(57–68)	J-A2/K^b	J-A2/K^b + M(57–68)	J-A2	J-A2 + M(57–68)	J-A2/K^b
1	18	21	28	0	38	16
6	35	32	58	10	42	29
3	19	7	33	9	54	45
4	15	22	40	2	42	58
0	12	9	23	1	44	37
3	6	12	11	1	41	37
15	35	58	53	10	64	51
0	8	12	16	0	42	22
3	9	6	20	11	28	29
4	12	24	17	6	27	25
0	16	10	26	0	68	75
4	16	24	28	0	25	11
4	10	7	18	0	20	37
6	14	25	19			
8	25	24	30			
9	27	30	41			
0	13	8	33			
9	49	35	68			
8	18	35	38			
5	35	33	53			
0	18	17	27			

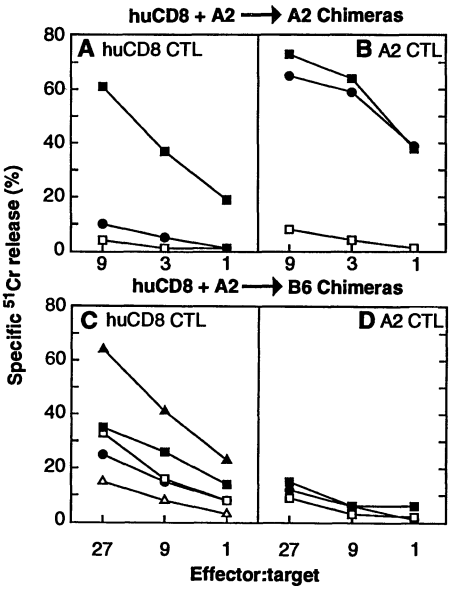

Fig. 3. Analysis of A2/K^b recognition by A2-restricted CTLs from double parent radiation chimeras. A2-Tg (**A** and **B**) or C57BL/(B6) (**C** and **D**) mice were lethally irradiated (1000 rads) and each recipient reconstituted by intravenous injection of a mixture of bone marrow cells derived from A2-Tg (3×10^6 cells) and huCD8-Tg mice (1×10^7 cells) that were depleted of mature T cells by treatment with antibody to Thy-1 plus complement. Spleen cells from PR8-primed mice were cultured with A2-Tg lipopolysaccharide (LPS) stimulators that had previously been incubated with matrix peptide M(58–66). After 6 days of culture, cells were treated with either a MAb to huCD8 (leu 2b) plus complement (A2 CTL) or a MAb to A2 (BB7.2) plus complement (huCD8 CTL) and assayed on EL4 (E) cells that had been transfected with A2 or A2/K^b: E-A2 targets (□), M(58–66)-pulsed E-A2 targets (■), and E-A2/K^b targets (●). For the experiment in (C), cells were also incubated with nucleoprotein peptide NP(365–380)-pulsed stimulators and assayed on EL4 (△) or NP(365–380)-pulsed EL4 (▲) targets.

interacted with A2 with low affinity.

If, as hypothesized, lysis of A2/K^b was attributable to a higher than normal TCR affinity for the A2 restriction element that was tolerated because of the decreased contribution of CD8 during thymic selection, then it would be predicted that the presence of a CD8 molecule in the thymus that could efficiently interact with A2 would reverse this effect and result in selection of an A2-restricted repertoire that had the normal lower affinity for the A2 restriction element and therefore would not lyse A2/K^b-bearing targets devoid of peptide antigen. To test this hypothesis, we utilized a transgenic murine line that expressed a functional human CD8 (huCD8) molecule and would be expected to interact with A2 during thymic development (*14*). Because initial attempts to obtain progeny that expressed both huCD8 and A2 were unsuccessful, we instead constructed double donor chimeras.

A mixture of bone marrow cells from both A2-Tg mice and huCD8-Tg mice were used to reconstitute the lymphoid system of lethally irradiated A2-Tg mice. Under these conditions, lymphoid cells that express huCD8 become tolerant of A2 and A2-restricted T cells of both huCD8 and A2 origin develop. Chimeras were immunized with virus and the specificity of each type of CTL was analyzed separately (Fig. 3). For these experiments the stimulating antigen was the more active form of matrix peptide, M(58–66). With this peptide we could obtain A2-restricted CTLs when using A2 (rather than A2/K^b) stimulators. Cells of A2 origin had the same specificity observed for A2 transgenic mice and lysed both antigen-pulsed A2 targets and nonantigen-pulsed A2/K^b targets to the same extent (Fig. 3B). In contrast, as predicted by the hypothesis, virus-specific CTLs of huCD8 origin did not lyse A2/K^b targets in the absence of antigen (Fig. 3A). To confirm that expression of A2 by the thymus was required for development of an A2-restricted T cell repertoire, we constructed double donor chimeras using conventional *H-2^b* hosts. Although an influenza-specific response restricted by H-2D^b was obtained when such mice were primed with influenza, no A2-restricted response was evident (Fig. 3, C and D).

Several laboratories have reported transgenic lines that express class I molecules unable to interact with CD8 due to alterations in their α3 domain (*15*). In each case, a repertoire was obtained that could respond to the corresponding wild-type molecule. These results were interpreted as evidence that, in the absence of interaction with CD8, a population of low-affinity anti-self MHC T cells evades negative selection. A difference between the anti-self CTLs previously described and the A2/K^b reactive cells described herein is that A2/K^b reactive cells are a part of the T cell repertoire positively selected by A2. The D^b-restricted CTLs from A2-Tg mice, such as those present in the cultures described in Fig. 1B, did not show A2/K^b reactivity. Considering that no evidence for positive selection by the transgene was reported in the prior studies, the anti-self T cells observed could have been positively selected by conventional class I molecules rather than the transgene product, thus making them analogous to alloreactive cells.

In summary, we conclude that, by decreasing the contribution of CD8, the receptor affinity required for both positive and

negative selection is increased. This results in an A2-restricted repertoire that contains receptors with higher than usual affinity for A2 per se. When provided with the A2/K^b molecule, murine CD8 can participate more fully in interaction with the restriction element, thus elevating the avidity of the interaction above the threshold required for the induction of detectable lytic activity in the absence of antigen. However, because CD8 is also required for T cell stimulation, our results are also consistent with a model in which only the affinity cutoff for negative selection is increased and the observed recognition of A2 per se is due to an increase in the affinity requirement for antigen responsiveness rather than positive selection.

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Comparative Genomic Hybridization for Molecular Cytogenetic Analysis of Solid Tumors

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Comparative genomic hybridization produces a map of DNA sequence copy number as a function of chromosomal location throughout the entire genome. Differentially labeled test DNA and normal reference DNA are hybridized simultaneously to normal chromosome spreads. The hybridization is detected with two different fluorochromes. Regions of gain or loss of DNA sequences, such as deletions, duplications, or amplifications, are seen as changes in the ratio of the intensities of the two fluorochromes along the target chromosomes. Analysis of tumor cell lines and primary bladder tumors identified 16 different regions of amplification, many in loci not previously known to be amplified.

The discovery of genetic changes involved in the development of solid tumors has proven difficult. Karyotyping is impeded by the low number of high-quality metaphase spreads and the complex nature of chromosomal changes (*1*). Molecular genetic studies of isolated tumor DNA have been more successful and have been used to detect common regions of allelic loss, mutation, or amplification (*2*, *3*). However, such molecular methods are highly focused; they target one specific gene or chromosome region at a time and leave the majority of the genome unexamined.

We have developed a molecular cytogenetic method, comparative genomic hybridization (CGH), that is capable of detecting and mapping relative DNA sequence copy number between genomes. A copy number karyotype can be generated for a tumor by the comparison of DNAs from malignant and normal cells, thereby identifying regions of gain or loss of DNA. In this application of CGH, biotinylated total tumor DNA and digoxigenin-labeled normal genomic reference DNA are simultaneously hybridized to normal metaphase spreads in the presence of unlabeled Cot-1 blocking DNA (*4*). Hybridization of tumor DNA is detected with green-fluorescing fluorescein isothiocyanate (FITC)–avidin, and the reference DNA hybridization is detected with red-fluorescing rhodamine antidigoxigenin (*5*). The relative amounts of tumor and reference DNA bound at a given chromosomal locus are dependent on the relative abundance of those sequences in the two DNA samples and can be quantitated by measurement of the ratio of green-to-red fluorescence. The reference DNA serves as a control for local variations in the ability to hybridize to target chromosomes. Thus, gene amplification or chromosomal duplication in the tumor DNA produces an elevated green-to-red ratio, and deletions or chromosomal loss cause a reduced ratio. The Cot-1 DNA included in the hybridization inhibits binding of the labeled DNA to the centromeric and heterochromatic regions, so these regions are excluded from the analysis. The fluorescence signals are quantitatively analyzed by means of a digital image analysis system (*6*). A software program integrates the green and red fluorescence intensities in strips orthogonal to the chromosomal axis, subtracts local background, and calculates intensity profiles for both colors and the green-to-red ratio along each chromosome.

The ability of CGH to quantitate changes in sequence copy number that affect an entire chromosome was tested with five fibroblast cell lines having one to five copies of the X chromosome and two copies of each autosome (*7*). Hybridization of DNA from a 45,X0 cell line (in green), together with normal female reference DNA (in red) to a normal male metaphase spread, resulted in a uniform green-red staining of the autosomes, whereas the X chromosome appeared more red (Fig. 1A). Hybridizations with DNA from cell lines carrying two, three, four, or five copies of the X chromosome resulted in increasingly strong green fluorescence from the X chromosome relative to the autosomes. The average green-to-red fluorescence ratio of the X chromosome (Fig. 1B), when normalized to the average ratio for the autosomes within the same metaphase spread, increased linearly with increasing number of X chromosomes [correlation coefficient (r) = 0.978]. Thus, CGH can quantitatively distinguish a change of plus or minus one copy of a chromosome at least up to four copies.

We used CGH to generate a complete copy number karyotype for a near-diploid breast cancer cell line, 600PE. According to the published karyotype (*8*), 600PE is near-diploid with five marker chromo-

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