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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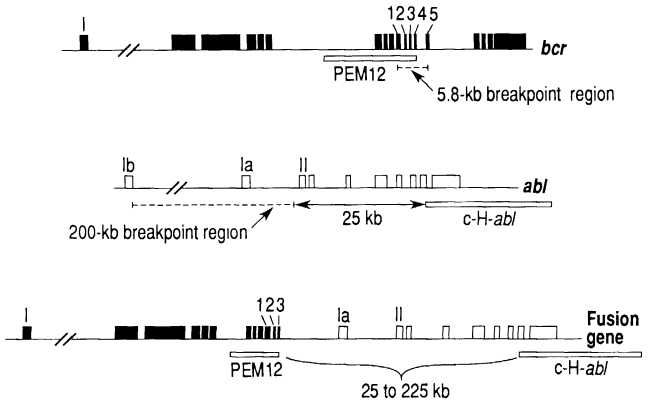

Fig. 1. Schematic representation of the *bcr* gene on chromosome 22, *abl* gene on chromosome 9 (*27*), and the *bcr-abl* fusion gene. Exons of the *bcr* gene are depicted as solid boxes. I refers to the first exon; the numerals 1 to 5 refer to the exons within the breakpoint cluster region, here indicated by the dashed line [map adapted from (*28*)]. The approximate location of the 18-kb phage PEM12 probe (*bcr* probe) is indicated by the open horizontal bar. Since the majority of breakpoints in CML occur between exons 2 and 4, 15 kb or more of target for PEM 12 will remain with the fusion gene. Exons of the *abl* gene are depicted as open vertical bars (not to scale). The numerals Ia and Ib refer to the alternative first exons, and II to the second exon [map adapted from (*28*)]. Exon II is approximately 25 kb upstream of the end of the 28-kb cosmid c-H-*abl* (*abl* probe). All CML breakpoints occur upstream of exon II, usually between exons Ib and Ia, within a region that is approximately 200 kb in length. Thus, c-H-*abl* will always be 25 to 200 kb away from the fusion junction. Therefore, on the *bcr-abl* fusion gene PEM12 will always lie at the junction and c-H-*abl* will be separated from it by 25 to 200 kb.

in a population without cell culture, and the genotype can be correlated directly with cell phenotype. This assay uses probes for sequences that flank the fusion in essentially all cases of CML (Fig. 1). One probe, c-H-*abl* (the *abl* probe), was a 35-kb cosmid (pCV105) clone selected to be telomeric to the 200-kb region of *abl* between exons Ib and II, in which the breaks occur on chromosome 9 (*9, 23*). Hybridization of the *abl* probe to normal metaphase cells yielded signals on both chromatids of chromosome 9 near the telomere of 9q. The second probe, PEM12 (*bcr* probe), was an 18-kb phage clone (in EMBL3) that contains part of, and extends centromeric to, the 5.8-kb breakpoint cluster region of the *bcr* gene on chromosome 22, in which almost all CML breakpoints occur. The *bcr* probe gave hybridization signals on the proximal long arm of chromosome 22 in normal metaphase cells. Two-color FISH was carried out with a biotin-labeled *abl* probe, detected with the fluorochrome Texas red, and a digoxigenin-labeled *bcr* probe, detected with the green fluorochrome fluorescein isothiocyanate (FITC) (*24*). Hybridization of both probes was observed simultaneously with a fluorescence microscope equipped with a double band-pass filter set (Omega Optical).

Two-color FISH with the *abl* (red) or *bcr* (green) probe to normal G_1 interphase nuclei typically resulted in two red and two green hybridization signals that were well separated and distributed randomly around the nucleus. A few cells showed two doublet hybridization signals, probably a result of hybridization to both sister chromatids of both homologs in cells that had replicated this region of DNA (those in the S or G_2 phase of cell cycle).

The genetic rearrangement of CML brings the binding sites of the *bcr* and *abl* probes to within 25 to 225 kb of each other on an abnormal chromosome, depending on the exact positioning of the breakpoints in the leukemic clone (Fig. 1). Dual-color hybridization with *abl* and *bcr* probes to interphase CML cells resulted in one red and one green hybridization signal located at random in the nucleus, and one red-green doublet signal in which the separation between the two colors was <1 μm. In some cases, the red-green doublet appeared yellow (Fig. 2). We ascribe the randomly located red and green signals to hybridization to the *abl* and *bcr* genes on the normal chromosomes, and the red-green doublet signal to hybridization to the *bcr-abl* fusion gene (Fig 3A). The distance between the red and green components of the fusion signal is consistent with interphase mapping studies, which show that DNA sequences separated by less than 250 kb should be within 1 μm of each other in two-dimensional interphase nuclei (*25*). Since the positions of the *bcr* and *abl* hybridization sites are distributed apparently randomly over the two-dimensional nuclear images in normal cells, it is to be expected that some normal cells will have red and green signals separated by <1 μm. We found such false positive cells at a frequency of about 1% (9 of 750 cells pooled from four normal individuals). The highest frequency of false positive fusion signals for an individual case was 3 of 150 cells analyzed. These results set a practical limit of about 1% for the detectable frequency of CML cells in a population with the use of this probe placement strategy.

The hybridization results for seven samples from six CML cases, along with data from PCR, Southern, and chromosome banding analysis are shown in Table 1. All six cases, including three that were found to be Ph^1-negative by banding analysis (CML-4, CML-5, and CML-6), showed red-green hybridization signals separated by <1 μm in more than 50% of nuclei. In most, the fusion event was visible in almost every cell. One case (CML-6) showed fusion signals in almost every cell even though PCR analysis failed to detect the presence of a fusion mRNA and banding analysis did not reveal a Ph^1. Hybridization to metaphase cells was performed in three cases (CML-1, CML-4,

Table 1. A summary of cytogenetic, FISH, and other analyses of *bcr-abl* rearrangements in six CML cases. CML-1 and CML-5 were bone marrow samples from patients with chronic phase CML who were receiving no treatment. CML-3a and CML-3b were from peripheral blood and bone marrow, respectively, of a CML patient in blast crisis, who was receiving hydroxyurea. CML-2 was from bone marrow in a blast crisis CML patient. CML-4 was bone marrow from a CML patient in blast crisis and receiving no treatment. CML-6 was from bone marrow in a chronic phase CML patient receiving hydroxyurea. Hybridization to metaphase cells was done on cases CML-1, CML-4, and CML-5. CML-1 and CML-4 both showed fusion gene signals localized near the end of a small acrocentric chromosome consistent with a classic Ph^1 resulting from a reciprocal translocation. CML-5 showed an interstitial fusion signal on 22q consistent with the *bcr-abl* fusion gene resulting from an insertional event. F, fusion; N, normal; D, double fusion; NI, not interpretable; and ND, not done.

Patient	Cytogenetics	Interphase FISH	Other analyses (reference)
CML-1	46,XX,t(9;22)(q34;q11)	80% F 2% D 18% NI	ND
CML-2	46,XY,t(9;22)(q34;q11)	60% F 40% NI	PCR+ (*30*)
CML-3a	46,XY,t(9;22)(q34;q11)	75% F 25% N	PCR+ (*29*)
CML-3b	46,XY,t(9;22)(q34;q11)	100% F	PCR+ (*29*)
CML-4	47,XY,+8,del(22)(q11)	100% F	PCR+ (*30*)
CML-5	46,XY,ins(22;9)(q11;q34q34)	100% F	PCR+ (*30*)
CML-6	46,XY,t(5;9)(q13;q34)	100% F	PCR− (*30*) Southern + (*31*)

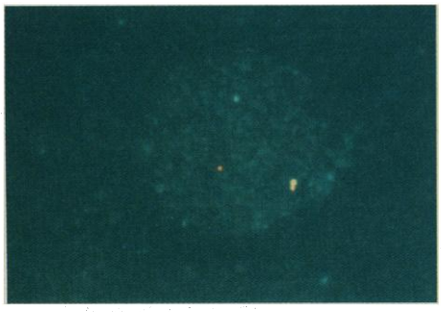

Fig. 2. Fluorescence in situ hybridization in interphase nuclei with *abl* (red) and *bcr* (green) probes visualized simultaneously through a double bandpass filter. Cells from a CML patient, 46,XY, t(9:22)(q34;q11), showing the red-green (yellow) signals resulting from the hybridization to the *bcr-abl* fusion gene, and single red and green hybridization signals to the *bcr* and *abl* genes on the normal chromosomes 22 and 9.

and CML-5). All of these showed red and green hybridization signals in close proximity on a single small acrocentric chromosome. In two cases (CML-1 and CML-4) scored as t(9:22)(q34:q11) by banding analysis, the red-green pair was in close proximity to the telomere of the long arm of a small acrocentric chromosome as expected for the Ph[1] (Fig. 3B). One case (CML-5) was suspected by banding analysis to have an insertion of chromosomal material at 22q11. Two-color hybridization to metaphase cells from this case showed the red-green pair to be centrally located in a small chromosome (Fig. 3C), consistent with formation of the *bcr-abl* fusion gene by an interstitial insertion. In one case (CML-1), two pairs of red-green doublet signals were seen in 3 of 150 (2%) interphase nuclei. This may indicate a double Ph[1] (or double fusion gene) in those cells that was not detected by banding analysis, which was limited to 25 metaphase cells. The acquisition of an additional Ph[1] is the most frequent cytogenetic event accompanying blast transformation, and its cytogenetic detection may herald disease acceleration. Samples CML-3a and CML-3b represent analysis of peripheral blood and bone marrow, respectively, from the same patient. The percentage of *bcr-abl* fusion-positive cells was higher in the bone marrow than peripheral blood.

Simultaneous hybridization with *abl* and *bcr* probes to metaphase cells of the CML-derived cell line K-562 showed multiple red-green hybridization sites along both arms of a single acrocentric chromosome. Hybridization to interphase nuclei showed that the red and green signals were confined to the same region of the nucleus (Fig. 3D). This is consistent with their being localized on a single chromosome. Eight to 16 hybridization pairs were seen in each of 250 nuclei enumerated, indicating corresponding amplification of the *bcr-abl* fusion gene. Fusion gene amplification was not seen in any of the normal controls or CML patients analyzed. These findings are consistent with previous Southern blot data showing amplification of the fusion gene in this cell line (*26*).

In summary, analysis of interphase cells from seven CML and four normal cell samples by dual-color FISH with *abl* and *bcr* probes suggests the utility of this approach for routine diagnosis and clinical monitoring of CML. One of the advantages of this technique is the ability to obtain genetic information from individual interphase or metaphase cells in less than 24 hours. It can be applied to all cells of a population, not just those that, fortuitously or through culture, happen to be in metaphase. The genotypic analysis can be associated with cell phenotype, as judged by morphology or other markers, which permits study of lineage specificity of cells carrying the CML genotype as well as assessment of the frequency of cells carrying the abnormality. In addition, counting of hybridization spots allowed us to determine the degree of *bcr-abl* gene amplification in the K-562 cell line. Quantitative measurement of fluorescence intensity may assist with this analysis in the future.

Random juxtaposition of red and green signals in two-dimensional images of normal cells, which occurs at a frequency of about 0.01 with the probe placement strategy described here, sets the practical limit for detection of rare CML cells in a population. The detection limit may be lowered in the future by quantitative measurement of the separation and fluorescence intensity of the hybridization signals in each nucleus, or by the use of differently placed probes. For example, a probe that hybridized to both sides of either breakpoint would produce two signals (or closely spaced doublets) in normal cells, but three in CML cells. Thus, a single-color hybridization could potentially eliminate false positives, but will give some false negatives. A more complete strategy with two-color hybridizations might use probes that hybridized to both sides of both breakpoints.

Use of other disease-locus–specific probes should allow this technique to be extended to other malignancies. Genetic events involving rearrangements, deletions, and amplifications are potentially detectable in individual cells.

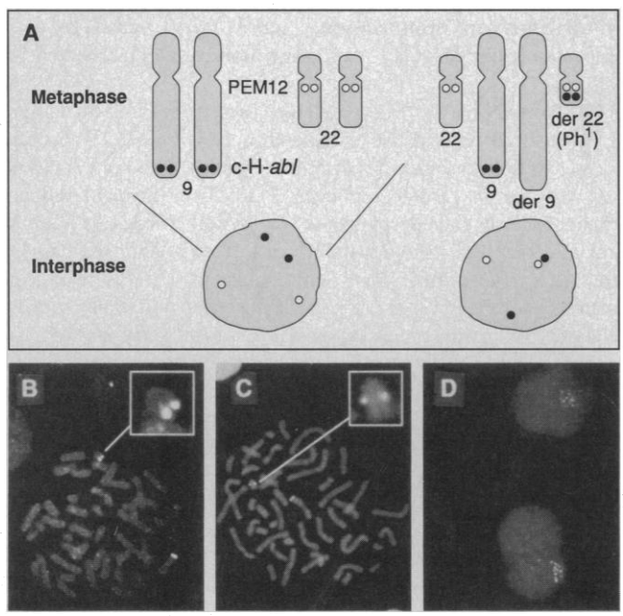

Fig. 3. (**A**) Illustration showing hybridization patterns for normal and CML metaphase and interphase cells. Closed circles represent red signals from c-H-*abl* (*abl* probe) and open circles represent green signals from PEM 12 (*bcr* probe). The left side of the figure shows a normal metaphase with c-H-*abl* staining near the end of 9q and PEM12 on proximal 22q. The corresponding interphase hybridization pattern shows random placement of all four signals. The right side of the figure shows a classic Ph[1] in CML. The *bcr-abl* fusion is represented by one set of red and green signals in close proximity in metaphase and interphase. (**B**) The *abl* staining localized to telomeric region of a classic Ph[1] in a case of CML (CML-1) with 46,XY,t(9:22)(q34;q11). (**C**) The *abl* staining is interstitial on the derivative 22 chromosome arising from an insertional event in a case of CML (CML-5) with 46,XY,ins(22;9)(q11;q34q34). (**D**) Interphase cells from the K-562 cell line showing multiple *abl* signals localized to a region of the interphase nucleus. The same staining pattern was seen with *bcr* probe indicating amplification of the *bcr-abl* fusion gene.

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24. Samples were prepared as follows. CML-3b and CML-6: Five to ten drops of marrow diluted with phosphate-buffered saline (PBS) to prevent clotting were fixed in methanol/acetic acid and dropped on slides. CML-1, CML-2, CML-4, and CML-5: Peripheral blood or bone marrow, or both, was cultured in RPMI 1640 supplemented with 10% fetal bovine serum, an antibiotic mixture (gentamycin 500 μg/ml), and 1% L-glutamine for 24 hours. Cultures were synchronized according to J. J. Yunis and M. E. Chandler [*Prog. Clin Path* 7, 267 (1977)], and chromosome preparations followed L. M. Gibas and L. G. Jackson [*Karyogram* **11**, 91 (1985)]. CML-3a: Peripheral blood was centrifuged for 5 min at 1100 rpm, the buffy coat was pipetted off and diluted with same volume of PBS, spun down, fixed in methanol/acetic acid, and dropped on slides. Hybridization followed procedures described by D. Pinkel *et al* [*Proc Natl Acad Sci U.S.A.* **85**, 9138 (1988)], Trask *et al*. (*25*), and J. B. Lawrence, C. A. Villnave, and R. H. Singer [*Cell* **42**, 51 (1988)], with modifications. The *bcr* probe was nick-translated (Bethesda Research Laboratories Nick-Translation System) with digoxigenin-11–dUTP (deoxyuridine 5′-triphosphate) (Boehringer Mannheim Biochemicals) with an average incorporation of 25%. The *abl* probe was similarly nick-translated with biotin-11–dUTP (Enzo Diagnostics). Cells were thermally denatured at 72°C for 5 min, dehydrated in an ethanol series, air-dried, and placed at 37°C. A hybridization mixture (10 μl) containing each probe (2 ng/μl), 50% formamide/2× standard saline citrate (SSC), 10% dextran sulfate, and human genomic DNA (1 mg/ml, sonicated to 200 to 600 bp) was heated to 70°C for 5 min, incubated for 30 min at 37°C, placed on the warmed slides, covered with a 20 mm by 20 mm cover slip, sealed with rubber cement, and incubated overnight at 37°C. Slides were washed three times in 50% formamide/2× SSC for 20 min each at 42°C, twice in 2× SSC at 42°C for 20 min each, and rinsed at room temperature in 4× SSC. All subsequent steps were performed at room temperature. Slides were blocked in 100 μl of 4× SSC/1% bovine serum albumin (BSA) for 5 min under a cover slip. The biotinylated *abl* probe was detected by applying 100 μl of Texas red–avidin (Vector Laboratories Inc., 2 μg/ml in 4× SSC/1% BSA) for 45 min. The slides were washed twice for 5 min in 4× SSC/1% Triton X-100 (Sigma). The signal was amplified by applying biotinylated goat antibody to avidin {Vector Laboratories Inc., 5 μg/ml in PNM [0.1 M NaH_2PO_4/0.1 M Na_2HPO_4, pH 8 (PN) containing 5% nonfat dry milk and 0.02% sodium azide and centrifuged to remove solids]}, washed twice in PN for 5 min, followed by another layer of Texas red–avidin in PNM. The digoxigenin-labeled *bcr* probe was detected by incubation with sheep antibody to digoxigenin (obtained from D. Pepper, Boehringer Mannheim Biochemicals, Indianapolis, IN; 15.4 μg/ml in PNM) for 30 min, washed twice in PN for 5 min, followed by a rabbit-antibody to sheep conjugated with FITC (Organon Teknika-Cappel, 1:50 in PNM). After washing twice for 5 min in PN, the signal was amplified by applying a sheep antibody to rabbit immunoglobulin G (IgG) conjugated to FITC (Organon Teknika-Cappel, 1:50 in PNM). The slides were then rinsed in PN. Slides were mounted in 10 μl of fluorescence antifade solution [G. D Johnson and J. G. Nogueria, *J. Immunol. Methods* **43**, 349 (1981)] containing 4′,6-amidino-2-phenylindole (DAPI) at 1 μg/ml as a counterstain. The slides were examined with an FITC/Texas red double-band pass filter set (Omega Optical) on a Zeiss Axioskop.
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29. We used the method of S. Kohler *et al* [*Leukemia* **4**, 8 (1990)] for *bcr-abl* PCR on CML-2, CML-4, and CML-5. The oligonucleotide primers used were as follows: *abl*X3 antisense downstream 5′-TTT CTC CAG ACT GTT GAC TGG-3′; *abl*X2 sense upstream 5′-CCT TCA GCG GCC AGT AGC AT-3′; CML *bcr* upstream 5′-ACA GCA TTC CGC TGA CCA TC-3′; CML *abl* antisense detection 5′-TAT GCT TAG AGT GTT ATC TCC ACT-3′.
30. Method used for *bcr-abl* PCR by S. Hegewisch-Becker *et al*. [*J. Biol. Chem. Suppl* **13E**, 289 (1989)] on cases CML-3a, CML-3b, and CML-6. The oligonucleotide primers used were as follows: sense primer (upstream of *bcr*) 5′-AGG GTG CAC AGC CGC AAC GGC-3′; antisense primer (*abl*) 5′-GGC TTC ACT CAG ACC CTG AGG-3′; probe for the identification of *bcr3/abl2* junction sequence 5′-GAA GGG CTT TTG AAC TCT G-3′; probe for the identification of *bcr2/abl2* junction sequence 5′-GAA GGG CTT CTT CCT TAT-3′. Exon 3 of *bcr* is joined to *abl* exon 2 if a 314-bp fragment is amplified. Exon 2 of *bcr* is joined to *abl* exon 2 if a 239-bp fragment is amplified.
31. Southern blot on case CML-6 showed a rearranged Bgl II band with an OSI Transprobe-1 Kit (Oncogene Science catalog no. TP88).
32. We thank B. Trask for expertise in hybridization techniques, R. Seagraves and C. Dana Bangs for technical help, and R. Wooten for art and photographic assistance. D.C.T. is a recipient of a Centennial Fellowship from the Medical Research Council of Canada. C.A.W. is a James S. McDonnell Scholar in Molecular Medicine in Cancer Research. This work was performed under the auspices of the U.S Department of Energy by Lawrence Livermore National Laboratory under contract W-7405-ENG-48 with support from PHS grants CA 45919, CA49605, and CA44700.

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Molecular Cloning and Functional Expression of the Cardiac Sarcolemmal Na^+-Ca^{2+} Exchanger

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The Na^+-Ca^{2+} exchanger of the cardiac sarcolemma can rapidly transport Ca^{2+} during excitation-contraction coupling. To begin molecular studies of this transporter, polyclonal antibodies were used to identify a complementary DNA (cDNA) clone encoding the Na^+-Ca^{2+} exchanger protein. The cDNA hybridizes with a 7-kilobase RNA on a Northern blot and has an open reading frame of 970 amino acids. Hydropathy analysis suggests that the protein has multiple transmembrane helices, and a small region of the sequence is similar to that of the Na^+- and K^+-dependent adenosine triphosphatase. Polyclonal antibodies to a synthetic peptide from the deduced amino acid sequence react with sarcolemmal proteins of 70, 120, and 160 kilodaltons on immunoblots. RNA, synthesized from the cDNA clone, induces expression of Na^+-Ca^{2+} exchange activity when injected into *Xenopus* oocytes.

THE Na^+-Ca^{2+} EXCHANGE TRANSporter of cardiac sarcolemma is a major pathway for transmembrane Ca^{2+} fluxes in cardiac myocytes (*1*). The exchanger uses the energy in the Na^+ gradient to move Ca^{2+} and is usually considered the dominant cellular Ca^{2+} efflux mechanism. The significance of Na^+-Ca^{2+} exchange in cardiac excitation-contraction coupling has recently attracted attention (*2*). Although both physiological and biochemical studies have defined the rate and magnitude of exchanger-mediated fluxes, molecular studies of the Na^+-Ca^{2+} exchanger have lagged because of the relatively low abundance and lability of the exchanger. We have previously correlated sarcolemmal Na^+-Ca^{2+} exchange activity with 70-, 120-, and 160-kD proteins (*3*). Here we report the molecular cloning, expression, deduced amino acid sequence, and apparent molecular size of the canine cardiac sarcolemmal Na^+-Ca^{2+} exchange protein.

We used a polyclonal antibody against a partially purified preparation of the exchanger (*3*) to screen an amplified λgt11 expression library (*4*). A 3.2-kb clone, designated A4, was isolated from the library for further study. Northern blot analysis of polyadenylated [poly(A)$^+$] RNA from dog heart tissue probed with DNA from A4 indicated that the complete exchanger transcript is 7 kb (*5*). Sequence analysis and our inability to express exchange activity in oocytes with RNA synthesized from the A4 clone indicated that this clone did not contain the entire coding region for the exchanger.

To obtain the 5′ end of the coding region of the exchanger clone, we constructed a

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