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N-Terminal Deletions in Rous Sarcoma Virus p60^{src}: Effects on Tyrosine Kinase and Biological Activities and on Recombination in Tissue Culture with the Cellular *src* Gene

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We have constructed deletions within the region of cloned Rous sarcoma virus DNA coding for the N-terminal 30 kilodaltons of p60^{src}. Infectious virus was recovered after transfection. Deletions of amino acids 15 to 149, 15 to 169, or 149 to 169 attenuated but did not abolish transforming activity, as assayed by focus formation and anchorage-independent growth. These deletions also had only slight effects on the tyrosine kinase activity of the mutant *src* protein. Deletion of amino acids 169 to 264 or 15 to 264 completely abolished transforming activity, and *src* kinase activity was reduced at least 10-fold. However, these mutant viruses generated low levels of transforming virus by recombination with the cellular *src* gene. The results suggest that as well as previously identified functional domains for p60^{src} myristylation and membrane binding (amino acids 1 to 14) and tyrosine kinase activity (amino acids 250 to 526), additional N-terminal sequences (particularly amino acids 82 to 169) can influence the transforming activity of the *src* protein.

Rous sarcoma virus (RSV) transforms cells in culture and causes tumors in animals as a result of the expression of the *src* gene, which encodes a plasma membrane-associated tyrosine protein kinase, p60^{src} (6, 7, 14, 21, 23, 24). The tyrosine kinase activity of p60^{src} can be mapped to a C-terminal domain of about 30 kilodaltons (kDa) (1, 2, 22). Tyrosine kinase activity is a required function for transformation (17, 32).

We have shown by deletion mapping that amino acids 1 to 14 are required for p60^{src} N-myristylation, which may be required for membrane association and cell transformation (10, 12a, 28). The remainder of the N-terminal half of p60 is important for p60^{src} function. Deletion of amino acids 173 to 227 results in a protein which is temperature sensitive for morphological transformation (4). The lesions of several temperature-sensitive mutants of RSV map by genetic recombination in the N-terminal half of the protein (34). These mutants are temperature sensitive for transformed morphology and for colony formation in soft agar. Several fusiform mutants of RSV with deletions within the N-terminal half of p60^{src} have been described previously (12, 20).

Most of these mutations have only moderate effects on the tyrosine kinase activity. However, N-terminal tyrosine phosphorylation can occur on incubation of p60^{src} in high ATP concentrations and correlates with activation of the kinase activity (5, 8, 30). Tyrosine kinase activity is activated by proteolytic removal of the N-terminal half of the protein (2, 22, 40).

There is a second functional open reading frame in the DNA encoding amino acids 136 to 199 in the *src* sequence, which might encode a 7-kDa peptide (26). It is not known whether the 7-kDa peptide is present in transformed cells or plays any role in transformation.

In this report we characterize mutants of RSV constructed in vitro with deletions in various portions of the N-terminal half of the p60^{src} coding sequence and examine the effect of the deletions on transformation and *src* kinase activity.

MATERIALS AND METHODS

Plasmid constructions and transfection. All methods for DNA manipulations were as previously described (10, 11, 25).

pSR-XD3, pSR-XD10, and pSR-XD18 were *Bgl*II linker insertions at the *Sma*I sites in the *src* sequence (35, 36). The insertion in pSR-XD3 was at the site in the codon for an arginine residue at position 264 (Arg-264), that in pSR-XD10 was at the site in the codon for Arg-169, and that in pSR-XD18 bridged a deletion between these two sites. pSR-XDR66 was a linker insertion at the *Rsa*I site in the codon for Tyr-149. pSR-XD11-1 was a linker insertion at the *Nae*I site in the codon for Arg-15 (the construction of this plasmid was described previously [11]).

Since all of these linker insertions were shown to be exact insertions of the 8-base-pair (bp) linker, without loss of DNA, by DNA sequencing (27), the following manipulations were done with these plasmids to create in-frame insertion-deletion mutations (these strategies have been described in detail previously [11]). For pSR-XD310, a 5′ fragment from pSR-XD11-1 was recombined with a 3′ fragment from pSR-XD10, resulting in an in-frame deletion of DNA encoding amino acids 15 to 169. For pSR-XD311, a 5′ fragment from pSR-XD11-1 was recombined with a 3′ fragment from pSR-XDR66, resulting in an in-frame deletion of DNA encoding amino acids 15 to 149. For pSR-XD312, a 5′ fragment from pSR-XD11-1 was recombined with a 3′ fragment from pSR-XD3, resulting in an in-frame deletion of DNA encoding amino acids 15 to 264. For pSR-XD320, pSR-XD10 and pSR-XDR66 were cut with *Bgl*II and treated with S1 nuclease, and a new *Bgl*II linker was inserted. Screening of the resultant clones by end-labeling and high-resolution restriction mapping identified a clone derived from pSR-XDR66 with an extra 2 bp inserted at the 5′ side of the linker relative to pSR-XDR66 and a clone derived from pSR-XD10 with an extra 2 bp inserted at the 3′ side of the linker relative to pSR-XD10. These two clones were recombined at their *Bgl*II sites, resulting in an in-frame deletion of DNA encoding

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TABLE 1. Tyrosine kinase activities of mutant *src* proteins

Virus	Deleted amino acids	Substituted amino acids[a]	Immunoglobulin G phosphorylation[b] (% of SRA)	In vivo P-Tyr[c] (% total PAA)
NY310	15–169	PRSG	86	ND[d]
NY311	15–149	PRSD	83	0.7
NY320	149–169	SQICG	95	0.6
NY18-3	169–264	PQICG	8	0.07
NY312	15–264	PRSG	4	ND
SRA	None		100	0.5
None			0.3	0.07

[a] C, Cysteine; D, aspartic acid; G, glycine; I, isoleucine; P, proline; Q, glutamine; R, arginine; S, serine.

[b] Cells were transfected as described previously (11). At 2 to 3 weeks after transfection, when cells were fully infected (data not shown), tyrosine kinase activities of the *src* proteins were analyzed. Cells were extracted in RIPA buffer lacking SDS, and immunoprecipitates with TBR serum were prepared from portions containing equivalent amounts of total cell protein and were assayed as described in Materials and Methods.

[c] The level of phosphotyrosine in total phosphoamino acids from infected cells was assayed as described.

[d] ND, Not done.

amino acids 149 to 169. For pSR-XD18-3, pSR-XD18 was treated by the *Bgl*II-S1-*Bgl*II linker reinsertion procedure described above, and pSR-XD18-3 was identified as a clone containing an extra 2 bp at both the 5′ and 3′ ends of the linker, resulting in an in-frame deletion of DNA encoding amino acids 169 to 264.

On transfection (11), infectious virus encoding each of these mutant *src* proteins was recovered. The virus name was the same as the plasmid name, except that the prefix NY was substituted for the prefix pSR-XD (e.g., virus NY18-3 was derived from transfection with pSR-XD18-3, ligated to pSR-REP to provide replicative functions).

Protein biochemistry. Cells were labeled with $^{32}P_i$ and [^{3}H]leucine as previously described (10, 18). Immunoprecipitations with tumor-bearing rabbit (TBR [3]) serum were as described previously (10). Cells were extracted in RIPA buffer lacking sodium dodecyl sulfate (SDS) and containing 10% glycerol, rather than with normal RIPA buffer (18), and immunoprecipitates were washed in 10 mM Tris-hydrochloride (pH 7.4)–1 mM EDTA–150 mM NaCl–1% Triton X-100 buffer rather than in RIPA buffer for determination of kinase activity. This procedure was found to give much higher kinase activities for some of the mutant *src* proteins than when cells were extracted and immunoprecipitates were washed in RIPA buffer (see below). Phosphorylations with membrane pellets were as described previously (13), except that cold ATP was added to a concentration of 20 μM. Gels were treated with alkali as described previously (9). Total phosphoamino acid levels were determined as described previously (16, 32). No large variation was seen in the levels of phosphothreonine or phosphoserine from cultures infected with different viruses.

RESULTS

Recovery of mutant virus encoding deleted *src* proteins. Mutants with deletions of various regions of the *src* gene were constructed in vitro. The deleted and substituted amino acids are indicated in Table 1. Transfection of chicken embryo fibroblasts (CEF) with these mutant plasmids was performed as described previously (11), and the transfected cultures were analyzed for the production of deleted *src* proteins by labeling and immunoprecipitation with TBR serum (Fig. 1, lanes 1 through 6). In all cases, a *src* protein of the predicted size (and proteolytic map; data not shown) was immunoprecipitated. The efficiency of labeling of these proteins was somewhat variable: the NY312 (del15–264) *src* protein was reproducibly less efficiently labeled than was p60^{src} from the wild-type (wt) Schmidt-Ruppin subgroup A strain of RSV (SRA) (data not shown), even after immunoprecipitation with different TBR sera. The NY312 *src* protein had lost 40% of the leucine residues of the wt sequence as a result of its extensive deletion and may have had an altered conformation, affecting its ability to be immunoprecipitated by TBR serum. The half-lives of these *src* proteins were determined to be 9, 8, 4, and 3 h for the SRA, NY311 (del15–149), NY320 (del149–169), and NY310 (del15–169) *src* proteins respectively (data not shown). Supernatants from these cultures contained infectious mutant virus.

Tyrosine kinase activities of mutant *src* proteins. Cultures of CEF infected with the mutant viruses were extracted and immunoprecipitated with TBR serum, and the phosphorylation of immunoglobulin G in the immune complex was assayed (6). When extracted in the absence of SDS, the kinase activities of NY310 (del15–169), NY311 (del15–149), and NY320 (del149–169) *src* proteins were close to the activity of wt p60^{src}, whereas the NY312 (del15–264) and NY18-3 (del169–264) *src* proteins were less than 1/10 as active (Table 1). These experiments were done with cells producing comparable levels (within twofold) of *src* proteins as estimated by [^{3}H]leucine labeling and immunoprecipitation. The activities of the NY310, NY311, and NY320 *src* proteins were somewhat labile to extraction with SDS, showing a 2- to 10-fold reduction in activity (data not shown). Even under the gentler conditions used for the experiments in Table 1, these *src* proteins gave a somewhat variable kinase activity from experiment to experiment, which is possibly also due to higher sensitivity to denatur-

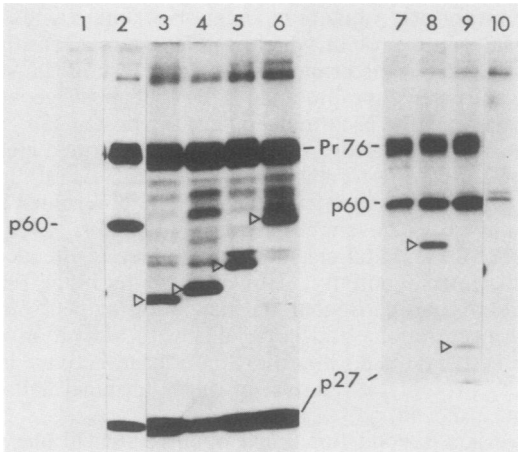

FIG. 1. Production of mutant *src* proteins. Transfected cells were labeled with [^{3}H]leucine for 4 h, and extracts were immunoprecipitated with TBR serum (a different TBR serum was used for lanes 7 through 10). Proteins were run on 10% SDS-polyacrylamide gels. Lanes contained cells infected with the following: 1, no virus; 2, SRA; 3, NY310 (del15–169); 4, NY311 (del15–149); 5, NY18-3 (del169–264); 6, NY320 (del149–169); 7, SRA, late passage; 8, NY18-3, late passage; 9, NY312 (del15–264), late passage; 10, no virus, late passage. The positions of the mutant *src* proteins are indicated by open triangles. In late-passage cultures infected by NY18-3 or NY312, full-length p60^{src} as well as the deleted *src* proteins (open triangles, lanes 8 and 9) were observed (see text).

ation than is observed with the wt SRA p60^{src} or with N-terminal deletion mutants with deletions restricted to the N-terminal 81 amino acids (10; data not shown). The kinase activities of the NY312 and NY18-3 *src* proteins were extremely low, regardless of the extraction conditions (data not shown).

The tyrosine kinase activities of these *src* proteins were also indirectly measured by the elevation of total phosphotyrosine levels in infected cells (16, 32) (Table 1). NY311 and NY320 elevate cellular phosphotyrosine levels as much as does wt SRA, whereas NY18-3 does not elevate cellular phosphotyrosine levels, approximately consistent with the in vitro tyrosine kinase activities.

Kinase activity in membrane fractions. Since the kinase activity of the mutant *src* proteins measured by the immune complex immunoglobulin G phosphorylation assay appeared to be subject to variable effects of detergent denaturation, we sought an in vitro assay in which the native conformation of the *src* protein would be better maintained. A tyrosine kinase activity attributable to p60^{src} that is associated with membrane vesicles from RSV-infected cells has been described previously (13). Since the *src* proteins of NY311 (del15–149) and NY320 (del149–169) are plasma membrane associated (12a), we tested membrane pellets for endogenous kinase activity essentially as described previously (13). However, since incubation of p60^{src} in high ATP concentrations elevates its kinase activity (5, 8, 30) and can result in N-terminal *src* phosphorylation, we added cold ATP to the reaction mixture to a concentration of 20 μM. If N-terminal *src* phosphorylation were observed, we hoped to roughly map it by using deletion mutants. After the reaction, the membranes were solubilized and the reaction products were run on duplicate polyacrylamide gels, one of which was soaked in alkali to enrich for tyrosine phosphorylation (9) (Fig. 2A). SRA, NY309 (with amino acids 15 to 81 deleted [10]), NY311, and NY320 all gave levels of alkali-resistant phosphorylation of various bands much above the level seen with membranes from uninfected cells, although the NY320 reaction was less efficient. This may reflect the degree of morphological transformation observed (see below). Immunoprecipitation showed that the mutant *src* proteins were also phosphorylated (data not shown). The phosphorylated NY320 *src* protein did not immunoprecipitate efficiently, possibly owing to its lower level of initial phosphorylation or instability or perhaps owing to increased susceptibility to phosphatases. The other *src* proteins were efficiently immunoprecipitated and on V8 protease mapping were found to contain label in both C-terminal and N-terminal fragments (Fig. 2B). Alkali treatment of the protease mapping gel indicated that the phosphorylations were on tyrosine (data not shown). The mobility of the N-terminal fragments was decreased in all cases, consistent with the report of Collett et al. (5, 8) that incubation in high ATP concentrations results in N-terminal tyrosine phosphorylation of p60^{src} with a decrease in electrophoretic mobility of the protein. The decrease seemed greatest for the NY309 *src* protein. Since the mobility decrease occurred with the NY311 (del15–149) *src* protein, some of the phosphorylated tyrosine residues are probably not within amino acids 15 to 149. However, the greater decrease in mobility of N-terminal fragments of the NY309 *src* protein might imply that tyrosine phosphorylation between amino acids 81 and 149 is significant in this phenomenon. We have mapped the *src* proteins labeled in membrane pellets and isolated from the gel of the total reaction products before alkali treatment, but the background from contaminating bands in the V1 region of the gel was too great to say with certainty whether V1 phosphorylation of the NY320 *src* protein occurred (data not shown).

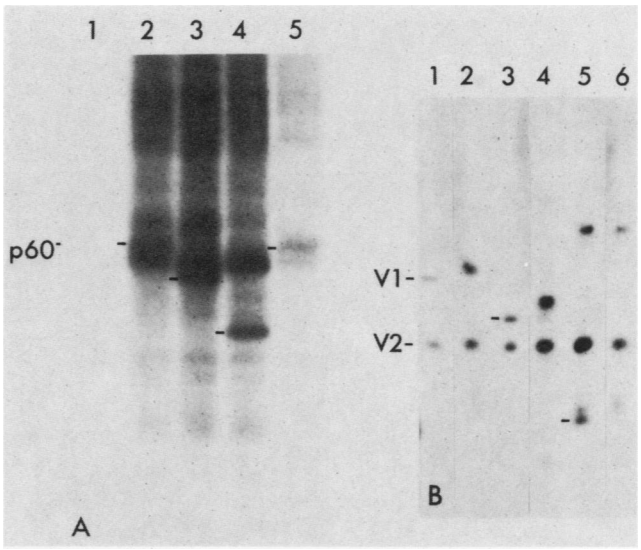

FIG. 2. Phosphorylation in membrane pellets from infected cells. A. Membrane pellets were prepared and phosphorylated as described previously (13), except that the ATP concentration was 20 μM. The products were run on a 10% polyacrylamide gel, which was soaked in alkali after electrophoresis to enrich for tyrosine phosphorylation (9). Lanes contained CEF infected with the following: 1, no virus; 2, SRA; 3, NY309 (del15–81); 4, NY311 (del15–149); 5, NY320 (del149–169). The positions of the phosphorylated *src* proteins are indicated (the identity of these proteins was confirmed by V8 protease mapping of comigrating bands from a parallel gel that was not alkali treated; see text). B. V8 protease mapping of *src* proteins phosphorylated in membrane pellets. After the in vitro phosphorylation, *src* proteins were immunoprecipitated with TBR serum. Excised bands were subjected to V8 proteolysis during reelectrophoresis. Lanes alternate between the *src* proteins metabolically labeled (lanes 1, 3, and 5) with [^{3}H]leucine (as in Fig. 1) and those labeled in vitro with ^{32}P (lanes 2, 4, 6). Lanes: 1 and 2, SRA; 3 and 4, NY309; 5 and 6, NY311. The positions of the V1 fragments of the [^{3}H]leucine-labeled *src* proteins are indicated.

Biological characterization of mutant viruses. The mutant viruses were tested for morphological alterations (Fig. 3) and anchorage-independent growth of infected cells (Table 2; Fig. 3). These results were similar at 37 and 41°C.

NY311 (del15–149) induces a fusiform morphology (Fig. 3D), which is much more pronounced than that induced by the fusiform mutant NY309 (del15–81) (10). Foci of NY311-infected cells can be difficult to detect. Infected cells grow to the same density as SRA-infected cells (about three times the uninfected cell level). NY311 induces colonies in soft agar (Fig. 3G) as efficiently as SRA (Fig. 3H), but the colonies are significantly smaller.

MY320 (del149–169) was considerably more defective than NY311 in morphological transformation. The virus did not induce foci, but cells in cultures infected with high titers of virus (Fig. 3C) were slightly more than uninfected CEF (Fig. 3A) and grew to about two- to threefold higher density. When cells were transferred several times after infection, the morphology became more highly fusiform. Virus stocks harvested from the more transformed, late-passage NY320-infected cultures induced subtle morphological changes similar to those induced by virus stocks harvested a short time after transfection. Therefore, this phenomenon may involve selection in culture of a subpopulation of cells which have altered morphology as a result of NY320 infection. The

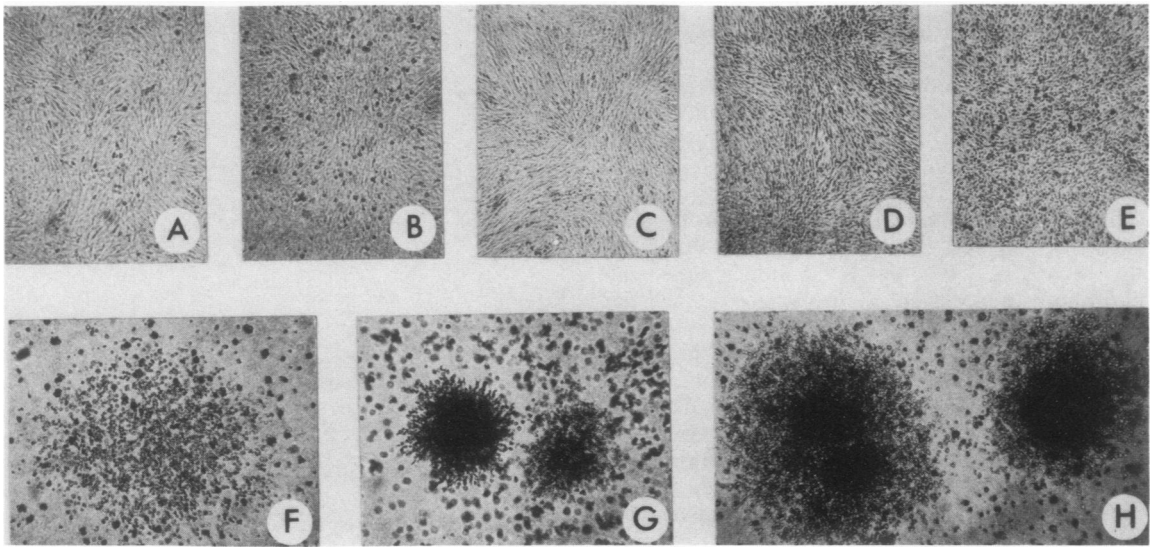

FIG. 3. Morphology of virus-infected cells in monolayer culture and morphology of soft agar colonies. CEF infected with the following: A, no virus; B, NY310 (del15–169); C, NY320 (del149–169); D, NY311 (del15–149); or E, SRA. Cultures were photographed 6 days, with one passage, after infection. Soft agar colonies of CEF infected with: F, NY320; G, NY311; or H, SRA. Colonies were photographed 2 weeks after seeding.

colony-forming activity of NY320 was similar to that of SRA, but colonies were frequently large and diffuse (Fig. 3F), in contrast to the compact SRA-induced colonies (Fig. 3H).

NY310 (del15–169) (Fig. 3B) was similar in its phenotype to NY320, except that it was quantitatively somewhat defective in inducing colony formation in soft agar. The "delayed" transformed phenotype described above for NY320 cell morphology also occurred with NY310.

NY312 (del15–264) and NY18-3 (del169–264) were transformation defective by both criteria. However, occasional foci were detected in cultures infected with these two viruses. This occurs because these viruses undergo recombination with the cellular *src* gene in tissue culture, giving rise to transforming virus with a repaired deletion (see below).

Production of recovered sarcoma virus in tissue culture. RSV derivatives with partial deletions in their *src* genes, which are transformation defective, give rise to fully transforming viruses after passage through chickens (15). This has been shown to be the result of recombination of the deleted *src* gene of these viruses with the cellular *src* gene (18, 19, 35–39). When passaged several times, cultures infected with NY312 (del15–264) and NY18-3 (del169–264) exhibited many foci of transformed cells, which can overgrow the culture. At this time, the cultures produced full-length $p60^{src}$ as well as the original deleted *src* protein (Fig. 1, lanes 8 and 9). Also, the *src* tyrosine kinase activity in the cells rose to the wt level (data not shown). These cultures also produced a high titer of transforming virus. Cells infected with virus from these cultures produced a full-length *src* protein, as well as the original deleted *src* protein (data not shown). Therefore, we conclude that the deletion in the *src* sequence in the transfected DNA was repaired after passage of the virus, probably as a result of recombination with the cellular *src* gene, and that transforming virus was produced as a result. To estimate the rate at which this event occurred and to delineate the sequence requirements, cells were infected with either NY312, NY18-3, NY313, NY321, or supernatant from cultures transfected with the pSR-REP DNA alone as a control. (NY313 and NY321 have deletions from amino acids 15 to 416 or from amino acid 15 to 105 bp after the *src* termination codon respectively [unpublished data].) Cultures were passaged under soft agar to select for transformed cells, and the number of foci was counted after each passage (Table 3). All the cultures infected with NY312, NY18-3, and NY313 eventually yielded subcultures which were positive for focus formation. This is presumed to be due to de novo generation of transforming virus, since the initial cultures were all negative, except for the NY18-3 culture, which had one focus. For this reason, a 10-fold dilution of NY18-3 was also carried through the experiment and shown to yield foci at a later passage. Cultures infected with NY321 or with the control (virus-free) supernatant from pSR-REP-transfected cells did not yield foci on subculture. This indicates that sequences at the 3′ end of the *src* gene are required for efficient recovery of transforming virus. A similar conclusion has been reached in studying the generation of rASV in chickens (38).

TABLE 2. Colony-forming activity of mutant viruses

Virus	CFU/unit of virus[a]
NY310	0.08
NY311	0.8
NY320	0.7
NY18-3	<0.0001
NY312	<0.0001
SRA	1.0

[a] CEF were infected with dilutions of virus and transferred into soft agar suspension. Colonies were counted 3 weeks later. Uninfected cells formed between 0 and 10 small colonies per 10^6 cells seeded. A unit of virus is defined by the amount of immunoglobulin G kinase activity induced 36 h after infection divided by the relative specific activity of the kinase (see Table 1; but note that the variability of kinase activity of some of these *src* proteins [see text] means that these comparisons are probably only accurate to within a factor of 2 to 3). The reference SRA stock induced 1.4×10^7 colonies per ml of virus stock when plated 18 h after infection.

DISCUSSION

Deletion mapping of functional regions in p60^{src}. We have shown previously that amino acids 1 to 14 are required for the myristylation and membrane association of p60^{src} and for transforming activity (10, 28). The absence of amino acids 15 to 81 in the *src* protein of NY309 had no effect on the myristylation and membrane association of the mutant *src* protein or on the biological activity of the mutant virus, except that infected cells were somewhat fusiform rather than round, and the virus had a reduced tumorigenicity (10).

In this work we have shown that amino acids 15 to 149, and to a greater extent amino acids 15 to 169, are required for some aspects of cell transformation by p60^{src} relating to focus formation and colony formation in soft agar. NY311 (del15–149), NY310 (del15–169), and NY320 (del149–169) are only partially transformation defective, however. The *src* proteins of NY311 and NY320 are myristylated and plasma membrane associated (12a). Although the tyrosine kinase activities of these *src* proteins are somewhat labile in vitro and *src* proteins with amino acids 149 to 169 deleted are more unstable than wt p60^{src}, these *src* proteins still induce comparable levels of phosphotyrosine in vivo (Table 1), suggesting that the total in vivo *src* tyrosine kinase activity is similar in cells infected with SRA, NY311, and NY320. However, as suggested by their increased sensitivity to detergents, the extensive deletions might affect the conformation of the mutant proteins and, as a result, their substrate specificity might have been altered, as was suggested for another N-terminal deletion mutant which was temperature sensitive for transformation (4). It is possible that the N-terminal portion of p60^{src}, past the myristylation signal (amino acids 1 to 14 [10, 28]), functions as a determinant of substrate specificity. This hypothesis could explain the rather variable phenotype observed with mutations in this region.

In contrast, the behavior of NY312 (del15–264) and NY18-3 (del169–264) shows that amino acids 169 to 264 are critical for transforming activity. The transformation defectiveness of NY18-3 and NY312 correlates with the loss of more than 90% of the tyrosine kinase activity of their *src* proteins measured in vitro. The retention of at least some transforming activity by NY311, NY310, and NY320, despite the loss of substantial portions of their *src* genes, correlates with the retention of tyrosine kinase activity by their *src* proteins. These data are thus consistent with the idea that p60^{src} tyrosine kinase activity is necessary for transformation by RSV (17, 32).

The C-terminal 30 kDa of p60^{src} constitutes a tyrosine kinase domain (2, 22). The loss of tyrosine kinase activity as a result of the deletion of amino acids 169 to 264 or 15 to 264 may imply that amino acid 264 is within the C-terminal tyrosine kinase domain. In this connection, it should be noted that deletion of amino acids 135 to 237 or 173 to 227 does not affect tyrosine kinase activity significantly (4, 20). Also, amino acid 264 is within the region of strong homology between *src* and *fps*, a distantly related tyrosine kinase (33). Alternatively, amino acid sequences 15 to 264 and 169 to 264 may have some indirect effect on the activity of the kinase domain. Consistent with this idea, the N-terminal mutations in the *src* proteins of NY310, NY311, and NY320 increase the lability of their kinase activities to SDS treatment and apparently alter the conformation of the *src* protein (12a). The observation that much more than 100% of the tyrosine kinase activity of p60^{src} was recovered in the C-terminal proteolytic fragment suggested that some N-terminal sequence is affecting the kinase activity (2, 22, 40). We have not observed an elevation of kinase activity above the wt level with any N-terminal deletion mutant.

Recently, Prywes et al. (29) showed that the minimum fibroblast-transforming region of v-*abl* consisted of a region homologous (31) to approximately amino acids 166 to 515 of p60^{src}. This result is essentially consistent with the results presented here. Transformation was defined by the induction of foci on NIH 3T3 cells. One contrast with our results is that a mutant with a deletion of the region of v-*abl* homologous to amino acids 137 to 165 of p60^{src} (pB1 [29]) had little or no kinase activity compared with wt v-*abl* when expressed in bacteria, whereas we can detect high kinase activity from the NY310 *src* protein, with amino acids 15 to 169 deleted, or NY320 *src*, with 149 to 169 deleted, although their kinase activities are less stable than that of wt SRA.

The second functional open reading frame in the *src* gene. Mardon and Varmus (26) showed that an open reading frame, encoding a 7-kDa peptide, is functional in *src* mRNA. This open reading frame is destroyed in the *src* gene of NY311 (del15–149). Therefore, the 7-kDa peptide is not required for fusiform morphological transformation or anchorage-independent growth.

Recovery of sarcoma virus from deletion mutants in culture. Mutants with partial deletions in *src* have been shown previously to give rise to recovered avian sarcoma virus on injection into chickens, as a result of recombination with the cellular *src* gene (15, 18, 19, 35–39). Similar recombinations were observed previously in tissue culture in cells infected with spontaneous deletion mutants td105, td108, and td113 (L.-H. Wang, T. Takeya, and H. Hanafusa, unpublished results). The results shown here with the constructed *src* deletion mutants confirm the same phenomenon in cell culture. The rate of the recombination is on the order of one

TABLE 3. Recovery of transforming virus in tissue culture

Virus	Inoculum (ml)[a]	Transfer[b]	No. of foci/plate[c]
NY18-3	0.1	0	1
		1	TMTC,[d] TMTC
	0.01	0	0
		1	8,12
		2	TMTC (8)
NY312	0.1	0	0
		1	1,0
		2	85 (1), 0 (0)
NY313	0.1	0	0
		1	1, 1
		2	TMTC, TMTC
NY321	0.1	0	0
		1	0, 0
		2	0, 0
None (REP sup[e])	0.1	0	0
		1	0, 0
		2	0, 0

[a] CEF (1.25×10^6) were infected with the indicated amount of virus stock from transfected cultures.

[b] At each transfer (10 days apart) cultures were split 1:3 (one or two subcultures per culture). The next day subcultures were overlaid with soft agar. Cultures were maintained at 40°C.

[c] Immediately before transfer, foci of morphologically transformed cells were counted. When two plates from one subculture showed different numbers of foci, both were transferred. The source culture is identified by the number of foci in the previous transfer, shown in parentheses.

[d] TMTC, Too many to count; >400 foci.

[e] Supernatant from control transfections with pSR-REP DNA alone was used for infection. This supernatant is virus free (unpublished data).

event per 5×10^6 cell divisions, starting with fully infected cells. A region of homology at the 3′ border of *src* with the cellular sequence is required. There was no substantial relationship between the size of the deletion and the rate of the recombination: NY18-3, with amino acids 169 to 264 deleted, gave only a slightly higher rate of recombination than did NY313, with amino acids 15 to 416 deleted.

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LITERATURE CITED

1. **Barker, W. C., and M. O. Dayhoff.** 1982. Viral *src* gene products are related to the catalytic chain of mammalian cAMP-dependent protein kinase. Proc. Natl. Acad. Sci. USA **79**:2836–2839.
2. **Brugge, J., and D. Darrow.** 1984. Analysis of the catalytic domain of the phosphotransferase activity of two avian sarcoma virus transforming proteins. J. Biol. Chem. **259**:4550–4557.
3. **Brugge, J. S., and R. L. Erikson.** 1977. Identification of a transformation specific antigen induced by an avian sarcoma virus. Nature (London) **269**:346–348.
4. **Bryant, D., and J. T. Parsons.** 1982. Site-directed mutagenesis of the *src* gene of Rous sarcoma virus: construction and characterization of a deletion mutant temperature sensitive for transformation. J. Virol. **44**:683–691.
5. **Collett, M. S., S. K. Belzer, and A. F. Purchio.** 1984. Structurally and functionally modified forms of $pp60^{v\text{-}src}$ in Rous sarcoma virus-transformed cell lysates. Mol. Cell. Biol. **4**:1213–1220.
6. **Collett, M. S., and R. L. Erikson.** 1978. Protein kinase activity associated with the avian sarcoma virus *src* gene product. Proc. Natl. Acad. Sci. USA **75**:2021–2024.
7. **Collett, M. S., A. F. Purchio, and R. L. Erikson.** 1980. Avian sarcoma virus transforming protein, $pp60^{src}$, shows protein kinase activity specific for tyrosine. Nature (London) **285**: 167–169.
8. **Collett, M. S., S. K. Wells, and A. F. Purchio.** 1983. Physical modification of purified Rous sarcoma virus $pp60^{v\text{-}src}$ after incubation with ATP/Mg^{2+}. Virology **128**:285–297.
9. **Cooper, J. A., and T. Hunter.** 1981. Changes in protein phosphorylation in Rous sarcoma virus-transformed chicken embryo cells. Mol. Cell. Biol. **1**:165–178.
10. **Cross, F. R., E. A. Garber, D. Pellman, and H. Hanafusa.** 1984. A short sequence in the $p60^{src}$ N terminus is required for $p60^{src}$ myristylation and membrane association and for cell transformation. Mol. Cell. Biol. **4**:1834–1842.
11. **Cross, F. R., and H. Hanafusa.** 1983. Local mutagenesis of Rous sarcoma virus: the major sites of tyrosine and serine phosphorylation are dispensable for transformation. Cell **34**:597–608.
12. **Fujita, D. J., J. Bechberger, and I. Nedic.** 1981. Four Rous sarcoma virus mutants which affect transformed cell morphology exhibit altered *src* gene products. Virology **114**:256–260.

12a. **Garber, E. A., F. R. Cross, and H. Hanafusa.** 1985. Processing of $p60^{v\text{-}src}$ to its myristylated membrane-bound form. Mol. Cell. Biol. **5**:2781–2788.

13. **Garber, E. A., J. G. Krueger, H. Hanafusa, and A. R. Goldberg.** 1983. Temperature-sensitive membrane association of $pp60^{src}$ in tsNY68-infected cells correlates with increased tyrosine phosphorylation of membrane-associated proteins. Virology **126**: 73–86.
14. **Hanafusa, H.** 1977. Cell transformation by RNA tumor viruses, p. 401–483. *In* H. Fraenkel-Conrat and R. Wagner (ed.), Comprehensive virology, vol. 10. Plenum Publishing Corp., New York.
15. **Hanafusa, H., C. C. Halpern, D. L. Buchhagen, and S. Kawai.** 1977. Recovery of avian sarcoma virus from tumors induced by transformation-defective mutants. J. Exp. Med. **146**:1737–1747.
16. **Hunter, T., and B. M. Sefton.** 1980. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. USA **77**:1311–1315.
17. **Iba, H., F. R. Cross, E. A. Garber, and H. Hanafusa.** 1985. Low level of cellular protein phosphorylation by nontransforming overproduced $p60^{c\text{-}src}$. Mol. Cell. Biol. **5**:1058–1066.
18. **Karess, R. E., and H. Hanafusa.** 1981. Viral and cellular *src* genes contribute to the structure of recovered avian sarcoma virus transforming protein. Cell **24**:155–164.
19. **Karess, R. E., W. S. Hayward, and H. Hanafusa.** 1979. Cellular information in the genome of recovered avian sarcoma virus directs the synthesis of transforming protein. Proc. Natl. Acad. Sci. USA **76**:3154–3158.
20. **Kitamura, N., and M. Yoshida.** 1983. Small deletion in *src* of Rous sarcoma virus modifying transformation phenotypes: identification of 207-nucleotide deletion and its smaller product with protein kinase activity. J. Virol. **46**:985–992.
21. **Krueger, J. G., E. A. Garber, and A. R. Goldberg.** 1983. Subcellular localization of $pp60^{src}$ in RSV-transformed cells. Curr. Top. Microbiol. Immunol. **107**:51–124.
22. **Levinson, A. D., S. A. Courtneidge, and J. M. Bishop.** 1981. Structural and functional domains of the Rous sarcoma virus transforming protein ($pp60^{src}$). Proc. Natl. Acad. Sci. USA **78**:1624–1628.
23. **Levinson, A. D., H. Oppermann, L. Levintow, H. E. Varmus, and J. M. Bishop.** 1978. Evidence that the transforming gene of avian sarcoma virus encodes a protein kinase associated with a phosphoprotein. Cell **15**:561–572.
24. **Levinson, A. D., H. Oppermann, H. E. Varmus, and J. M. Bishop.** 1980. The purified product of the transforming gene of avian sarcoma virus phosphorylates tyrosine. J. Biol. Chem. **255**:11973–11980.
25. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Mardon, G., and H. E. Varmus.** 1983. Frameshift and intragenic suppressor mutations in a Rous sarcoma provirus suggest *src* encodes two proteins. Cell **32**:871–879.
27. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
28. **Pellman, D., E. A. Garber, F. R. Cross, and H. Hanafusa.** 1985. Fine structural mapping of a critical NH_2-terminal region of $p60^{src}$. Proc. Natl. Acad. Sci. USA **82**:1623–1627.
29. **Prywes, R., J. G. Foulkes, and D. Baltimore.** 1985. The minimum transforming region of v-*abl* is the segment encoding protein-tyrosine kinase. J. Virol. **54**:114–122.
30. **Purchio, A. F., S. K. Wells, and M. S. Collett.** 1983. Increase in the phosphotransferase specific activity of purified Rous sarcoma virus $pp60^{v\text{-}src}$ protein after incubation with ATP plus Mg^{2+}. Mol. Cell. Biol. **3**:1589–1597.
31. **Reddy, E. P., M. J. Smith, and A. Srinivasan.** 1983. Nucleotide sequence of Abelson murine leukemia virus: structural similarity of its transforming gene product to other *onc* gene products with tyrosine-specific kinase activity. Proc. Natl. Acad. Sci. USA **80**:3623–3627.
32. **Sefton, B. M., T. Hunter, K. Beemon, and W. Eckhart.** 1980. Evidence that the phosphorylation of tyrosine is essential for transformation by RSV. Cell **20**:807–816.
33. **Shibuya, M., and H. Hanafusa.** 1982. Nucleotide sequence of Fujinami sarcoma virus: evolutionary relationship of its transforming gene with transforming genes of other sarcoma viruses. Cell **30**:787–795.
34. **Stoker, A. W., P. J. Enrietto, and J. A. Wyke.** 1984. Functional domains of the $pp60^{v\text{-}src}$ protein as revealed by analysis of temperature-sensitive Rous sarcoma virus mutants. Mol. Cell. Biol. **4**:1508–1514.
35. **Takeya, T., R. A. Feldman, and H. Hanafusa.** 1982. DNA sequence of the viral and celluar *src* gene of chickens. I. Complete nucleotide sequence of an *Eco*RI fragment of recovered avian sarcoma virus which codes for gp37 and $pp60^{src}$. J. Virol. **44**:1–11.

36. **Takeya, T., and H. Hanafusa.** 1982. DNA sequence of the viral and cellular *src* gene of chickens. II. Comparison of the *src* genes of two strains of avian sarcoma virus and of the cellular homology. J. Virol. **44**:12–18.
37. **Takeya, T., and H. Hanafusa.** 1983. Structure and sequence of the cellular gene homologous to the *src* gene of Rous sarcoma virus and the mechanism for the generation of the viral transforming gene. Cell **32**:881–890.
38. **Wang, L.-H., M. Beckson, S. M. Anderson, and H. Hanafusa.** 1984. Identification of the viral sequence required for the generation of recovered avian sarcoma viruses and characterization of a series of replication-defective recovered avian sarcoma viruses. J. Virol. **49**:881–891.
39. **Wang, L.-H., C. C. Halpern, M. Nadel, and H. Hanafusa.** 1978. Recombination between viral and cellular sequences generated transforming sarcoma virus. Proc. Natl. Acad. Sci. USA **75**:5812–5816.
40. **Wells, S. K., and M. S. Collett.** 1983. Specific proteolytic fragmentation of $p60^{v\text{-}src}$ in transformed cell lysates. J. Virol. **47**:253–258.