Two Independent Mutations Are Required for Temperature-Sensitive Cell Transformation by a Rous Sarcoma Virus Temperature-Sensitive Mutant

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We molecularly cloned the *src* coding region of tsNY68, a mutant of Rous sarcoma virus temperature sensitive (ts) for transformation, and constructed a series of ts wild-type recombinant *src* genes. DNA containing the hybrid genes was transfected into chicken cells together with viral vector DNA and helper viral DNA, and infectious transforming viruses were recovered. Characterization of these recombinant viruses indicated that at least two mutations are present in the 3' half of the mutant *src* gene, both of which are required for ts. Nucleotide sequence analysis revealed three differences in the deduced amino acid sequence compared with the parental virus. Two of these changes, a deletion of amino acids 352 to 354 and an amino acid substitution at position 461, are responsible for the ts phenotype.

The *src* gene of the highly oncogenic avian retrovirus Rous sarcoma virus (RSV) encodes a tyrosine-specific protein kinase, p60^{src} (2, 5). The carboxy-terminal domain of the protein is highly conserved among the *src*-related oncogenes and other protein kinases, whereas the amino terminus contains sequences required for membrane association (1, 23, 24). Association of the protein with the plasma membrane appears to be required for transformation (7, 22, 35b).

Many mutants of RSV temperature sensitive (ts) for transformation have been isolated (16, 26, 28, 46). tsNY68 is one of the most widely used of these strains. Focus formation and soft agar colony formation of infected chicken embryo fibroblasts (CEF) are greatly reduced at the nonpermissive temperature (41°C), generally less than 10⁻³ of that at 37°C. The phenotype of infected cells is rapidly reversible after temperature shift in either direction (16).

The mechanism of ts of tsNY68 is unknown. It was reported that, in tsNY68-infected cells maintained at the nonpermissive temperature, p60^{src} is found almost exclusively as a soluble cytoplasmic protein tightly bound to two cytosolic proteins, p50 and p90, which have been implicated in transport of wild-type (wt) *src* protein to the plasma membrane (3, 6, 37; see reference 21 for a review). The failure of tsNY68 to transform CEF at 41°C could be explained by (i) an amino-terminal mutation leading to decreased membrane association, (ii) decreased kinase activity, or (iii) a change in protein structure resulting in aberrant interaction with the p50-p90 complex.

We molecularly cloned the tsNY68 genome and the *src* coding region of TK-15, a virus strain derived from tsNY68. To identify the sites of mutation, we sequenced the ts *src* gene and constructed chimeric genes with ts and wt *src* sequences. In addition, the availability of a cloned ts *src* gene will be useful in studies of transformation in systems in which viral infection is not practical.

MATERIALS AND METHODS

Cells and virus. Endogenous virus-free CEF were prepared as previously described (11, 17). Some fertilized eggs were supplied by Nippon Institute of Biological Science, Tokyo, Japan. TK-15 is a mutant of RSV defective in packaging its own genomic RNA as characterized previously (17, 20, 33). This mutant contains the ts *src* gene of its parental virus, tsNY68.

Molecular cloning. The ts *src* gene derived from tsNY68 was cloned from both integrated TK-15 proviral DNA and tsNY68 viral circular DNA. Chicken genomic DNA was prepared from TK-15-infected CEF by sodium dodecyl sulfate-proteinase K treatment and phenol extraction. This was digested with *Eco*RI and fractionated by agarose gel electrophoresis. Fragments of approximately 3.1 kilobases were recovered by glass powder adsorption (47), ligated to λ gtWES $\cdot$ λ B *Eco*RI arms, and packaged in vitro. Plaques were screened with the *src*-specific insert of pPvuII-E (9). The insert was subcloned into pBR322, and this plasmid, pTK15EB-2, was used for restriction mapping, in vitro recombination, and sequence analysis. The cloning and characterization of the full-length genome of tsNY68 will be described elsewhere. Briefly, tsNY68 viral circular DNA was partially digested with *Eco*RI and cloned into the *Eco*RI site of λ gtWES $\cdot$ λ B, a clone containing the entire viral genome was isolated, and the biological activity of the insert was confirmed by transfection of CEF. The 3.1-kilobase *Eco*RI B fragment subcloned into pBR322, p68-13-1, was used for sequence determination.

Plasmid construction. Recombinant *src* genes were constructed from pTK15EB-2 and pTT107 (45), a subclone of the wt Schmidt-Ruppin A (New York) strain of RSV (SR-A[NY]) containing the *Eco*RI B fragment. The parental plasmids were digested with restriction endonucleases which cleave twice in the *src*-containing insert (*Stu*I, *Mlu*I) or with *Eco*RI plus enzymes recognizing a single site (*Hinc*II, *Hgi*AI, *Bgl*II). Fragments were purified by agarose gel elec-

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FIG. 1. Structures of the plasmids used for the transfection assay. Bold lines represent virally derived sequences, and lighter lines indicate plasmid vector DNA.

trophoresis, and appropriate ts and wt derived fragments were ligated with T4 ligase to reconstruct hybrid *src* genes (see Fig. 2). pICI-1 was constructed by exchanging the *EcoRI-HgiAI* 5' fragment of the pB-2 insert with the equivalent fragment of pS-2. pICI-2 was generated by ligation of the *StuI* fragment of pTK15EB-2 with *StuI*-digested pHg-2.

Transfection assay. We developed a transfection assay system to determine the biological activity of the hybrid *src* genes. The plasmids used in this system are shown in Fig. 1. pYAV-e is a complete helper virus clone of Y73-associated virus. This clone produces helper virus very efficiently on transfection of CEF without restriction digestion. pRV-1 is a retroviral vector plasmid containing all necessary *cis*-acting viral sequences, including the packaging signal, putative dimer linkage, and polypurine tract required for initiation of plus-strand viral DNA synthesis. Uncut pYAV-e, *XhoI*-digested pRV-1, and *EcoRI*-digested hybrid *src* plasmid were combined in equal molar ratio, and 1 μ g of the mix was transfected onto CEF in a 60-mm dish by the dimethyl sulfoxide and polycation method described previously (18). As indicated by Miller and Temin (30), transfected cells mediate ligation of physically unlinked DNAs at high frequency. We expected that a fraction of the *src*-containing *EcoRI* fragments would ligate to the *XhoI* site of pRV-1 in an appropriate orientation so that the *src* gene would be transcribed into viral genomic RNA. In that case, transforming virus would be recovered as a replication-defective virus in the presence of helper YAV virus. However, we later found that, even in the absence of pRV-1 plasmid, infectious transforming virus could be recovered, although at a lower efficiency. Analysis of the virus thus recovered suggested that pYAV-e recombined with the *EcoRI* fragment to produce a replication-competent transforming virus, possibly mediated by homologous sequences present in both DNAs. Therefore, the transforming viruses recovered from the transfected cultures and used for determination of ts of the recombinant *src* genes would be a mixture of replication-defective and replication-competent viruses. The structures of the recovered viruses, efficiency of viral recovery of this system, and construction of pYAV-e and pRV-1 will be published elsewhere. The titer of the recovered viruses was assayed at 37 and 41°C.

DNA sequence analysis. The entire *src* coding regions of pTK15EB-2 and p68-13-1 were sequenced by the Sanger dideoxy method with M13 mp8, mp18, and mp19 as cloning-sequencing vectors (29).

RESULTS

Sequences required for ts. We did not detect any differences between the restriction maps of the ts and wt *src* clones (data not shown); this indicates that the lesions

involved in ts are small alterations. This is in agreement with the results of previous studies on tsNY68-infected cells, which showed that the apparent molecular weight of the mutant *src* gene product in sodium dodecyl sulfate-polyacrylamide gels after immunoprecipitation with tumor-bearing rabbit serum is indistinguishable from that of wt RSV. To define the regions of the gene responsible for the ts phenotype, we constructed a series of plasmids in which fragments of the pTK15EB-2 insert were substituted with the corresponding wt fragments from pTT107. Figure 2 summarizes the plasmids constructed in the present study.

Transfection of CEF with the *src*-containing *EcoRI* B fragment (which contains no promoter) induces transformation at a very low frequency. In addition, the morphology of transfectants varies, probably owing to differences in the level of *src* transcription. Therefore, to test the transforming activity of the chimeric *src* genes, we developed a convenient transfection system which results in the production of infectious transforming viruses. Recombinant *src*-containing plasmids were transfected onto CEF together with complete helper virus DNA and retroviral vector sequences, and infectious transforming viruses containing the chimeric *src* genes were produced. Viruses collected from plasmid-transfected cultures were assayed for focus formation on CEF at the permissive and nonpermissive temperatures. Transfection with *src*-containing *EcoRI* fragments derived from wt and ts mutant RSV (pTT107 and pTK15EB-2) reproducibly produced wt and ts transforming viruses, respectively, indicating that this system is useful for testing the ts of recombinant *src* gene products (Fig. 2). Clones pH-1, pM-1, and pS-1 generated wt viruses with similar focus-forming ability at 37 and 41°C, whereas the reciprocal constructs pH-2, pM-2, and pS-2 generated ts transforming viruses. These results indicate that the mutations responsible for ts reside in the carboxy-terminal half of the ts *src* gene. Unexpectedly, the reciprocal ts-wt constructs exchanged at the *HgiAI* (pHg-1, pHg-2) and *BglII* (pB-1, pB-2) sites all generated wt transforming viruses. There were no morphological differences between cells transformed by these recombinant viruses and wt RSV. To confirm the structures of these four plasmids, we reconstructed the original ts *src* sequence from pHg-1 and pHg-2 and from pB-1 and pB-2. The reconstructed plasmids generated ts transforming viruses (not shown). These results define two regions of the ts *src* gene, both of which are required for ts. The first is bounded by the *StuI* and *HgiAI* sites, and the second extends from the *BglII* site to the carboxy terminus. This was confirmed by the construction of two additional chimeric plasmids, pICI-1 and pICI-2.

Nucleotide sequence of the ts *src* gene. The nucleotide sequence of the tsNY68-derived *src* gene was determined

FIG. 2. Construction of hybrid *src* genes and ts of focus-forming ability. Restriction sites used in construction of the plasmids are shown at the top of the figure. Shaded portions were derived from the ts *src* subclone pTK15EB-2; open portions were derived from the wt *src* subclone pTT107 (45). CEF were transfected with *EcoRI* fragments containing the hybrid *src* genes in the presence of pRV-1 and pYAV-e DNAs by the polybrene and dimethyl sulfoxide method (18). Transfected cultures were agar overlaid the day after transfection. Seven days later, the agar was removed and cultures were transferred. Culture fluids were collected 12 days after transfection and were used to infect fresh CEF to make fully transformed cultures. Stocks harvested from these fully transformed cultures were subjected to titration assay at the permissive (37°C) and nonpermissive (41°C) temperatures. Titers of these virus stocks ranged from 1×10^5 to 1.5×10^6 focus-forming units per ml at 37°C.

twice, with two independently cloned isolates of the gene (pTK15EB-15 and p68-13-1). The entire sequence of the ts *src* coding region is shown in Fig. 3. The location of the translation initiation and termination codons of wt p60^{src} are well established (8, 38, 42). We found a corresponding open reading frame which uses the same initiation and termination codons. The nucleotide sequences revealed a deletion of nine bases and six (pTK15EB-2) or seven (p68-13-1) point mutations in the ts *src* coding region compared with the wt. In addition, several base substitutions and a five-base insertion were found in the noncoding flanking sequences (not shown). Of these mutations, only the nine-base deletion and two point mutations affect the deduced amino acid sequence of p60^{src}. The first, a C-to-T transversion, would lead to the substitution of isoleucine for threonine at amino acid position 96. However, since this position is 5' to the *HincII* site, the in vitro recombination experiments described above demonstrated that this mutation is not involved in ts. The second lesion is a deletion of nine bases resulting in the deletion of three amino acid residues, Gly-Glu-Met, at positions 352 to 354. This position is located between the *StuI* and *HgiAI* sites. The third mutation is a G-to-A base change, resulting in the substitution of methionine for valine at position 461. This change is located between the *BglI* site and the carboxyl terminus of the *src* coding region. The sequence of the two independently isolated ts *src* genes differed at only one position within the coding region (nucleotide 1371); the deduced amino acid sequences were identical.

DISCUSSION

In this study we report that two mutations in the putative tyrosine kinase domain are essential for the ts phenotype of

RSV mutant tsNY68. Chimeric *src* genes which contain either of the two mutations alone are indistinguishable from the wt in their transforming activity, suggesting that the two mutations interact to affect protein structure at the nonpermissive temperature. A similar phenomenon has been observed in hybrid viral-cellular *src* genes. C-*src*, the cellular homolog of the viral *src* gene (*v-src*), contains seven scattered amino acid changes compared with *v-src* and a substitution of 19 amino acids for the carboxy-terminal 12 amino acids of *v-src* (43, 44). When c-*src* is highly expressed in CEF it is nontransforming and has low kinase activity (12-14). However, chimeric genes in which the c-*src* coding sequence is replaced by the homologous *v-src* sequence either upstream or downstream of the *BglI* site have full transforming activity (12, 14). The observation that elimination of one of the two essential mutations of tsNY68 is sufficient to restore the wt phenotype is supported by previous recombination experiments with transformation-defective *src* deletion mutants (15). It was found that td108, which retains 225 bases of the 3' *src* coding sequence (48), and td109, which retains 296 bases of 3' *src* information (35), could generate temperature-insensitive transforming viruses when coinfecting with tsNY68. Both of these transformation-defective mutants would be able to repair the downstream but not the upstream mutation of tsNY68 through recombination events. The partial ts phenotype of the recombinant viruses between tsNY68 and td107 which was reported previously (15) could be explained by a secondary mutation caused by the recombination or preexisting in the transformation-defective virus.

It has been reported that p60^{src} in tsNY68-infected cells is found primarily in the cytoplasm at 41°C; at the permissive temperature, however, the majority is localized on the

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-30          src
  GCGGTAGCTGGGACGTGCAGCCGACCACCATGGGGAGTAGCAAGAGCAAGCCTAAGGACCCAGCCAGCGCCGGCGC
      C
49      (20)          (40)
  AGCCTGGAGCCACCCGACAGCACCCACCACGGGGATTCCCAGCCTCGCAGACCCCAACAAGACAGCAGCCCCCGAC

127          (60)
  ACGCACCGCACCCCCAGCCGCTCCTTCGGGACCGTGGCCACCGAGCCCAAGCTCTTCGGGGACTTCAACACTTCTGAC

205          (80)
  ACCGTTACGTCGCCGACGCTGCCGGGGCACTGGCTGGCGGCGTCACCACTTTCGTGGCTCTCTACGACTACGAGTCC

283      (100)          HincII          (120)
  TGGATTGAAACGGACTTGTCTTCAAGAAAGGAGAACGCCTGCAGATTGTCAACAACACGGAAGGTAAGTGGTGGCTG
      C
361          (140)
  GCTCATTCCGTGACTACAGGACAGACGGGCTACATCCCCAGTAAGTATGTCGCGCCCTCAGACTCCATCCAGGCTGAA
      A      C
439          (160)
  GAGTGGTACTTTGGGAAGATCACTCGTCGGGAGTCCGAGCGGCTGCTGCTCAACCCCGAAAACCCCGGGGAACCTTC
      T
517          (180)
  TTGGTCCGGGAGAGCGAGACGACAAAAGGTGCCTATTGCCTCTCCGTTTCTGACTTTGACAACGCCAAGGGGCTCAAT

595 (200)          (220)
  GTGAAGCACTACAAGATCCGCAAGCTGGACAGCGCGGCTTCTACATCACCTCACGCACACAGTTTCAGCAGCCTGCAG

673          (240)
  CAGCTGGTGGCCTACTACTCCAACATGCTGATGGCTTGTGCCACCGCTGACCAACGTCTGCCCCACGTCCAAGCCC

751          MluI (260)
  CAGACCCAGGGACTCGCCAAGGACGCGTGGGAAATCCCCCGGGAGTCGCTGCGGCTGGAGGTGAAGCTGGGGCAGGGC

829          (280)          (300)
  TGCTTTGGAGAGGTCTGGATGGGGACCTGGAACGGCACCACCAGAGTGGCCATAAAGACTCTGAAGCCCGGCACCATG

907          StuI          (320)
  TCCCCGAGGCCCTTCTGCAGGAAGCCCAAGTGATGAAGAAGCTCCGGCATGAGAAGCTGGTTCAACTGTACGCAGTG

985          (340)
  GTGTCGGAAGAGCCCATCTACATCGTCATTGAGTACATGAGCAAGGGGAGCCTCCTGGATTTCTGAAGGGAGAGATG
      GGAGAGATG
1063          (360)          (380)
  GGCAAGTACCTGCGGCTGCCACAGCTCGTTGATATGGCTGCTCAGATTGCATCCGGCATGGCCTATGTGGAGAGGATG

1141          HgiAI          (400)
  AACTACGTGCACCGAGACCTGCGGGCGGCCAACATCCTGGTGGGGGAGAACCTGGTGTGCAAGGTGGCTGACTTTGGG

1219          (420)          BglI
  CTGGCACGCCTCATCGAGGACAACGAGTACACAGCACGGCAAGGTGCCAAGTTCCCCATCAAGTGGACAGCCCCCGAG

1297          (440)
  GCAGCCCTCTATGGCCGGTTCACCATCAAGTCGGATGTCTGGTCCTTCGGCATCCTGCTGACTGAGCTGACCACCAAG

1375 (460)          (480)
  GGCCGGATGCCATACCCAGGGATGGGCAACGGGGAGGTGCTGGACCGGTGGAGAGGGGCTACCGCATGCCCTGCCCG
      G
1453          (500)
  CCCGAGTGCCCCGAGTCGCTGCATGACCTTATGTGCCAGTGCTGGCGGAGGGACCTGAGGAGCGGCCCACTTTTGAG

1531          (520)          end
  TACCTGCAGGCCAGCTGCTCCCTGCTTGTGTGTTGGAGGTCGCTGAGTAGTGCGCGAGCAAAATTTAAGCTACAACA
      T

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FIG. 3. Nucleotide sequence of the tsNY68-derived *src* gene. The numbering of the nucleotides (left end of each line) or amino acids (in parentheses) begins with the first nucleotide or amino acid of the *src* coding region. The nucleotide sequence of the wt *src* gene of the New York strain of SR-A (42, 43) is shown under the sequence of the ts *src* gene only where they differ. Asterisks mark amino acid differences. The shaded box indicates the nine-base-pair deletion found in the ts *src* gene.

plasma membrane (3, 6, 10). Since membrane association has been correlated with transforming activity, and since the amino-terminal domain of the protein is involved in membrane binding (7, 22), we looked for an amino-terminal lesion

in tsNY68. Although we found a substitution at position 96 (in the amino-terminal domain, our results demonstrate that this mutation does not affect the transforming activity of the gene. It should be noted that the *src* protein of the San

Francisco strain of SR-A [SR-A(SF)] contains isoleucine at position 96, as does tsNY68 (8). In addition, recent results indicate that the amino-terminal 14 amino acids of p60^{src} are sufficient for myristylation and membrane association of a hybrid protein (35a, 35b). These residues are identical in tsNY68 and wt RSV. It therefore seems unlikely that the ts membrane association of tsNY68 is due to intrinsic inability to bind to the membrane at 41°C.

Newly synthesized wt *src* protein is transiently complexed with cellular proteins p50 and p90 before association with the plasma membrane (3, 6). At both the permissive and nonpermissive temperatures, a larger proportion of p60^{src} from tsNY68-infected cells is found in this soluble complex compared with the wt. At 41°C, virtually all of the ts *src* protein is complexed with p50 and p90 (3, 6). It is possible that the two essential mutations of tsNY68 affect the structure of the protein in a manner that renders the complex much more stable than in the case of wt p60^{src}, especially at the nonpermissive temperature. The site of complex binding is considered to be within the carboxyl domain of the *src* protein, since antisera raised against the carboxy terminus of p60^{src} do not precipitate the complex as well as does tumor-bearing rabbit serum (40), and in vitro-constructed *src*

mutants with extensive amino-terminal deletions have been reported to associate transiently with the complex (7). In addition, the *fps* and *yes* transforming proteins, which contain tyrosine kinase domains homologous to that of *src*, have been found associated with p50 and p90 (27), suggesting that it is the conserved kinase domain which is recognized.

In addition to its aberrant subcellular localization, the *src* protein of tsNY68 has been reported to have lower in vitro kinase activity (4, 10, 25, 39) and to be more thermolabile in vitro (34) compared with the wt. The two essential mutations we have defined are both within the conserved kinase domain. When the amino acid sequences of the conserved domains of the kinase oncogene products are compared, it is noticed that there are variable, not strictly conserved amino acids as well as invariable, strictly conserved residues. The strictly conserved amino acids may be essential for the function of these proteins. On the other hand, structural changes caused by substitution or deletion of the variable amino acids may be nonlethal. A comparison of amino acid sequences in the vicinity of the two mutations of the tsNY68 *src* gene with the corresponding regions of other kinase oncogenes (Fig. 4) shows that the deletion (Fig. 4A) and substitution (Fig. 4B) occurred at amino acids which are not strictly conserved. In this sense, it may not be surprising that both mutations are required for ts; either of the two alone may not cause a structural alteration profound enough to bring about a change in phenotypic expression of the *src* gene.

Since kinase activity and complex binding both map to the same region of the protein, the inability of the *src* protein of tsNY68 to transform at the nonpermissive temperature could therefore be due to either decreased kinase activity or more stable binding to the p50-p90 complex. To distinguish between these possibilities, analyses of the protein kinase activity and p50-p90 complex formation of the products of the ts-wt recombinant *src* genes would be helpful.

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(A)	ts- <i>src</i> :	Leu Lys	■■■■	■■■■	■■■■	Gly Lys
	wt- <i>src</i> :	(350) Leu Lys Gly Glu Met Gly Lys				
	<i>yes</i> :	(634) Leu Lys Glu Gly Glu Gly Lys				
	<i>fgr</i> :	(487) Leu Lys Asp Gln Glu Gly Gln				
	<i>ros</i> :	(341) Leu Arg Gly Gln Lys Phe Gln				
		Ala Arg Lys				
	<i>fps</i> :	(1018) Leu Arg Ser Lys Gly Pro Arg				
	<i>erbB</i> :	(222) Ile Arg Glu His Lys Asp Asn				
	<i>abl</i> :	(446) Leu Arg Glu Cys Asn Arg Gln				
(B)	ts- <i>src</i> :	Gly Arg Met Pro Tyr				
	wt- <i>src</i> :	(459) Gly Arg Val Pro Tyr				
	<i>yes</i> :	(743) Gly Arg Val Pro Tyr				
	<i>fgr</i> :	(696) Gly Arg Val Pro Tyr				
	<i>ros</i> :	(462) Gly Gln Gln Pro Tyr				
	<i>fps</i> :	(1127) Gly Ala Val Pro Tyr				
	<i>erbB</i> :	(331) Gly Ser Lys Pro Tyr				
	<i>abl</i> :	(555) Gly Leu Asn Pro Tyr				

FIG. 4. Comparison of the amino acid sequences of the protein kinase oncogenes in the vicinity of the two carboxy-terminal mutations of the tsNY68-derived *src* gene. Amino acids were aligned to give maximum homology. In A, the *ros* protein sequence contains an insertion of three amino acids; the shaded boxes represent amino acids deleted in the ts *src* protein. Amino acid numbers are given in parentheses. The deduced amino acid sequences are from the following references: *src* (42), *yes* (19), *fgr* (31), *ros* (32), *fps* (41), *erbB* (49), and *abl* (36).

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