recessive and for MC an autosomal dominant inheritance with 100% penetrance were assumed (all unaffected individuals were beyond the age of risk). The prevalence of these two diseases was assumed (*1*) to be 1:50,000 in GM and 1:23,000 in MC.
18. After receipt of informed consent, seven families (a total of 14 affected and 13 nonaffected siblings) with the diagnosis of GM and 4 families with MC (20 affected, 7 nonaffected, and 7 spouses) were studied. The age of onset for individuals with GM was 2 to 10 years, and 1 to 18 years for MC. All individuals in a family received a thorough neurological examination. In addition, a standardized forearm cooling test was performed on MC individuals to exclude paramyotonia congenita [K. Ricker *et al.*, *Arch. Neurol.* **47**, 268 (1990)]. For the purpose of the study, it was assumed that GM and MC are single-gene disorders, where variation in symptoms probably results from different mutations in the same locus.
19. M. C. Koch *et al.*, data not shown.
20. A genomic library in λFIXII was constructed with lymphocyte DNA from a GM patient homozygous for the unusual Nsi I site (Fig. 2), and another genomic human library in λFIXII was purchased (Stratagene). Phages hybridizing to human *CLC*-1 cDNAs were purified, and their inserts were isolated, digested with Nsi I, and subcloned into pGem5Zf. The Nsi I fragment of interest was identified by hybridization to successively smaller cDNA fragments that still recognized the unusual Nsi I site detected by genomic Southern (DNA) analysis, and the region surrounding the restriction site was sequenced with the chain termination method. The sequences shown in Fig. 3 were obtained by means of internal sequencing primers located in the intron preceding the D8 coding sequence.
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27. We thank G. Grahmann and C. Schmekal for technical assistance, L. C. Tsui for the *TCRB* gene probe, and all families for their collaboration. Supported by grants from the Deutsche Forschungs-gemeinschaft (M.C.K. and T.J.J.), the Bundesministerium für Forschung und Technologie (T.J.J.), the Muscular Dystrophy Association (T.J.J.), and the Deutsche Gesellschaft Bekämpfung der Muskelkrankheiten.

12 March 1992; accepted 17 June 1992

Hypovirulence of Chestnut Blight Fungus Conferred by an Infectious Viral cDNA

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Strains of the chestnut blight fungus *Cryphonectria parasitica* that contain viral double-stranded RNAs often exhibit reduced virulence. Such hypovirulent strains act as biocontrol agents by virtue of their ability to convert virulent strains to hypovirulence after anastomosis. Transformation of virulent *C. parasitica* strains with a full-length complementary DNA copy of a hypovirulence-associated viral RNA conferred the complete hypovirulence phenotype. Cytoplasmic double-stranded RNA was resurrected from the chromosomally integrated complementary DNA copy and was able to convert compatible virulent strains to hypovirulence. These results establish viral double-stranded RNA as the causal agent of hypovirulence and demonstrate the feasibility of engineering hypovirulent fungal strains.

The North American chestnut blight epidemic, initiated by the unintentional introduction of the Asian fungus *Cryphonectria (Endothia) parasitica* at the turn of the century, resulted in the destruction of several billion mature American chestnut trees (*1–4*). The potential for biological control of chestnut blight effected by naturally occurring strains of *C. parasitica* that exhibit reduced levels of virulence (hypovirulence) has been demonstrated (*5–7*). Whereas virulent *C. parasitica* strains penetrate and destroy bark and cambium layers and cause wilting and death, hypovirulent strains usually produce superficial cankers that eventually heal. Hypovirulence is correlated with the presence of cytoplasmically replicating unencapsidated double-stranded RNAs (dsRNAs) (*8*). The ability of these genetic elements and the hypovirulence phenotype to be transmitted to virulent fungal strains after anastomosis (physical fusion of hyphae) provides the basis for disease control (*7*).

The largest dsRNA present in hypovirulent *C. parasitica* strain EP713, large dsRNA (L-dsRNA), was recently cloned and characterized (*9–12*). The similarity of the L-dsRNA genetic organization and expression strategy to those of several viral genomes (*11*) and an apparent evolutionary relationship to the plant potyviruses (*13*) prompted the introduction of the term hypovirulence-associated virus (HAV) to denote this class of genetic elements (*11*). Efforts to rigorously demonstrate HAV dsRNAs as the causal agent of hypovirulence have been hampered by the inability of these viral elements to initiate infection by an extracellular route, a common property of mycoviruses and fungus-associated unencapsidated viral-like RNAs (*14–16*). We describe the construction of a full-length cDNA clone of EP713 L-dsRNA that, when introduced into virulent *C. parasitica* strains by DNA-mediated transformation, generated a resurrected, cytoplasmically replicating dsRNA form from the integrated cDNA copy.

One strand of L-dsRNA contains a 3′ polyadenylate [poly(A)] tail that is base-paired to a stretch of polyuridine [poly(U)] present at the 5′ terminus of the complementary strand (*17*). The molecule consists of 12,712 bp, excluding the poly(A):poly(U) homopolymer domain and contains two large open reading frames (ORF) within the poly(A) strand that were designated ORF A (622 codons) and ORF B (3,165 codons) (*11*). To construct a full-length cDNA clone of L-dsRNA, we first generated several large intermediate clones from a set of overlapping partial cDNA clones (Fig. 1). A four-factor ligation-transformation was then performed to generate plasmid pLDST that contained a full-length cDNA copy of L-dsRNA inserted between the Xba I and Hind III sites of pUC19. Unique Ssp I and Spe I sites were introduced at the 5′ terminus and a 22-residue-long stretch of poly(A):poly(U) to simulate the natural homopolymer tail followed by Spe I and Hind III sites at the 3′ terminus. Restriction sites used in the four-factor ligation to form pLDST are indicated by an asterisk.

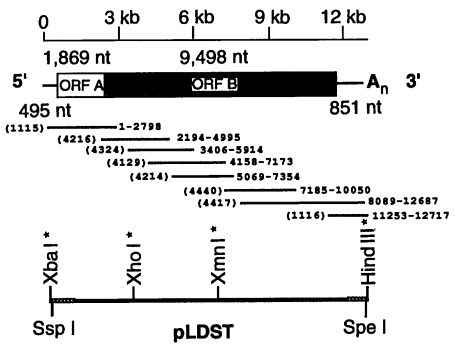

Fig. 1. Genetic organization of the hypovirulence-associated virus RNA, L-dsRNA, present in hypovirulent *C. parasitica* strain EP713 [American Type Culture Collection (ATCC) number 52571] and construction of the full-length cDNA clone pLDST. The general organization of the coding sense strand of L-dsRNA is indicated at the top; overlapping cDNA clones that span the entire molecule are represented by the horizontal lines below. Clone designations are indicated at the left of each line, and the map coordinates (*11*) covered by each clone are indicated to the right of each line. Modifications of the terminal regions of the L-dsRNA cDNA (indicated by diagonal stripes in pLDST) included the addition of Xba I and Ssp I sites at the 5′ terminus and a 22-residue-long stretch of poly(A):poly(U) to simulate the natural homopolymer tail followed by Spe I and Hind III sites at the 3′ terminus. Restriction sites used in the four-factor ligation to form pLDST are indicated by an asterisk.

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duced at the termini of the L-dsRNA cDNA by polymerase chain reaction (PCR) (*18*) early in the construction sequence. This allowed for the release of the intact L-dsRNA cDNA from pLDST and subsequent subcloning into transformation vector pCPXHY1 (*19*) to yield plasmid pXH9. The L-dsRNA cDNA was thus placed under the control of the C. *parasitica* glyceraldehyde-3-phosphate dehydrogenase gene (*gpd-1*) promoter and terminator in a plasmid that also contained the *Escherichia coli* hygromycin B phosphotransferase gene as a selectable marker (*20*).

Hypovirulent strain EP713 exhibits traits (termed hypovirulence-associated traits) in addition to reduced virulence, all of which distinguish it from the virus-free isogenic virulent strain EP155 (*21*). These traits include reduced production of orange pigments, suppressed conidiation, and reduced production of laccase and provide useful phenotypic markers for transformation studies. We recently showed that introduction of the L-dsRNA ORF A coding domain into virulent strain EP155 by DNA-mediated transformation conferred these hypovirulence-associated traits but failed to confer hypovirulence (*19*). As indicated in Fig. 2A, most of the hygromycin-resistant colonies selected after transformation of strain EP155 with plasmid pXH9 also exhibited hypovirulence-associated traits (that is, transformants CN2, CN3, CN6, and CN7). Hygromycin-resistant colonies that resembled untransformed strain EP155 were also observed among the pXH9 transformants at a rate of approximately 10%, as exemplified by transformants CN4 and CN5 (Fig. 2A). Southern (DNA) analysis (*22*) revealed that those transformants that exhibited hypovirulence-associated traits contained the intact viral cDNA and flanking vector sequences integrated into the chromosomal DNA. In contrast, transformants CN4 and CN5 contained the L-dsRNA cDNA sequence integrated in a deleted or rearranged form, respectively.

Because pXH9 contained the entire L-dsRNA sequence, including the putative RNA-dependent RNA polymerase and RNA helicase coding domains (*13*), the possibility existed that transcripts generated from the integrated cDNA copy could function as both mRNA and as a template for RNA-dependent replication. Consistent with this proposal, pXH9 transformants CN2, CN3, CN6, and CN7 were all found to have a dsRNA species that comigrated with L-dsRNA extracted from EP713 (Fig. 2B). These transformants did not contain the internally deleted forms of L-dsRNA—that is, the predominant medium dsRNA (M-dsRNA) species (Fig. 2B)—that are invariably present in hypovirulent strain EP713 (*23*). As expected from the results of the Southern analysis, transformants CN4 and CN5 lacked any detectable dsRNA species.

Conversion of a virulent C. *parasitica* strain to hypovirulence requires anastomosis with a hypovirulent strain because HAV dsRNA is not infectious by an extracellular route (*7*). However, there are restrictions in the ability of strains to undergo anasto-

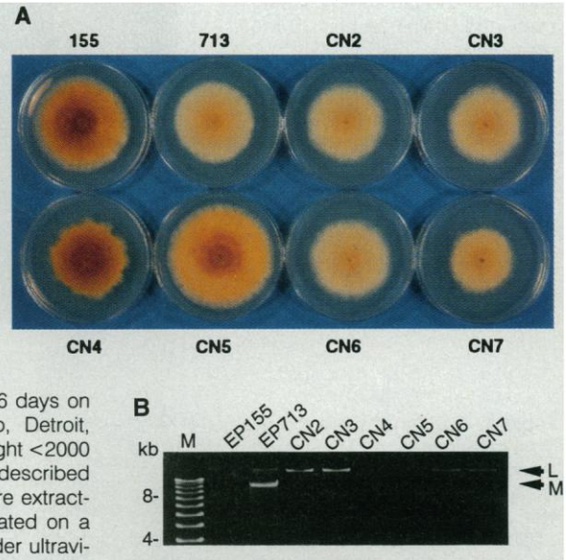
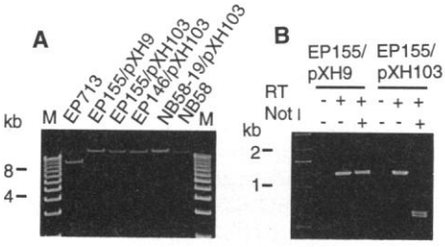
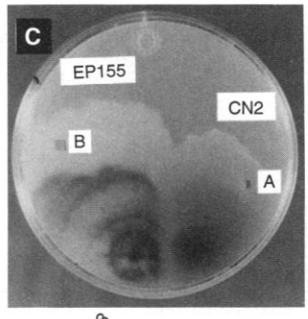


Fig. 2. Colony phenotype of transformants that resulted from transformation of strain EP155 with plasmid pXH9 and agarose gel analysis of extracted dsRNAs. (**A**) Spheroplasts prepared from the virus-free virulent *C. parasitica* strain EP155 (ATCC number 38755) were transformed with plasmid pXH9 (*19*) followed by selection on hygromycin B–containing medium (40 μg/ml). Randomly selected transformants (CN2 through CN7 shown in this figure) were grown in parallel with untransformed EP155 and the isogenic hypovirulent strain EP713 for 6 days on potato dextrose agar (PDA) (Difco, Detroit, Michigan) on the laboratory bench (light <2000 lux; temperature was 22° to 24°C) as described (*21*). (**B**) Double-stranded RNAs were extracted from fungal mycelia (*21*), separated on a 0.8% agarose gel, and detected under ultraviolet light after staining with ethidium bromide. The lane marked M contained the 1-kb DNA ladder [Bethesda Research Laboratories (BRL)]. The migration positions of the L-dsRNA (L) and the internally deleted M-dsRNA (M) species (*23*) are indicated at the right.





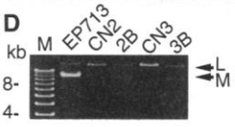


Fig. 3. Characterization of resurrected L-dsRNAs from transformed virulent *C. parasitica* strains of different vegetative compatibility groups and from strains converted by anastomosis with transformed stains. (**A**) Plasmid pXH103, derived from pXH9 by introduction of a unique Not I site at L-dsRNA map position 12,038 (*11*), was used to transform three virulent *C. parasitica* strains: EP155, vegetative compatibility (VC) group 40 (*29*); EP146 (ATCC number 64671), VC group 9 (*30*); and NB58-19 (ATCC number 76221), a virus-free single conidial isolate of strain NB58 (*31*) of unknown VC group. Designations used for the resulting transformants consisted of fungal strain followed by the transformation vector. Transformants that were resistant to hygromycin and phenotypically similar to hypovirulent strain EP713 had L-dsRNA. The viral dsRNAs present in natural hypovirulent strains EP713 and NB58 are also included for reference. (**B**) To test the resurrected L-dsRNAs for the presence of the sequence corresponding to the introduced Not I site, we used combined reverse transcription and PCR (*23*) to generate an amplified DNA fragment spanning L-dsRNA map positions 11,406 to 12,697 from deoxyribonuclease-treated dsRNA samples prepared from pXH9- and pXH103-transformed strain EP155. Control reactions lacked reverse transcriptase (RT). The 1291-bp amplified fragment was then digested with restriction enzyme Not I, and the products were analyzed by agarose gel electrophoresis. (**C**) Virulent strain EP155 and pXH9 transformants CN2 were inoculated 2 cm apart at the edge of a petri dish that contained PDA. The plate was photographed on day 10. Mycelial samples were taken from the areas of the plate marked by the letters A and B for further culturing and analysis. (**D**) We extracted dsRNA from cultures derived from positions of a paired plating corresponding to letters A (lanes CN2 and CN3) and B (lanes 2B and 3B) shown in (C) and analyzed it by agarose gel electrophoresis. In (A) and (D), the lanes marked M contained the 1-kb DNA ladder (BRL); in (D), L, L-dsRNA, and M, M-dsRNA.

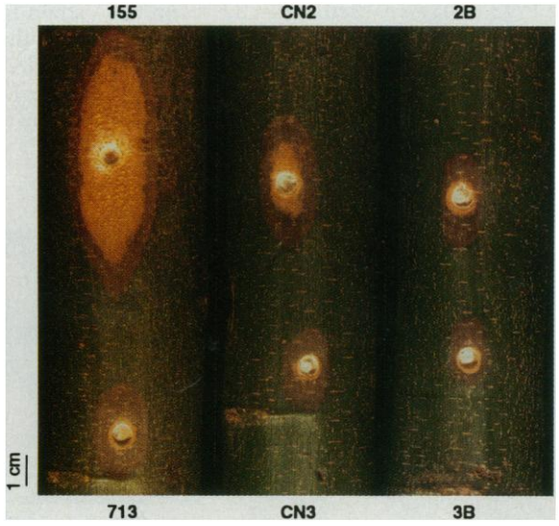


Fig. 4. Virulence assay on dormant American chestnut stems (*25, 26*). Representative examples of cankers formed 11 days after inoculation with virulent strains EP155, hypovirulent strain EP713, pXH9 transformants CN2 and CN3, and the corresponding converted strains 2B and 3B are presented.

Table 1. Results of virulence assays of wild-type, transformed, and converted *C. parasitica* strains on dormant chestnut stems. Virulence assays were performed on dormant chestnut stems (*26*) with measurements taken on days 11 and 21 after inoculation. Data are presented as mean canker area (square centimeters) based on five replicates except for strains CN3 and 2B, for which values were calculated from four replicates. Statistical analysis was performed with the aid of the Statistical Analysis System (SAS Institute, Cary, North Carolina) system for personal computer, release 6.04. Within each data set, mean values followed by the same letters were not significantly different according to Tukey's method of multiple comparison test.

Strain	Mean canker area (cm^2)	
	Day 11	Day 21
EP155	10.28 ± 3.26 a	21.79 ± 8.47 a
EP713	1.65 ± 1.10 b	2.93 ± 2.24 b
CN2	2.73 ± 1.11 b	5.06 ± 2.96 b
CN3	2.62 ± 1.27 b	5.06 ± 2.31 b
2B	1.63 ± 0.72 b	1.92 ± 1.32 b
3B	1.64 ± 0.60 b	2.61 ± 1.08 b

mosis imposed by a vegetative incompatibility system operating in C. *parasitica* (*24*). Thus, HAV dsRNAs are transmitted efficiently only to compatible virulent strains in the population, consequently limiting dissemination. It was reasoned that this barrier to transmission could be circumvented by the transformation of strains of different vegetative compatibility groups with the infectious cDNA copy of L-dsRNA.

To determine whether strains other than EP155 could support the resurrection of L-dsRNA from an integrated cDNA copy, we transformed virulent strains EP155, EP146, and NB58-19, each representing a different vegetative compatibility group, with plasmid pXH103. This plasmid is a modified version of pXH9 in which the viral cDNA sequence was tagged by introduction of a Not I linker at the unique Sna BI site within the 3′ noncoding region at L-dsRNA map position 12,038 (*11*). Most hygromycin-resistant transformants exhibited the white phenotype characteristic of hypovirulent strain EP713 and had L-dsRNA (Fig. 3A). The presence of the introduced Not I site in the L-dsRNA was demonstrated by combined reverse transcription and PCR (Fig. 3B). Thus, the host range of the HAV L-dsRNA was not limited to virulent strains closely related to EP155, and the L-dsRNA present in transformants was directly resurrected from the integrated transformation plasmid.

To test further the biological activity of resurrected L-dsRNA, we examined whether the dsRNA and hypovirulence-associated traits could be cytoplasmically transmitted through anastomosis. Anastomosis-mediated conversion of virulent strain EP155 by HAV L-dsRNA can be monitored visually in vitro after paired inoculation on agar plates (Fig. 3C). The hyphae of strain EP155 that were converted as a result of anastomosis appeared as a wedge of white mycelium that initiated at the interface between the two colonies and extended along the periphery of the orange virulent colony. Samples taken from the CN2 colony at the position marked A were hygromycin-resistant, produced white colonies, and contained L-dsRNA (Fig. 3D). In contrast, samples taken from the orange portion of the EP155 colony were hygromycin-sensitive, produced orange colonies, and were dsRNA-free (*22*). Samples taken from the converted periphery of the EP155 colony in the vicinity of position B were also hygromycin-sensitive (lacked integrated pXH9) but produced white colonies and contained L-dsRNA (Fig. 3D). Significantly, strains converted by anastomosis with pXH9 transformants exhibited the entire range of hypovirulence-associated traits even though they contained only cytoplasmically replicating viral dsRNA in the absence of integrated viral cDNA.

The dsRNA samples extracted from several converted strains contained species in addition to L-dsRNA that were not found in the original CN2 transformant (Fig. 3D). Similar results were obtained after anastomosis of pXH9 transformant CN3 with EP155 (Fig. 3D). Although the new dsRNAs present in these converted strains resembled the internally deleted M-dsRNA species present in strain EP713 (*23*), they were clearly different in terms of electrophoretic mobility, number of species, and relative concentration. The relationship between anastomosis and the generation of internally deleted defective forms of L-dsRNA is currently unclear.

The level of virulence exhibited by pXH9 transformants and converted strains that contained resurrected L-dsRNA was examined by inoculation of dormant chestnut stems (*25, 26*). In such assays, virulent strain EP155 produced prominent expanding cankers within several days after inoculation, whereas cankers incited by the isogenic hypovirulent strain EP713 expanded at a slow rate (Fig. 4). Cankers incited by the pXH9 transformants CN2 and CN3 and the corresponding converted strains 2B and 3B were not significantly different in size (area) from cankers incited by hypovirulent strain EP713 (Fig. 4 and Table 1). Thus, the infectious cDNA clone of HAV L-dsRNA was able to confer hypovirulence as well as hypovirulence-associated traits.

This study clearly demonstrates that a HAV dsRNA is the causal agent of hypovirulence in the chestnut blight fungus. The introduction of an artificial cDNA intermediate into the replication cycle of an HAV dsRNA could also have an impact on efforts to restore the American chestnut. Although hypovirulence has been shown to control chestnut blight in Europe, attempts to apply this biocontrol strategy to North American forest ecosystems have met with limited success (*1, 4*). It is generally accepted that this difference in effectiveness is related to the greater complexity of the vegetative incompatibility structure of C. *parasitica* populations in North America as compared to those in Europe (*1, 4, 27*). Because the hypovirulence phenotype is transmitted efficiently only after anastomosis with compatible virulent strains and has not been reported to be transmitted during mating (*1, 28*), the proportion of virulent strains that would be subject to conversion would be directly related to the vegetative compatibility diversity in the population. The fact that C. *parasitica* transformants

representing different vegetative compatibility groups were found to support replication of resurrected L-dsRNA (Fig. 3A) suggests the possibility that transformation vectors similar to pXH9 could be used to engineer hypovirulence in field isolates that represent the range of vegetative compatibility groups present in a specific ecosystem. Moreover, because sexual compatibility in C. *parasitica* is determined by a single mating-type locus with two alleles (*29*), it is also likely that the integrated viral cDNA copy would spread through the virulent strain population by nuclear inheritance as the result of mating, irrespective of the barriers normally imposed by the vegetative compatibility system. Subsequent resurrection of cytoplasmic L-dsRNA from the inherited viral cDNA could then result in expanded vegetative dissemination. Because the surviving root systems of blight-infested American chestnut trees continue to produce sprouts throughout the natural range (*1*), it is conceivable that the release of improved, genetically engineered hypovirulent C. *parasitica* strains could lead to the restoration of this valuable forest species.

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Identification of a Protein That Binds to the SH3 Region of Abl and Is Similar to Bcr and GAP-rho

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A Src homology 3 (SH3) region is a sequence of approximately 50 amino acids found in many nonreceptor tyrosine kinases and other proteins. Deletion of the SH3 region from the protein encoded by the c-*abl* proto-oncogene activates the protein's transforming capacity, thereby suggesting the participation of the SH3 region in the negative regulation of transformation. A complementary DNA was isolated that encoded a protein, 3BP-1, to which the SH3 region of Abl bound with high specificity and to which SH3 regions from other proteins bound differentially. The sequence of the 3BP-1 protein is similar to that of a COOH-terminal segment of Bcr and to guanosine triphosphatase–activating protein (GAP)–rho, which suggests that it might have GAP activity for Ras-related proteins. The 3BP-1 protein may therefore be a mediator of SH3 function in transformation inhibition and may link tyrosine kinases to Ras-related proteins.

The c-*abl* proto-oncogene is one of many genes that encode nonreceptor tyrosine kinases that contain SH3 regions (*1*). These regions also occur in a wide variety of other proteins, such as phospholipase C-γ and the cytoskeletal proteins, myosin1, spectrin, and ABP-1, an actin binding protein from yeast (*1*). SH3 regions might interact with the actin cytoskeleton and mediate their functions through protein-to-protein interactions (*2*). However, we did not detect any direct binding of the SH3 region of Abl to filamentous actin (F-actin) (*3*). Deletion or mutation of the SH3 region in both Src and Abl activates their transforming abilities, which indicates that this region has a negative regulatory function in transformation (*4*). One possible mode of action of the SH3 region might be to bind to another protein that mediates a negative effect on transformation. We therefore searched for proteins that bind to the Abl SH3 region.

We used the pGEX bacterial expression vector to create a fusion protein that contained the glutathione-S-transferase (GST) of pGEX and the 55–amino acid SH3 region of Abl (*5*). This fusion protein was biotinylated (*6*) and used to probe a λgt11 cDNA expression library made from the mouse pre-B cell line 22D6. Six positive clones out of approximately 7×10^6 were identified (*7*), and five were plaque-purified, yielding cDNA fragments that represented two independent clones. To verify that the β-galactosidase fusion proteins from the recombinant λgt11 vectors were responsible for the reactivity, we produced lysogens from these clones (*8*). These lysogens were induced with isopropylthio-β-D-galactoside (IPTG), and the induced and uninduced proteins were fractionated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and probed with biotinylated SH3-GST fusion peptides (*9*). The GST–Abl SH3 probe recognized a protein of approximately 130 kD from the induced lysate of clone 1 but not from its uninduced lysate and a protein of approximately 150 kD from the induced lysate of clone II but not from its uninduced lysate (Fig. 1). A GST fusion protein probe that contained the SH3 region of murine Src also recognized the 150-kD protein of clone II but did not recognize the 130-kD protein of clone I (Fig. 1). A biotinylated control GST probe reacted only with background proteins of the induced and uninduced lysates. The IPTG-induced 130-kD and 150-kD β-galactosidase fusion proteins of clones I and II

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