15. These cDNA clones terminated in the exon flanked by introns 4 and 5. Poly A+ RNA from *S. pombe* was reverse transcribed using primer M2-B14 (CCTTGGAAAAATCCATTGAAGCCACATGTG). The resulting cDNA was ligated to oligonucleotide pGGGCCGTGTTGGCCTAGTTCTCTGCTCddA using T4 RNA ligase and amplified by two rounds of PCR: in the first round, we used primers M2-B14 and Adapt-Sfi (GAGGAGGAGAAGAGCAGAGAACTAGGCCAACACGGCCC), and in the second, we used primers M2-B15 (AAAGTGGTATGCCAGAAATCTGAAGGTAAT) and Adapt-Sfi.
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19. A lambda cDNA library from the human 293 cell line, which has high levels of telomerase activity, was partitioned into 25 pools containing ~200,000 plaques each. These were screened by PCR with primers LT5 (CGGAAGAGTGTCTGGAGCAA) and LT6 (GGATGAAGCGGAGTCTGGA) to the 5′ region of the #712562 clone insert. Six subpools of one positive primary pool were further screened by PCR. One positive subpool was then screened by plaque hybridization with a probe from the 5′ region of clone #712562. One phage was positively identified and the ~4 kbp insert from this clone was excised and subcloned into the pBluescript II SK+ vector (Stratagene) as an Eco RI fragment.
20. Polyadenylated RNAs from human testis and from the 293 cell line were amplified using a nested PCR strategy. The first primer set was TCP1.1 (GTGAAGGCACTGTTCAGCG) and TCP1.15 (CGCGTGGGTGAGGTGAGGTG); the second primer set was TCP1.14 (CTGTGCTGGGCCTGGACGATA) and bTCP6 (AGCTTGTTCTCCATGTCGCCGTAG).
21. hTRT mRNA was amplified using oligonucleotide primers LT5 and LT6 (*19*) for 31 cycles (94°C for 45 s, 60°C for 45 s, 72°C for 90 s). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was amplified using primers K136 (CTCAGACACCATGGGGAAGGTGA) and K137 (ATGATCTTGAGGCTGTTGTCATA) for 16 cycles (94°C for 45 s, 55°C for 45 s, 72°C for 90 s). hTR was amplified using primers F3b (TCTAACCCTAACTGAGAAGGGCGTAG) and R3c (GTTTGCTCTAGAATGAACGGTGGAAG) for 22 cycles (94°C for 45 s, 55°C for 45 s, 72°C for 90 s). TP1 mRNA was amplified using primers TP1.1 (TCAAGCCAAACCTGAATCTGAG) and TP1.2 (CCCGAGTGAATCTTTCTACGC) for 28 cycles (cycles same as for hTRT). Reaction products were resolved on an 8% polyacrylamide gel, stained with SYBR Green I (Molecular Probes, Eugene, OR).
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28. *h+/h− leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-M210/ade6-M216 his3-D1/his3-D1 trt1+/trt1−::his3+*.
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Contrasting Genetic Influence of *CCR2* and *CCR5* Variants on HIV-1 Infection and Disease Progression

Michael W. Smith,* Michael Dean,* Mary Carrington,* Cheryl Winkler, Gavin A. Huttley, Deborah A. Lomb, James J. Goedert, Thomas R. O'Brien, Lisa P. Jacobson, Richard Kaslow, Susan Buchbinder, Eric Vittinghoff, David Vlahov, Keith Hoots, Margaret W. Hilgartner, Hemophilia Growth and Development Study (HGDS), Multicenter AIDS Cohort Study (MACS), Multicenter Hemophilia Cohort Study (MHCS), San Francisco City Cohort (SFCC), ALIVE Study, Stephen J. O'Brien†

The critical role of chemokine receptors (CCR5 and CXCR4) in human immunodeficiency virus–type 1 (HIV-1) infection and pathogenesis prompted a search for polymorphisms in other chemokine receptor genes that mediate HIV-1 disease progression. A mutation (*CCR2-64I*) within the first transmembrane region of the *CCR2* chemokine and HIV-1 receptor gene is described that occurred at an allele frequency of 10 to 15 percent among Caucasians and African Americans. Genetic association analysis of five acquired immunodeficiency syndrome (AIDS) cohorts (3003 patients) revealed that although *CCR2-64I* exerts no influence on the incidence of HIV-1 infection, HIV-1–infected individuals carrying the *CCR2-64I* allele progressed to AIDS 2 to 4 years later than individuals homozygous for the common allele. Because *CCR2-64I* occurs invariably on a *CCR5*-+−bearing chromosomal haplotype, the independent effects of *CCR5-Δ32* (which also delays AIDS onset) and *CCR2-64I* were determined. An estimated 38 to 45 percent of AIDS patients whose disease progresses rapidly (less than 3 years until onset of AIDS symptoms after HIV-1 exposure) can be attributed to their *CCR2*-+/+ or *CCR5*-+/+ genotype, whereas the survival of 28 to 29 percent of long-term survivors, who avoid AIDS for 16 years or more, can be explained by a mutant genotype for *CCR2* or *CCR5*.

The nexus of chemokine immunobiology and AIDS pathogenesis has revealed untapped avenues for resolving patterns of HIV-1 disease progression, for clarifying epidemiologic heterogeneity, and for design of therapies (*1–6*). Identification of the CC-chemokines, RANTES, MIP1α and MIP1β, as suppressor factors produced by CD8 cells that counter infection by certain HIV-1 strain infections (*7*) previewed the critical identification of two chemokine receptor molecules, CXCR4 (formerly named LESTR/fusin) and CCR5 (formerly CKR5), as cell surface coreceptors with CD4 for HIV-1 infection (*8–13*). Additional chemokine receptors CCR2 and CCR3 also have been implicated as HIV-1 coreceptors on certain cell types (*12–14*). HIV-1–infected patients harbor predominantly macrophage-tropic HIV-1 isolates during early stages of infection, but accumulate increasing amounts of T cell–tropic strains just before accelerated T cell depletion and progression to AIDS. The identification of "dual"-tropic HIV-1 strains over the course of infection suggests that such strains may represent an intermediate between macrophage- and T cell–tropic populations (*11–13, 15*). This tropic transition indicates that viral adaptation from CCR5 to CXCR4 receptor use may be a key step in progression to AIDS (*16*).

A common 32–base pair (bp) deletion mutation in the *CCR5* gene that causes truncation and loss of CCR5 receptors on lymphoid cell surfaces of homozygotes was recently described (*17–19*). Genotype analysis of more than 4000 individuals from multiple AIDS cohorts demonstrated that (*CCR5-Δ32/Δ32*) deletion homozygosity was not uncommon (1 to 5%) among exposed uninfected individuals, but exceedingly rare (<0.1%) among infected individuals, indicating the *CCR5-Δ32/Δ32* homozygotes strongly resist HIV-1 infection (*19–23*). Further, the onset of AIDS was postponed 2 to 4 years in individuals heterozygous for *CCR5-Δ32* and the normal *CCR5-+* allele in several large AIDS cohort studies (*19, 21, 22*).

Despite the *CCR5* data plus several *HLA* associations that influence HIV-1 exposure outcome (*24–27*), identified genetic factors can account for only a small proportion of the "long-term survivors" who continue to resist AIDS-defining illness 10 to 20 years after HIV-1 infection. For example, 80% of highly exposed uninfected individuals in these studies are not *CCR5-Δ32/Δ32* homozygotes (*21*), and more than 60% of long-term survivors are homozygous for the common allele (*CCR5-+/+*) (*19, 21, 22*).

To identify alterations in chemokine receptor genes implicated as HIV-1 coreceptors (*8, 12–14, 28–30*), we screened the entire *CCR2* gene for variants by means of the single-strand conformation polymorphism (SSCP)/heteroduplex mobility assay (*31*). A G-to-A nucleotide substitution was detected at position 190 (counting from the ATG start codon) that substitutes the *CCR2-+* amino acid residue valine at position 64 to isoleucine (*CCR2-64I*), a conservative change located within the first transmembrane domain of the CCR2 receptor. That domain has a completely conserved amino acid sequence identity with CCR5, which suggests functional constraints on mutational variation. The mutant *CCR2-64I* allele has a transmembrane sequence that is identical to the *CCR5* normal allele. Using both SSCP and sequence-directed polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) assay (*32*), we determined the allele and genotype frequencies of 3003 individuals enrolled in five prospective AIDS cohorts. The *CCR2-64I* alteration was common in all ethnic groups with the following allele frequencies: 0.098 in Caucasians (n = 1847 individuals); 0.151

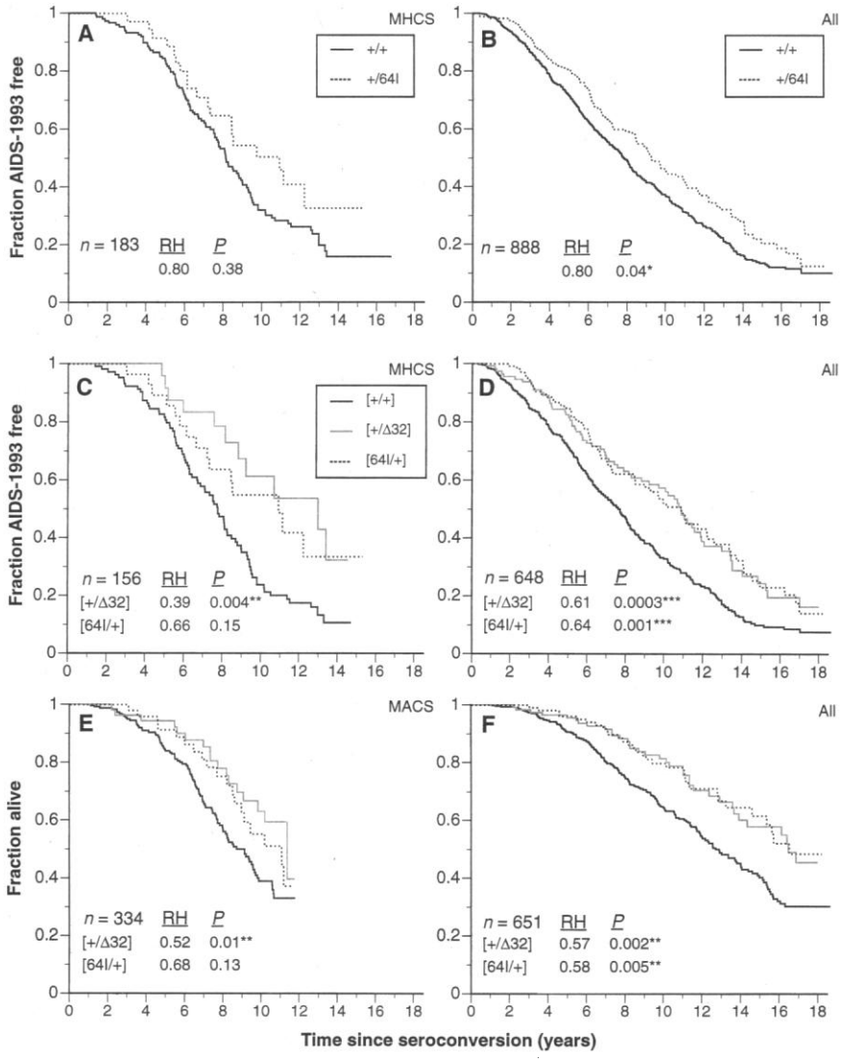

Fig. 1. Kaplan-Meier survival curves demonstrate the dependence of progression to AIDS-1993 on *CCR2* genotype in (**A**) MHCS and (**B**) combined "All" cohort analyses among seroconverters (*33, 41*). *CCR2-+/+* genotype survival is compared to *CCR2-+/64I* plus *CCR2-64I/64I* individuals, because *CCR2-64I/64I* individuals comprise few (~1%) members of each cohort. Number of patients (n), statistical P value (P), and relative hazard (RH) based on the Cox proportional hazard models (*35*) are given. Summary statistics for each cohort for each AIDS endpoint is presented in Table 1. (**C**) Kaplan-Meier survival plots of time to AIDS-1993 are shown for MHCS seroconverters genotyped for *CCR2-64I* and *CCR5-Δ32* polymorphisms. Curves represent compound genotypes of the *CCR2/CCR5* compound locus as defined in text. (**D**) Survival analysis of combined "All" cohorts for AIDS-1993, based on compound locus gentoypes as in (C); (**E**) survival plots for MACS seroconvertors time-to-death, based on compound locus genotypes as in (C); and (**F**) survival analysis of combined "All" cohorts time-to-death, based on compound locus genotypes as in (C) are shown. Summary statistics for each cohort and each AIDS endpoint are presented in Table 2 (*CCR2* and *CCR5* genotypes separated). (C) through (F) are for Caucasians only.

M. W. Smith, M. Carrington, C. Winkler, D. A. Lomb, Science Applications International Corp. Frederick, National Cancer Institute, Frederick, MD 21702–1201, USA.
M. Dean, G. A. Huttley, S. J. O'Brien, Laboratory of Genomic Diversity, National Cancer Institute, Frederick, MD 21702–1201, USA.
J. J. Goedert and T. R. O'Brien, Viral Epidemiology Branch, National Cancer Institute–Executive Plaza North, 6130 Executive Boulevard, Bethesda, MD 20892, USA.
L. P. Jacobson, Department of Epidemiology, The Johns Hopkins University School of Hygiene and Public Health, Room E-7008, 615 North Wolfe Street, Baltimore, MD 21205–1999, USA.
R. Kaslow, Department of Epidemiology, University of Alabama at Birmingham, 720 South 20th Street, Tidwell Hall 212C, Birmingham, AL 35294, USA.
S. Buchbinder and E. Vittinghoff, AIDS Office, Department of Public Health, 25 Van Ness Avenue, Suite 500, San Francisco, CA 94102–6033, USA.
D. Vlahov, Department of Epidemiology, The Johns Hopkins School of Hygiene and Public Health, Room E-6008, 615 North Wolfe Street, Baltimore, MD 21205, USA.
K. Hoots, Departments of Pediatrics and Internal Medicine, University of Texas Medical School at Houston, Houston, TX 77030, USA.
M. W. Hilgartner, Division of Pediatric Hematology and Oncology, New York Hospital–Cornell Medical Center, 525 East 68th Street, Payson 695, New York, NY 10021, USA.

*These authors contributed equally to this study.
†To whom correspondence should be addressed. E-mail: obrien@ncifcrf.gov

in African Americans (n = 899); 0.172 in Hispanics (n = 207), and 0.250 in Asians (n = 40).

There were no significant differences in *CCR2* allele or genotype frequencies in comparisons of exposed (or high-risk) uninfected (HIV–) versus infected (HIV+) patients in any of five clinically defined AIDS cohorts (*33*–*39*). A collection of 58 extremely high-risk, exposed uninfected individuals (those with documented receipt of HIV-1–contaminated clotting factor, or frequent unprotected sexual encounters with high-risk partners) (*19*, *21*, *37*, *38*) also showed *CCR2* allele/genotype frequencies not significantly different from those of HIV-1–infected individuals (*40*). The *CCR2* genotype frequencies in each cohort and each HIV-1 infection category conformed to expectations of Hardy-Weinberg equilibrium, further excluding any significant effect of *CCR2-64I* on HIV infection.

A subgroup of 891 seroconverter patients (those whose date of HIV-1 infection could be estimated precisely, because they enrolled in the cohort before converting from HIV-1 antibody-negative to HIV-1 antibody-positive) from four cohorts (MACS, SFCC, MHCS, and ALIVE) (*41*) was analyzed by comparing the rate of progression to AIDS among different *CCR2* genotypes, using a Cox proportional hazards model (*42*). Three endpoints or AIDS definitions, two of which had been stipulated by CDC (*43*) (reflecting increasing morbidity) were considered: (i) AIDS-1993 definition includes HIV-1 infection plus AIDS-defining illness, decline of CD4-T-lymphocytes to ≤200 cells/mm^3 or death; (ii) the more stringent AIDS-1987 definition includes HIV-1 infection plus development of AIDS-defining illness or death; and (iii) death during follow-up for an HIV-1–infected patient (97% of these had AIDS-1993). The results of these analyses are illustrated in Fig. 1 and tabulated in Table 1.

For three cohorts (MACS, MHCS, and SFCC) plus the combined cohort analysis, a consistent 2- to 3-year postponement in median time to AIDS outcome (when the median was reached by each definition) was observed for *CCR2-+/64I* plus *CCR2-64I/64I* genotypes compared with patients homozygous for the normal *CCR2* +/+ allele. The *CCR2* genotypic protection was statistically significant for combined analysis with AIDS-1987, AIDS-1993, and death endpoints. The ALIVE cohort, which is composed of 94% African Americans, did not show a *CCR2* genotype association in the survival analysis (*44*). When Caucasian participants alone were examined, the combined analysis showed significant (or highly significant) postponement of AIDS among *CCR2-+/64I* plus *CCR2-64I/64I* genotypes for each clinical AIDS endpoint definition. The significant relative hazards for Caucasian seroconverters ranged from 0.69 to 0.74, indicating that individuals with a *CCR2-+/+* genotype progress to AIDS 40% more rapidly than patients carrying the *CCR2-64I* allele.

The protective effect exerted by the *CCR2-64I* allele and included genotypes is also apparent from a defined disease category analysis, which allows the inclusion of both seroconverters and HIV-1 seroprevalent individuals (Fig. 2). Each individual cohort plus combined cohorts were divided into relatively rapid progressors versus slow progressors and nonprogressors on the basis of the midpoint of their survival distribution. The *CCR2-64I* allele frequency was consistently lower among rapid progressors to AIDS than in the slow or nonprogressor group who avoid AIDS (by each clinical definition) for greater than 6 to 12.5 years after infection. This analysis included 1746 patients, and in every case, the cohorts showed a 30 to 80% increase in *CCR2-64I* allele frequency in the slow and nonprogressor category (Fig. 2). Similarly, when the frequency of *CCR2-+/64I* genotypes is compared among disease categories, the heterozygote genotype frequency is greater in the slow and nonprogressor categories for 11 of 12 comparisons (four cohorts, three AIDS outcomes; Fig. 2) (*45*).

CCR5-Δ32/+ heterozygotes also demonstrate a slower progression to AIDS based on studies of these same and other cohorts (*19*, *21*, *22*). Because the *CCR2* and *CCR5* loci are very tightly linked (~10 kb apart) on chromosome 3 (*19*, *29*, *30*), we examined the co-occurrence and genotypic independence of *CCR5* and *CCR2* alleles among patients from the same cohorts. Analysis of the two locus genotypes for 2916 patients showed that three genotypes (*CCR2-64I/64I*, *CCR5-+/Δ32*; *CCR2-+/64I*, *CCR5-Δ32/Δ32*; and *CCR2-64I/64I*, *CCR5-Δ32/Δ32*) were absent, indicating that the mutant alleles of the two genes are in strong, perhaps complete, linkage disequilibrium with each other (g = 54.01; 4 df; $P < 0.0001$). This means that *CCR5-Δ32* invariably occurs on a chromosomal haplotype (linked-gene allele combination) that is *CCR2-+*, whereas *CCR2-64I* occurs on a haplotype that contains

Table 1. Survival analysis for progression to AIDS among HIV-1–infected individuals as a function of *CCR2* genotype as in Fig. 1. Seroconverters of all racial groups were analyzed for the ALIVE, MACS, MHCS, and SFCC cohorts and the combination of all the cohorts (*41*). HGDS was excluded since this cohort has only seroprevalent individuals. A χ^2 (1 df), P, and relative hazard (RH) were calculated for each variable in the analysis of AIDS outcomes. Time to AIDS-1993, AIDS-1987, and death were calculated from the midpoint of their last HIV-1–negative and first antibody-positive test (*41*, *53*). Seroconvertors were analyzed in a Cox proportional hazard analysis (*42*). Log Survival Time versus Log Time plots were examined for proportionality and they were reasonably parallel especially after the first few years of outcomes. The significance of the Kaplan-Meier log-rank Chi-square results obtained for the analyses were essentially the same as Cox proportional hazards models (*54*). Analyses were age adjusted for those individuals <30, 30 to 40, or >40 years old.

Cohort	*CCR2-+/+* versus *CCR2-+/64I* or *CCR2 64I/64I*		
	n/events	χ^2/*P* value	RH
	AIDS-1993		
ALIVE	126/57	0.00/0.96	1.02
MACS	403/253	1.97/0.96	0.80
MHCS	183/124	0.76/0.38	0.80
SFCC	176/120	1.74/0.19	0.75
All	888/554	4.42/0.04	0.80
Caucasian	675/449	6.08/0.01	0.74
African American	154/78	0.42/0.52	1.19
	AIDS-1987		
ALIVE	126/37	0.00/0.99	1.00
MACS	406/202	1.99/0.16	0.77
MHCS	183/90	0.05/0.83	0.94
SFCC	176/78	2.08/0.14	0.66
All	891/407	4.41/0.04	0.76
Caucasian	678/335	6.12/0.01	0.69
African American	154/53	0.12/0.73	1.12
	Death		
ALIVE	126/28	0.01/0.94	0.96
MACS	406/153	0.81/0.37	0.83
MHCS	183/70	0.40/0.53	0.79
SFCC	176/54	1.22/0.27	0.67
All	891/305	3.09/0.08	0.76
Caucasian	678/258	4.05/0.04	0.70
African American	154/36	0.01/0.91	1.05

CCR5-+. As a consequence of the tight chromosomal linkage plus alternative haplotypes carrying each mutant allele, the two mutant haplotypes (*CCR2-+*)-(*CCR5-Δ32*) and (*CCR2-641*)-(*CCR5-+*) plus the two-locus normal haplotype (*CCR2-+*)-(*CCR5-+*) can be considered as three alleles of a combined *CCR2/CCR5* compound locus producing three recognizable genotypes designated as: [+/+]; [+/Δ32]; and [641/+] representing (*CCR2-+/+*; *CCR5-+/+*); (*CCR2-+/+*; *CCR5-+/Δ32*); and (*CCR2-+/641*; *CCR5-+/+*) compound genotypes, respectively.

A survival analysis of the effect of both *CCR2* and *CCR5* genotypes on progression to AIDS-1993 and on progression to death for two cohorts (MHCS and MACS, respectively) plus a combined cohort analysis of seroconverters is presented (Fig. 1, C through F). This analysis allows the independent evaluation of *CCR2-641* and *CCR5-Δ32* containing genotypes as separate categories compared to [+/+], but removing the mutant-bearing genotypes at one locus from the analysis of the other. The Cox proportional hazards model revealed highly significant postponement of AIDS onset for *CCR2-641*–containing genotypes and also for *CCR5-+/Δ32* heterozygotes compared to the [+/+] individuals, homozygous for normal alleles at each locus (Fig. 1, C through F).

An analytical summary for each cohort's survival curves is presented (Table 2) for *CCR5-+/Δ32* versus *CCR5-+/+* for all seroconvertors; compound *CCR2-CCR5* locus genotype classes, [+/+] versus [+/Δ32] and [+/+] versus [641/+] (also illustrated in Fig. 2); and combined analysis of *CCR2-+/+*; *CCR5-+/+* versus all mutant genotypes at either locus. These data reaffirm the protective effects of *CCR5* (*17–19*) and show that the *CCR2-641* protection is as strong as and independent of the *CCR5-+/Δ32* influence. The *CCR2* effect increases when the *CCR2-641/+* and *CCR5-+/Δ32* subjects are evaluated as separate categories, because the relative hazards for each cohort in Table 2 (*CCR2* and *CCR5* genotypes separated) are lower than in Table 1, which includes *CCR5-Δ32/+* heterozygotes in the *CCR2-+/+* group. The results are highly significant for combined cohort analyses for each AIDS endpoint (Table 2). The *CCR5-Δ32* protective effect is also strengthened when *CCR2-641*–containing genotypes are evaluated separately (Table 2; *CCR2* and *CCR5* separated versus *CCR5* only). When mutant genotypes for *CCR2* and *CCR5* are combined as a single genetic category (Table 2, *CCR2* and *CCR5* grouped), the statistical significance for genetic influence reaches as low as 6.3×10^{-6} for combined cohort progression to AIDS-1993 (Table 2). Individuals with a mutant genotype at either *CCR2* or *CCR5* show hazards relative to normal of 0.51 to 0.71 in statistically significant analyses.

A defined disease category analysis using compound two-locus genotypes (Fig. 2) shows that the heterozygote frequencies for both *CCR2* and *CCR5* mutant haplotypes were elevated in slow or nonprogressors relative to the rapid progressors. As for the analysis for *CCR2* separately, the frequency of *CCR2* and *CCR5* mutant genotypes was significantly greater among slow progressors for both AIDS-1993 and death with combined cohort analyses plus for the MACS cohort alone. The mutant alleles *CCR2-641* and *CCR5-Δ32*, and genotypes containing them, are less frequent in the more rapid progressors in all three cohorts and when all three AIDS outcomes (17 of 18 comparisons) (*46*) are considered, affirming the protective effects shown in the survival analysis.

The cumulative effects of *CCR2* and *CCR5* mutant alleles over six intervals after HIV-1 infection (Fig. 3) show a marked increase of mutant genotypes for both *CCR2* and *CCR5* among patients that survive without progressing to AIDS over longer periods. The elevated heterozygote frequency for the two loci in long-term survivors (patients surviving more than 16 years and free of AIDS) emphasize the protective effect of both loci. The difference in observed mutant genotype (either *CCR2* or

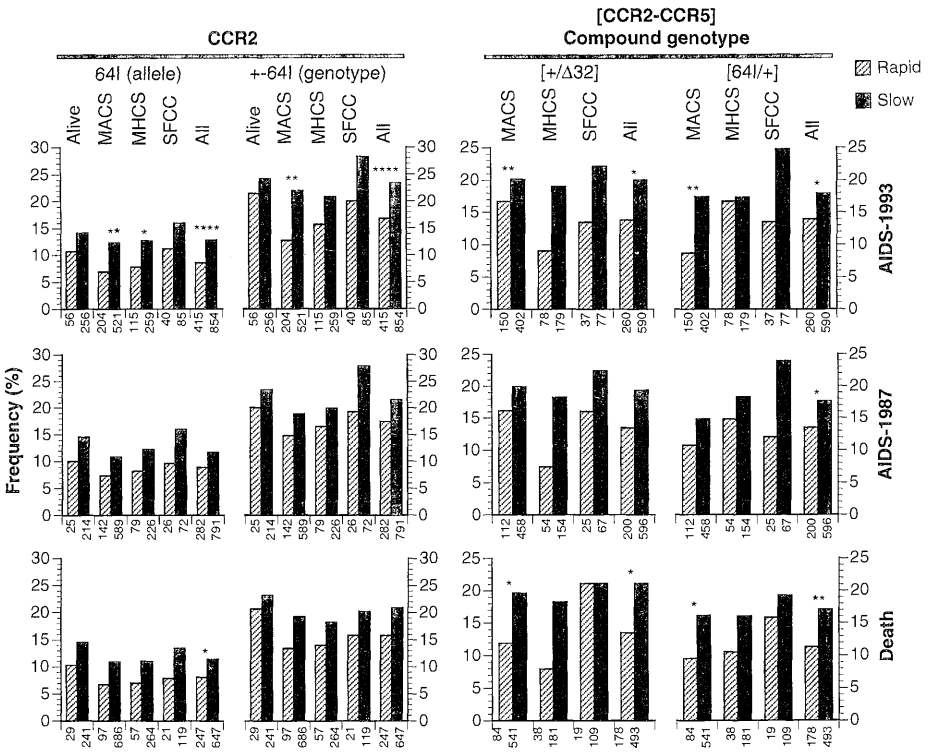

Fig. 2. Analysis of *CCR2-641* allele and genotype frequencies (all races) and *CCR2/CCR5* compound locus genotypes (Caucasians only) with reference to progression to AIDS in each cohort and in all patients based on three AIDS endpoints. Seroconverters who progressed to designated outcomes before the cutoff time (n = 640) were compared with seroconverters plus seroprevalents (n = 660; those who were HIV-1 antibody-positive when enrolled in the cohort) who survived outcome-free for at least that long. Cutoffs, in years, were chosen as the time where approximately half the seroconverters had progressed to the outcome. Times for the outcomes and cohorts were : (for AIDS-1993) MACS, 6 years; MHCS, 9 years; SFCC, 12.5 years; and All, 8 years; (for AIDS-1987) MACS, 7.5 years; MHCS, 11.5 years; SFCC, 14.5 years; and All, 10 years; (for death) MACS, 8 years; MHCS, 11.5 years; SFCC, 14 years; and All, 12 years (*34–38*). Number of patients in each disease category is listed below the bar graph. The χ^2 analyses of alleles and genotypes had one and two degrees of freedom, respectively. P values are indicated by * (<0.05), ** (<0.01), and **** (<0.0001). For *CCR2* allele and genotype (left panel), all patients are included, regardless of *CCR5* genotype. Slow or nonprogressors to AIDS had higher *CCR2-64I* allele frequencies than did more rapid progressors in every cohort-outcome combination. Similarly, in all comparisons, the *CCR2+/64I* and *64I/64I* genotypes are more frequent in the longer-term survivors who have a binomial sign test, $p = 6 \times 10^{-8}$. *CCR2-+/+*; *CCR5-+/Δ32* (right panel) equals [+/Δ32] compound genotype prevalence in defined disease categories and *CCR2-+/64I*; *CCR5-+/+* equals [64I/+] compound genotype prevalence in defined disease categories. *CCR2-64I* and *CCR5-Δ32* allele frequencies were also elevated in slow progressors in analyses of individual and combined cohorts (*54*).

Table 2. Effects of *CCR2* and *CCR5* genotypes on progression to AIDS outcomes in survival analyses of Caucasian seroconverters. Non-Caucasians were excluded because of the absence of *CCR5-Δ32* allele in these populations (*18–22*) plus the potentially different *CCR2* survival (*44*). Cox proportional hazard analyses were performed as described in Table 1. *CCR5;CCR2* genotypes were analyzed in three ways: (i) *+/Δ32* versus *+/+* for *CCR5* alone; (ii) *CCR5* and *CCR2* separated into the compound genotypes [*64I/+*] or [*64I/64I*], and [*+/Δ32*] versus [*+/+*] normal at both loci, illustrated in Fig. 2, C through F; and (iii) *CCR5* and *CCR2* grouped together: [*+/+*] versus [*+/64I*], [*64I/64I*], [*+/Δ32*], or [*64I/Δ32*]. Caucasians from the ALIVE cohort are included in the analyses of combined "All" cohorts. Sample sizes for the three analyses described above vary slightly because: (i) analysis of *CCR5* alone includes some individuals without *CCR2* genotypes; (ii) separate analysis of the CCR2 and CCR5 genotypes excludes 17 (*CCR2-+/64I*; *CCR5-+/Δ32*) double heterozygotes because too few were available for separate analysis, and combining them with either single locus heterozygote category would be arbitrary; and (iii) group analysis of *CCR2* and *CCR5* includes patients with both *CCR2* and *CCR5* genotypes including (*CCR2-+/64I*; *CCR5-+/Δ32*). A complete *CCR2* and *CCR5* separate analysis that included the 17 double heterozygotes as a separate composite genotypic category was performed as well. Relative hazards (*P* values) for the combined cohorts of this analysis including four composite genotypes [*+/+*]; [*64I/64I*] or [*64I/+*]; [*+/Δ32*]; and [*64I/Δ32*] for three outcomes were: AIDS-1993, RH = 0.72 (*P* = 0.07); AIDS-1987, RH = 0.81 (*P* = 0.07); death, RH = 0.79 (*P* = 0.06). RH and *P* values for genotypes other than the double heterozygotes were identical to the values listed in the *CCR2* and *CCR5* separated genotype analysis. The co-occurrence of *CCR2* and *CCR5* protective variants does not appear to confer additional protection, but the double heterozygote is rare in these groups.

Cohort	*CCR5* only			*CCR2* and *CCR5* genotypes separated					*CCR2* and *CCR5* grouped		
	CCR5: [+/+] versus [+/Δ32]			*CCR2*: [+/+] versus [64I/+] *or* [64I/64I]			*CCR5*: [+/+] *versus* [+/Δ32]		[+/+] *versus* [+/Δ32], [64I/+], [64I/Δ32], *or* [64I/64I]		
	n/events	χ^2/*P* value	RH	*n*/events	χ^2/*P* value	RH	χ^2/*P* value	RH	*n*/events	χ^2/*P* value	RH
					AIDS-1993						
MACS	356/228	2.23/0.14	0.77	331/215	3.88/0.05	0.68	3.12/0.08	0.71	339/222	6.25/0.01	0.70
MHCS	169/115	7.75/0.005	0.45	156/107	2.03/0.15	0.66	8.40/0.004	0.39	159/109	8.49/0.004	0.52
SFCC	159/112	1.14/0.28	0.78	153/109	3.95/0.05	0.59	2.35/0.13	0.69	159/112	5.19/0.02	0.64
All	692/456	9.69/0.002	0.68	648/432	10.81/0.001	0.64	13.11/0.0003	0.61	665/444	20.42/0.0000063	0.63
					AIDS-1987						
MACS	359/182	1.67/0.20	0.78	334/173	2.99/0.08	0.67	1.99/0.16	0.74	342/178	4.47/0.03	0.71
MHCS	169/84	1.64/0.20	0.67	156/78	1.54/0.22	0.64	3.14/0.08	0.52	159/80	3.29/0.07	0.62
SFCC	159/73	0.65/0.42	0.79	153/71	4.33/0.04	0.47	1.63/0.20	0.68	159/73	4.72/0.03	0.58
All	695/339	3.78/0.05	0.76	651/322	10.21/0.001	0.58	6.75/0.009	0.67	668/331	13.84/0.0002	0.64
					Death						
MACS	359/142	4.76/0.03	0.60	334/136	2.30/0.13	0.68	6.22/0.01	0.52	342/140	7.25/0.007	0.60
MHCS	169/65	1.08/0.30	0.70	156/60	3.55/0.06	0.37	2.84/0.09	0.50	159/62	4.45/0.04	0.51
SFCC	159/52	0.85/0.36	0.73	153/51	1.31/0.25	0.63	1.00/0.32	0.70	159/52	2.26/0.13	0.64
All	695/259	5.85/0.02	0.67	651/247	7.76/0.005	0.58	9.42/0.002	0.57	668/254	14.50/0.0001	0.59

CCR5) frequency in patients with different times to AIDS from the frequency in all 849 patients (arrow in Fig. 3) allows an estimate of the fraction of rapid progressors (AIDS in <3.5 years) and long-term survivors (≥16 years without progressing to AIDS) that can be attributed to *CCR2/CCR5* genetic factors (*47*). For long-term survivors, the attributable risk conferred by [+/Δ32] and [+/64I] genotypes is 29% for AIDS-1993; 28% for AIDS-1987 and 28% for death. For rapid progressors, the estimate for [+/+] genotypes is 42% for AIDS-1993; 38% for AIDS-1987, and 45% for death.

The protective effect of the *CCR2-64I* allele in delaying the onset of AIDS among prospective cohorts is of similar magnitude and in addition to the protection observed in *CCR5-+/Δ32* heterozygotes. Approximately one quarter of the HIV-1–infected long-term survivors who avoided AIDS for more than 16 years can be attributed to their *CCR2/CCR5* genotype (Fig. 3). However, there are important differences between *CCR2* and *CCR5* that bear on their effects and interpretations.

First, the *CCR2-64I* mutation has no noticeable effect on HIV-1 infection, whereas *CCR5-Δ32* homozygotes are strongly resistant (*19–23*). Second, the *CCR5-Δ32* mutation results in a truncated

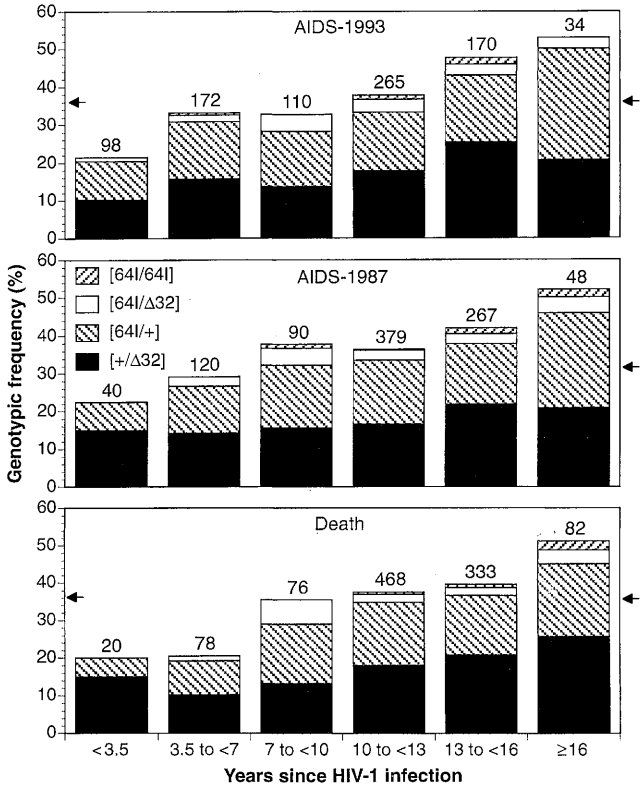

Fig. 3. Frequencies of the protective genotypes ([*+/Δ32*], [*+/64I*], [*64I/Δ32*], and [*64I/64I*]) in six categories of increasing survivorship during HIV-1 infection in Caucasians. Genotypic frequencies were calculated separately for time to AIDS-1993, AIDS-1987, and death, from seroconverters which were <3.5, 3.5 to <7, and 7 to <10 years. In addition, genotypic frequencies were calculated for seroconverters and seroprevalents whose time to the outcome or in study without developing AIDS was 10 to <13, 13 to <16, and ≥16 years. The number of people observed in each category is shown above each column. The average frequency of these variants in Caucasians (36%) is shown as an arrow for comparison of progression categories. Contingency tests of the three common genotypes ([*+/+*],[*+/Δ32*], and [*+/64I*]) were performed for time to AIDS-1993 [χ^2 (10) = 25.44, *P* = 0.005], AIDS-1987 definition [χ^2 (10) = 14.64, *P* = 0.015], and death [χ^2 (10) = 19.27, *P* = 0.04]. Contingency tests of [*+/+*] versus all others for time to AIDS-1993 [χ^2 (5) = 24.22, *P* = 0.0002], AIDS-1987 definition [χ^2 (5) = 14.21, *P* = 0.01], and death [χ^2 (5) = 19.57, *P* = 0.002] were also performed.

protein removing the HIV-1 coreceptor from cells (*17*, *48*), whereas *CCR2-64I* encodes a conservative amino acid substitution. Third, the *CCR5-Δ32* mutation is unique to the Caucasian ethnic group, whereas *CCR2-64I* was found in every ethnic group tested. This suggests that the *CCR2-64I* mutation may be much older in human history than the relatively recent *CCR5-Δ32* deletion (*49*). Fourth, CCR2 and CCR5 bind to different chemokine ligands (CCR2 binds MCP-1, -2, and -3; CCR5 binds RANTES, MIP1α, and MIP1β), although the distribution of CCR2 and CCR5 in cells and tissue is very similar (*50*, *51*).

The mechanism for *CCR2-64I* delay of AIDS in patients is not immediately apparent. Three possible modes of restriction include: (i) *CCR2-64I*–bearing individuals stem viral spread and pathogenesis directly by altering the kinetics of HIV-1 infection. This mechanism gains support from the demonstration that CCR2 serves as a critical coreceptor for certain macrophage-tropic, T cell–tropic, and dual-tropic strains of HIV (*13*, *14*, *51*). (ii) CCR2 concentration and physiological variation can indirectly affect the availability of CCR5 on target cells for HIV-1. There is precedence for cross-regulation of different cytokine receptors and indirect evidence for the same among chemokine receptors (*14*, *52*). Furthermore, the amount of CCR5 on lymphocytes varies markedly among individuals with the same *CCR5* genotype, suggesting that factors other than *CCR5* genotype contribute to CCR5 abundance (*53*). These could be other genes (for example, *CCR2*), ligand binding, or other chemokine receptors. (iii) The *CCR2-64I* mutation could be tracking a linked mutation through linkage disequilibrium. This may involve a regulatory or structural mutation in the *CCR1-5* gene cluster, but not *CCR5-Δ32* because the *CCR2-64I* mutation travels on a different haplotype. We sequenced the complete coding regions of several *CCR5* and *CCR2* mutant homozygotes and did not find any other nucleotide changes (*31*).

The *CCR5-Δ32* and *CCR2-64I* polymorphisms are present in the protective heterozygous or homozygous states in 30 to 40% of people in every ethnic group. The demonstration that having mutant alleles at these genes is protective against progressing to AIDS has important implications for therapy, because chemokine receptors are required cellular ports for HIV-1 cell entry and spread.

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31. The coding region of the *CCR2* gene was amplified with primers CCR2F3: 5′ ATGCT GTCCA CATCT CGTTC and CCR2R3: 5′ CCCAA AGACC CACTC ATTTG (1 to 327 bp); CCR2F4: 5′ ATTAC TCTCC CATTG TGGGC and CCR2R4: 5′ GGAAA TTATT CCATC CTCGTG (277 to 604 bp); CCR2F1: 5′ TTCTG TTTAT GTCTG TGGCC and CCR2R6: 5′ GATTG ATGCA GCAGT GAGTC (555 to 904 bp); and CCR2F5: 5′ CCAAG CCACG CAGGT GACAG and CCR2R5: 5′ TTATA AACCA GCCGA GACTT (852 to 1083 bp). The products were resolved on 6% acrylamide gels (37.5:1 acrylamide:bis-acrylamide) containing 10% glycerol at room temperature. The entire *CCR2* coding region was examined by SSCP in 127 individuals. One common synonymous nucleotide substitution was discovered (N260N; f = 0.46), and three additional variants (*CCR2*: V52V, P47L, and S87A) were found in fewer than 1% of chromosomes. *CCR2-64I* homozygotes (one Caucasian and one African American) were sequenced through the entire coding region of *CCR2* (including the *CCR2A* exon) and *CCR5*, as well as 500 bp of the upstream region of both genes. No nucleotide alterations were identified. More than 100 individuals have been screened by SSCP across the *CCR5* gene. A total of 16 additional variants in the coding region have been identified (M. Carrington *et al.*, in preparation). All of these variants are rare (≤4%) and none is found exclusively on the *CCR2-64I* haplotype.
32. Genotypes were determined by SSCP and with a PCR-RFLP assay using a Bsa BI site introduced into the PCR primer next to the C-T transition that encodes the *CCR2-64I* polymorphism. Amplification with the primers CKR2_1A: 5′TTGTGGGCAACAT-GaTGG, which has a cytosine substituted with an adenine (in lower case) and CKR2_1Z: 5′GAGC-CCACAATGGGAGAGTA generated a 128-bp product. Digestion with Bsa BI yields 110- and 18-bp fragments when an isoleucine was present instead of valine at position 64 in *CCR2*. These products were genotyped on 4% AMRESCO 3:1 biotechnology-grade agarose TBE gels.
33. The cohorts and date of first enrollments were: the AIDS Link to the Intravenous Experience (ALIVE-1988) (*34*), Human Growth and Development (HGDS-1989) (*35*), Multicenter AIDS Cohort Study (MACS-1984) (*36*), Multicenter Hemophiliac Cohort Study (MHCS-1985) (*37*) and San Francisco City Clinic Study (SFCC-1978) (*38*). Patient genotypes were determined from DNA extracted from immortal lymphoblastoid B-cell lines established for each patient (*19*). The HGDS cohort did not include seroconvertor patients.
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39. Of 2993 typed individuals in five cohorts (*33*), 445 were high-risk uninfected and 2548 were HIV-1 infected. No significant difference in *CCR2* allele or genotype frequency was apparent between high-risk exposed uninfected individuals versus HIV-1–infected patients (alleles: $\chi^2 = 0.31$; $P = 0.86$; genotypes g = 1.12; $P = 0.57$). The same lack of difference was observed when individual cohorts were examined.
40. G = 0.44; $P = 0.5$. Only Caucasian individuals were examined in this analysis. *CCR5-Δ32/Δ32* homozygotes were excluded to remove known protective effects (*19–22*) because none of these would have *CCR2-64I* genotypes due to linkage disequilibrium (see text).
41. Seroconverter patients included 891 subjects with a maximum interval of 3 years between an HIV-1 antibody–negative test date and their first HIV-1 antibody–positive test date. Seroconversion date was the midpoint between the last HIV-1 antibody–negative and first positive clinic visits. Ninety patients enrolled in the SFCC study before 31 December 1980 were included using imputed seroconversion dates based on their date of first HIV-1 antibody positive, because the likelihood of infection before 1 January 1978 (a 3-year window of infection) was extremely low (≤0.01). Seroconversion dates for the imputed SFCC subjects were set at 60 days, 120 days, and 180 days before the date for first antibody positive visit for patients enrolled in 1978, 1979, and 1980, respectively (*38*).
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44. The ALIVE cohort is composed of 94% African Americans, and the large racial difference between ALIVE and other cohorts (MACS, MHCS, SFCC, and HGDS are composed of 6%, 13%, 4%, and 10%

African Americans, respectively) may contribute to the absence of a survival effect of *CCR2* genotypes seen in Table 1. Alternatively, the results may reflect the relatively shorter period of follow-up, because the ALIVE began enrollment in 1988 (*33*). In support of the latter explanation is the elevated *CCR2-+/64I* "protective" genotype frequency among ALIVE slow progressors relative to rapid progressors to AIDS for the three AIDS endpoints (Fig. 2). Because this result is not statistically significant, the conclusion remains tentative until longer follow-up of African American cohorts becomes available.

45. Because the four cohorts show no significant differences in CCR2 allele or genotype frequency, they were pooled to test for significant differences between rapid and slow or nonprogressors, which were apparent. In addition, *CCR2-64I* containing genotypes were higher in all cohorts for 24 of 24 comparisons (two genotypes, four cohorts, three AIDS outcomes). Because these comparisons are interdependent, we applied a sign test to eight comparisons (four cohorts, two genotypes) to detect $P \leq 0.004$.
46. When a conservative sign test to three genotypes and three cohorts was applied for only one outcome, there was a significant excess of [+/Δ32], [64I/+], and [64I/64I] (Fig. 2 and (*54*) for [64I/64I]) ($P = 0.002$).
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AIB1, a Steroid Receptor Coactivator Amplified in Breast and Ovarian Cancer

Sarah L. Anzick, Juha Kononen, Robert L. Walker, David O. Azorsa, Minna M. Tanner, Xin-Yuan Guan, Guido Sauter, Olli-P. Kallioniemi, Jeffrey M. Trent, Paul S. Meltzer*

Members of the recently recognized SRC-1 family of transcriptional coactivators interact with steroid hormone receptors to enhance ligand-dependent transcription. AIB1, a member of the SRC-1 family, was cloned during a search on the long arm of chromosome 20 for genes whose expression and copy number were elevated in human breast cancers. AIB1 amplification and overexpression were observed in four of five estrogen receptor–positive breast and ovarian cancer cell lines. Subsequent evaluation of 105 unselected specimens of primary breast cancer found AIB1 amplification in approximately 10 percent and high expression in 64 percent of the primary tumors analyzed. AIB1 protein interacted with estrogen receptors in a ligand-dependent fashion, and transfection of AIB1 resulted in enhancement of estrogen-dependent transcription. These observations identify AIB1 as a nuclear receptor coactivator whose altered expression may contribute to development of steroid-dependent cancers.

Gene amplification is a frequent mechanism of increased gene expression in human cancers. In breast cancer, commonly amplified chromosomal regions are derived from 17q12, 8q24, and 11q13 and encode *erbB-2*, c-*myc*, and cyclin D1, respectively (*1*). Molecular cytogenetic studies of breast cancers have revealed the occurrence of additional regions of increased DNA copy number whose target genes are unknown, including 20q (*2*). Recently, we used chromosome microdissection and hybrid selection to clone expressed sequences from 20q in an attempt to identify genes of biological significance (*3*). In this fashion, we isolated partial cDNAs for a candidate target gene termed AIB1 (amplified in breast cancer-1), which was ubiquitously expressed in normal human tissues (*3*). We now report that AIB1 is a member of the SRC-1 family of nuclear receptor (NR) coactivators, that it is amplified and overexpressed in breast and ovarian cancer cell lines as well as in breast cancer biopsies, that it interacts with estrogen receptor (ER), and that it functions to enhance ER-dependent transcription.

Sequence analysis of partial AIB1 cDNAs provided the first evidence of similarity between AIB1 and the SRC-1 family. SRC-1 and TIF2 are closely related transcriptional coactivators recently isolated on the basis of their affinity for NRs (*4*, *5*). Although the mechanism of action of SRC-1 has not been completely elucidated, in addition to interacting with NRs, SRC-1 binds to the transcriptional integrators CREB binding protein (CBP) and the closely related p300, which interact directly with the basal transcription machinery (*6*).

To further characterize AIB1, the full-length cDNA was cloned and sequenced (*7*), revealing an open reading frame that encodes a protein of 1420 amino acids with a predicted molecular mass of 155 kD (Fig. 1). Database searches with BLASTP identified a highly significant similarity of AIB1 with TIF2 (45% amino acid identity) and SRC-1 (33% amino acid identity) (*8*). Like TIF2 and SRC-1, AIB1 contains a basic helix-loop-helix (bHLH) domain preceding a PAS (Per/Arnt/Sim) region, serine- and threonine-rich regions, and a charged cluster. There is also a glutamine-rich region that, unlike SRC-1 and TIF2, contains a polyglutamine tract. AIB1 also contains three copies of the conserved LXXLL motif (L = leucine, X = any amino acid), which was recently demonstrated to be critical to the coactivator receptor interaction (*9*, *10*).

Because of this strong sequence similarity, we evaluated the amplification and expression of AIB1 in a series of ER-positive and -negative breast and ovarian cancer cell lines (*11*). AIB1 gene copy number was determined by fluorescence in situ hybridization (FISH) (Fig. 2). High-level amplification of AIB1 (>20-fold) was observed in three ER-positive breast carcinoma cell lines (BT-474, MCF-7, and ZR75-1) and in one ovarian carcinoma cell line (BG-1) (Fig. 2, A and B). Overall, AIB1 was amplified in four of five ER-positive cell lines tested and in zero of six ER-negative cell lines (*12*). To determine whether AIB1 amplification also occurred in uncultured cells from tumor biopsies, we screened 105 unselected breast cancer specimens for AIB1 amplification by FISH. Ten specimens of primary tumors (9.5%) demonstrated amplification of AIB1, although the amplification levels were not as high as in the cell lines (*13*).

Previous interphase FISH studies have

S. L. Anzick, J. Kononen, R. L. Walker, D. D. Azorsa, X.-Y. Guan, O.-P. Kallioniemi, J. M. Trent, P. S. Meltzer, Laboratory of Cancer Genetics, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, USA.
M. M. Tanner, Laboratory of Cancer Genetics, Institute of Medical Technology, University of Tampere and Tampere University Hospital, Post Office Box 607, FIN-33101 Tampere, Finland.
G. Sauter, Institute for Pathology, University of Basel, Schönbeinstrasse 40 4003 Basel, Switzerland.

*To whom correspondence should be addressed. E-mail: pmeltzer@nhgri.nih.gov