Neutralization of the AIDS Retrovirus by Antibodies to a Recombinant Envelope Glycoprotein

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Mammalian cell lines have been engineered to produce a secreted form of the AIDS retrovirus envelope glycoprotein. The recombinant protein has been isolated from growth-conditioned culture media and used to immunize animals. Antibodies directed against the recombinant molecule were found to react with the envelope glycoprotein produced in virus-infected cells. Furthermore, these antibodies were able to directly inactivate the AIDS retrovirus in a neutralization assay in vitro. The expression system reported here should provide sufficient quantities of the AIDS retrovirus envelope protein for biological and vaccination studies.

THE ACQUIRED IMMUNE DEFICIENCY syndrome (AIDS) is recognized as an epidemic in several areas of the world, including the United States (*1*). The disease generally manifests itself as severe immunosuppression typically leading to devastating, often fatal, opportunistic infections and neoplasia. Several laboratories have shown AIDS to be associated with a retrovirus, variously termed lymphadenopathy virus (LAV) (*2*), human T cell leukemia virus type III (HTLV-III) (*3*), or AIDS-related virus (ARV) (*4*). The AIDS retrovirus appears to cause immune dysfunction by selectively infecting and eventually destroying the $OKT4^+$ helper-inducer subset of T cells (*5*). Because there is evidence that heterosexual transmission can occur as a result of sexual contact, a considerable percentage of the population may be at risk for infection with this virus (*6*). Alterations in the life-styles of certain high-risk groups may help to reduce the spread of the disease, but the development of a reliable vaccine against the AIDS retrovirus would be more effective in arresting the current epidemic (*1, 7*).

We previously demonstrated the feasibility of using genetically engineered mammalian cells for the production of an efficacious subunit vaccine for type 1 and 2 herpes simplex virus infections (*8*). This work showed that glycoprotein D (gD) of these viruses could be constitutively secreted from Chinese hamster ovary (CHO) cell lines transfected with a gD gene truncated so as to delete the transmembrane domain that normally binds the protein to the viral or infected cell surface (*8*). We have taken a similar approach to produce a potential recombinant vaccine for AIDS. In initial studies, the full-length AIDS retrovirus envelope protein, as well as variants truncated so as to delete COOH-terminal hydrophobic domains, showed inefficient expression in CHO cells. Examination of the protein sequence (*9*) revealed one factor that might account for the poor expression. The AIDS retrovirus envelope glycoprotein contains a novel signal sequence in that an unusually long hydrophobic domain at the NH_2-terminus of the protein is preceded by a highly charged region (*9, 10*). Other viral envelope antigens, such as gD, usually contain a signal sequence at the NH_2-terminus possessing a 15 to 20 residue long hydrophobic domain containing a small core of charged amino acids (*11*).

To improve the expression of a secreted variant of the AIDS retrovirus envelope, we constructed a truncated form of the gene that used the gD signal sequence (Fig. 1). The first 50 amino acids of the gD protein were joined in phase to amino acid 61 of the retroviral envelope antigen. Correct cellular processing of the gD signal sequence would result in envelope antigen containing 25 amino acids of gD at its NH_2-terminus (*12*) and lacking 30 residues from the mature processed form of gp120 (*10*). Expression of the full-length envelope-gD signal sequence chimera in CHO cells resulted in the production of a highly glycosylated protein that was retained in one of the intracellular compartments with no evidence of proteolytic processing or export to the cell surface

Table 1. Neutralization of AIDS retrovirus by antisera elicited to recombinant gp130.Neutralizing antibodies were measured by using a modification of previously published techniques (*16*). $TCID_{50}$ measurements were performed as described (*16*). Briefly, neutralization assays were performed by incubating 1:5 diluted, heat-inactivated (56°C, 30 minutes) sera with titered aliquots [corresponding to 20 $TCID_{50}$ units of $HTLV\text{-}III_B$ (*3*)] of virus for 1.5 hours at 4°C. The serum-virus mixture was then inoculated onto Polybrene-treated H9 cells (1×10^6 cells per milliliter) (*3*). Cultures were incubated for either 7 or 10 days, at which time the cells were harvested by centrifugation, fixed with acetone-methanol, and stained for $HTLV\text{-}III_B$ antigens by indirect immunofluorescence using high titer polyclonal human antiserum. Reverse transcriptase activity was measured by standard techniques (*16*).

Serum or IgG	Immunogen	Indirect immunofluorescence (% cells fluorescing)		Reverse transcriptase (10^5 count/min)	
		Day 7	Day 10	Day 7	Day 10
Guinea pig					
2	Preimmune	20	74	2.02	25.14
6	Preimmune	30	73	4.36	28.56
9	Preimmune	27	84	1.92	24.15
10	Preimmune	16	41	1.76	25.19
1	gp130	<1	36	0.07	5.40
2	gp130	0	0	0.02	0.37
3	gp130	<1	1	0.05	0.31
4	gp130	<1	36	0.24	5.90
Rabbit					
4	Preimmune	13	84	1.4	20.00
25	Preimmune	6	61	0.31	16.00
26	Preimmune	6	70	0.72	17.00
27	Preimmune	6	66	1.00	15.00
19	gp130	6	54	1.16	19.00
25	gp130	4	49	0.26	10.60
26	gp130	<1	7	0.19	0.11
27	gp130	0	<1	0.04	0.03
Rabbit IgG*					
26	Preimmune	26	ND	6.24	ND
26	gp130	0	ND	0.0	ND
27	gp130	<1	ND	0.0	ND

*The IgG was purified by using a Protein A–Sepharose column and the concentration was 10 mg/ml. The IgG was used at a 1:5 dilution; 100 $TCID_{50}$ units of virus were used in this experiment; ND, not done.

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during the labeling period (*13*). So that the envelope antigen would be secreted from the cell, we deleted the entire putative transmembrane region (that is, gp41) from the retrovirus envelope gene. The truncation site was within the first major hydrophobic domain at amino acid 531 of the envelope, 20 amino acids COOH-terminal to the actual 160-kD precursor processing site (*14*), resulting in a chimeric envelope gene of 520 amino acids in length (Fig. 1).

Transfection of this plasmid into CHO cells and subsequent selection permitted the isolation of many stable clones that contained the retrovirus envelope expression plasmid (pAIDSenv/trDHFR). Several of the clones were labeled with [^{35}S]methionine, and the cell lysates and growth conditioned media were analyzed by radioimmunoprecipitation (RIPA) with the use of serum from a healthy homosexual male who had high antibody titers against the native envelope antigens of the AIDS retrovirus (Fig. 2). RIPA of the cell lysate from one transfected cell line with serum from this healthy homosexual male showed an antibody-specific protein at approximately 100 kD (Fig. 2D). This protein did not appear in control experiments where normal human serum was used for RIPA of the lysates from transfected cell lines, nor was the protein present when serum from the same healthy homosexual male was reacted with lysates from the parental CHO cell line (for example, Fig. 2B). When the cell-conditioned supernatants from the transfected cells were examined (Fig. 2C), a larger, more diffuse immunoprecipitable band was found that migrated at approximately 130 kD (gp130). This protein, which was immunoprecipitated by sera from several individuals positive for AIDS virus antibodies, was specifically secreted from cells transfected with the AIDS retrovirus expression plasmid and was only immunoprecipitable by antibodies to the AIDS retrovirus (for example, Fig. 2A). Thus, the transfected cells secreted a truncated form of the envelope protein that was capable of interacting with antibodies elicited against the authentic virus envelope antigen.

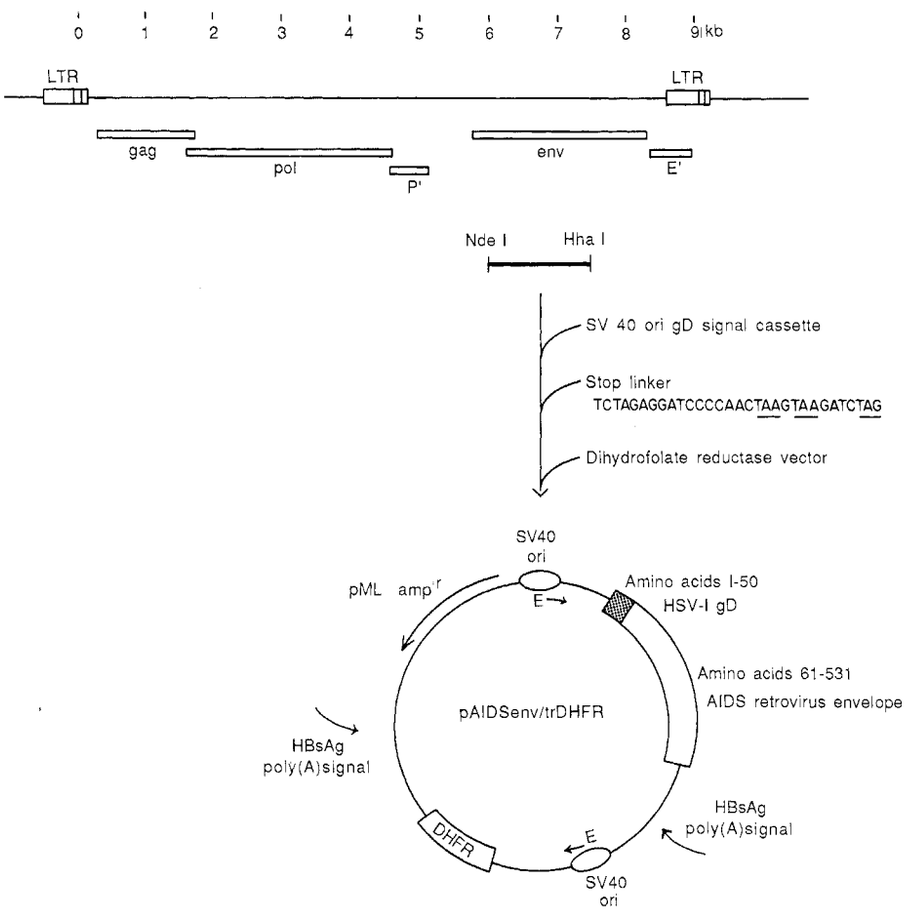

Fig. 1. Construction of a plasmid for the synthesis of a secreted form of the AIDS retrovirus envelope in mammalian cells (pAIDSenv/trDHFR). The viral genome is shown at the top with the location of each of the five major open reading frames of the virus (*9*). The *env* reading frame encodes the viral envelope glycoprotein antigen described herein (*10*). A Nde I–Hha I envelope fragment encoding amino acid residues 61–531 (*9*) was used for expression of a secreted envelope protein by in-frame ligation to a herpes simplex virus (HSV-1) gD signal sequence fragment (*8*). Envelope translation was terminated by the inclusion of a synthetic piece of DNA (stop-linker) containing termination codons in all three reading frames. These fragments were incorporated into a vector that contained the components necessary for proper expression of this integrated envelope gene as well as for selection in mammalian cells. "SV40 ori" contains an early promoter from the SV40 virus (E) which is used to drive transcription of either the AIDS retrovirus envelope or the dihydrofolate reductase (DHFR) complementary DNA (cDNA). Transcription termination and message polyadenylation are accomplished by using signals derived from the 3′ nontranslated region of the hepatitis B virus surface antigen gene [HBsAg poly(A) signal] (*20*). Replication of the plasmid in *Escherichia coli* is accomplished by the inclusion of an origin of replication derived from the plasmid pML (*21*) and a β-lactamase gene that confers resistance to ampicillin (amp^r). Finally, a murine DHFR cDNA clone is included as a selectable marker for selection in DHFR-deficient CHO cells (*22*).

The size differences between the intracellular material and the extracellular material appeared to be due to differences in glycosylation. To explore this question we conducted endoglycosidase digestion studies. Neuraminidase treatment of the secreted envelope protein reduced its size from 130 kD to approximately 110 kD, suggesting that the secreted antigen possessed the complex form of *N*-linked carbohydrate with terminal sialic acid residues (Fig. 2E). Treatment with endoglycosidase H showed that the extracellular material was resistant to this enzyme, whereas the intracellular form of the envelope protein was sensitive and generated a 69-kD species. These results demonstrated that the intracellular protein possessed the simple form of *N*-linked carbohydrate (high mannose), and that approximately 61 kD of the protein can be attributed to carbohydrate, a result similar to that found for authentic envelope antigen synthesized in AIDS retrovirus–infected cells (*10*).

The secreted protein was partially purified from CHO cell–conditioned media by using an immunoaffinity column prepared with antibody purified from the serum of a healthy homosexual male possessing a high titer of antibodies to the viral envelope protein (*15*). The secreted proteins that bound to this column were eluted at high salt concentrations and at *p*H 3.3. Sodium dodecyl sulfate (SDS)–polyacrylamide gel analysis of the eluted proteins revealed the presence of a 130-kD band that comprised approximately 20% of the total proteins bound to the affinity column. This protein appeared to be identical in size to the radioactively labeled secreted envelope protein (~130 kD, Fig. 3C). In Western blot analyses, this 130-kD secreted protein reacted with serum from an AIDS retrovirus–seropositive individual. From gel electrophoretic analyses we estimate that the amount of gp130 produced is greater than 1 mg per liter.

The affinity-purified antigen was next for-

mulated with complete Freund's adjuvant and used to immunize rabbits and guinea pigs. The animals were inoculated with approximately 100 μg of the partially purified envelope antigen. They subsequently received two booster injections of affinity-purified gp130 incorporated in incomplete Freund's adjuvant. Sera from these animals immunoprecipitated the CHO cell–derived envelope antigen, demonstrating that the recombinant protein was immunogenic. Serum from guinea pigs vaccinated with recombinant gp130 was then used to immunoprecipitate [^{35}S]methionine-labeled AIDS retrovirus proteins (Fig. 3). Sera from four gp130-vaccinated guinea pigs immunoprecipitated both the gp160 and gp120 envelope antigens from AIDS retrovirus–infected T cells (Fig. 3, B to E). These same two antigens, in addition to the 55-kD *gag* precursor, were immunoprecipitated by human antisera to AIDS virus (Fig. 3F). Thus, the recombinant envelope protein is able to elicit antibodies reactive with a soluble, presumably nondenatured form of the virus-derived protein.

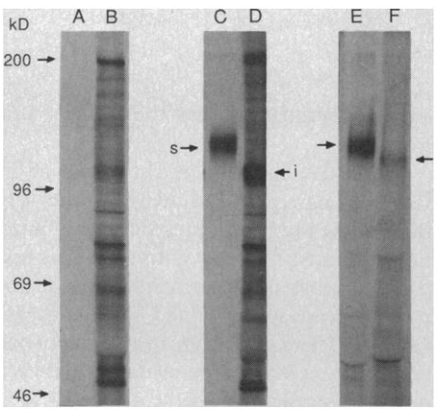

Fig. 2. Radioimmunoprecipitation (RIPA) of the intracellular and secreted forms of the AIDS retrovirus envelope from transfected cells. Control cells (CHO, DHFR-deficient) and cells transfected with pAIDSenv/trDHFR were labeled with [^{35}S]methionine, and the intracellular and secreted proteins were subjected to RIPA with serum from an AIDS retrovirus–seropositive individual. The precipitated proteins were then subjected to SDS-PAGE and resolved by autoradiography. (A) Culture supernatants of DHFR$^-$ CHO control cells. (B) Cell lysates from DHFR$^-$ CHO control cells. (C) Culture supernatants from cells transfected with the pAIDSenv/trDHFR. (D) Cell lysates from cells transfected with the pAIDSenv/trDHFR (s, secreted envelope antigen; i, intracellular envelope antigen). The nature of the glycosylation of the secreted envelope antigen was investigated by neuraminidase digestion and RIPA. (E) Untreated material secreted from envelope-producing cell lines. (F) Neuraminidase-treated material secreted from envelope-producing cell lines. The CHO cells were transfected with plasmid DNA (Fig. 1) and selected as described (*8, 22, 23*). Control cells or methotrexate amplified transfected cells (100 n*M* methotrexate) were labeled for 4 hours with [^{35}S]methionine (70 μCi per milliliter). The medium was collected and the cells were lysed in RIPA buffer as described (*8*). Antiserum from an AIDS retrovirus–seropositive individual was used for RIPA with Protein A–Sepharose beads. The precipitated proteins were resolved on 7.5% polyacrylamide SDS gels and autoradiographed as described (*8*). Secreted envelope protein was subjected to RIPA with antibodies to AIDS retrovirus and treated with 10 mU of neuraminidase for 2 hours at 37°C. The proteins were resolved by SDS-PAGE.

The ability of antibodies to the recombinant envelope protein to inactivate the AIDS retrovirus was tested in a neutralization assay in vitro (*16*). In these studies, AIDS retrovirus [derived from the original HTLV-III$_B$ isolate of Gallo *et al.* (*3*)] was incubated with coded serum samples from either gp130-vaccinated animals or control animals and then added to cultures of human T cells [H9 cell line (*3*)]. After 7 or 10 days of culture, the cells were fixed and assayed for retrovirus infection by indirect immunofluorescence with human serum that showed a high titer of antibodies to AIDS retrovirus proteins. In addition, supernatants of these H9 cell cultures were assayed for viral reverse transcriptase activity. Neutralization assays were performed with sera at a dilution of 1:5 with 20 TCID$_{50}$ units (50% tissue culture infective dose) of AIDS retrovirus. All of the guinea pig antisera to gp130 showed neutralizing activity on day 7 in both assays (Table 1). By day 10, however, some virus growth was detected in cultures treated with sera from guinea pigs 1 and 4. This shows that these sera inhibited the growth of virus but did not completely inactivate all the virus in the culture system. Two other sera (guinea pigs 2 and 3), however, substantially prevented virus growth in both the 7- and 10-day cultures. This result demonstrates that recombinant gp130 possesses antigenic determinants that stimulate the production of antibodies that can inhibit the growth of the AIDS retrovirus in vitro, even in relatively long-term cultures. Analyses of the rabbit sera showed similar results. Virus treated with serum from rabbit No. 26 gave no evidence of growth on day 7, but did indicate a small amount of virus growth on day 10. Serum from rabbit 27, however, completely prevented virus growth on both days 7 and 10. It is interesting that sera from rabbits 19 and 25 were apparently ineffective in inhibiting virus growth despite the fact that sera from both of these animals had antibodies that reacted with authentic viral gp120 on Western blots and in RIPA. Finally, purified immunoglobulin G (IgG) from rabbits 26 and 27 was able to neutralize 100 TCID$_{50}$ units of the AIDS retrovirus, demonstrating that antibodies were responsible for neutralization in vitro (Table 1). In conclusion, these results show that antibodies to the recombinant gp130 antigen can inhibit AIDS retrovirus infectivity in vitro.

Recently, Clapham and Weiss (*17*) tested the rabbit and guinea pig sera described above in a VSV pseudotype neutralization assay (*18*). In these studies sera from rabbits 25 and 27 and from guinea pigs 2 and 3 reduced the pseudotype titer greater than 80%. Sera from guinea pigs 1 and 4 reduced the pseudotype titer to a somewhat lesser extent. These results confirm, by means of a completely independent assay, that antibodies elicited against recombinant gp130 can neutralize the AIDS retrovirus.

Although it is premature to suggest that the recombinant antigen described here will be effective as an AIDS retrovirus vaccine, our results demonstrate that useful quantities of the AIDS retrovirus envelope protein can be produced in a safe, retrovirus-free mammalian cell system. Our finding that the isolated, recombinant gp130 envelope antigen is able to elicit virus-neutralizing antibodies demonstrates that at least some of the epitopes involved in neutralization of the virus in vitro and, presumably, in vivo (*16*)

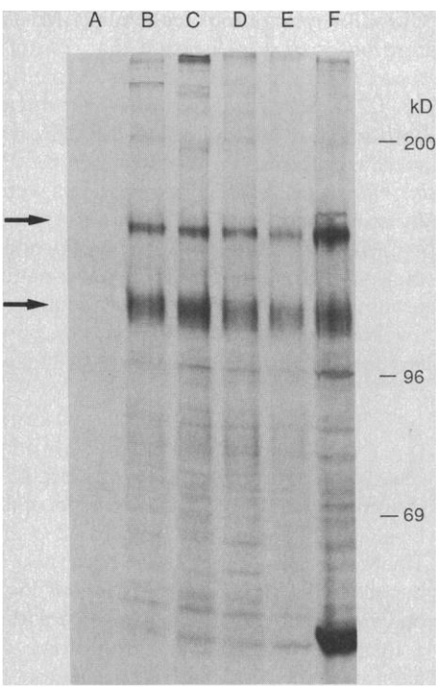

Fig. 3. Radioimmunoprecipitation of [^{35}S]methionine-labeled AIDS retrovirus proteins with guinea pig and AIDS retrovirus–seropositive human sera. (A) Control guinea pig serum. (B to E) Sera from guinea pigs vaccinated with recombinant gp130 antigen. (F) Serum from human AIDS retrovirus–seropositive individual. The arrows show the 160-kD envelope precursor and proteolytically processed 120-kD envelope proteins of the virus. Viral antigens were labeled overnight in infected H9 T cells with 500 μCi/ml of [^{35}S]methionine (*3*). Radioimmunoprecipitations were performed as described in Fig. 2.

are located on the viral gp120 envelope antigen, and that these epitopes are also found on the recombinant form of the envelope glycoprotein.

These results point to one possible route toward the production of a safe and efficacious vaccine for the prevention of infection by the AIDS retrovirus. While the demonstration here of virus neutralization in vitro is encouraging, the use of an animal model, such as chimpanzees, will be a necessary next step (*19*).

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15. An immunoaffinity column was produced by incubating the IgG fraction isolated from an AIDS retrovirus–seropositive patient with CNBr-activated Sepharose beads. The serum-free medium conditioned by an envelope-producing cell line was concentrated 100-fold by ultrafiltration using an Amicon ym-10 filter and passed over this column at room temperature at a flow rate of 0.5 ml/min. The column was washed with high and low salt containing buffers and was eluted at *p*H 3.3 with acetate buffer. The eluted material was immediately neutralized, dialyzed, and then concentrated.
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The Chronic Myelogenous Leukemia–Specific P210 Protein Is the Product of the *bcr/abl* Hybrid Gene

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Chronic myelogenous leukemia (CML) is a human disease associated with a consistent chromosomal translocation that results in sequences from the c-*abl* locus on chromosome 9 being fused to sequences in a breakpoint cluster region (*bcr*) on chromosome 22. CML cells have two novel products: an 8.5-kilobase RNA transcript containing both *abl* and *bcr* and a 210-kilodalton phosphoprotein (P210) recognized by v-*abl*–specific antisera. To test whether the P210 is the product of the novel 8.5-kilobase *bcr/abl* fusion transcript, antibodies were prepared against c-*abl* and *bcr* determinants. By using these reagents and v-*abl*–specific antisera, it was demonstrated that the P210 in CML cells is indeed the protein product of the 8.5-kilobase transcript. By analogy to the *gag/abl* fusion protein of Abelson murine leukemia virus, the replacement of amino terminal c-*abl* sequences by *bcr* sequences in P210 may create a transforming protein involved in CML. A 190-kilodalton phosphoprotein that is a candidate for the normal *bcr* protein was identified in both HeLa and K562 cells.

CHRONIC MYELOGENOUS LEUKEMIA (CML) cells contain the Philadelphia chromosome, a product of a reciprocal exchange of distal segments of the long arms of chromosomes 9 and 22 (*1, 2*). The breakpoints of the t(9;22) occur near to but at variable distances from the c-*abl* locus on chromosome 9, and are clustered within a small (6-kb) region of chromosome 22 designated the breakpoint cluster region, or *bcr* (*3*). The translocation is associated with two novel products: a *bcr*/c-*abl* transcript of 8.5 kb (*4*), larger than the normal c-*abl* messenger RNA (mRNA) sizes of 6 and 7 kb seen in most tissues (*5*); and a phosphoprotein that cross-reacts with v-*abl*–specific antisera and has a molecular mass of 210 kD, larger than the normal c-*abl* protein which has a molecular mass of 145 kD (*6*). From sequences of complementary DNA (cDNA), the 8.5-kb mRNA represents a fusion of *bcr* sequences to c-*abl* sequence at just the point where the multiple 5′ exons of c-*abl* are fused to the common body of the c-*abl* mRNA. The exon at which the fusion occurs, the most upstream of the common set of c-*abl* exons, has been called the "common exon" (*7*).

It was suggested that the 210-kD phosphoprotein (P210) found in CML cells is the protein product of the hybrid *bcr/abl* transcript (*8*). If P210 is the product of the CML-specific 8.5-kb transcript, it should contain, in addition to determinants which cross-react with v-*abl* antisera, c-*abl* common exon determinants as well as *bcr* determinants. We have prepared a set of immunologic reagents specific to either c-*abl* or *bcr* and have used them to test the relationship of P210 to the 8.5-kb *bcr/abl* transcript. The results indicate that P210 represents a fusion of parts of two normal cellular proteins and is the product of the *bcr/abl* hybrid transcript.

In Fig. 1A, the proposed structure of the P210 coding region is shown with the positions of the five peptides used for immunization indicated. The *bcr* antiserum was prepared against a β-galactosidase/*bcr* fusion protein expressed in a pEX vector (*9, 10*) (Fig. 1B). The antiserum to the c-*abl* common exon was prepared against a synthetic peptide representing the sequence close to the 5′ border of the exon (Fig. 1C). The specificity of this serum was demonstrated by radioimmunoassay (*11*). The v-*abl*–specific antisera pEX2, pEX4, and pEX5 were described previously (*12*). pEX2 recognizes the protein kinase domain of v-*abl*; pEX4 and pEX5 are directed against carboxyl terminal determinants of v-*abl*.

The specificity of the c-*abl* antiserum was examined by immunoprecipitation of the normal c-*abl* protein from HeLa cells. Extracts immunoprecipitated with c-*abl* antisera were processed for in vitro kinase assay with γ-^{32}P-ATP as described (*13*) and ^{32}P-labeled proteins were displayed by electrophoresis. Under these conditions, v-*abl* antisera (pEX4 and pEX5) and the antiserum to the c-*abl* common exon precipitated the same phosphorylated protein of 140 kD previously described as the P145 c-*abl* protein (*13*) (Fig. 2, lanes 1 and 2). The immunoprecipitation of this 140-kD phosphoprotein with the antiserum to c-*abl* common exon peptide could be completely inhibited by its cognate peptide (Fig. 2, lane

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