JOURNAL OF VIROLOGY, Dec. 1986, p. 858–867
0022-538X/86/120858-10$02.00/0

Vol. 60, No. 3

Genetic Lesions Involved in Temperature Sensitivity of the *src* Gene Products of Four Rous Sarcoma Virus Mutants

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Received 9 June 1986/Accepted 26 August 1986

The *src* genes of four Rous sarcoma virus (RSV) mutants temperature-sensitive (*ts*) for cell transformation were analyzed. The mutant *src* genes were cloned into a replication-competent RSV expression vector, and the contribution of individual mutations to the *ts* phenotype was assessed by in vitro recombination with wild-type *src* sequences. Three of the mutants, which were derived from the Schmidt-Ruppin strain of RSV, each encoded two mutations within the conserved kinase domain. In all three cases, one of the two mutations was an identical valine to methionine change at amino acid position 461. Virus encoding recombinant *src* genes containing each of these mutations alone were not *ts* for transformation, demonstrating that two mutations are required for temperature sensitivity. The sequence of the *src* gene of the Bryan high-titer strain of RSV was determined and compared with that of the fourth *ts* mutant which was derived from it, again revealing two lesions in the kinase domain of the mutant.

Numerous studies have demonstrated that the transforming gene product of Rous sarcoma virus (RSV) $p60^{src}$ is a protein kinase (7, 25) that specifically catalyzes the phosphorylation of tyrosine residues (8, 20, 26). Temperature-sensitive (*ts*) mutants of RSV were extremely useful in demonstrating the existence of $p60^{src}$ (23, 28) and that its kinase activity correlates with cell transformation (7, 34–36). Infection with RSV induces a wide range of cellular changes (17), and the complexity of the transformed phenotype has been investigated by using *ts* mutants in which one or more parameters of transformation have been dissociated as a function of temperature (1, 2, 22, 41).

Lesions within both the C-terminal kinase domain and the N-terminal half of $p60^{src}$ have been shown to confer temperature sensitivity. In vivo recombination experiments with a series of 14 *ts* RSV mutants mapped a majority of the *ts* lesions to the 3′ end of the gene, which encodes the conserved kinase domain; the remainder mapped to the 5′ 60% of the gene (12). Recently, two of these C-terminal mutants have been sequenced, revealing a single amino acid change in each case (13). In another study, Nishizawa et al. (30) reported the nucleotide sequence of the *src* gene of *ts*NY68, in which two lesions were identified within the carboxy-terminal kinase domain. Both of these mutations were shown to be required for temperature sensitivity. In addition, two in vitro deletion mutants containing large deletions within the amino-terminal half of the protein were found to be *ts* (3, 31).

In this study we determined the nucleotide sequences of three additional *ts src* genes, those of *ts*NY72-4, PA101, and PA104. Although all are *ts* for cell transformation, these mutants differ in their biological properties. *ts*NY72-4, like *ts*NY68, is nonpermissive for transformation at 41°C; however, *ts*NY72-4 is more stringently *ts* for stimulation of cell proliferation than *ts*NY68, and infected cells have a slightly less rounded and refractile morphology at 37°C, the permissive temperature (H. Hanafusa and B. Mayer, unpublished data). PA101 and PA104 were both selected on the basis of their ability to stimulate the proliferation of neuroretina cell cultures at 37°C in the absence of morphological alteration (4, 5, 32). As described in an accompanying paper (22), we subsequently found that both mutants induce morphological alteration and stimulate anchorage-independent growth at 34°C. PA101 is less defective than PA104 in several transformation parameters, especially at 37°C (22). Despite these differences, we found that the genetic lesions that render these mutant *src* proteins *ts* are similar. In each case, two mutations within the C-terminal kinase domain of $p60^{src}$ conferred temperature sensitivity. Furthermore, we identified a common mutation which was present in three of the mutants that acts cooperatively with a second C-terminal mutation to abolish transforming activity at high temperatures.

MATERIALS AND METHODS

Cell culture and viruses. Chicken embryo fibroblasts (CEFs) were prepared and cultured as described previously (16). The isolation and properties of *ts*NY68 have been reported previously (23). *ts*NY72-4 is a *ts* mutant derived by nitrosoguanidine treatment of the New York stock of Schmidt-Ruppin RSV, subgroup A [SRA(NY)] (32; H. Hanafusa, unpublished data). PA101 and PA104 have been described previously (4, 5, 22).

Molecular cloning. The cloning of the *src* genes of PA101 and PA104 are described in an accompanying paper (22). The full-length genomes of *ts*NY68 and *ts*NY72-4 were molecularly cloned from unintegrated circular viral DNA. QT6 cells were infected at a multiplicity of infection of >4, and low-molecular-weight circular DNA was isolated 36 h postinfection by the method of Hirt (19). DNA was passed over a Biogel A-5M column to remove contaminating RNA; in our hands enriching for closed circular DNA by the acid-phenol method was unnecessary. Since there are no known restriction endonucleases that cleave only once in the SRA(NY)

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PA101   TAGTAAACTTGTAGACTTGGCTACAGCATAGAGTATCTTCTGTAGCTCTGATGACTGCTAGGAAATAATGCTA
BH-RSV  TAGTAAACTTGT*****TGGCTACAGCATAGAGTATCTTCTGTAGCTCTGATGACTGCTAGGAAATAATGCTA
SR-A    TAG********TGCGCGAGCAAAATTTAAGCTACAACAAGGCAAGGCTTGGCCGACAATTGCATGAAGAATCT
PR-C    TAA**********************GTACGAGGCGTGACCTACAATTGGCTAAATAATGCTTCTGTAGAAATT

PA101   CGGATAATGTGGGGAGGGCAAGGCTTGCGAATCGGGTTGTAACGGGGCAA******GGCTTGACTGAGGGGAC
BH-RSV  CGGATAATGTGGGGAGGGCAAGGCTTGCGAATCGGGTTGTAACGGG*CAA******GGCTTGACTGAGGGGAC
SR-A    GCTTAGGGTTAGGCGTTTTGCGCTGCTTCGCGATGTACGGGCCAGATATA******CGCGTATCTGAGGGGAC
PR-C    GTTTAGCATTAGGCGTCCTGCGTTGCTCCGCGATGTACGGGTCAGGTATAATGTGCAGTTTGACTGAGGGGAC
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FIG. 1. Comparison of the 3'-flanking sequences of the PA101, BH-RSV (24; this study), SRA(NY) (37, 38), and PRC (33) strains of RSV. The sequences begin with the termination codon of $p60^{v\text{-}src}$. Asterisks denote deleted bases or gaps imposed to maximize alignment of homologous sequences. Colons above each line indicate homology with the PA101 sequence.

genome, it was necessary to use partial digestion conditions to obtain full-length genomic clones. The purified viral DNA was digested with *Eco*RI, and the success of partial digestion was assayed by Southern blot hybridization. The partially digested low-molecular-weight DNA was ligated to λgtWES · λB *Eco*RI arms and packaged in vitro, and recombinant phage were screened in situ by hybridization to a nick-translated RSV genomic probe. Full-length clones were identified by restriction mapping and Southern blot analysis.

The biological activity of the clones was assayed by calcium phosphate-mediated transfection (42) of CEFs. Lambda DNA was partially digested with *Eco*RI, and the full-length insert was isolated by agarose gel electrophoresis and electroelution of the DNA fragments. The 3.1-kilobase (kb) *src*-containing *Eco*RI fragments from the biologically active clones were subcloned into the *Eco*RI site of pBR322, generating p68-13-1 and p72-12-1.

DNA sequencing. Nucleotide sequences were determined by the dideoxy chain-termination method of Sanger, essentially as described previously (27), by using M13mp8 as a cloning-sequencing vector. The plasmids used to subclone into M13 for sequence analysis were as follows: p72-12-1 for *ts*NY72-4; pVS101A (22) for PA101; pVS104A (22) for PA104; pBH-beta (24) for Bryan high-titer RSV (BH-RSV); pTT107 (40) for SRA(NY).

Transfection assay system. Cloned *src* genes were expressed by using the two-plasmid RSV vector system described by Cross and Hanafusa (9). Briefly, all mutant and recombinant *src* genes were cloned into the homologous site of pSR-XD2 (9), which contains the wild-type (wt) SRA *src* gene. The pSR-XD2 derivatives were cleaved with *Sal*I together with pBH-REP (21), which contains the RSV replicative gene sequences. The two plasmids were then ligated and transfected into CEFs by calcium phosphate coprecipitation. This system resulted in generation of infectious virus expressing the cloned *src* gene. Culture media from transfected cultures were harvested 7 to 12 days posttransfection as high-titer virus stocks ($>10^7$ focus-forming units per ml with pSR-XD2) and were used to reinfect CEFs for assay of the biological activity of the various *src* gene products.

Plasmid constructions. Standard recombinant DNA techniques were used to construct the *src*-containing plasmids used in this study (see Fig. 4). pSR-XD2 contains the wt v-*src* gene of SRA. pXD68 and pXD72, which contain the *ts src* genes of *ts*NY68 and *ts*NY72-4, respectively, were constructed by ligating the 1.7-kb *Nco*I-partial *Mlu*I fragment from p68-13-1 or p72-12-1 with the 7.5-kb *Mlu*I-partial *Nco*I fragment of pSR-XD2. pXD568 and pXD572, containing 5' *ts* and 3' wt sequences, were constructed by ligation of the 1.5-kb *Mlu*I-partial *Nco*I fragment from p68-13-1 or p72-12-1 with the 7.7-kb *Nco*I-partial *Mlu*I fragment of pSR-XD2. pXD368 and pXD372, containing 3' *ts* and 5' wt sequences, were constructed by ligation of the 0.9-kb *Mlu*I fragment of p68-13-1 or p72-12-1 with the 8.3-kb *Mlu*I fragment of pSR-XD2 (treated with bacterial alkaline phosphatase to minimize self-ligation). pXDM72, encoding only the mutation at amino acid 461 (Met-461), was made by ligation of the 6.0-kb *Cla*I-partial *Sph*I fragment from pSR-XD2 with the 3.2-kb *Cla*I-partial *Sph*I fragment from pXD372. pXDS72, the reciprocal construct encoding only the Ser-503 mutation of *ts*NY72-4, was made by ligation of the 6.0-kb *Cla*I-partial *Sph*I fragment of pXD372 with the 3.2-kb *Cla*I-partial *Sph*I fragment of pSR-XD2. pXD104 contains the *ts src* gene of PA104 and is described in an accompanying paper (22). pXDP104, encoding only the

TABLE 1. Nucleotide differences between the SRA(NY) and BH-RSV *src* genes

Nucleotide no.	Amino acid no.	Nucleotides in SRA(NY)	Nucleotides in BH-RSV
48	16	CGC (Arg)	CGT (Arg)
129	43	ACG (Thr)	ACA (Thr)
157	53	ACC (Thr)	GCC (Ala)
210	70	GTT (Val)	GTC (Val)
244	82	GGC (Gly)	AGT (Ser)
254	85	ACT (Thr)	ATT (Ile)
272	91	GAC (Asp)	GGC (Gly)
283	95	TGG (Trp)	CGG (Arg)
349	117	AAC (Asn)	GAC (Asp)
370	124	GTG (Val)	CTC (Leu)
476	159	GAG (Glu)	GGG (Gly)
696	232	AAA (Lys)	AAG (Lys)
830	277	TGC (Cys)	TAC (Tyr)
1092	364	GTT (Val)	GTC (Val)
1095	365	GAT (Asp)	GAA (Glu)
1257	419	CGG (Arg)	CGA (Arg)
1259	420	CAA (Gln)	CCA (Pro)
1268	423	AAG (Lys)	AGG (Arg)
1276	426	ATC (Ile)	GCC (Ala)
1382	461	GTG (Val)	GCC (Ala)
1400	467	GGC (Gly)	GTC (Val)
1413	471	GTG (Val)	GTA (Val)
1502	501	AGG (Arg)	AAG (Lys)
1567	523	GAG (Glu)	AAG (Lys)
1578	526	GAG (Glu)	GAA (Glu)

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BH-RSV 1   MGSSKSKPKDPSQRRRSLEPPDSTHHGGFPASQTPNKTAAPDTHRTPSRSFGAVATEPKLFGDF
PA101                                                          T    L

BH-RSV 65  NTSDTVTSPQRAGALAGSVTIFVALYGYESRIETDLSFKKGERLQIVNNTEGDWWLAHSLTTGQ
PA101                          T                   R

BH-RSV 129 TGYIPSNYVAPSDSIQAEEWYFGKITRRESGRLLLNPENPRGTFLVRESETTKGAYCLSVSDFD
PA101

BH-RSV 193 NAKGLNVKHYKIRKLDSGGFYITSRTQFSSLQQLVAYYSKHADGLCHRLTNVCPTSKPQTQGLA
PA101

BH-RSV 257 KDAWEIPRESLRLEVKLGQGYFGEVWMGTWNGTTRVAIKTLKPGTMSPEAFLQEAQVMKKLRHE
PA101

BH-RSV 321 KLVQLYAVVSEEPIYIVIEYMSKGSLLDFLKGEMGKYLRLPQLVEMAAQIASGMAYVERMNYVH
PA101             M

BH-RSV 385 RDLRAANILVGENLVCKVADFGLARLIEDNEYTARPGARFPAKWTAPEAALYGRFTIKSDVWSF
PA101

BH-RSV 449 GILLTELTTKGRAPYPGMVNGEVLDRVERGYRMPCPPECPESLHDLMCQCWRKDPEERPTFEYL
PA101

BH-RSV 513 QAQLLPACVLKVAE
PA101                I
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FIG. 2. Comparison of the amino acid sequences of PA101 and BH-RSV p60$^{v\text{-}src}$. Only amino acid changes relative to BH-RSV are noted. Closed circles indicate changes that have been shown by in vitro recombination to be involved in temperature sensitivity of PA101 (22).

Pro-325 mutation of PA104, was made by three-part ligation of the 3.0-kb *Cla*I-*Bgl*I and 4.9-kb *Sac*I-*Cla*I fragments from pXD104 with the 1.3-kb *Bgl*I-*Sac*I fragment of pSR-XD2.

The structures of pXDM72, pXDS72, and pXDP104 were verified by restriction analysis. *Nci*I cleaves within the *src* gene at the codon for amino acid 461; the Met-461 mutation of *ts*NY72-4 and PA104 abolishes this site. Likewise, *Ava*II cuts at the position corresponding to amino acid 503, and the Ser-503 mutation of *ts*NY72-4 destroys this recognition site. Recombinant plasmids were screened by digestion with each of these enzymes in parallel with pSR-XD2 and either pXD372 or pXD104 and run on 2% agarose gels in Tris borate-EDTA buffer to separate the small DNA fragments.

RESULTS

Molecular cloning. The full-length genomes of *ts*NY68 and *ts*NY72-4 were molecularly cloned from unintegrated closed circular viral DNA by standard methods. Because viral replicative gene or regulatory sequences could contribute to the mutant phenotypes, the complete genomes were cloned. For each virus, eight lambda clones were isolated containing inserts that hybridized to a RSV probe. Restriction digestion and Southern blot analysis identified two *ts*NY68 clones and one *ts*NY72-4 clone that contained the full-length genome. The biological activity of the cloned viral DNA was assayed by transfection. Full-length insert was isolated from partially digested lambda DNA and was transfected into CEFs. On transfection, one clone of each mutant, λ68-13 and λ72-12, produced infectious virus with a *ts* phenotype indistinguishable from that of the parental strain (data not shown), demonstrating that the authentic *ts* viruses were cloned. The molecular cloning of the *src*-containing *Eco*RI fragments of PA101 and PA104 from proviral DNA is described in an accompanying paper (22).

TABLE 2. Nucleotide differences between the BH-RSV and PA101 *src* genes

Nucleotide no.	Amino acid no.	Nucleotides in BH-RSV	Nucleotides in PA101
157	53	GCC (Ala)	ACC (Thr)
173	58	CCC (Pro)	CTC (Leu)
201	67	TCT (Ser)	TCC (Ser)
246	82	AGT (Ser)	AGC (Ser)
254	85	ATT (Ile)	ACT (Thr)
313	105	GGA (Gly)	AGA (Arg)
615	205	CGC (Arg)	CGT (Arg)
798	266	TCG (Ser)	TCA (Ser)
982	328	GTG (Val)	ATG (Met)
1570	524	GTC (Val)	ATC (Ile)

Sequence of the BH-RSV *src* gene. *ts*NY68, *ts*NY72-4, and PA104 were derived by mutagenization of the SRA(NY) strain of RSV. As expected, the nucleotide sequences of the *src* genes of these three viruses differed only slightly from that of SRA(NY) (discussed below). The *src* gene of PA101, however, differed at numerous positions from that of SRA(NY), and the 3′-flanking sequences of the two genes diverged radically. Comparison of the 3′ *src* flanking sequences of PA101 with those of the BH-RSV (24), SRA(NY) (37, 38), and Prague subgroup C (PRC) (33) strains of RSV (Fig. 1) revealed that the PA101 flanking sequence is virtually identical to that of BH-RSV, the only differences being two insertions of one and five nucleotides within the 150-base-pair region that was compared. Although PA101 was isolated as a spontaneous mutant from a subgroup D SR-RSV-transformed mammalian cell line (4, 5), these results strongly suggest that PA101 is derived from contaminating BH-RSV. This possibility is supported by the observation that PA101, like BH-RSV, is a replication-defective virus which has suffered an extensive deletion of the *env* gene (4, 22, 24). Restriction analysis demonstrated that the 0.3-kb *Kpn*I-*Nco*I fragments spanning the *env* deletions of the two viruses were indistinguishable by polyacrylamide gel electrophoresis (R. Jove, unpublished data).

Because these results strongly imply that BH-RSV is the parental strain of PA101, we sequenced the BH-RSV *src* gene to identify mutations in PA101 that could be responsible for the *ts* phenotype. The results of the sequence analysis are summarized in Table 1, which lists nucleotide differences between the SRA(NY) (37, 38) and BH-RSV *src* genes. There are 28 nucleotide differences coding for 17 amino acid differences between the two genes.

TABLE 3. Corrections to the nucleotide sequence of the SRA(NY) *src* gene

Nucleotide	Amino acid no.	Published nucleotides[a]	Corrected nucleotides
287	96	ACT (Thr)	ATT (Ile)
363	121	GCA (Ala)	GCT (Ala)
375	125	ACC (Thr)	ACT (Thr)
474	158	TCT (Ser)	TCC (Ser)
1551	517	CTT (Leu)	CTC (Leu)

[a] See references 37 and 38.

Identification of mutations in *ts src* genes. Comparison of the *src* sequences of BH-RSV and PA101 revealed a close relationship between the two *src* genes, further supporting the theory that PA101 is derived from BH-RSV. Nucleotide differences between the *ts* mutant and its parental wt gene are listed in Table 2. There are 10 nucleotide differences coding for six amino acid changes; the majority of these mutations are found in the amino-terminal half of the protein. The deduced amino acid sequences of the BH-RSV and PA101 *src* gene products are compared in Fig. 2. Only two amino acid differences, a valine to methionine change at position 328 and a valine to isoleucine change at position 524, were found in the carboxy-terminal kinase domain of the *ts* protein. As described in an accompanying paper (22), construction of chimeric wt-PA101 *src* genes has demonstrated that the carboxy-terminal half of PA101 $p60^{src}$, when fused with the amino-terminal half of SRA, is sufficient to confer temperature sensitivity to the hybrid protein.

When comparing the nucleotide sequences of *ts*NY68 (30), *ts*NY72-4, and PA104 with that of their parent strain SRA(NY) (37, 38), it was noted that several mutations relative to the published SRA(NY) sequence were common to all three mutants. Four common changes at the third base, which did not affect the predicted amino acid sequence, were found in the three mutants; these are assumed to be anomalies in the original SRA(NY) sequence. In addition, two changes resulting in amino acid substitutions were found to be common to all three *ts* mutants. To determine whether these were actually common mutations or errors in the published SRA(NY) sequence, the SRA(NY) *src* gene was resequenced in the region of each of these two putative mutations. The results indicate that SRA(NY) actually codes for isoleucine, not threonine as previously reported, at amino acid position 96. The second common mutation, at position 461, was found to be genuine (see below). The corrections to the SRA(NY) *src* sequence which emerged from this study are summarized in Table 3; all comparisons in this report to the SRA(NY) *src* gene are to this corrected sequence. It should be noted that in our previous report of the *ts*NY68 *src* sequence (30), isoleucine at position 96 was listed incorrectly as a mutation, based on the published SRA(NY) sequence.

The mutations found in the *src* genes of *ts*NY72-4 and PA104 relative to their parental wt strain are listed in Table 4. The deduced amino acid sequences of these two viruses, as well as *ts*NY68 (30), are compared with that of SRA(NY) in Fig. 3. Remarkably, all three *ts src* genes encode the same valine to methionine mutation at position 461. Resequencing of SRA(NY) confirmed that the parental virus encodes valine at this position; both chicken c-*src* and the v-*src* genes of PRA and PRC RSV encode valine in this position as well (13, 33, 39). These data, together with results of the in vitro recombination experiments described below, indicate that this is a genuine mutation that is required for temperature sensitivity. In addition to the mutation at position 461, each of the three *ts* mutants contains a second mutation in the region of the gene encoding the conserved kinase domain. *ts*NY72-4 contains a proline to serine substitution at position 503, while PA104 encodes a leucine to proline substitution at position 325. As reported previously (30), the *ts*NY68 protein has suffered a deletion of three amino acids at position 352 to 354. Compared with the revised SRA(NY) sequence, *ts*NY68 and PA104 encode no amino-terminal mutations; *ts*NY72-4 contains a deletion of six nucleotides that results in the replacement of Asn-Pro-Glu at positions 164 to 166 with lysine, as well as a leucine to phenylalanine change at position 197 and the substitution of asparagine for aspartate at position 208.

Properties of hybrid *ts*-wt *src* genes. The contribution of individual amino acids changes identified by sequence analysis to the overall *ts* phenotype of these mutants was examined by in vitro recombination. All mutant and recombinant *src* genes were constructed into a replication-competent RSV vector and transfected into CEFs, and the phenotypes of the infectious virus produced were assayed. The two-plasmid vector system used has been described previously (9). The recombinant *src* genes constructed for this study, the nomenclature used for the virus derived from each plasmid, and the phenotype of each virus are shown in Fig. 4. Initially, we replaced the wt *src* gene of pSR-XD2 with the corresponding molecularly cloned *ts*NY68 or *ts*NY72-4 sequences. The viruses generated by transfection with these plasmids were phenotypically identical to the parental mutants, demonstrating that the *ts* properties are due entirely to the mutant *src* gene products.

The *src* genes of *ts*NY68 and *ts*NY72-4 were next bisected at the *Mlu*I site (corresponding to amino acid 259) and exchanged with the corresponding wt sequences. As expected from results of a previous study (30) and the predicted amino acid sequence, NY568 (which contains the 5′ half of the *ts*NY68 *src* gene) was phenotypically wt, while NY368 (which contains the 3′ half of the mutant gene) was

TABLE 4. Nucleotide differences between the SRA(NY), *ts*NY72-4, and PA104 *src* genes

Nucleotide	Amino acid no.	Nucleotides in SRA(NY)	Nucleotides in *ts*NY72-4	Nucleotides in PA104
306	102	TTC (Phe)	TTC (Phe)	TTT (Phe)
490–498	164–166	AACCCCGAA (Asn Pro Glu)	AAA (Lys)	AACCCCGAA (Asn Pro Glu)
589	197	CTC (Leu)	TTC (Phe)	CTC (Leu)
622	208	GAC (Asp)	AAC (Asn)	GAC (Asp)
974	325	CTG (Leu)	CTG (Leu)	CCG (Pro)
1257	419	CGG (Arg)	CGA (Arg)	CGG (Arg)
1381	461	GTG (Val)	ATG (Met)	ATG (Met)
1507	503	CCT (Pro)	TCT (Ser)	CCT (Pro)

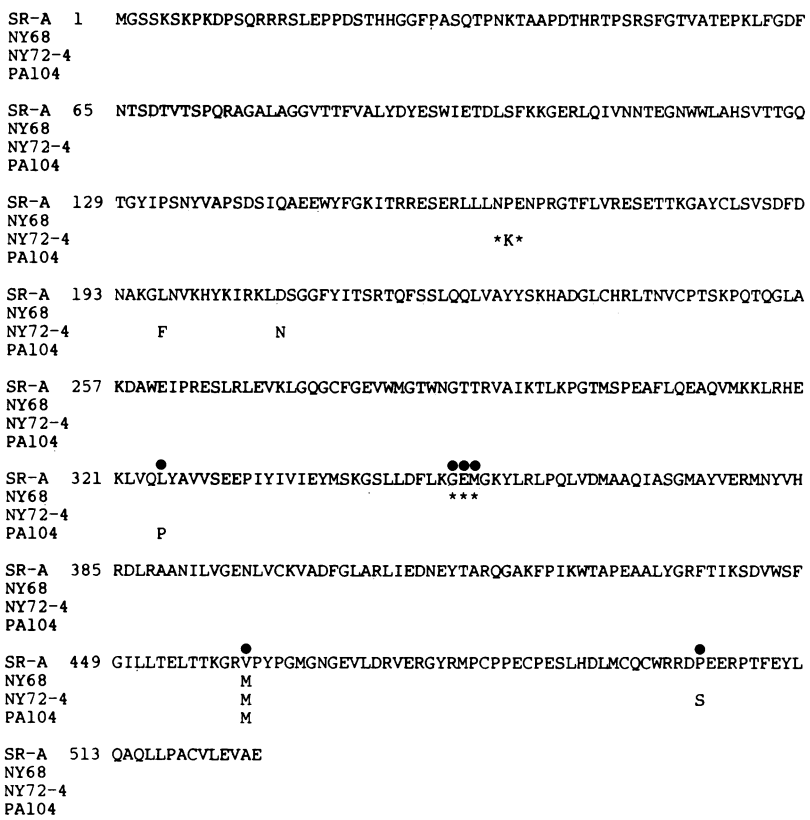

FIG. 3. Comparison of the amino acid sequences of *ts*NY68 (30), *ts*NY72-4, and PA104 $p60^{v\text{-}src}$ with that of wt SRA(NY) (37, 38). Only amino acid changes relative to SRA(NY) are noted. Asterisks denote deleted amino acids. Closed circles indicate amino acid changes which have been shown by in vitro recombination to be required for temperature sensitivity of the mutants.

indistinguishable from *ts*NY68. The same result was obtained with *ts*NY72-4. The virus containing only the three N-terminal mutations (NY572) was indistinguishable from SRA, whereas the virus containing only the two carboxy-terminal mutations of the *ts*NY72-4 *src* gene (NY372) was phenotypically identical to the parental *ts* mutant (Fig. 5). Because the three N-terminal mutations of *ts*NY72-4 are phenotypically silent, it is possible that they arose on passage of the virus prior to molecular cloning.

Previous work has shown that virus containing either of the two C-terminal mutations of *ts*NY68, in the absence of the second, are no longer *ts* for transformation (30), demonstrating that both mutations are essential for expression of the phenotype. We separated the two C-terminal mutations of *ts*NY72-4 and found that, as in the case of *ts*NY68, both mutations are required for full temperature sensitivity. Figure 5 illustrates the temperature sensitivity of morphological alteration induced by the various wt-*ts*NY72-4 hybrid viruses. Consistent with previous results, NYM72 (which contains only the Met-461 mutation) is indistinguishable from the wt at all temperatures by the criteria of morphological alteration and soft-agar colony stimulation. NYS72, which encodes only the Ser-503 mutation, confers a slightly flatter morphology than the wt, especially at higher temperatures. However, NYS72 induces approximately the same number of both transformed foci and colonies in soft agar at 41 as at 37°C (data not shown), and infected cells maintained under soft agar for long periods often appear fully transformed at 41°C.

PA104 is more defective in its ability to transform than either *ts*NY68 or *ts*NY72-4. The permissive temperature for colony formation in soft agar is 34 versus 37°C for the other two viruses, and morphological alteration at 34°C is subtle and highly dependent on culture conditions (22). We constructed a virus, termed NYP104, which encodes only the Pro-325 mutation of PA104. Figure 6 illustrates the ability of NYP104 and NYPA104 (which contains the *ts src* gene of PA104) to stimulate anchorage-independent growth of infected CEFs. The permissive temperature for colony formation of NYP104 is shifted upward significantly relative to that of NYPA104, with some activity remaining at 41°C; however, the number of colonies induced at 41°C is approximately fivefold less than that at 34°C. The colonies induced by NYP104 are much smaller than those induced by wt RSV, and morphological alteration by NYP104 is extremely subtle at all temperatures (data not shown).

DISCUSSION

In this study we have compared the sequences of four molecularly cloned *ts src* genes and have confirmed by in vitro recombination the identity of amino acid changes that confer *ts* transforming activity. The four mutants analyzed in this study have different biological activities, varying in their permissive temperatures, transformed phenotypes at the permissive temperature, and basal levels of activity at the nonpermissive temperature. Nevertheless, the mutations in these four *ts src* genes follow a similar pattern. Each mutant contains two amino acid changes in the C-terminal kinase domain of the protein; in three of these mutants, the same

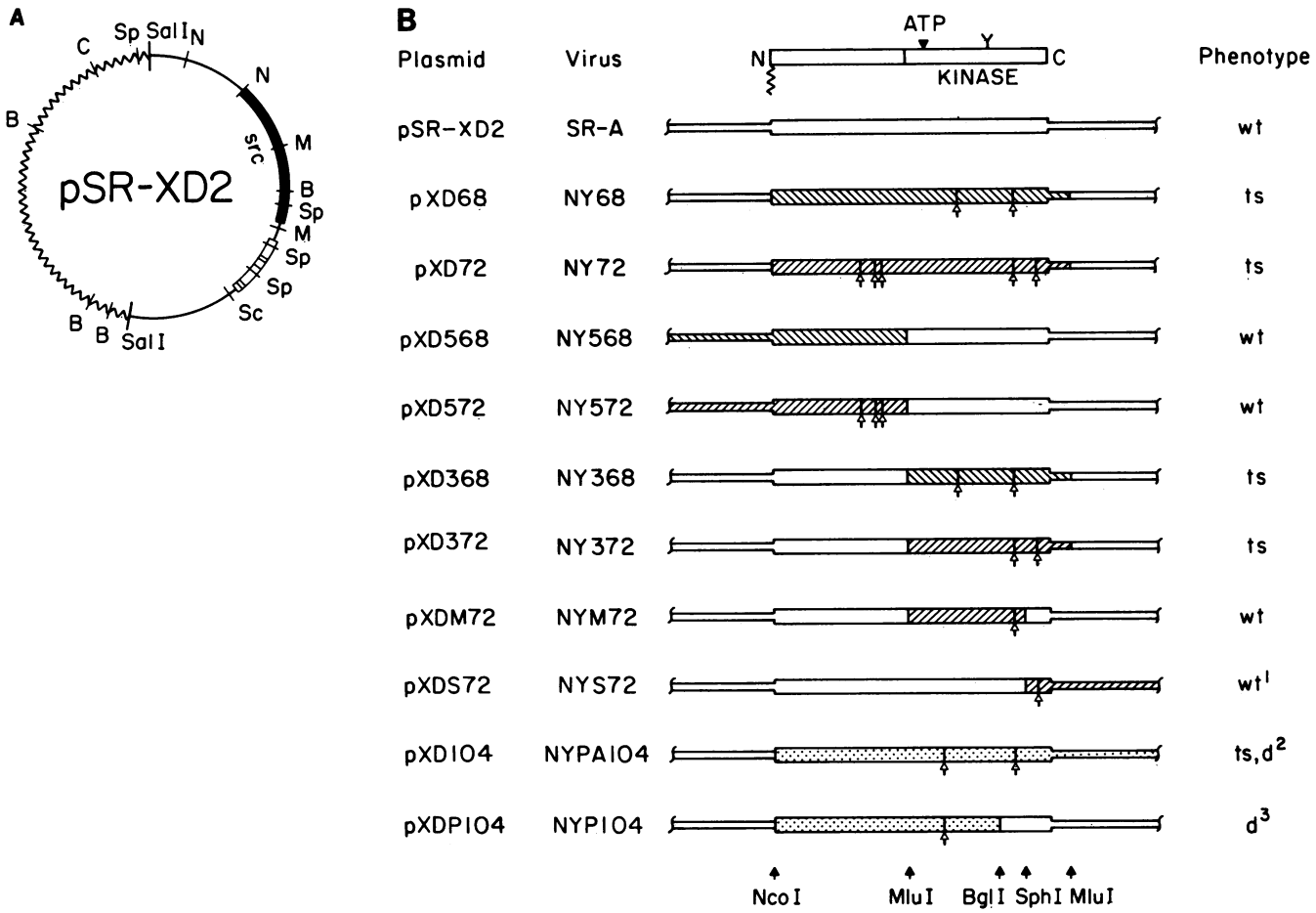

FIG. 4. Recombinant *src*-containing plasmids and the viruses derived from them. (A) pSR-XD2, which contains the wt SRA v-*src* gene. Wavy line denotes pBR322 sequences; the remaining sequences are derived from RSV. Cleavage sites for restriction enzymes used to construct *ts* and *ts*-wt hybrid *src* genes into the homologous site of pSR-XD2 are indicated: Abbreviations: B, *BglI*; C, *ClaI*; M, *MluI*; N, *NcoI*; Sc, *SacI*; Sp, *SphI*. (B) Recombinant *src* genes, the corresponding replication-competent viruses derived from them, and the phenotypes of the viruses. The structure of $p60^{src}$ is indicated at the top of the figure. Abbreviations and symbols: wavy line, myristic acid linked to glycine 2; ATP, ATP binding site at lysine 295; Y, major site of tyrosine phosphorylation at tyrosine 416. Below structure of chimeric *src* genes: ▧, sequences derived from *ts*NY68; ▨, sequences derived from *ts*NY72-4; stippled areas, sequences derived from PA104; open areas, derived from wt SRA; open arrowheads, mutations relative to wt SRA; [1], NYS72 is slightly *ts* (see text); [2], NYPA104 is both *ts* and quite transformation-defective (see text); [3], NYP104 is similar to NYPA104 but is much less *ts* (see text).

valine to methionine change is found at position 461. These results suggest that multiple mutations in the kinase domain of $p60^{src}$ are commonly required for strongly *ts* transforming activity. Although Fincham and Wyke (13) have reported that two PRA-derived *ts* mutants each encode a single C-terminal mutation relative to the parental strain, it is difficult to compare these results since PRA and SRA differ at seven amino acid positions and PRA and BH-RSV differ at 12 positions within this region.

The system that we used to assay the transforming activity of the *ts* and *ts*-wt recombinant *src* proteins allows the unambiguous correlation of phenotypes with specific amino acid changes. All *src* genes in this study were inserted into the same replication-competent RSV vector, eliminating the effects of potential secondary mutations in the viral replicative sequences of the parental *ts* viruses. In addition, the use of a system that generates infectious virus allows the assay of the average phenotype in fully infected mass cultures, as opposed to the study of individual transfected cell clones in which the phenotype may reflect variations in the levels of $p60^{src}$ expression or other cellular factors. Using this assay system, we have demonstrated that only mutations within the C-terminal kinase domain contribute to temperature sensitivity. Hybrid *src* proteins encoding the wt SRA N-terminal half fused to the C-terminal half of *ts*NY68, *ts*NY72-4, PA101, or PA104 were fully *ts* (22, 30; this study).

*ts*NY68, *ts*NY72-4, and PA104 each contain a change from valine to methionine at position 461, in addition to a second, strain-specific C-terminal mutation. A previous study demonstrated that $p60^{src}$ molecules containing only the deletion of *ts*NY68 were indistinguishable from the wt in their biological activity (30). In this study we also found that a protein containing only the second mutation (Ser-503) of *ts*NY72-4 is virtually like the wt in colony formation and morphological alteration; however, the morphology of infected cells, especially at higher temperatures, was slightly flatter and less refractile than that of wt-transformed cells. The Pro-325 mutation of PA104 had a stronger effect on $p60^{src}$ activity. Although soft-agar colony formation was stimulated at all temperatures, the colonies were very small compared with the wt, particularly at high temperatures. While the Met-461 mutation has no observable effect alone, the presence of this

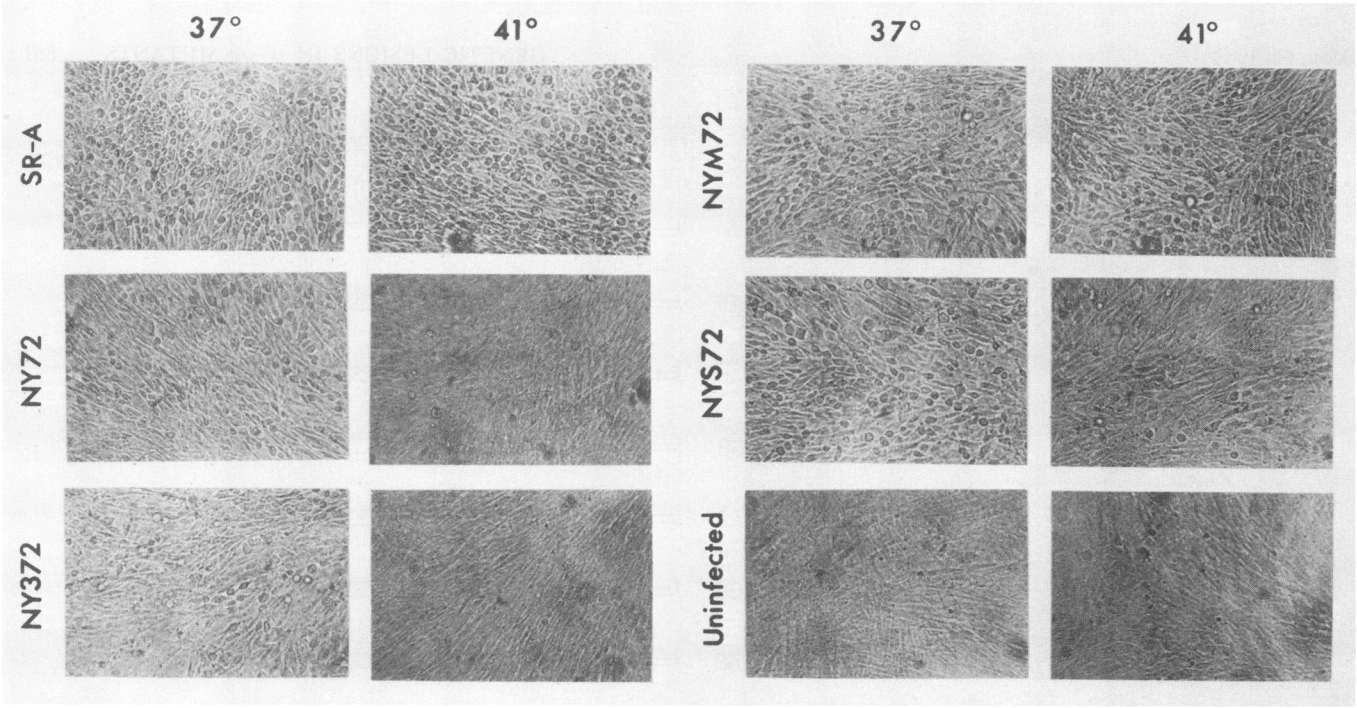

FIG. 5. Morphology of CEFs infected with molecularly cloned *ts*NY72-4-derived viruses. Cells were infected at 37°C, overlaid with Scherer soft agar supplemented with 5% calf serum–10% tryptose phosphate broth–0.5% bovine embryo extract, and maintained at 37 or 41°C for 5 days before photography.

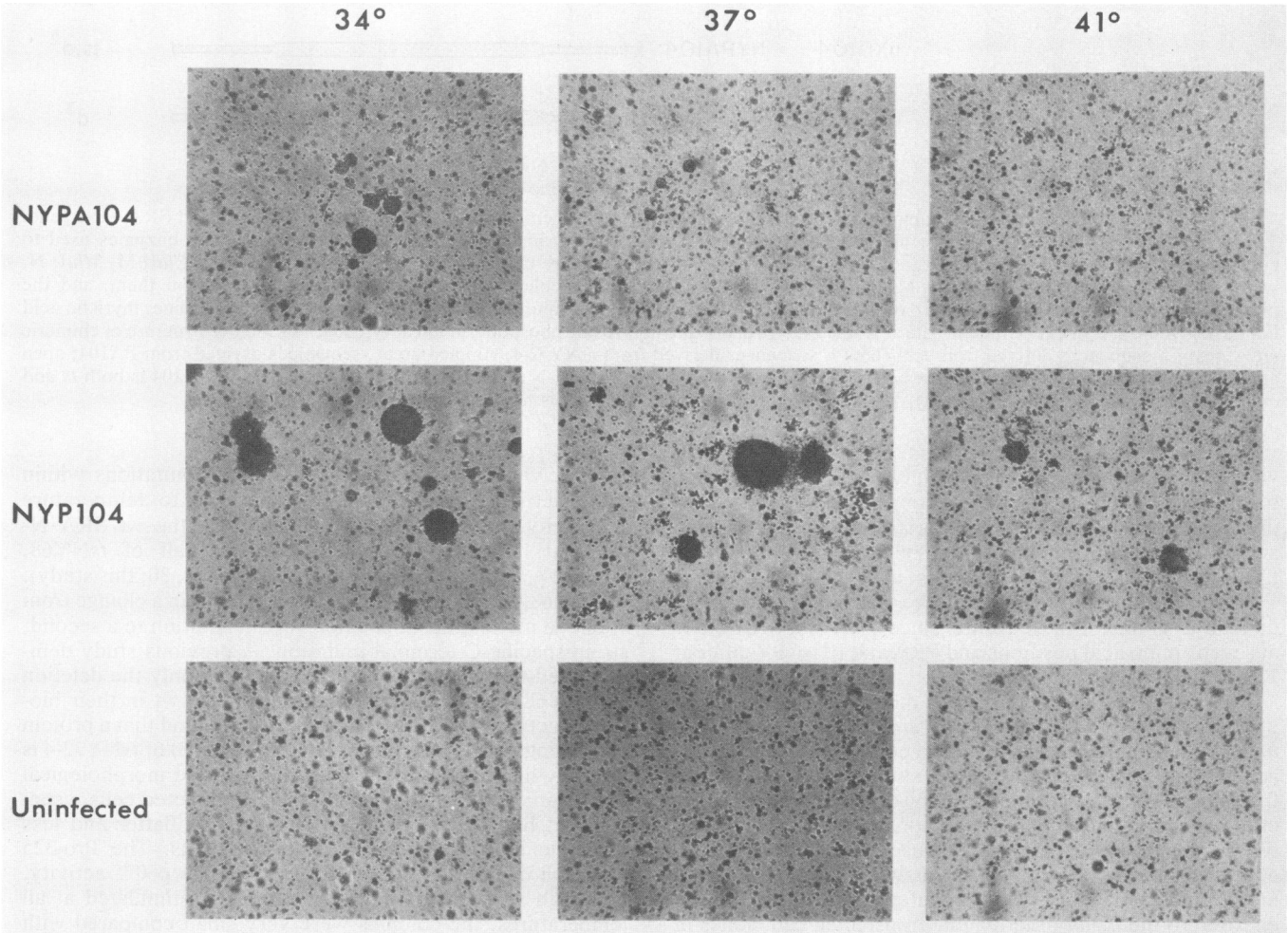

FIG. 6. Stimulation of anchorage-independent growth by NYPA104 and NYP104. CEFs were infected at low multiplicities of infection, trypsinized, and replated in agar-containing Eagle minimum essential medium supplemented with 10% calf serum–1% chicken serum–10% tryptose phosphate broth. Plates were maintained at 34, 37, or 41°C for 19 days before photography.

```
c-src   1 MGSSKSKPKDPSQRRRSLEPPDSTHHGGFPASQTPNKTAAPDTHRTPSRSFGTVATEPKLFGGF
BH-RSV                                                      A         D
SR-A                                                                  D
PR-C                    H                   DE     A  N              W

c-src  65 NTSDTVTSPQRAGALAGGVTTFVALYDYESRTETDLSFKKGERLQIVNNTEGDWWLAHSLTTGQ
BH-RSV                      S  I     G    I
SR-A                                    WI                   N      V
PR-C                                    W

c-src 129 TGYIPSNYVAPSDSIQAEEWYFGKITRRESERLLLNPENPRGTFLVRESETTKGAYCLSVSDFD
BH-RSV                                  G
SR-A
PR-C                                                    K   A

c-src 193 NAKGLNVKHYKIRKLDSGGFYITSRTQFSSLQQLVAYYSKHADGLCHRLTNVCPTSKPQTQGLA
BH-RSV
SR-A
PR-C          P       Y  Y            G

c-src 257 KDAWEIPRESLRLEVKLGQGCFGEVWMGTWNGTTRVAIKTLKPGTMSPEAFLQEAQVMKKLRHE
BH-RSV                        Y
SR-A
PR-C                    A                 D

c-src 321 KLVQLYAVVSEEPIYIVTEYMSKGSLLDFLKGEMGKYLRLPQLVDMAAQIASGMAYVERMNYVH
BH-RSV                      I                               E
SR-A                        I
PR-C                        I

c-src 385 RDLRAANILVGENLVCKVADFGLARLIEDNEYTARQGAKFPIKWTAPEAALYGRFTIKSDVWSF
BH-RSV                                          P  R  A
SR-A
PR-C

c-src 449 GILLTELTTKGRVPYPGMVNREVLDQVERGYRMPCPPECPESLHDLMCQCWRKDPEERPTFEYL
BH-RSV                A       G    R
SR-A                        G G    R                            R
PR-C                                                                   K

c-src 513 QAFLEDYFTSTEPQYQPGENL*
BH-RSV      QLLPACVLKVAE*
SR-A        QLLPACVLEVAE*
PR-C        QLLPACVLEVAE*
```

FIG. 7. Comparison of the amino acid sequence of $p60^{c\text{-}src}$ with $p60^{v\text{-}src}$ of various strains of RSV. Only amino acid changes relative to c-*src* are noted. Asterisks denote the termination codon of $p60^{src}$. Open boxes indicate mutations shared by two strains of RSV. Closed box indicates the mutation shared by all 3 strains. c-*src* encodes lysine, not arginine as reported previously (39), at position 501 (27).

mutation together with each of the above changes (i.e., in the parental *ts* mutants) dramatically increases the temperature sensitivity of the protein.

We used the method of secondary structure prediction developed by Chou and Fasman (6) to examine the potential effects of these mutations on the conformation of $p60^{src}$. In all three cases, the second, strain-specific mutation is predicted by Chou-Fasman analysis to have a significant effect on the secondary structure. The deletion of *ts*NY68 and the leucine to proline mutation of PA104 occur in regions that are predicted to have an α-helical structure; the mutations would shorten or break these α helices, respectively. The proline to serine change of *ts*NY72-4 is predicted to convert an eight-amino-acid coil and β-turn region to an α helix. In contrast, the predicted effect of the common Met-461 change is much more subtle. This residue is within a moderately hydrophobic 11-amino-acid region containing four glycines and two prolines that is predicted to have β-turn character. The relatively conservative valine to methionine mutation changes neither the hydropathy nor the predicted secondary structure significantly, but the unbranched side chain and the large size of the sulfur atom of methionine might be expected to alter subtly the Van der Waal's contacts of the residues in a hydrophobic pocket of the protein. It is interesting that PA101, as well as its parental strain BH-RSV, encodes alanine and not valine at position 461. It is possible that this difference contributes to the *ts* phenotype of PA101, but is masked in wt BH-RSV in a manner similar to that of the NYM72 virus.

In general, the stabilization energy of the folded conformation of proteins is quite small relative to the individual components of stabilization and destabilization, such as hydrophobic interactions and chain entropy (18, 29). The x-ray crystal structures of several *ts* point mutants of T4 lysozyme have been determined, and the structural differences relative to the wt enzyme were found to be very subtle (14, 15). It is therefore thought that minor differences in such parameters as hydrogen bonding or hydrophobic interactions can have drastic effects on the thermodynamic stability of proteins. The dramatic increase in the temperature sensitivity of $p60^{src}$ observed when the common valine to methionine mutation was present supports this view, although our results suggest that a second C-terminal mutation, which significantly alters the secondary structure of $p60^{src}$, is required to interact cooperatively with the Met-461 mutation for expression of temperature sensitivity. Because it is statistically improbable that precisely the same mutation could have been generated randomly without selection in all three *ts* mutants, our results also suggest that the region around position 461 is uniquely critical to the stability of the kinase activity of $p60^{src}$.

In this study we have also reported the sequence of the *src* gene of BH-RSV. Information about the evolutionary relationship between various v-*src* genes and c-*src*, the cellular protooncogene from which they evolved, can be derived from a comparison of their predicted amino acid sequences. In Fig. 7 the amino acid sequences of the v-*src* genes of the BH-RSV, SRA(NY) (37, 38), and PRC (33) strains of RSV are compared with that of c-*src* (39). The BH-RSV amino acid sequence differs from that of c-*src* at 16 positions, in

addition to the carboxy-terminal 12 residues that are common to all v-*src* genes. BH-RSV and SRA(NY) contain five mutations in common relative to c-*src*, while SRA(NY) and PRC contain two common mutations and BH-RSV and PR-C share a single common mutation. This change, the replacement of threonine by isoleucine at position 338, is the only internal mutation shared by the five wt v-*src* genes that have been sequenced to date (the *src* gene of the San Francisco stock of SRA has been sequenced [10, 11] and differs from that of SRA(NY) only at amino acid position 124; the PRA strain of RSV has also been sequenced recently [13] and is closely related to PRC). These results suggest that the common position 338 mutation and the v-*src*-specific C-terminus were the only two mutations present in the *src* gene of the ancestral RSV from which these strains evolved.

ACKNOWLEDGMENTS

We thank Ellen Garber and Sumio Sugano for critically reading this manuscript and Berkley Lynch for helpful discussions about protein structure. R.J. gratefully acknowledges Thambi Dorai for expert help in sequencing BH-RSV *src*.

This work was supported by Public Health Service grants CA14935 and CA18213 from the National Cancer Institute and grant MV128C from the American Cancer Society. B.J.M. was supported by a National Science Foundation Graduate Fellowship. R.J. is a recipient of Damon Runyon-Walter Winchell Cancer Fund Fellowship DRG-786.

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