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Site-Directed Point Mutation in the *src* Gene of Rous Sarcoma Virus Results in an Inactive *src* Gene Product

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Site-directed mutagenesis techniques were used to construct defined point mutations within the *src* gene of the Prague A strain of Rous sarcoma virus. Bisulfite mutagenesis at a *BglI* restriction site in the *src* gene yielded three mutations which contained the same single base change, a guanine-to-adenine transition. The resulting genomes encoded an *src* protein containing a substitution of threonine for alanine at amino acid position 433. Transfection of chicken cells with mutagenized DNA did not result in cellular transformation even though the cells produced a pp60^{src}. Immune complexes containing mutant pp60^{src} did not phosphorylate immunoglobulin G heavy chain or casein.

Transformation of cells by Rous sarcoma virus (RSV) requires the functional expression of the RSV *src* gene (11). The *src* gene encodes a 60,000-molecular weight (M_r) phosphoprotein (pp60^{src}) (2, 19) which exhibits a unique tyrosine-specific protein kinase activity in vitro, catalyzing the phosphorylation of tyrosine residues in a variety of substrates (4, 5, 13, 16). In vivo cellular transformation results in the tyrosine-specific phosphorylation of defined cellular proteins (1, 6, 8, 20, 22, 23) and an increase in the level of intracellular phosphotyrosine (13, 23). To define important structural and functional domains of pp60^{src}, we used site-directed mutagenesis to introduce point mutations within molecularly cloned Prague A (PrA) RSV DNA (12). Mutagenesis at a *BglI* site 39 base pairs downstream from the codon for tyrosine 416 (21) (the major site of tyrosine phosphorylation [27]) yielded three clones which contained the same single base change within the *BglI* recognition site. The viral genome containing the single base mutation codes for a *src* protein containing a substitution of threonine for alanine at amino acid position 433. Transfection of chicken cells with mutagenized DNA did not result in cellular transformation even though the cells produced a 60,000-M_r phosphoprotein which could be immunoprecipitated with antibodies to pp60^{src}. Mutant pp60^{src} did not phosphorylate immunoglobulin G (IgG) heavy chain or casein in vitro or a cellular substrate, 34,000-M_r protein, in vivo. Therefore, the alteration of the *src* polypeptide sequence containing alanine 433 appears to decrease protein kinase activity and abolish the pp60^{src}-mediated cellular transformation.

PrA RSV DNA contains *BglI* restriction enzyme recognition sites in both the *gag* gene and the *src* gene (12). In addition, there are three *BglI* sites in the plasmid vector pBR322 (27). As a prelude to mutagenesis, two of the *BglI* sites were removed from pBR322, and the modified vector was used to clone a *SalI* fragment containing the complete viral genome, yielding the recombinant plasmid, pΔB Sal 102 (Fig. 1). Mutations at the *BglI* sites in pΔB Sal 102 were constructed by bisulfite mutagenesis (25, 29). Single-stranded nicks in pΔB Sal 102 DNA were introduced by digestion with *BglI* in the presence of ethidium bromide, and form II DNA was purified by agarose gel electrophoresis. The single-stranded nicks were expanded to small gaps utilizing the 5′ → 3′, 3′ → 5′ exonuclease activity of *Escherichia coli* DNA polymerase I. The exposed single-stranded DNA regions were mutagenized with sodium bisulfite, a single-strand-specific mutagen which produces deamination of cytosine residues (25, 26). The gapped region was then repaired with DNA polymerase I and four deoxynucleoside triphosphates. Mutagenized pΔB Sal 102 DNA was used to transform *E. coli* HB101 (9), and the resulting transformants were screened by *BglI* digestion of the plasmid DNA. Restriction enzyme analysis of the DNA from 25 individual transformants identified three plasmids missing the *BglI* site in the *src* gene. Direct DNA sequence analysis (17) revealed that each of these plasmids, pCH1, pCH7, and pCH20, contained the same mutation, a G-C → A-T transition at nucleotide position 1297 (21), a result of a C → T transition in the noncoding strand. The mutation at base pair 1297 gives rise to an alanine-to-threonine change at position 433 in the amino acid sequence of pp60^{src}.

To determine the biological activity of these

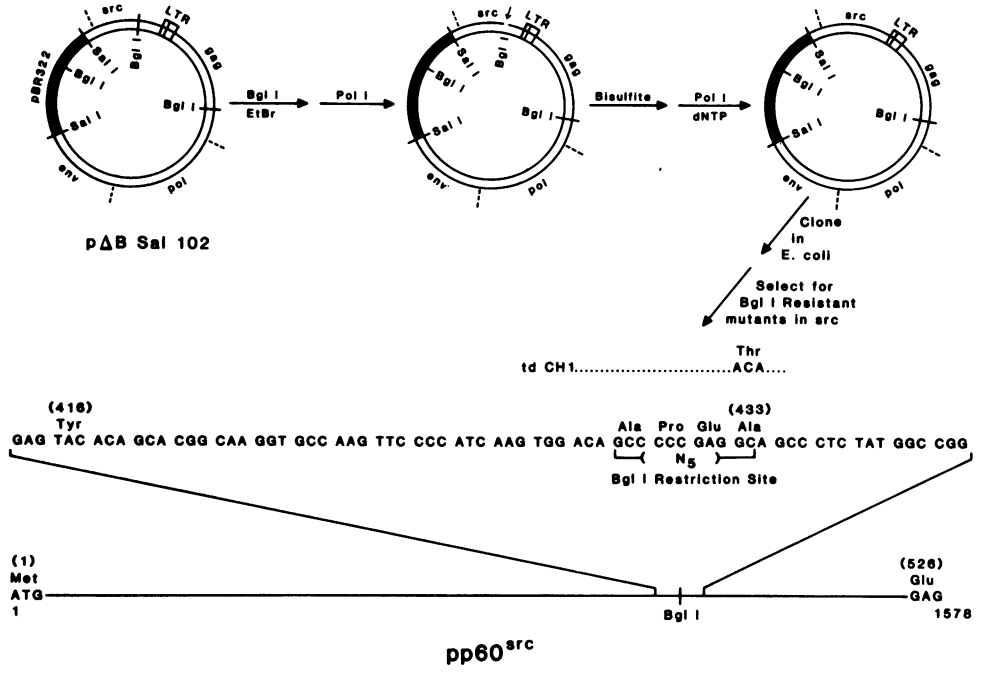

FIG. 1. Construction of mutations at the *BglI* site in the RSV *src* gene and DNA sequence analysis of mutant DNA. A modified pBR322 vector (pΔB322) containing a single *BglI* site (at position 3481) (27) was constructed by partial *BglI* (New England Biolabs) digestion of pBR322 DNA, isolation of a 4.1-kilobase pair *BglI* fragment (a result of cleavage at the *BglI* sites at positions 928 and 1162) (27), exonuclease digestion, and ligation with T4 DNA ligase (New England Biolabs) (12). The resulting DNA was cloned in *E. coli* HB101 (7), DNA from the ampicillin-resistant, tetracycline-sensitive transformants was screened by digestion with *BglI*, and plasmids containing a single *BglI* site were identified. One such plasmid, pΔB322, was used as a vector to reclone a *SalI* fragment containing a complete copy of the PrA RSV DNA genome (12). DNA from the resulting plasmid, designated pΔB Sal 102, was subjected to bisulfite mutagenesis (25, 26) as follows. The *BglI*-specific conversion of pΔB Sal 102 DNA from form I to form II (nicked circles) was carried out by incubation of 8 μg of form I DNA with 320 U of *BglI* and 6 μg of ethidium bromide in 500 μl of 6 mM Tris-hydrochloride, pH 8.0, 0.1 M NaCl, and 6 mM $MgCl_2$ for 2 h at 23°C. Form II DNA was purified by agarose gel electrophoresis, electroelution, and DEAE-cellulose chromatography and incubated with *E. coli* DNA polymerase I (Bethesda Research Labs) to generate gaps at single-strand nicks. Gapped DNA was incubated with sodium bisulfite as described by Shortle and Nathans (25, 26), and after mutagenesis, the DNA was repaired with *E. coli* DNA polymerase I (9.6 U of DNA polymerase, 0.18 μmol of deoxynucleotide triphosphates in 100 μl of 50 mM Tris-hydrochloride, pH 7.8, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 50 μg of bovine serum albumin per ml; at 15°C for 60 min). Repaired DNA was ligated with T4 ligase (12) and used to transform *E. coli* HB101 (7). DNA from individual transformants was isolated and digested with *BglI*, and the restriction fragments were resolved by agarose gel electrophoresis on 0.85% agarose gels. Three mutations missing the *BglI* site in the *src* gene were identified, pCH1, pCH7, pCH20 (see text). For DNA sequence analysis, a 917-base pair *TaqI* fragment spanning the *src BglI* site was isolated, end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs), and subjected to Maxam-Gilbert DNA sequence analysis (17). The numbering of the *src* nucleotide sequence and the deduced amino acid sequence is based on the nucleotide sequence of PrC RSV (21).

mutants, the viral DNA insert of pCH1, pCH7, and pCH20 was removed from the plasmid and used to transfect chicken embryo cells as described previously (12). In contrast to cells transfected with wild-type viral DNA, cells transfected with either CH1, CH7, or CH20 DNA remained morphologically normal. Cells transfected with CH1, CH7, or CH20 were resistant to superinfection with PrA RSV, indicating complete infection of the cultures. Culture media from cells transfected with the mutagenized DNA could be used to infect normal cells, indicating that the mutants were not defective for virus replication.

To examine the expression of the altered *src* gene, chicken embryo cells were infected with PrA RSV or with tdCH20 (either viral DNA or virus from transfected cells) and labeled with [^{35}S]methionine. Labeled cell extracts, prepared as described in the legend to Fig. 2, were im-

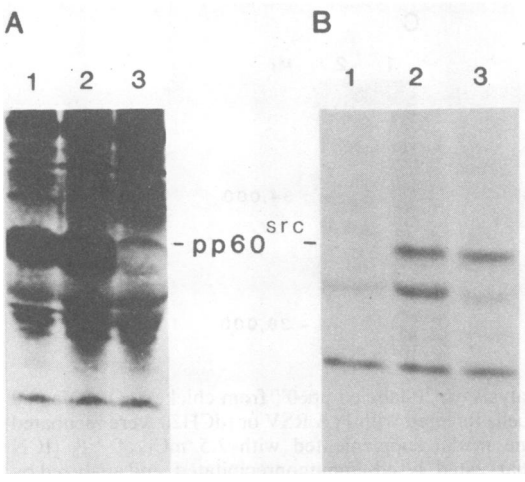

FIG. 2. Immunoprecipitation of pp60^{src} from chicken cells infected with PrA RSV and tdCH20. Chicken embryo cells, uninfected or infected with either PrA RSV or tdCH20, were incubated in methionine-free media for 2 h and then in the same media supplemented with 300 μCi of [^{35}S]methionine (Amersham Corp.) per ml for 4 h. Labeled cells were harvested at 4°C, lysed in 2 ml of 10 mM Tris-hydrochloride, pH 7.2–0.1 M NaCl–1 mM EDTA–1% Nonidet P-40–0.5% deoxycholate (18), and centrifuged for 30 min at 100,000 × *g*. A portion of each cell extract was incubated with (A) 2 μl of rabbit antibacterial p60^{src} sera (9) or (B) 20 μl of ascites fluid containing monoclonal antibody against p60^{src} at 0°C for 60 min and then with *Staphylococcus aureus* protein A for 60 min at 0°. The immune complexes were collected by centrifugation and washed twice with lysis buffer, once with 10 mM Tris-hydrochloride, pH 7.2–1 M NaCl–0.1% Nonidet P-40, and finally with the lysis buffer. The immunoprecipitates were suspended in 50 μl of sample buffer (70 mM Tris-hydrochloride, pH 7.2, 5% 2-mercaptoethanol, 3% sodium dodecyl sulfate, 11% glycerol, 0.01% bromophenol blue), incubated at 100°C for 15 min, and subjected to electrophoresis on a 9.5% polyacrylamide gel (14). The position of pp60^{src} was determined relative to the position of known molecular weight standards (92,000, phosphorylase B; 66,000, bovine serum albumin; 45,000, ovalbumin; 31,000, carbonic anhydrase). (A) Lane 1, PrA RSV-infected cells; lane 2, tdCH20-infected cells; lane 3, uninfected cells. (B) Lane 1, uninfected cells; lane 2, PrA RSV-infected cells; lane 3, tdCH20-infected cells. Samples immunoprecipitated with normal rabbit serum contained no visible bands upon autoradiography (data not shown).

munoprecipitated with rabbit anti-p60^{src} sera (Fig. 2A) (9) or a monoclonal antibody directed against p60^{src} (18a) (Fig. 2B) and analyzed by polyacrylamide gel electrophoresis. Cells infected with tdCH20 contained a 60,000-M_r *src* protein present at a level comparable to that observed in PrA RSV-infected cells (Fig. 2A and B). Identical results were obtained with tdCH1- and tdCH7-infected cells (data not shown). Therefore, cells infected with mutant virus express an altered p60^{src} which is unable to mediate cellular transformation.

Wild-type pp60^{src} is phosphorylated on both serine and tyrosine residues (4). To determine the phosphorylation state of the altered p60^{src}, cells infected with tdCH20 or PrA RSV were labeled with $^{32}P_i$ and immunoprecipitated with antisera to pp60^{src} (Fig. 3A and B). Although mutant- and wild-type-infected cells contain similar levels of p60^{src}, we consistently observed a decreased level of p60^{src} phosphorylation in tdCH20-infected cells (Fig. 3A and B). To determine whether both serine and tyrosine residues were phosphorylated in the altered pp60^{src}, the ^{32}P-labeled *src* proteins (Fig. 3B) were excised from the gel and subjected to partial V8 protease cleavage (Fig. 3C). Both mutant and wild-type pp60^{src} yielded similar amounts of a 34,000-M_r peptide (containing phosphoserine) and a 26,000-M_r peptide (containing phosphotyrosine) (4, 27). Therefore, the observed underphosphorylation of tdCH20 pp60^{src} does not arise from a specific block in the phosphorylation of either serine or tyrosine.

To assess the degree to which the mutations in tdCH1 and tdCH20 altered pp60^{src} kinase activity, infected cell lysates were incubated with tumor-bearing rabbit sera, and the immune complexes were collected on *Staphylococcus aureus* protein A-Sepharose and assayed for their ability to phosphorylate the heavy chain of IgG. Figure 4A shows that immune complexes from either tdCH1- (lane 3) or tdCH20-infected (lane 4) cells exhibited little kinase activity above the level present in uninfected cells (lane 1). In contrast, immune complexes isolated from PrA RSV-infected cells (lane 2) readily phosphorylated IgG heavy chain. In a parallel experiment, each of the immune complexes was tested for the ability to phosphorylate casein. Immune complexes from PrA RSV-infected cells readily catalyzed phosphorylation of casein, whereas immune complexes from tdCH1- and tdCH20-infected cells exhibited little activity greater than that observed in uninfected cells (data not shown).

A number of cellular proteins, including a 34,000-M_r protein, have been suggested as in vivo substrates for pp60^{src} in that they are phosphorylated on tyrosine residues in cells transformed with RSV (1, 6, 8, 20, 22, 23). To examine the 34,000-M_r protein, uninfected cells and cells infected with tdCH20 or PrA RSV were labeled with $^{32}P_i$, and cell extracts were immunoprecipitated with antisera directed against the 34,000-M_r protein. Figure 4B shows that 34,000-M_r protein is phosphorylated to approximately the same extent in uninfected (lane 1) and mu-

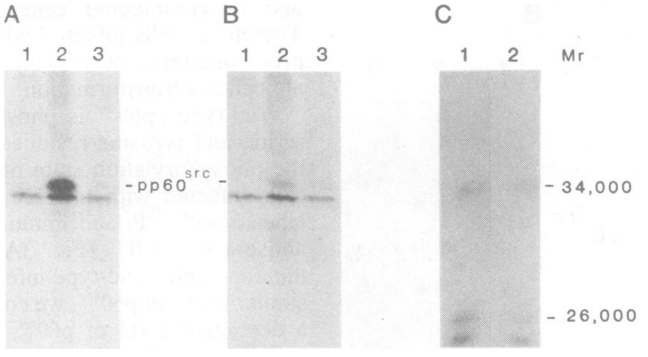

FIG. 3. Immunoprecipitation and phosphorylation analysis of ^{32}P-labeled pp60^{src} from chicken cells infected with PrA RSV and tdCH20. Uninfected chicken cells or cells infected with PrA RSV or tdCH20 were incubated in phosphate-free media for 2 h and then in the same media supplemented with 2.5 mCi of $^{32}P_i$ (ICN Pharmaceuticals, Inc.) per ml for 4 h. Labeled cells were harvested, lysed, immunoprecipitated, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described in the legend to Fig. 2. (A) Immunoprecipitation with rabbit anti-p60^{src} sera. (B) Immunoprecipitation with monoclonal antibody directed against p60^{src}. For both (A) and (B), lane 1, uninfected cells; lane 2, PrA RSV-infected cells; lane 3, tdCH20-infected cells. (C) The pp60^{src} bands were excised from (B) and reelectrophoresed in the presence of 10 ng of *Staphylococcus* V8 protease on a 12.5% polyacrylamide gel as described by Cleveland et al. (3). Lane 1, pp60^{src} from PrA RSV-infected cells; lane 2, pp60^{src} from tdCH20-infected cells. M_r markers are shown on the right.

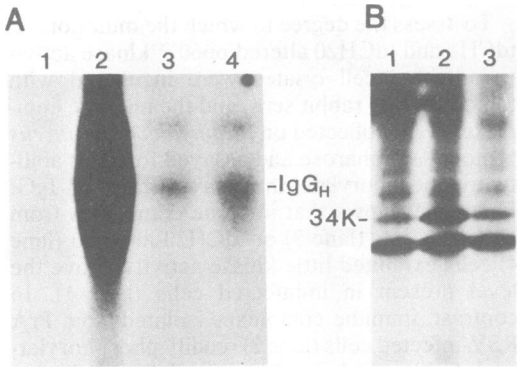

FIG. 4. Phosphorylation of IgG heavy chain (IgG_H) in vitro and 34,000-M_r protein (34K) in vivo. (A) Chicken embryo cells, uninfected or infected with PrA RSV, tdCH1, or tdCH20, were lysed and incubated with tumor-bearing rabbit sera, and the immune complexes were collected on *S. aureus* protein A-Sepharose and washed as described in the legend to Fig. 2. After the third wash, the immune complexes were washed once in phosphate-buffered saline and suspended in 50 μl of kinase buffer (20 mM potassium phosphate, pH 7.2, 0.1 M NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM 2-mercaptoethanol, 1 μCi of [γ-^{32}P]ATP [3,000 Ci/mmol; New England Nuclear Corp.). Mixtures were incubated at 37°C for 30 min, mixed with sample buffer, boiled, and subjected to electrophoresis on a 9.5% polyacrylamide gel and autoradiography. Lane 1, uninfected cells; lane 2, PrA RSV-infected cells; lane 3, tdCH1-infected cells; lane 4, tdCH20-infected cells. (B) Uninfected chicken cells or cells infected with PrA RSV or tdCH20 were labeled with $^{32}P_i$ as described in the legend to Fig. 3. The cells were harvested, lysed, immunoprecipitated, and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography as described in the legend to Fig. 2, with the exception that cell lysates were immunoprecipitated with rabbit anti-34,000-M_r sera. Lane 1, uninfected cells; lane 2, PrA RSV-infected cells; lane 3, tdCH20-infected cells.

tant-infected (lane 3) cells, whereas the level of phosphorylation is increased in PrA RSV-infected cells (lane 2).

In vitro mutagenesis of a region of the RSV *src* gene proximal to the major site of tyrosine phosphorylation (27) has yielded viruses defective for cellular transformation. Direct sequence analysis of the mutagenized DNA revealed a single G-C → A-T change, which gives rise to an alanine-to-threonine change in the amino acid sequence of pp60^{src}. Two experimental observations are consistent with our assumption that this alteration in the pp60^{src} protein sequence is responsible for defective *src* protein. First, repeated passage of mutant virus in chicken cells results in the formation of focus-forming (revertant) virus within three to four passages. DNA blot analysis (28) of *Bgl*I-digested cellular DNA, isolated from cells transformed with revertant virus, revealed the presence of a *Bgl*I site within the *src* gene. Therefore, the recovery of wild-type virus is consistent with the reversion of a single point mutation residing within the *Bgl*I site. Second, DNA sequence analysis of the regions flanking the *Bgl*I site, as well as restriction enzyme analysis with a variety of restriction enzymes with four base recognition sites, has

revealed no additional alterations in the *src* DNA sequence. Thus, it appears unlikely that a second mutation elsewhere in the *src* gene is responsible for the transformation defectiveness of the virus mutants.

In the mutants reported here, we have engineered an alteration in the nucleotide sequence of the pp60^{src} gene that results in the production of a functionally inactive *src* gene product, as judged by the inability of mutagenized DNA or virus to induce cell transformation. The low levels of IgG kinase activity observed in vitro, as well as the lack of 34,000-M_r phosphorylation in vivo, would suggest that the alteration of the alanine at position 433 in the amino acid sequence to threonine results in an inactivation of the kinase activity of pp60^{src}. This conclusion would be consistent with recent observations suggesting that the kinase activity resides within the carboxy terminal half of pp60^{src} (15). DNA sequence analysis of the Fujinami sarcoma virus oncogene, *fps*, and the Snyder-Theilen and Gardner-Arnstein feline sarcoma virus oncogene, *fes*, has revealed a high degree of amino acid sequence homology (about 40%) between the carboxy terminal portions of pp60^{src} and the putative transforming proteins encoded by *fps* and *fes* (10, 24). Interestingly, the alanine at position 433 of pp60^{src} resides within a heptapeptide sequence (lys-trp-thr-ala-pro-glu-ala$_{433}$) which is identical in these three transforming proteins.

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