strips. The strips were washed, incubated with a 1 5200 dilution of alkaline phosphatase–labeled goat antibodies to mouse IgG and developed with nitroblue tetrazolium 5-bromo 4-chloro-indolyl phosphate.

22. The OspA-N40 gene amplified by PCR was cloned into Eco RI and Bam HI sites of pGEX-2T (Pharmacia) in frame with the glutathione *S*-transferase gene The recombinant plasmid was used to transform *E. coli* strain JM109. Recombinant fusion protein production was induced with 1 mM IPTG. Cells were washed in PBS, suspended in 1/100 the volume PBS with 1% Triton and lysed by sonication. The lysate was centrifuged at 13,000 rpm and the supernatant was placed over a glutathione–Sepharose 4B column (Pharmacia). The glutathione *S*-transferase OspA-N40 fusion protein was eluted with 5 mM glutathione. Purified glutathione *S*-transferase was prepared in a similar fashion from *E coli* transformed with pGEX-2T.
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Probing Immunosuppressant Action with a Nonnatural Immunophilin Ligand

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The immunosuppressants FK506 and rapamycin bind to the same immunophilin, FK506 binding protein (FKBP), and inhibit distinct signal transduction pathways in T lymphocytes. A nonnatural immunophilin ligand, 506BD, which contains only the common structural elements of FK506 and rapamycin, was synthesized and found to be a high-affinity ligand of FKBP and a potent inhibitor of FKBP rotamase activity. Whereas 506BD does not interfere with T cell activation, it does block the immunosuppressive effects of both FK506 and rapamycin. Thus, the common immunophilin binding element of these immunosuppressants, which is responsible for rotamase inhibition, is fused to different effector elements, resulting in the inhibition of different signaling pathways. Inhibition of rotamase activity is an insufficient requirement for mediating these effects.

IN ADDITION TO THEIR THERAPEUTIC potential, the immunosuppressive agents cyclosporine A (CsA), FK506, and rapamycin are useful probes of the T cell activation process (Fig. 1) (*1–4*). CsA and FK506 inhibit a T cell receptor (TCR)–mediated signal transduction pathway that results in the transcription of early T cell activation genes, including interleukin-2 (IL-2). Despite the structural similarity to FK506, rapamycin has no effect on the production of IL-2, yet potently inhibits the response of the T cell to activation through the IL-2 receptor (*2*). Although they specifically inhibit different T cell activation events, rapamycin and FK506 have also been shown to inhibit each other's actions (*3, 4*).

These three immunosuppressants bind to proteins termed immunophilins with high affinity. CsA (K_d = 30 nM) is bound by human cyclophilin (*5*), and both FK506 (K_d = 0.4 nM) and rapamycin (K_d = 0.2 nM) are bound by human FK506 binding protein (FKBP) (*4, 6, 7*). Although unrelated in amino acid sequence (*8*), cyclophilins and FKBPs catalyze the interconversion of the cis- and trans-rotamers of the peptidyl-prolyl amide bond of peptide and protein substrates. These rotamase enzymes are inhibited by their respective immunosuppressant ligands; FK506 and rapamycin do not inhibit cyclophilin and CsA does not inhibit FKBP (*6–8*).

The ability of FK506 and rapamycin to inhibit each other's actions suggests a role for either a single immunophilin or separate immunophilins that share a common receptor site (hereafter referred to as the "common receptor site") in mediating the actions of these drugs (*4*). Rapamycin and FK506 appear to be mimics of a leucyl-prolyl twisted amide bond (*9, 10*), and may therefore mimic the action of an endogenous ligand that contains a peptidyl (perhaps leucyl)-prolyl dipeptide fragment as a structural motif for the recognition of an immunophilin regulatory site (the "common receptor site"). We explored the hypothesis that FK506 and rapamycin are comprised of a common immunophilin binding element that is fused to distinct effector elements that determine the T cell activation signaling pathway with which the drug will interfere.

We designed a molecule, termed 506BD, to adopt a geometry similar to that of the putative immunophilin binding domain found in FK506 (Fig. 1). To emulate the conformation of the cis-rotamer of FK506 [observed in the solid state by x-ray diffractometry (*11*)], a scaffolding element was installed to limit the conformational freedom of 506BD. 506BD lacks the putative effector elements of both rapamycin and FK506. Therefore, 506BD should not exhibit the T cell inhibitory properties that are characteristic of either FK506 or rapamycin, yet, by virtue of its anticipated immunophilin binding properties, 506BD should inhibit the actions of both FK506 and rapamycin.

The isopropyl substituent of 506BD was included to introduce a conformational constraint, involving the avoidance of a −gauche/+gauche (*syn*-pentane) local conformation, that is present in FK506. The trans-enoate spacer was chosen to fix the distance of the attachment sites in the same geometry observed in the solid-state conformation of FK506. We also synthesized a truncated 506BD that lacked the cyclohexyl moiety (des CyH-506BD) to assess its role.

An efficient synthetic pathway was developed that provided 506BD in useful quantities and in a stereochemically homogeneous form (Fig. 2). The asymmetric synthesis began with the six-carbon chain **1**, which had previously been prepared from mannitol in four steps (*12*). The pyranose **2** was obtained as a mixture of anomers from **1** in four steps; only the major β-anomer is shown. Stereoselective introduction of the allylic methoxyl group in **3** followed subjection of **2** to a one-pot reduction-Grignard addition sequence (*13*), whereas the benzyloxy bearing stereocenter in **4** was introduced by a stereoselective ketone reduction (*14*). After ring opening and homologation of **4** into **5**, the three dioxolane stereocenters of **6** were produced in a stereospecific fashion by an asymmetric osmylation reaction (*15*) and then acetal protection. The coupling partner **8** was prepared by metalation and hydroxyalkylation of the vinyl bromide **7**, which we have reported earlier in the course of a total synthesis of FK506 (*16*). The coupling of **6** and **8** was achieved by amide bond formation, while macrocyclization of the resultant **9** was accomplished by an intramolecular Horner-Emmons reaction (*17*) that produced **10** as a single E-olefin diastereomer. Following selective deprotection of the cyclic acetal and oxidation of the resultant diol to a diketone, a final deprotection reaction resulted in a transannular hem-

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iketalization to afford the target system. 506BD was isolated with the seven-membered hemiketol isomer in a 2:1 ratio. Although these compounds did not interconvert in $CDCl_3$ and could be separated by high-performance liquid chromatography (HPLC), they underwent a more facile equilibration in polar solvents, including H_2O. In a related series of transformations, targets lacking the appended trisubstituted olefin and cyclohexyl moieties (des CyH-506BD) and the macroring were also prepared.

The analysis of the solution conformation of 506BD was undertaken by ^1H NMR methods. The cis-rotamer (>8:1) predominates by comparison of the chemical shifts of the hydrogens adjacent to nitrogen on the pipecolinyl rings of 506BD and the corresponding hydrogens of the major cis- and minor trans-rotamers (3:1 ratio in $CDCl_3$) of FK506. The similarity in conformation of the pyranose rings and neighboring appendages in FK506 and 506BD was evident upon comparison of the vicinal coupling constants (Fig. 3). These data suggest that the solution conformation of 506BD is similar to that anticipated by application of the previously described design principles.

Equilibrium binding and rotamase inhibition studies with human recombinant FKBP (*8*) demonstrated a high-affinity interaction of 506BD with this immunophilin. In a competition binding assay (*5*), 506BD could effectively displace (K_d = 20 nM) [^{3}H]dihydro-FK506 from FKBP. 506BD inhibited the rotamase activity of FKBP with K_i = 5 nM (Fig. 3C). By comparison to the binding (K_d = 1.8 μM) and inhibition (K_i = 0.3 μM) of des CyH-506BD, the cyclohexyl containing appendage of 506BD, and thus presumably of FK506 and of rapamycin, contributes significantly to the binding to FKBP. 506BD also bound to FKBP with higher affinity than a closely related acyclic analogue of 506BD (*18*), indicating the importance of the macroring constraint. As expected, 506BD had no measurable affinity to the CsA binding immunophilin cyclophilin.

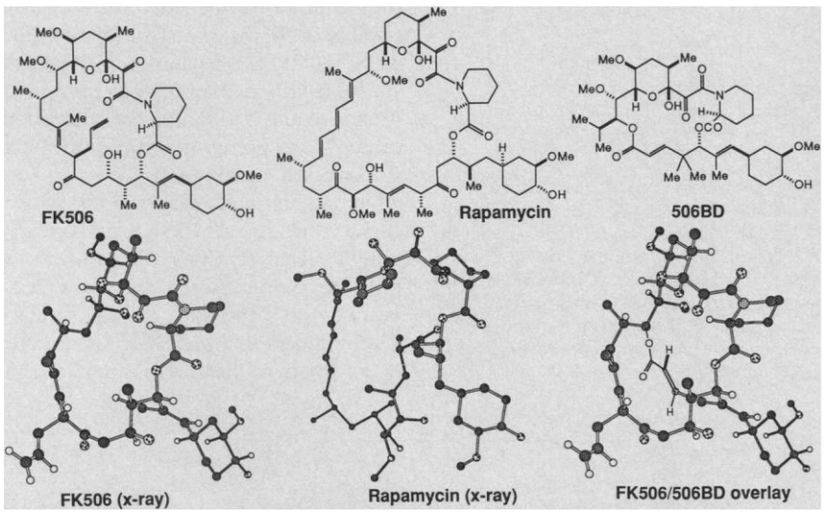

Fig. 1. Structural analysis of immunophilin ligands. Chemical structures for FK506, rapamycin, and 506BD are shown. Three-dimensional structures for FK506 and rapamycin were obtained from solid-state x-ray crystal structures, whereas the three-dimensional representation of 506BD is derived from the crystal structure of FK506. The trans-enoate scaffolding element of 506BD is shown superimposed on the crystal structure of FK506, demonstrating how the two anchor points of the FK506 binding domain are fixed in three-dimensional space.

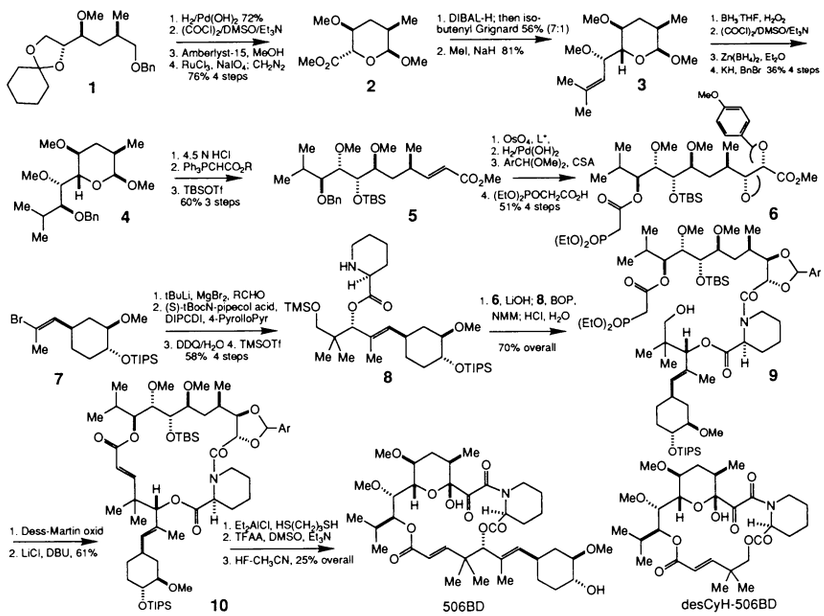

Fig. 2. Synthetic pathways to 506BD and des CyH-506BD. Abbreviations: DIBAL-H, diisobutylaluminum hydride; L*, dihydroquinidine acetate; RCHO, 2,2-dimethyl-3-(4-methoxybenzyloxy)propanal; DIPCDI, diisopropylcarbodiimide; DDQ, 2,3-dichloro-5,6-dicyano-1,4-benzoquinnone; BOP, benzotriazol-1-yloxy-tris(dimethylamino)-phosphonium hexafluoro-phosphate; NMM, 4-methylmorpholine; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; TFAA, trifluoroacetic anhydride; TIPS, triisopropylsilyl; and TBS, tert-butyldimethylsilyl.

The biological effects of 506BD were first tested with a murine antigen-specific T cell hybridoma (*19, 20*) that responds to stimulation of its antigen receptor by producing IL-2 (Fig. 4A). Incubation with FK506, 506BD, or a control nonstimulatory monoclonal antibody (MAb) to Thy 1 did not induce the production of IL-2. Unlike 1 nM FK506, 1 μM 506BD did not prevent IL-2 production upon stimulation with a MAb to CD3 (anti-CD3), which triggers the TCR-CD3 complex. However, 1 μM 506BD largely reversed FK506-mediated inhibition of IL-2 production.

TCR-CD3 mediated stimulation of the hybridoma also results in activation-induced cell death, or apoptosis, characterized by DNA fragmentation to multimers of 180 base pairs (Fig. 4B). Incubation of the hybridoma with anti-CD3 induced apoptosis; the induction of apoptosis was prevented, however, in the presence of 1 nM FK506. While 2 μM 506BD alone did not effect anti-CD3–mediated DNA fragmentation, it effectively inhibited the ability of FK506 to prevent apoptosis. CsA also prevents the induction of apoptosis (*21*); however, 2 μM 506BD was unable to inhibit the effects of CsA. Thus, the ability of 506BD to reverse inhibition of IL-2 production and activation-induced cell death is specific for FK506 and, like rapamycin, distinguishes the biological actions of FK506 and CsA.

Proliferation of an IL-2 dependent murine T cell clone, CTLL-20 (*22*), stimulated by exogenous IL-2 in the presence or ab-

sence of rapamycin and 506BD, was tested. Rapamycin inhibited IL-2–induced proliferation, whereas 506BD had no effect. However, a 1000- to 2000-fold excess concentration of 506BD effectively reversed rapamycin-induced inhibition of IL-2–dependent proliferation (Fig. 4C).

Both FK506 and rapamycin inhibit stimulation of T cell proliferation induced by anti-CD3 MAb, albeit by different mechanisms (discussed above). To confirm that 506BD could reverse the inhibition of both FK506 and rapamycin, we tested anti-CD3–stimulated proliferation of human peripheral blood mononuclear cells (PBMC). Whereas 1 μM 506BD alone did not inhibit proliferation of PBMC or reverse the inhibition of proliferation mediated by CsA, it prevented the inhibition mediated by either 0.5 nM FK506 or 0.5 nM rapamycin. Indeed, when both FK506 and rapamycin were added to culture, their inhibition of proliferation was increased. This inhibition was also reversed by the addition of 1 μM 506BD (Fig. 4D).

In each of these assays, a greater than 100-fold excess of 506BD was required to inhibit the actions of FK506 and rapamycin. This is reminiscent of the mutual inhibition exhibited by FK506 and rapamycin (*3, 4*); in these studies a greater than tenfold excess of one drug is required to inhibit the actions of the other. Our explanation of this phenomenon (*4*) is based on the abundance of FKBP, which is present at ~5 nM in the cytosol of Jurkat cells (*23*). As these drugs completely inhibit signal transmission pathways at 1 nM, the immunophilin-drug complex appears to be responsible for the inhibition. The abundant immunophilin serves to buffer the ability of these immunophilin ligands

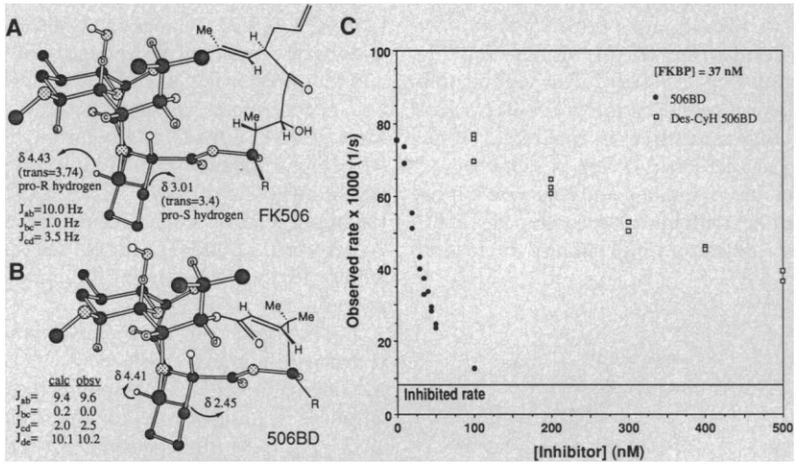

Fig. 3. Conformational analysis of FK506 and 506BD. (**A**) Solution-state conformation of FK506 substructure based on ^{1}H NMR spectroscopic characterization (500 MHz, $CDCl_3$). (**B**) Conformation of 506BD substructure based on ^{1}H NMR spectroscopic analysis (500 MHz, $CDCl_3$). Calculated coupling constants obtained from MM2-minimized 506BD structure based originally on the FK506 crystal structure. Similarities to the local conformation of FK506 are highlighted. (**C**) 506BD and des CyH-506BD inhibition of the rotamase activity of FKBP; data gathered with use of the modified assay of Fischer (*4, 8*). Start and end points for the spectrophotometric analyses were chosen to minimize the standard deviation in the rate constant over six half-lives. The reactions were run in duplicate for 506BD and in triplicate for des CyH-506BD.

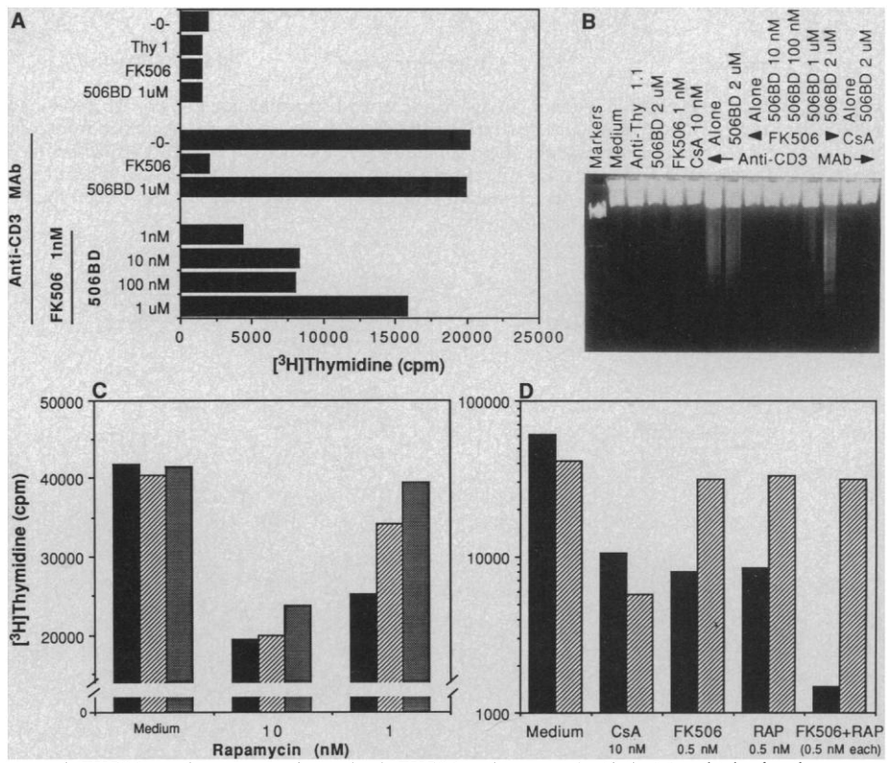

Fig. 4. The inhibitory properties of immunophilin ligands. (**A**) 506BD reversed FK506-mediated inhibition of IL-2 production. The antigen-reactive murine T cell hybridoma 16.CD2-15.20 (*19, 20*) (10^6 cells per well) was cultured in a 24-well plate, in the absence or presence of a nonstimulatory MAb to Thy 1 or the activating MAb to murine CD3 145-2C11 (*25*), in the absence or presence of 1 nM FK506 or 506BD at the indicated concentrations. At 20 hours, culture supernatants were harvested and assayed for the presence of IL-2 by their ability to support the proliferation of an IL-2 dependent murine T cell line, CTLL-20 (*22*), as described (*4, 19*). (**B**) Whereas it has no effect alone, 506BD reversed the FK506-, but not CsA-, mediated inhibition of activation-induced cell death. Cells of the murine hybridoma 16.CD2-15.20 were cultured as described in (A). After 20 hours, DNA was extracted and electrophoresed on a 2% agarose gel as described (*4, 21*). Anti-CD3–stimulated cell death resulted in fragmentation of DNA to characteristic multimers of 180-base pair fragments. (**C**) 506BD reversed rapamycin-mediated inhibition of IL-2–dependent proliferation of CTLL-20. The IL-2–dependent T cell line CTLL-20 (5×10^3 cells per well) was cultured with human recombinant IL-2 (20 U) in the absence or presence of rapamycin or 506BD at the indicated concentrations. Proliferation was assessed by the incorporation of [^{3}H]thymidine in a 6-hour pulse after an 18-hour incubation as described (*4*). Black bar, medium; striped bar, 1 μM 506BD; gray bar, 2 μM 506BD. (**D**) 506BD effectively reversed FK506- and rapamycin-, but not CsA-, mediated inhibition of proliferation of PBMC stimulated with anti-CD3. Freshly isolated PBMC (10^5 cells per well) were stimulated with either anti-CD3 (OKT3) at a 1:40,000 dilution of ascites fluid in the presence of medium, 10 nM CsA, 0.5 nM FK506, 0.5 nM rapamycin, or both FK506 and rapamycin (0.5 nM each) in the absence or presence of 1 μM 506BD. Cells were cultured in triplicate and harvested at 72 hours after an 8-hour pulse with [^{3}H]thymidine. Black bar, medium; striped bar, 1 μM 506BD. Experiments were performed from two to four times; a representative for each is shown (*26*).

to reverse the inhibitory properties of the immunosuppressants. In the studies reported herein, the approximately tenfold lower affinity of 506BD for FKBP, relative to FK506 and rapamycin, results in antagonism at tenfold higher concentrations than that required in the FK506/rapamycin antagonism studies (*24*). The excess of FKBP in the cell at effective drug concentrations also provides independent evidence that the inhibition of rotamase activity of FKBP is not central to the biological actions of these drugs.

These studies demonstrate that inhibition of the rotamase activity of FKBP is insufficient for mediating the biological effects of either FK506 or rapamycin. The ability to dissect the structural elements involved in FKBP binding by FK506 and rapamycin from those elements that are essential for their specific biological actions supports the view of these drugs as dual-domain agents. The effects of these drugs on separate T cell activation pathways is probably the result of a common mode of drug binding and subsequent interactions of the resultant complexes with different target molecules. The specificity of these latter interactions is determined by the precise geometry of the immunophilin/drug complexes, which in turn is determined by the unique effector elements found on each of these immunosuppressants. These studies suggest that versatile immunophilin binding modules can be constructed that will allow attachment of different effector cassettes in order to inhibit different signal transduction pathways.

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24. We note that, since only the mutual inhibition of FK506 and rapamycin, and not the T cell activation inhibition mediated by these agents, is subject to the "buffer effect," synergistic inhibition is observed (Fig. 4D). Thus, FK506 and rapamycin may yet be considered as a viable combination for therapeutic applications.
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Detection of *bcr-abl* Fusion in Chronic Myelogeneous Leukemia by in Situ Hybridization

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Chronic myelogeneous leukemia (CML) is genetically characterized by fusion of the *bcr* and *abl* genes on chromosomes 22 and 9, respectively. In most cases, the fusion involves a reciprocal translocation t(9;22)(q34;q11), which produces the cytogenetically distinctive Philadelphia chromosome (Ph^1). Fusion can be detected by Southern (DNA) analysis or by in vitro amplification of the messenger RNA from the fusion gene with polymerase chain reaction (PCR). These techniques are sensitive but cannot be applied to single cells. Two-color fluorescence in situ hybridization (FISH) was used with probes from portions of the *bcr* and *abl* genes to detect the *bcr-abl* fusion in individual blood and bone marrow cells from six patients. The fusion event was detected in all samples analyzed, of which three were cytogenetically Ph^1-negative. One of the Ph^1-negative samples was also PCR-negative. This approach is fast and sensitive, and provides potential for determining the frequency of the abnormality in different cell lineages.

FUSION OF THE PROTO-ONCOGENE *abl* from the long arm of chromosome 9 with the *bcr* gene of chromosome 22 is a consistent finding in CML (*1–3*). This genetic change leads to formation of a *bcr-abl* transcript that is translated to form a 210-kD protein present in virtually all cases of CML (*4–6*). This fusion can be detected by Southern analysis for *bcr* rearrangements (*7–9*) or by in vitro amplification (PCR) of a complementary DNA (cDNA) transcript copied from CML mRNA (*10–16*). In approximately 95% of cases the fusion gene results from a reciprocal translocation involving chromosomes 9 and 22, producing a cytogenetically distinct small acrocentric chromosome called Ph^1 (*17–22*). In the remaining cases the genetic rearrangement is more complex, and the involvement of the *bcr* and *abl* regions of chromosomes 9 and 22 may not be apparent during analysis of banded metaphase chromosomes. Southern, PCR, and banding analysis provide complementary but incomplete information on CML. They do not permit a genetic analysis on a cell by cell basis in a format in which the results can be related to cell phenotype as judged by morphology or other markers. Thus, assessment of the distribution of the CML genotype among cells of different lineages and maturity is not possible.

We describe here the use of two-color fluorescence in situ hybridization (FISH) for detection of the *bcr-abl* fusion in metaphase and interphase cells. The *bcr-abl* fusion status can be determined rapidly for each cell

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