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Fine structural mapping of a critical NH_2-terminal region of $p60^{src}$

(viral *src* gene/membrane association/myristic acid/Rous sarcoma virus/*in vitro* mutagenesis)

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ABSTRACT **We have recently demonstrated that an NH_2-terminal sequence required for myristylation and membrane association of the Rous sarcoma virus transforming protein, $p60^{src}$, is contained within amino acids 2–14 [Cross, F. R., Garber, E. A., Pellman, D. & Hanafusa, H. (1984) *Mol. Cell. Biol.* 4, 1834–1842]. This sequence is also required for cell transformation. We have now constructed five mutants of Rous sarcoma virus that contain alterations in the *src* sequence coding for these 14 amino acids. Mutants encoding *src* proteins with a peptide insertion between amino acids 1 and 2, or peptide substitutions for amino acids 2–4, 3–4, or 7–15, were transformation-defective. The *src* proteins of these mutants differed from the wild-type protein in that they were not myristylated and did not fractionate with the plasma membrane of infected cells. The fifth mutant encoded a *src* protein with a short peptide substituted for amino acids 11–15. This protein was myristylated and plasma membrane associated, and the virus transformed cells. We therefore conclude that a sequence required for myristylation and membrane association of $p60^{src}$ is located within the first 7–10 amino acids of the *src* protein, and that $p60^{src}$ myristylation and membrane association are required for cell transformation. Consistent with this idea, we have isolated four transforming revertants from one of the transformation-defective mutants. The *src* proteins of all four revertants were found to be myristylated and membrane associated.**

The *src* gene product, $p60^{src}$, of Rous sarcoma virus (RSV) is a tyrosine-specific protein kinase that has been shown to be associated with the plasma membranes of infected chicken embryo fibroblasts (refs. 1 and 2; see ref. 3 for review). $p60^{src}$ is covalently modified with a fatty acid that is attached within the NH_2-terminal 16 kDa (4–6). The kinase activity is contained within a COOH-terminal 30-kDa domain, which is separable from an NH_2-terminal membrane binding domain (7–10). Since the NH_2-terminus of $p60^{src}$ does not contain a sequence of hydrophobic amino acids (11), the covalently bound fatty acid [recently shown to be myristic acid (6)] may be required for the interaction of $p60^{src}$ with the plasma membrane.

Recently, we have demonstrated that of the NH_2-terminal 81 amino acids of $p60^{src}$, only amino acids 2–14 are required for myristylation and membrane association (12). While a mutant *src* protein with a deletion of amino acids 15–81 was myristylated, membrane associated, and transformed cells, mutant *src* proteins with deletions of amino acids 2–81 or 2–15 were not myristylated and were defective for both membrane association and cellular transformation. It has recently been determined that the glycine at position 2 of the $p60^{src}$ primary sequence is *N*-myristylated (6), which could explain the lack of myristylation of the *src* proteins with amino acids 2–81 or 2–15 deleted, because the second amino acid of these proteins was not glycine (12). In this study, we have constructed five mutants that contain alterations in the sequence coding for the first 14 amino acids of $p60^{src}$, in order to further define the sequence requirements for myristylation and membrane association and to test the correlation between myristylation, membrane association, and transformation. We have also isolated four transforming revertants from one of the transformation-defective mutants, and characterized their *src* proteins.

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MATERIALS AND METHODS

Restriction enzyme digestion, linker insertion, BAL-31 digestion, gap filling, and recombinations between clones with linkers inserted in different locations were done as described (12–14).

The constructions of three of the mutants studied are diagrammed in Fig. 1.

pSR-XD316 and pSR-XD317 contain insertion/deletion mutations in the *src* gene. The strategy for their construction is similar to a strategy that has been described (12), except that the final constructs contain deletions in the 5′ rather than the 3′ direction. Briefly, the parent clone was pSR-XD11-1, a *src*-containing plasmid with a *Bgl* II linker inserted into the *Nae* I site interrupting the codon for the arginine residue at position 15 (11, 14). This plasmid was digested with *Bgl* II and BAL-31, and a new *Bgl* II linker was inserted at the site of the deletion. Two plasmids, one with BAL-31 digestion to nucleotide 20 of the *src* gene (with nucleotide 1 being the first nucleotide of the codon for the initiator methionine), the other with digestion to nucleotide 30, were recombined at the *Bgl* II site with the appropriate plasmids to yield in-frame deletions 5′ to DNA coding for arginine-16. In pSR-XD316, the 3′ arm is derived from pSR-XD11-1, and in pSR-XD317, the 3′ arm is derived from pSR-XD11-4 (14). The resulting sequence changes are shown in Fig. 2.

Conditions for cell culture, preparation and assay of virus, protein biochemistry, and cell fractionation have been described (12, 14).

RESULTS

Virus Recovery and Expression of Mutant *src* Genes. We have made mutations in the sequence encoding the first 14 amino acids of *src* from both the 5′ and 3′ sides. All mutations were verified by DNA sequence analysis.

Replication-competent virus identical to the Schmidt–Ruppin subgroup A strain of RSV (SRA), except for the *src* gene mutations, was recovered from chicken embryo fibroblasts transfected with the mutant plasmids ligated to pSR-REP, as described (14). Two weeks and three transfers

Abbreviations: RSV, Rous sarcoma virus; TBR, tumor-bearing rabbit; wt, wild type.

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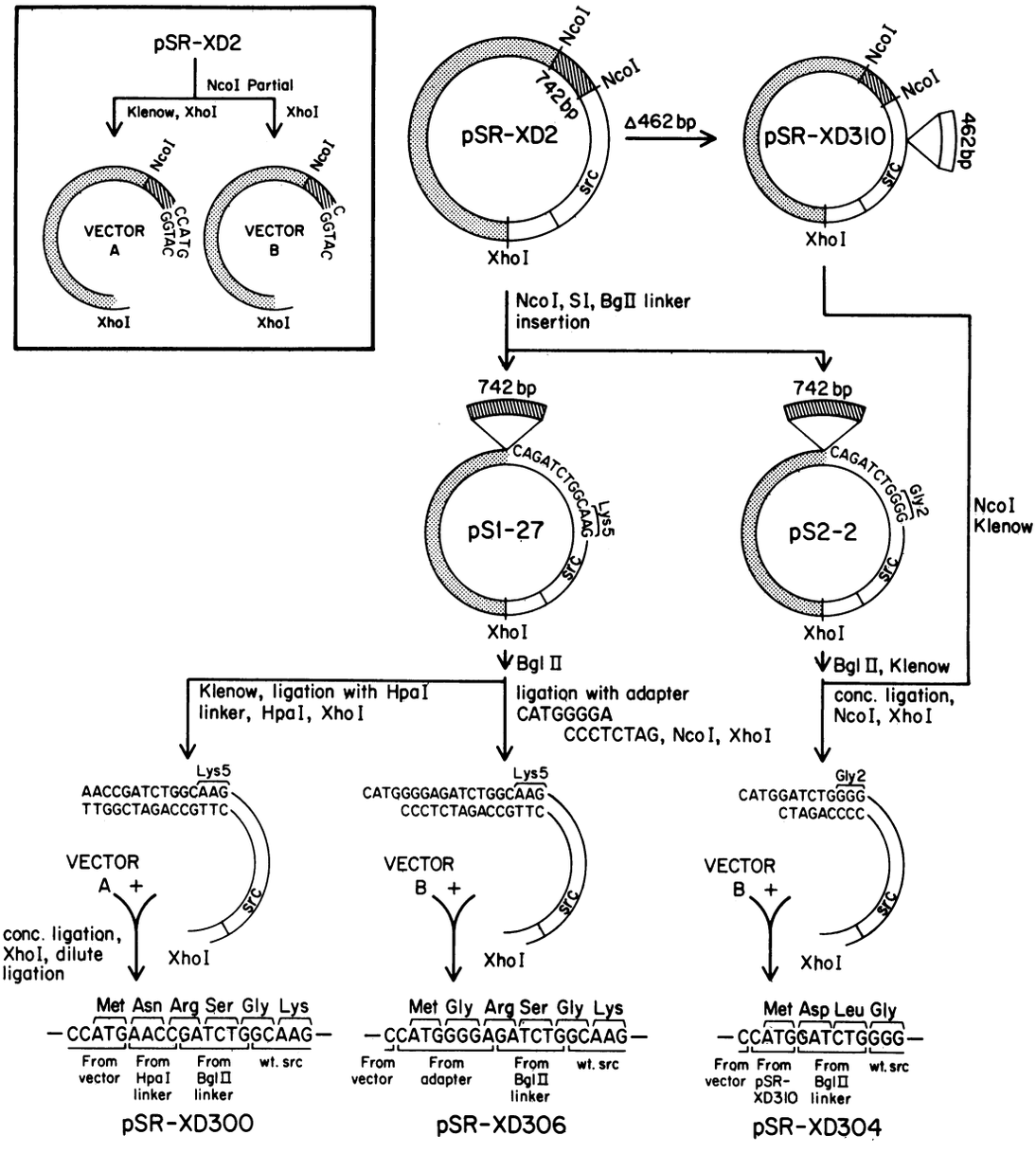

FIG. 1. Construction of pSR-XD300, pSR-XD304, and pSR-XD306. Starting material: pS2-2 and pS1-27. pSR-XD2 was digested with *Nco* I, followed by S1 nuclease digestion and *Bgl* II linker (C-A-G-A-T-C-T-G) insertion. pS2-2 and pS1-27 both contained *Bgl* II linkers and deletion of the 742-base-pair (bp) *Nco* I fragment and had lost the *Nco* I site, which spans the initiator methionine codon of $p60^{src}$ (11). In pS2-2, S1 nuclease digestion removed only the single-stranded overhang from the *Nco* I digestion. In pS1-27, S1 nuclease overdigestion resulted in deletion of the first 10 bp of *src*. pSR-XD300: pS1-27 was digested with *Bgl* II, gap-filled with the large fragment of *Escherichia coli* DNA polymerase I, and ligated to *Hpa* I linker (G-G-T-T-A-A-C-C). This DNA was digested with *Hpa* I and *Xho* I, and a 2.9-kilobase *Hpa* I/*Xho* I insert fragment was isolated. This insert was missing the sequence coding for the initiator methionine. To restore this sequence, the insert was ligated to form concatemers with a 6.3-kilobase *Xho* I/*Nco* I vector fragment gap-filled at the *Nco* I site (vector A). Plasmid length DNA was liberated by digestion with *Xho* I and circularized by ligation at a concentration of 1 μg/100 μl. pSR-XD306: *Bgl* II-digested pS1-27 DNA was ligated to a *Bgl* II/*Nco* I oligonucleotide adapter (see above) made on an Applied Biosystems model 380A synthesizer according to the manufacturer's instructions and purified by fast polynucleotide liquid chromatography (15). Ligated DNA was digested with *Nco* I and *Xho* I, and a 2.9-kilobase *Nco* I/*Xho* I insert was isolated and ligated to a 6.3-kilobase *Xho* I/*Nco* I vector fragment (vector B). pSR-XD304: *Bgl* II-digested pS2-2 DNA was gap-filled and the blunt ends were converted to sticky-ended *Nco* I sites as follows: pSR-XD310 DNA (a pSR-XD2 derivative with a 462-bp deletion in *src*; unpublished data) was digested with *Nco* I and gap-filled. This DNA was ligated with the gap-filled pS2-2 DNA. The 5′ guanosine from the gap-filled *Bgl* II site, after ligation to the gap-filled *Nco* I site, becomes the 3′ guanosine in the *Nco* I recognition sequence. The ligated DNA was digested with *Nco* I and *Xho* I and a 2.9-kilobase insert fragment was isolated. The insert was ligated to vector B.

after transfection, cultures were fully infected, at which time virus stocks were collected. Transfected cultures (2–3 weeks after transfection) or infected cultures were used for the biochemical analysis, with similar results. We refer to the virus recovered from transfection of pSR-XD2 (the wt plasmid), pSR-XD300, pSR-XD304, pSR-XD306, pSR-XD316, or pSR-XD317 ligated to pSR-REP, as SRA, NY300, NY304, NY306, NY316, or NY317, respectively.

Infected cultures produced *src* proteins of the expected sizes after [^{3}H]leucine labeling and immunoprecipitation (Fig. 3). Judging both from the relative amounts of *src* protein between the cultures, and from the ratio of the amount of *src* protein to the amount of Pr76, the viral structural protein precursor, the rate of synthesis of the mutant *src* proteins was similar to that of wt $p60^{src}$ (Fig. 3; data not shown). The half-lives of all the mutant *src* proteins

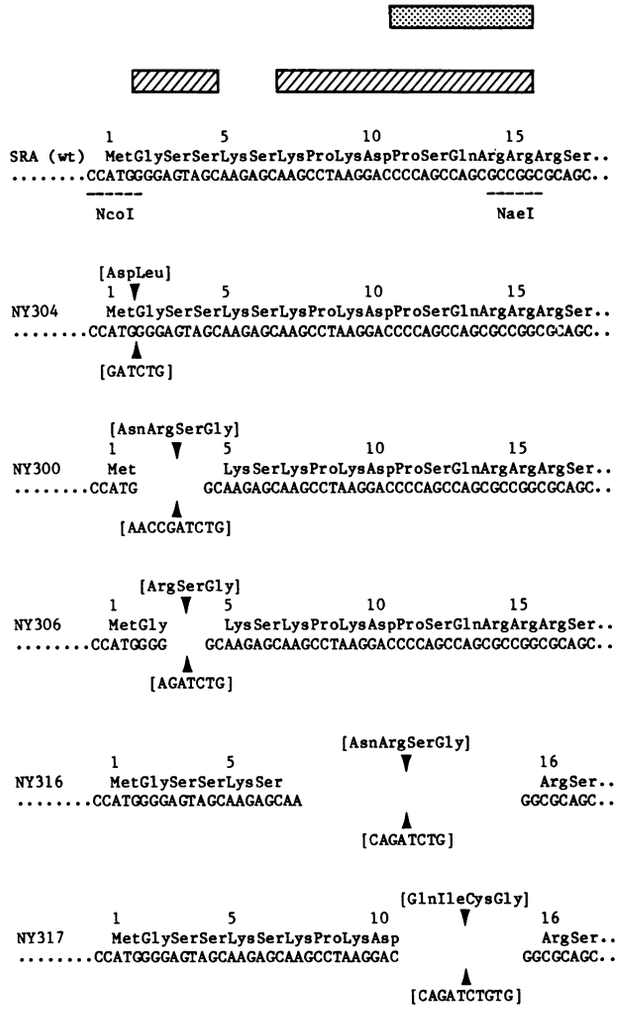

FIG. 2. Deduced sequences across alterations in mutant *src* genes. All mutations were confirmed by partial DNA sequence analysis. SRAγ contains the wild-type (wt) *src* gene (11, 14). Amino acids are numbered according to the wt sequence, and sequences that are inserted or altered relative to the wt sequence are delimited by brackets. Note that the GGG sequence encoding glycine-2 in the NY306 sequence, although it is identical to the wt sequence, is actually derived from the oligonucleotide adapter used in the NY306 construction (see Fig. 1). Hatched boxes indicate wt sequences that are required for myristylation; the dotted box indicates a sequence that is not required.

were within ≈25% of that of wt p60^{src}, except for that encoded by NY306, which was ≈50% reduced (data not shown). We consider this variation in half-life to be unlikely to significantly affect the biological activity of the mutant viruses. The half-life of the *src* protein of the (fully transforming) Prague strain of RSV was reported to be ≈25% of that of the Schmidt–Ruppin strain *src* protein (16).

Kinase Activity of the Mutant *src* Proteins. p60^{src} has been shown to phosphorylate the heavy chain of IgG in immunoprecipitates using TBR antiserum (1, 17). Using this *in vitro* assay, we compared the kinase activities of the mutant *src* proteins to that of the wt protein (Table 1). We assayed the specific activity and the total level of *src* tyrosine kinase activity by performing the immunoprecipitations in antigen or antibody excess, respectively. The specific activity of the mutant *src* proteins appears comparable to that of wt p60^{src} (Table 1, antigen excess) and mutant-infected cells contained levels of p60^{src} kinase activity similar to that found in cells infected with wt virus, except in the case of NY306 infection (Table 1, antibody excess). The reduction with NY306

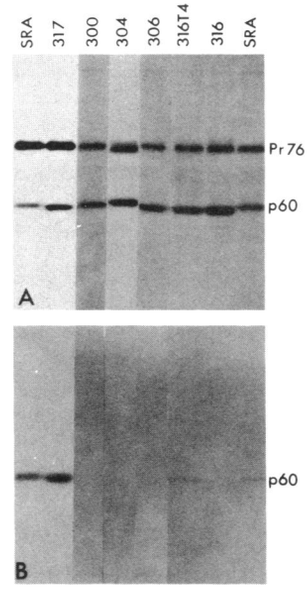

FIG. 3. Myristylation of mutant *src* proteins. Cells infected with various viruses were labeled with [^{3}H]leucine (*A*) or [^{3}H]myristic acid (*B*), and *src* proteins were immunoprecipitated with tumor-bearing rabbit (TBR) antiserum. Positions of wt p60^{src} and viral structural protein precursor Pr76 are indicated. The figure is a composite from a number of autoradiograms of various exposure times. NY316-T4 is one of the revertant transforming viruses isolated from NY316. The other three revertants encoded myristylated *src* proteins of the same molecular weight as NY316-T4.

infection may be related to the reduced half-life of the NY306 *src* protein.

Myristylation of the Mutant *src* Proteins. Cells infected with the wt or mutant RSVs were labeled with [^{3}H]leucine or [^{3}H]myristic acid. Cell extracts were prepared and immunoprecipitated with TBR antiserum (Fig. 3). Both the SRA and NY317 *src* proteins labeled well with [^{3}H]myristic acid; the *src* proteins of the other mutants were not detectably labeled (even after long exposure). These data suggest that the NH_2-terminal sequence required for myristylation of p60^{src} is contained within amino acids 1–10.

Subcellular Localization of the Mutant *src* Proteins. Infected cells were fractionated into an S100 and a P100 (7). The *src* protein was immunoprecipitated from these fractions, and its distribution was quantitated by measuring the tyrosine kinase activity in the immune complexes or by determining the radioactivity associated with the *src* protein band from [^{3}H]leucine-labeled cells. Consistent with previous reports (3), ≈80% of the wt p60^{src} fractionated with the membrane pellet. The distribution of the *src* protein of NY317, the only mutant whose *src* protein could be labeled with [^{3}H]myristic acid, was similar to that of wt p60^{src}. However, the *src* proteins of the other four mutants were found primarily in the cytosolic fraction (Table 2).

To assay for association of the *src* proteins with specific cellular membranes, we performed a further fractionation of the crude membrane pellets from infected cells by flotation

Table 1. Tyrosine kinase activity of mutant *src* proteins relative to wt p60^{src}

Virus	IgG phosphorylation (fraction of SRA)	
	Ab excess	Ag excess
NY300	1.0	1.2
NY304	0.8	1.1
NY306	0.4	0.7
NY316	0.6	0.8
NY317	0.9	0.9
SRA	1.0	1.0
None	0.002	ND

Immunoprecipitates were assayed for kinase activity by quantitating total ^{32}P incorporated into IgG, as described (18). Antiserum was titrated to determine the amount required for antibody (Ab) excess or antigen (Ag) excess. A wt SRA sample was included as a standard in each experiment. ND, not done.

Table 2. Fractionation of mutant *src* proteins

Virus	% *src* protein in P100 determined by [^{3}H]Leucine	Kinase activity
SRA	78	81
NY300	20	24
NY304	13	20
NY306	21	32
NY316	22	20
NY317	77	76
NY316–T4	ND	79

Infected cells were Dounce homogenized, and a post-nuclear supernatant was fractionated into crude membrane (P100) and cytosolic (S100) fractions (7). Recovery was greater than 90% when the *src* protein was assayed by kinase activity. The percentage values of the *src* protein found in the P100 are shown, assuming 100% recovery. Cells were labeled and the amount of [^{3}H]leucine in immunoprecipitated *src* protein was quantitated after gel electrophoresis. The amount of tyrosine kinase activity in fractions was determined after immunoprecipitation, as in Table 1. NY316–T4 is one of the transforming revertants isolated from NY316 (see text). The other three revertants gave similar results. ND, not done.

in discontinuous sucrose gradients. The distribution of the SRA and NY317 *src* proteins was similar. Both proteins were enriched in the plasma membrane fraction (10%/30%) (Table 3). We found no enrichment for the *src* proteins of NY300, NY304, NY306, or NY316 in any membrane fraction. The small amount of the *src* protein found in crude membrane pellets prepared from cells infected with these viruses was distributed in proportion to the total protein in each membrane fraction.

Biological Characterization of the Mutant Viruses. After transfection with the wt pSR-XD2 plasmid and pSR-XD317 ligated to pSR-REP, similar numbers of foci of round morphologically transformed cells were observed within 2 weeks. In contrast, no foci were observed 2 weeks after transfection of pSR-XD300, pSR-XD304, pSR-XD306, or pSR-XD316. After 1.5 weeks of further incubation, a few foci were observed in cultures transfected with pSR-XD316, but the number of foci was <1% of the number seen with pSR-XD2. These foci were shown to be the result of reversion of the mutant to the wt phenotype (see below). Although few or no foci were observed after transfection with pSR-XD300, pSR-XD304, pSR-XD306, or pSR-XD316, transfected cells were producing mutant *src* proteins and

Table 3. Fractionation of sedimentable *src* kinase activity on discontinuous sucrose gradients

Virus	Specific activity of IgG kinase in membrane fractions 10%/30%	30%/35%	35%/45%	45%/60%
NY300	1.2	0.7	0.8	1.0
NY304	0.9	0.4	1.3	1.0
NY306	0.7	0.7	0.8	1.0
NY316	1.1	1.5	1.7	0.9
NY317	55.0	11.3	6.1	1.5
SRA	117.3	48.7	14.2	1.0

Cells infected with the indicated viruses were labeled with [^{3}H]leucine for 4 hr, Dounce homogenized, and a crude membrane pellet was prepared, fractionated on a sucrose gradient, and analyzed for specific activity of *src* kinase as described (12). Data are normalized to the specific activity of the SRA kinase activity found in the 45%/60% interface. Specific activity is defined as the ^{32}P cpm in IgG in a kinase assay divided by the total amount of protein in the fraction as indicated by trichloroacetic acid-precipitable [^{3}H]leucine cpm.

Table 4. Biological characterization of mutant viruses

Virus	ffu/ml	cfu/ml	Kinase titer*	Colonies per unit of virus†
NY300	<10	10	0.36	4.6×10^{-5}
NY304	5	60	0.34	1.5×10^{-4}
NY306	5	130	1.22	1.8×10^{-4}
NY316	280	160	0.32	4.2×10^{-4}
NY317	3×10^6	2×10^6	2.56	1.2
SRA	2–5 $\times 10^6$‡	9×10^5	1.0	1.0

All virus stocks were collected approximately 2 weeks and 3 transfers after transfection. ffu, Focus-forming units; cfu, colony-forming units. cfu assay was read 19 days after infection. The value for SRA represents the average of two experiments.

*This figure represents the amount of tyrosine kinase activity induced 36 hr after infection of 1.25×10^6 cells with 0.1 ml of virus stock, divided by the amount induced by 0.1 ml of wt SRA stock ($\approx 5 \times 10^6$ ffu/ml).

†One unit of virus produces kinase activity 36 hr after infection equal to that given by 1 ml of reference SRA stock.

‡SRA stock used for kinase titer and colony formation was not titered for focus formation. This figure gives the range of focus-forming titers seen with these SRA stocks.

infectious virus (see above). We recovered virus stocks from these cultures and standardized their infectivity by measuring the p60^{src} kinase activity 36 hr after infection with a small aliquot of the virus stock. The rationale for the use of this procedure for assay of this class of transformation-defective viruses has been described (12).

Table 4 shows the quantitative characterization of the transforming activity of these virus stocks. Focus formation by NY300, NY304, and NY306 was decreased by a factor of $\approx 10^6$; focus formation by NY316 was decreased by a factor of $\approx 10^4$ compared with wt SRA. NY317 was wt with respect to focus formation. Colony formation in soft agar by NY300, NY304, NY306, and NY316 was decreased by a factor of 10^3–10^4 relative to NY317 and SRA. NY300, NY304, NY306, and NY316 were equally defective in transformation, because the low level of focus formation observed with NY316 was shown to be due to revertant virus present at a low level in the stocks (see below).

Four Revertants Isolated from NY316. Although cultures transfected with pSR-XD316 initially produced a low level of transforming virus, after continued passage the transforming titer increased. It is therefore likely that the foci observed with early NY316 stocks are induced by a small population of revertants. To confirm this, four independent cultures were infected with a dilution of NY316 past the end point of transforming virus (Table 4), and after transfer one focus was picked from each culture. A high titer of transforming virus was recovered from each focus. The *src* proteins encoded by all four revertants had the same apparent molecular weight as the *src* protein expressed in cells infected with NY316 (slightly lower than that of wt p60^{src}), but the *src* proteins of the revertants were labeled with [^{3}H]myristic acid (Fig. 3) and fractionated primarily with the membrane pellet (Table 2). We have also isolated a transforming revertant from NY306 and found that it encoded a myristylated membrane-associated *src* protein (data not shown).

DISCUSSION

Sequence Requirements for p60^{src} Myristylation. The mutations we introduced into the *src* gene were made with the assumption that the linkage of myristic acid to p60^{src} might be identical to the linkage of myristic acid to several other proteins. The catalytic subunit of cAMP-dependent protein kinase (19), murine leukemia virus p15 (20), and calcineurin

B (21) are *N*-myristylated at glycine (myristic acid is bound by an amide bond to the NH_2-terminal glycine residue). It has recently been determined that glycine-2 of $p60^{src}$ is in fact *N*-myristylated (6). We have constructed five mutants to evaluate the importance of glycine-2, and to test whether other amino acids within the critical sequence 2–14 (12) were also required.

NY304 was constructed to encode a *src* protein with the entire wt amino acid sequence except that the peptide Asp-Leu is interposed between the initiator methionine and glycine-2. This insertion might be expected to prevent an amide linkage of myristic acid to the protein. NY300 *src* protein has a short peptide substituted for amino acids 2–4, and it also lacks a glycine in position 2. The *src* protein encoded by NY306 is identical to that encoded by NY300 except that asparagine-2 of the NY300 *src* protein is changed to glycine. This mutant was designed to test the hypothesis that the lack of myristylation of the NY300 *src* protein was solely due to the absence of the attachment site. NY316 and NY317 encode *src* proteins with deletions of amino acids 7–15 and 11–15, respectively. The *src* protein of NY317 is myristylated, while the *src* proteins encoded by the other four mutants are not. These data indicate that sequences within amino acids 2–4 and 7–14 are required for myristylation and that amino acids 11–15 are not. Since we have previously shown that amino acids 15–81 are also not required for myristylation (12), the NH_2-terminal sequences required for myristylation may be contained within amino acids 1–10. These results are summarized in Fig. 2.

The lack of myristylation of the *src* proteins of NY304 and NY300 is likely due to the absence of a glycine in position 2. However, the *src* proteins of NY306 and NY316 contain a glycine in position 2, but they are not myristylated, suggesting that the sequence of amino acids COOH-terminal to glycine-2 is also important for $p60^{src}$ myristylation. The additional sequences may be required for the *src* protein to be recognized as a target for myristylation.

The lack of myristylation of the *src* proteins of NY300, NY304, NY306, and NY316 could be due to inhibitory effects of the inserted peptides. However, we have constructed other RSV mutants whose *src* proteins contain similar inserted peptides at amino acid 15 (but retain amino acids 1–14) and are myristylated (12).

Myristylation and Membrane Association of $p60^{src}$, and Cell Transformation. Using *in vitro* constructed mutants, we have found a correlation between $p60^{src}$ myristylation, membrane association, and cell transformation (ref. 12; this work). A further confirmation of this correlation comes from analysis of the transforming revertants that we isolated from NY316. The size of the *src* proteins of the revertants indicates that they are new isolates and not the wt virus generated by recombination with the cellular *src* gene (22). These viruses were selected for their ability to transform chicken embryo fibroblasts, yet their *src* proteins are myristylated and membrane associated.

On the basis of the lack of transforming activity of the mutants encoding soluble *src* proteins, we have proposed that myristylation of $p60^{src}$ is the initial event that is required for membrane association, which in turn is required for transformation, perhaps because some critical targets for $p60^{src}$ are restricted to the membrane (12). However, the soluble mutant *src* proteins might be altered in their substrate specificity as well as their subcellular localization.

A Signal for Myristylation? Our data indicate that the first 10 amino acids of $p60^{src}$ are required for its myristylation. Amino acids 11–15 or 15–264 of $p60^{src}$ can be deleted with no effect on myristylation (ref. 12; unpublished data), but most of the NH_2-terminal 10 amino acid sequence must remain intact for myristylation to occur. Therefore, the NH_2-terminal 10 amino acids may contain a recognition sequence for myristylation. The previous construction of *Bgl* II linker insertions in the codon for arginine-15, with the linker in all three reading frames (14), allows this hypothesis to be tested by fusing the sequence coding for amino acids 1–14 with 3′ sequences of heterologous genes.

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