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Neither Arginine nor Histidine Can Carry Out the Function of Lysine-295 in the ATP-Binding Site of p60^{src}

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All 15 protein kinases whose amino acid sequence is known contain a lysine residue at a position homologous to that of lysine-295 in p60^{src}, the transforming protein of Rous sarcoma virus. The ATP analog *p*-fluorosulfonyl 5′-benzoyl adenosine inactivates both p60^{src} and the catalytic subunit of the cyclic AMP-dependent protein kinase by modification of this lysine. We used oligonucleotide-directed mutagenesis to examine the possible functions of this residue. Lysine-295 in p60^{src} was replaced with a glutamic acid, an arginine, or a histidine residue, and mutant p60^{src} proteins were characterized in chicken cells infected by mutant viruses. None of these three mutant p60^{src} proteins had tyrosine protein kinase activity in vitro, and none induced morphological transformation of infected cells. Since neither a histidine nor an arginine residue can replace the function of lysine-295, we suggest that it carries out the specialized function of proton transfer in the phosphotransferase reaction. All three mutant viruses underwent reversion to wild type during passage in tissue culture. Because the rate with which this occurred differed significantly among the mutants, reversion appears to have resulted from errors in transcription, rather than from recombination with the cellular *src* gene.

Oncogenic transformation by Rous sarcoma virus (RSV) results from the expression of p60^{src} (22), a virally encoded phosphoprotein that possesses tyrosine-specific protein kinase activity (7, 14). p60^{src} is one of 12 members of the tyrosine protein kinase family. Partial tryptic digestion has been used to isolate a 30-kilodalton (kDa) catalytic domain from the carboxy terminus of p60^{src} which functions autonomously as a tyrosine protein kinase, indicating that it contains all amino acids necessary for catalysis (19). The catalytic domain of serine protein kinases has both structural and functional homology with the kinase domain of tyrosine protein kinases. Direct sequence comparison of residues 259 to 485 of p60^{src} reveals 22% sequence homology with residues 33 to 258 of the cyclic AMP-dependent serine protein kinase (1). Functional homology within the active sites of these two kinases was recently established by demonstration that the ATP analog *p*-fluorosulfonyl 5′-benzoyl adenosine reacts with a lysine found in a homologous position in both p60^{src} (lysine-295) and the cyclic AMP-dependent protein kinase (lysine-72) (16, 29). This lysine is, in fact, 1 of 12 residues which are strictly conserved within the catalytic domains of every protein kinase sequenced to date (13).

What is the exact function of this lysine? Lysine-295 of p60^{src} may have one of four functions. First, it could be present on the surface of p60^{src} and aid in orienting this region of the enzyme toward the hydrophilic cytosol. Second, lysine-295 might be the cationic half of a salt bridge which stabilizes the structure of p60^{src} in or near the ATP-binding site. A third possiblity is that lysine-295 is present within the ATP-binding site and stabilizes directly the negatively charged phosphates of ATP. Fourth, lysine-295 might remove or donate a proton at the active site of p60^{src} and therefore be one of the catalytic residues.

We used oligonucleotide-directed mutagenesis in an attempt to determine which of these functions lysine-295 performs. Codons for glutamic acid, arginine, and histidine residues were substituted for the codon for lysine-295 in a clone of RSV DNA. If only a charged residue was needed at position 295, without regard to sign, all three substituted amino acids should be acceptable. On the other hand, the ability of lysine-295 to form a salt bridge or bind ionicly the phosphates of ATP might be unaffected by the substitutions of arginine or histidine but could not be replaced by glutamic acid. In contrast, the more specialized catalytic functions of proton transfer would be dramatically affected by any substitutions at position 295. Our results show that mutants of p60^{src} containing glutamic acid, arginine, or histidine at position 295 are inactive tyrosine protein kinases and cannot transform chicken embryo fibroblasts. We suggest that lysine-295 is a catalytic residue and most likely performs the role of proton transfer in the active site of p60^{src}.

MATERIALS AND METHODS

Site-specific mutagenesis. Oligonucleotide-directed mutagenesis of the codon for lysine-295 of p60^{src} was performed by using viral DNA from the Prague strain of RSV subgroup C which had been cloned originally in pBR322 from unintegrated circular viral DNA (17). This clone is designated pATV-8. To accomplish the mutagenesis, a 2.7-kilobase *Sst*I fragment encompassing the v-*src* gene and 3′ long terminal repeat was removed from pATV8 and inserted into the polylinker of M13mp10. Site-directed mutagenesis (30) was performed with single-stranded M13 virion DNA, using three oligonucleotides, each 19 residues long, which encompassed the codon for lysine-295. Oligonucleotide CTTCAGAGT TTCTATGGCC was used to create the glutamic acid mutant, CTTCAGAGTTCTTATGGCC was used for the arginine mutant, and CTTCAGAGTGTGTATGGCC was used for the histidine mutant.

Isolation of mutant viruses. To isolate mutant virus, we used the mutagenized 2.7-kilobase *Sst*I fragment, excised from the replicative form of M13, to replace the wild-type fragment in the original plasmid, pATV8. The completely reconstructed viral DNA insert was then excised by partial digestion with restriction endonuclease *Hin*dIII. Because the

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viral genes are permuted in pATV8, the excised DNA was concatamerized before transfection into chicken embryo fibroblasts (10). Virus stocks were harvested from transfected cultures after 3 weeks.

Biosynthetic labeling. Labeling with methionine was performed by growing cells for 16 h in the presence of 100 μCi of [^{35}S]methionine (1,400 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) in 1.0 ml of Dulbecco-Vogt modified Eagle medium containing 20% of the normal concentration of methionine and 4% calf serum. Cells were labeled with $^{32}P_i$ by incubation for 16 h in the presence of 1.0 mCi of $^{32}P_i$ (ICN Pharmaceuticals Inc., Irvine, Calif.) in 1.0 ml of Dulbecco-Vogt modified Eagle medium lacking phosphate which had been supplemented with 4% calf serum dialyzed against water.

Angiotensin assays. Immunoprecipitates of p60^{src} were formed from a Nonident P-40 (NP-40) buffer (150 mM NaCl, 10 mM sodium phosphate [pH 7.2], 1% NP-40, 1% Trasylol, 2 mM EDTA) extract of infected cells by using an antipeptide serum specific for the carboxy-terminal six amino acids of p60$^{v\text{-}src}$ and denoted anti-*src*-c (23). After three washes in NP-40 buffer, the immunoprecipitates were washed once in phosphate-buffered saline (10 mM sodium phosphate [pH 7.2], 150 mM NaCl) and suspended in 10 μl of kinase buffer (10 mM sodium phosphate [pH 7.2], 5 mM $MgCl_2$) containing 1 μM ATP, 5 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham), and 2 mM [Val^5]angiotensin II (Sigma Chemical Co., St. Louis, Mo.). The mixture was incubated at 30°C for 10 min followed by heating at 90°C to inactivate p60^{src}. All samples were analyzed by one-dimensional electrophoresis at pH 3.5 on cellulose thin-layer plates as described elsewhere (28).

Gradient centrifugation. To determine the extent of complex formation, we separated the population of p60^{src} which is physically associated with hsp 90 and p50 from the monomeric pool of p60^{src} using velocity sedimentation in glycerol gradients. Cells were labeled overnight with 100 μCi of [^{35}S]methionine as described above, lysed in NP-40 buffer, clarified by centrifugation at 20,000 × *g* for 1 h, layered over 5-ml gradients of 5 to 20% glycerol in NP-40 buffer, and subjected to centrifugation for 17 h at 100,000 × *g*. Fractions (0.25 or 0.40 ml) were collected, and p60^{src} was isolated by immunoprecipitation with anti-*src*-c serum (23). The anti-carboxy-terminal peptide serum immunoprecipitates both the complexed and monomeric forms of wild-type p60^{src} efficiently in NP-40 buffer; however, the complexed form does not precipitate in RIPA buffer with this serum. The immunoprecipitates were analyzed on sodium dodecyl sulfate-polyacrylamide gels.

Reverse transcriptase assays. Culture medium (5 ml) was spun at 100,000 × *g* for 45 min. The supernatant was aspirated, and the pelleted viruses were assayed for reverse transcriptase activity with poly(rC) as a synthetic RNA template and [^{3}H]dGTP as the incorporated nucleotide by the method of Verma and Baltimore (26). After a 60-min incubation at 37°C, portions of each reaction were assayed by spotting them on DEAE-cellulose filter disks. The disks were washed twice in 0.36 M NaCl–20 mM sodium phosphate (pH 7.7)–2 mM EDTA, once in 95% ethanol, and then dried. Radioactivity was quantified by scintillation spectroscopy.

RESULTS

Glu-295, Arg-295, and His-295 mutants of p60^{src} are transformation defective. Using site-directed mutagenesis we replaced the codon for lysine-295 (AAG) with one for glutamic acid (GAA), arginine (AGA), or histidine (CAC) and designated the viruses encoding these mutant *src* proteins SD1, SD2, and SD3, respectively. These specific codon changes were verified by Maxam-Gilbert DNA sequence analysis (19a). In addition, the sequence of 100 flanking nucleotides was determined and found to be unchanged. Chicken embryo fibroblasts infected by SD1, SD2, and SD3 and producing titers of virus 70 to 90% as great as that of wild-type-transformed cells remained morphologically normal (data not shown).

SD1, SD2, and SD3 encode mutant p60^{src} proteins which lack tyrosine protein kinase activity. To examine whether these mutant proteins possessed tyrosine protein kinase activity, they were immunoprecipitated from infected chicken cells with anti-*src*-c serum, which is specific for the carboxy-terminal hexapeptide of p60$^{v\text{-}src}$. This serum was used to minimize the possible interference between the antibody and the catalytic region of p60^{src}. The ability of these immunoprecipitates to phosphorylate the tyrosine residue in [Val^5]angiotensin II, a well-characterized peptide substrate of the wild-type p60^{src} (28), was quantified. The relative abundance of p60^{src} in each immunoprecipitate was determined by immunoprecipitation of p60^{src} from parallel cultures labeled with [^{35}S]methionine and measurement of the amount of radioactivity in p60^{src}. The extent of [Val^5]angiotensin II phosphorylation was normalized to the amount of p60^{src}. The mutant proteins lacked any detectable tyrosine protein kinase activity (Table 1).

Mutant proteins are less abundant than wild-type p60^{src}. We next asked whether substitutions at position 295 affected the structure of p60^{src}. The metabolic stability of mutant p60^{src} proteins was estimated by comparing their steady-state abundance to that of the wild type in infected cells. For these comparisons, we measured the extent of infection of the cultures by assaying viral reverse transcriptase in the culture medium. All cultures were found to be infected by mutant viruses to an extent at least 70% that of the wild-type virus. p60^{src} was isolated by immunoprecipitation from chicken cells infected by SD1, SD2, or SD3 virus in three separate experiments (Fig. 1). The carboxy-terminal antiserum does not immunoprecipitate the complexed form of p60^{src} in RIPA buffer (23), and consequently the p50 and hsp 90 cellular proteins found in this complex are not evident on

TABLE 1. Mutant p60^{src} proteins lack kinase activity and are phosphorylated poorly

Virus (amino acid substitution)	Relative incorporation in p60^{src} [a]		Relative kinase activity[b] (^{32}P-Val^5 AngII/unit[c])
	[^{35}S]methionine	$^{32}P_i$/[^{35}S]methionine	
SD1 (Glu)	9	33	<2
SD2 (Arg)	23	28	<1
SD3 (His)	14	29	<1
SDWT[d] (Lys)	100	100	100

[a] In all cases, [^{35}S]methionine incorporation into p60^{src} was normalized to that of hsp 90 to account for differences in the specific activity of proteins derived from cells infected with each of the four viruses. Each value represents the average from three independent experiments.

[b] Average values from two independent experiments.

[c] Arbitrary units normalized to p60^{src} from densitometric scans of autoradiograms. Val^{5}AngII, [Val^5]angiotensin II. In all cases, immunoprecipitates prepared from uninfected cells were used to estimate background, which was subtracted in calculating these values.

[d] WT, Wild type.

this gel. The abundance of all three mutant proteins was substantially less than that of the wild-type protein (Table 1). A direct pulse-chase analysis demonstrated that the half-life of the histidine mutant, encoded by SD3, was reduced from 5.6 to 2.4 h.

The extent of phosphorylation of p60^{src} was used as a second criterion to judge the structural integrity of the mutant proteins. All of the mutant proteins were found to contain phosphate. However, the extent of phosphorylation, as measured by the ratio of incorporation of $^{32}P_i$ and [^{35}S]methionine, was approximatley 30% that of the wild-type protein (Fig. 1; Table 1). Partial proteolysis with *Staphylococcus aureus* V8 protease was used to examine the location of the phosphorylation sites in the mutant proteins. At low concentrations, *S. aureus* V8 protease cleaves p60^{src} into a major 34-kDa amino-terminal fragment which contains phosphoserine and a 26-kDa carboxy-terminal species which encompasses tyrosine-416, the major site of tyrosine phosphorylation (8). The 34-kDa fragment derived from the amino terminus is subsequently cleaved to two overlapping amino-terminal fragments of 20 and 18 kDa (8). Partial proteolysis of the mutant p60^{src} proteins by *S. aureus* V8 protease revealed an almost complete lack of phosphate in the 26-kDa carboxy-terminal fragment which contains the normal site of tyrosine phosphorylation, tyrosine-416. All mutant proteins contained phosphate in their amino-terminal 18-kDa domain, presumably at serine-17 (Fig. 2).

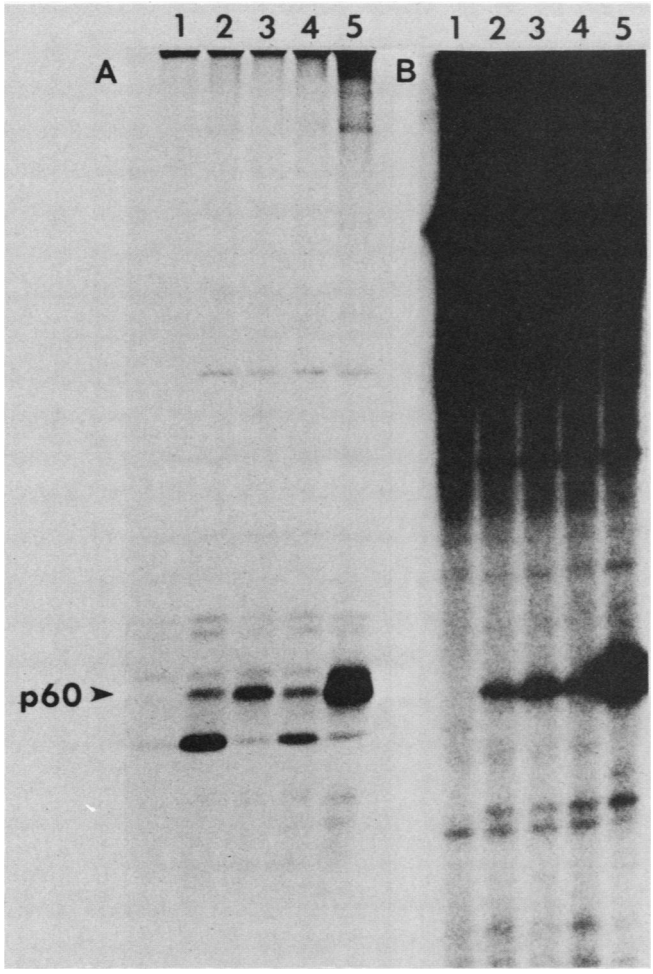

FIG. 1. Abundance and phosphorylation of mutant and wild-type p60^{src}. p60^{src} was isolated by immunoprecipitation from chicken embryo fibroblasts labeled for 16 h [^{35}S]methionine (A) or $^{32}P_i$ (B). To control for the possibility of differences in the specific activity of labeled protein among the labeled cultures, the phosphoprotein hsp 90 was immunoprecipitated quantitatively from a fraction of both [^{35}S]methionine- and $^{32}P_i$-labeled lysates. Variations in the specific activity of hsp 90 were measured, and the amount of p60^{src} sample loaded on each gel was normalized for any such changes. Cells were infected with: lanes 1, no virus; lanes 2, SD1 virus; lanes 3, SD2 virus; lanes 4, SD3 virus; lanes 5, wild-type virus.

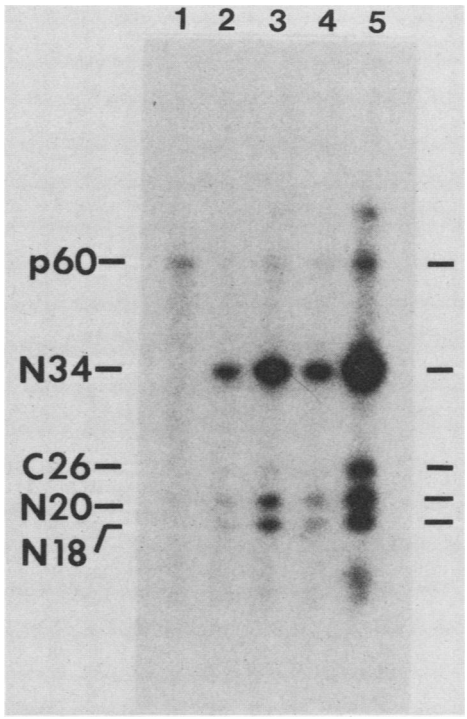

FIG. 2. Mutant p60^{src} proteins lack phosphate in their carboxy-terminal 26-kDa fragment. Mutant and wild-type p60^{src} proteins were isolated by immunoprecipitation from infected chicken embryo fibroblasts labeled for 16 h in the presence of $^{32}P_i$. Partial proteolysis of the p60^{src} proteins with *S. aureus* V8 protease was performed by a modified Cleveland method, and the resulting peptides were separated by gel electrophoresis. p60 designates undigested p60^{src} protein, N34 designates the amino-terminal 34-kDa fragment containing phosphoserine, and C26 designates the carboxy-terminal 26-kDa fragment which contains mostly phosphotyrosine. N20 and N18 designate the overlapping amino-terminal fragments which are derived from N34. Chicken embryo fibroblasts were infected with: lane 1, no virus; lane 2, SD1 virus; lane 3, SD2 virus; lane 4, SD3 virus; lane 5, wild-type virus.

Finally, we examined the association p60^{src} with two cellular proteins, p50 and hsp 90 (3). The complex of p60^{src}, p50, and hsp 90 sedimented as a 200-kDa species during centrifugation through 5 to 20% glycerol gradients. In the case of wild-type p60^{src} from this stock of Prague RSV-C, 20 to 40% of p60^{src} could be found in this complex. Approximately 40 to 60% of each of the mutant proteins were found in this rapidly sedimenting complex (Fig. 3).

Rapid reversion occurs in cultures infected with SD1, SD2, and SD3 virus. The experiments described here could only be performed during a narrow window of time. Invariably, revertant transformation-competent viruses arose in the infected cultures. In fact, revertants arose so quickly in SD1

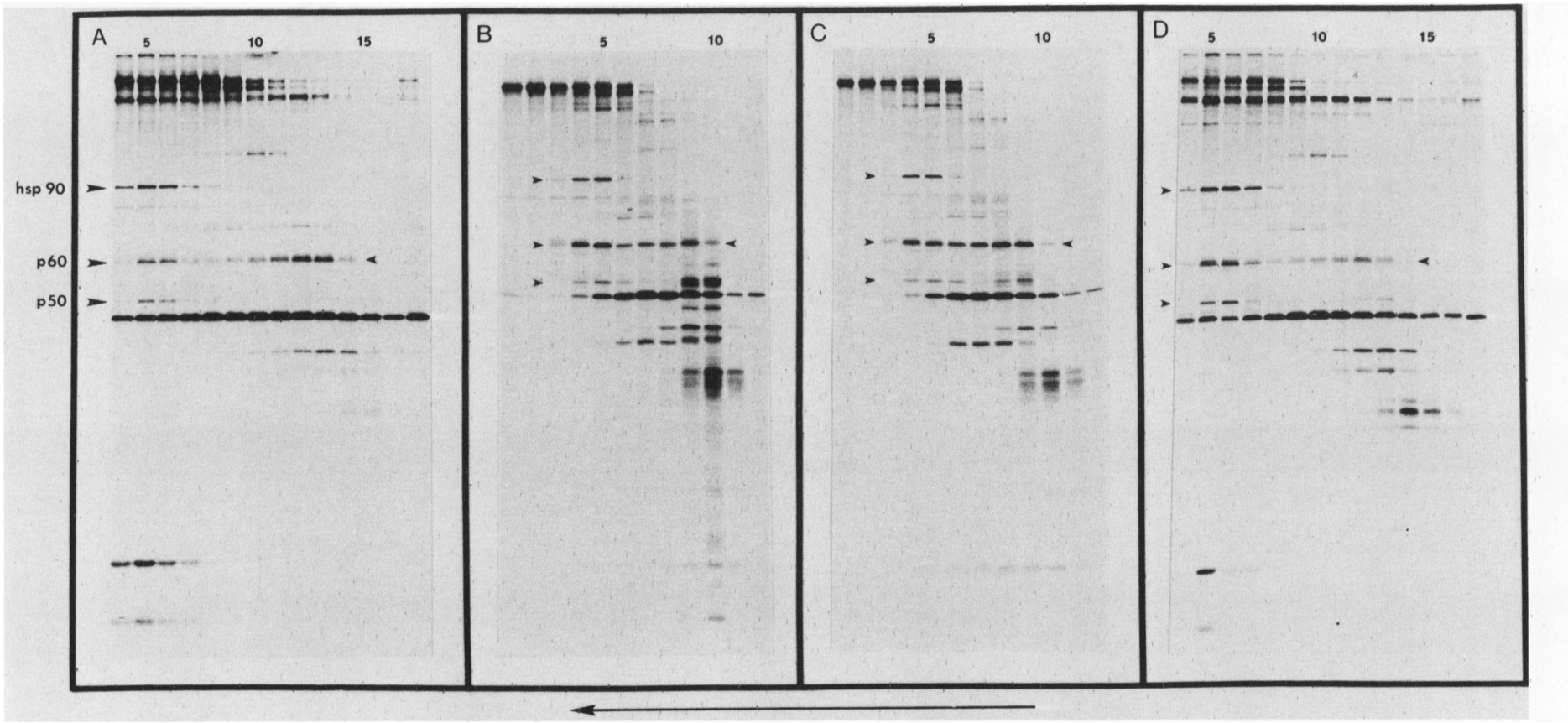

FIG. 3. Mutant $p60^{src}$ proteins form a complex with p50 and hsp 90. Infected chicken cells were labeled for 16 h with [^{35}S]methionine. Cells were lysed in NP-40 buffer (see the text), and the lysates were sedimented at 150,000 × *g* for 19 h at 4°C in gradients of 5 to 20% glycerol in NP-40 buffer. Proteins were immunoprecipitated from gradient fractions with anti-*src*-c serum, analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and visualized by fluorography. $p60^{src}$, p50, and hsp 90 are indicated with arrows. The direction of sedimentation was from right to left and is indicated at the bottom of the figure. Fraction numbers are indicated at the top of the fluorograms. In panels A and D, the 5-ml gradients were divided into 17 fractions, whereas in panels B and C, only 12 fractions were collected. Proteins were immunoprecipitated from cells infected with: panel A, wild-type virus; panel B, SD1 virus; panel C, SD2 virus; panel D, SD3 virus.

and SD2 virus-infected cultures that biological characterization needed to be performed on cells which had been transfected with mutant viral DNA. Reversions could result from recombination with the cellular c-*src* gene, from mistakes made by either reverse transcriptase or RNA polymerase during viral replication and propagation, or from errors introduced by DNA polymerase within the integrated provirus during the replication of host-cell DNA. To distinguish among these alternatives, we compared the reversion rates of the three mutant viruses. Duplicate plates of chicken cells were transfected with the DNAs of the three mutants, and the time required for the unequivocal appearance of revertant virus was measured. Transformed areas appeared in the cells transfected with either SD1 or SD2 DNA in 15 to 21 days, an average of 6 days after the cells were fully infected. In contrast, transformed cells did not appear in the cultures of cells transfected with SD3 viral DNA until an average of 47 days (Table 2).

TABLE 2. SD1 and SD2 revert rapidly in tissue culture

Mutant virus	Mutant codon[a]	Minimum mutation required to regenerate any lysine codon	Observed reversion time (days)[b]
SD1	GAA (Glu)	1 transition (AAG or AAA)	3
SD2	AGA (Arg)	1 transition (AAA)	6
SD3	CAC (His)	2 transversions (AAG or AAA)	35

[a] The wild-type codon is AAG (Lys).
[b] Observed time for the production of transforming virus from chicken embryo fibroblasts fully infected by the nontransforming mutants.

DISCUSSION

Properties of mutant p60^{src}s encoded by SD1, SD2, and SD3. Our data indicate that lysine-295 in p60^{src} is absolutely essential for the protein kinase activity of p60^{src}. Even an arginine or a histidine residue cannot replace it. Substitutions at lysine-295 did not affect the avid association of p60^{src} with the two cellular proteins p50 and hsp 90. In fact, a larger percentage of the mutant p60^{src} was bound to p50 and hsp 90 than is the case with the wild-type protein. Consequently, changes in the sequence of p60^{src} at position 295 apparently do not disturb significantly the surface region which interacts with p50 and hsp 90.

All three mutant p60^{src} proteins contained less phosphate than does wild-type p60^{src}. Tyrosine-416, the major site of tyrosine phosphorylation in p60^{src}, was not phosphorylated at all. This suggests, but does not prove, that this residue is phosphorylated exclusively by p60^{src} itself. In addition, each mutant p60^{src} protein contained less phosphoserine than did the wild-type protein. In the Prague-C strain of RSV used in these experiments, approximately 80% of the total phosphate is on serine, with serine-17 being the major site of phosphorylation. Consequently, if serine phosphorylation continued unabated, each mutant protein should contain 80% as much total phosphate as wild-type p60^{src}. Instead, we observed an average of only 30% as much as the wild-type protein. A 50 to 95% reduction in total phosphorylation of p60^{src} has also been observed in point mutations made within a highly conserved Ala-Pro-Glu-Ala sequence at positions 430 to 433 in the kinase domain of p60^{src} which eliminate tyrosine protein kinase activity (4, 5). Phosphorylation of serine-17 in p60^{src} by cAMP-dependent protein kinase appears to be easily perturbed by substitutions at highly conserved residues within the carboxy-terminal kinase domain.

Each mutant p60^{src} was less abundant than the wild-type protein under the conditions of our assays. The reduced abundance of the mutant proteins results in part from a shorter half-life. The histidine-295 mutant, for example, has a half-life 40% that of the wild-type protein.

Snyder et al. (24) have replaced lysine-295 of p60^{src} with a methionine residue. This transformation-defective p60^{src} variant possessed no kinase activity and was not phosphorylated in vivo at any site. They proposed that serine-17 was not phosphorylated because the binding of ATP was required to allow p60^{src} to assume a conformation in which serine-17 was available for phosphorylation. We found that mutant p60^{src} proteins encoded by SD1, SD2, and SD3 contain less phosphate than does wild-type p60^{src} but that each mutant is phosphorylated to the same reduced extent. Therefore, the extent of phosphorylation is independent of either the size or the charge of the substituted amino acid. Both the charge and size of residues which replace lysine-295 should, however, have very different effects on the efficiency with which p60^{src} can bind ATP. Consequently, our data are most consistent with the notion that the phosphorylation of serine-17 is reduced in p60^{src} proteins containing mutations at position 295 as a result of localized distortion of the ATP-binding region of the protein and is independent of the binding of ATP per se.

What is the role of lysine-295 in the function of p60^{src}? Lysine residues perform three passive roles in proteins. A total of 95% of lysine residues and 90% of arginine residues are found on the surface of enzymes (15). The charged side chains stabilize the tertiary structure of the protein by interacting with the surrounding aqueous compartment. Acidic amino acids perform this passive function as well. Since no other charged amino acid can replace lysine-295, it is unlikely that lysine-295 serves this function.

Lysine residues can also form salt bridges with acidic amino acids. Since both lysine and arginine residues are virtually 100% protonated at the surface of a protein, their interchange represents a classical conservative substitution. However, since neither arginine nor histidine at position 295 in p60^{src} creates a viable enzyme, it is improbable that lysine-295 forms a salt bridge.

A third role of lysine, which can also be carried out by arginine and histidine, is the stabilization of the negative charges of phosphate-containing ligands. For example, phosphofructose kinase from *Bacillus stearothermophilus* contains three distinct binding sites for phosphate-containing ligands: one for fructose-6-phosphate, a second for ATP, and an allosteric activating site which binds ADP. Within the active site, a histidine residue and two arginine residues serve to bind the phosphate moiety of phosphofructose, and a single arginine residue interacts with the phosphates of ATP (11). Three arginines are located in the allosteric activation site which binds ADP; however, two of these three arginine residues have been replaced by lysine residues in the sequence of the enzyme isolated from sheep heart (27). Since arginine cannot replace lysine-295 in p60^{src}, it is unlikely that ligand binding is the only function of this lysine residue.

Finally, the unique chemistry of the primary amino group of a lysine residue allows it to perform proton exchange within the active site of an enzyme. This function cannot be catalyzed by the guanidino group of an arginine residue. The most likely function of lysine-295 in p60^{src}, therefore, is proton transfer, since an arginine substitution at position 295 produces an inactive kinase.

Lysine-295 as a catalytic residue. Lysine-295 could assume

a number of roles in catalyzing the phosphotransferase reaction. In its charged state, it could activate the γ phosphate of ATP for nucleophilic attack by placing the positive charge of its ε amino group adjacent to the oxygen which bridges the β and γ phosphates of ATP. Nucleophilic attack by a lone pair of electrons from the hydroxyl oxygen of a tyrosine residue could then follow, with the bond between the bridging oxygen and γ phosphorus atom of ATP breaking and the electrons within that bond then forming an OH bond with the hydrogen atom transported into the active site by lysine-295. The final products would be ADP and phosphotyrosine. Lysine-295 would be left as an uncharged residue and acquire a new proton from the cytosol.

Either reverse transcriptase or RNA polymerase mediates the reversion of viral point mutations. These experiments also showed that the rapid reversion of point mutants in $p60^{src}$ is probably a consequence of mistakes made by reverse transcriptase or RNA polymerase during virus replication, rather than the result of homologous recombination with the chicken c-*src* gene. If the reversions of SD1, SD2, and SD3 viruses were attributable to homologous recombination, then the rates of reversion should be equivalent. In contrast, if reversion was to occur because of errors in transcription by either reverse transcriptase or by RNA polymerase, the rate of reversion should be a function of the ease with which either of the lysine codons, AAA and AAG, can be regenerated from the respective GAA (SD1), AGA (SD2), and CAC (SD3) codons. The rate of mutation made by *Escherichia coli* DNA polymerase I has been studied both in vitro (18) and in vivo (25). Transition mutations occur 10 to 30 times more frequently than transversion mutations. Reverse transcriptase is 100 times more error prone than DNA polymerase I when assayed on homopolymeric substrates (2, 20) and 10 times more so on φX174 single-stranded DNA (12). Not surprisingly, retroviruses have been found to undergo high rates of mutations when passaged in vitro (6). The minimal mutation required for reversion of SD1 and SD2 is a single transition mutation. The observed reversion rates of these two viruses are extremely high, with transforming variants arising within 3 to 6 days of complete infection of cultured chicken cells (Table 2). In contrast, the minimal change required for the reversion of SD3 is two transversions, and we found that cells infected with this virus must be maintained in tissue culture approximately 35 days after the cells are completely infected before transformants are found (Table 2).

The reversion of these mutations is not unique. Nontransforming point mutations have also been made in $p60^{src}$ around a conserved Ala-Pro-Glu sequence at positions 431 to 433 (4, 5) and at glycine-2, the site of myristylation. All of these replication-competent mutant viruses revert during growth in chicken cells (4; M. Kamps, unpublished data).

When DNA encoding mutant oncogene proteins is introduced into mammalian cells, where viral replication does not occur, the problem of reversion is largely avoided. Mutations created at the lysine residue in the *fps* and *mos* oncogenes homologous to lysine-295 or $p60^{src}$ have been studied in Rat-2 and NIH3T3 cells, respectively. Both remain transformation defective (12a; G. Weinmaster, personal communication). We would argue that mutations such as these do not undergo reversion in transfected mammalian cells because many cycles of virus replication do not occur. The fact that such point mutants of the *fps* and *mos* oncogenes can exist stably in mammalian cell lines eliminates the possibility that the reversions we observed resulted from frequent errors made by eucaryotic DNA polymerase during chromosomal replication. It would appear to be wise to introduce multiple transversion mutations when performing site-directed mutagenesis of retroviral DNA which is intended to yield an infectious virus stock.

The fairly rapid appearance of random mutations during retroviral replication may be an evolutionary asset. Rapid mutation apparently allows some retroviruses to evade the immune system. The replication of equine infectious anemia virus, for example, progresses through cycles in infected horses, with each peak in virus replication reflecting a change in the antigenicity of its envelope glycoprotein (21). Similarly, five independent isolates of the acquired immune deficiency syndrome retrovirus all display striking sequence heterogeneity in the external region of the envelope glycoprotein, indicating that random mutation followed by a positive selection for varients which can escape immune detection may have occurred (9).

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