JOURNAL OF VIROLOGY, Feb. 1989, p. 481–492
0022-538X/89/020481-12$02.00/0

Vol. 63, No. 2

Transduction of the Cellular *src* Gene and 3′ Adjacent Sequences in Avian Sarcoma Virus PR2257

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Received 19 August 1988/Accepted 21 October 1988

When injected into chickens, a transformation-defective mutant of the Prague C strain of Rous sarcoma virus induced tumors at low incidence and after a long latency. One such tumor released a replication-defective virus designated PR2257. We molecularly cloned and sequenced the proviral DNA from quail fibroblasts transformed by PR2257. Comparison of PR2257 sequence with that of Prague C, cellular *src*, and 3′ adjacent cellular DNA showed that the spliced version of the c-*src* gene and about 950 base pairs (bp) of 3′-flanking cellular DNA were transduced into PR2257. This transduction eliminated nearly all replicative genes, since the *gag* gene splice donor site was linked to the splice acceptor site of the *src* gene and, on the 3′ side, recombination occurred in the end of *env* gene. Insertion of two extra cytosines 23 bp before and 19 bp after the c-*src* stop codon resulted in an extension of the coding portion up to 587 amino acids, divergence of sequences after Pro-525 and replacement of Tyr-527 by a valine residue. In addition, it appears that the 5′ and 3′ untranslated regions of PR2257 result from multiple recombinations between exogenous and endogenous virus genomes. Limited digestion of $p66^{src}$ encoded by PR2257 with *Staphylococcus aureus* V8 protease yielded a V2 peptide (C-terminal moiety) with an apparent molecular mass of 31 kilodaltons, consistent with the 5.7-kilodalton increase expected from the DNA sequence. The structure of PR2257 suggests that the first step in the capture of c-*src* gene by avian lymphomatosis viruses is the *trans* splicing of the viral leader mRNA to exon 1 of c-*src*.

It is widely accepted that acutely transforming retroviruses arose by transduction of cellular genes called proto-oncogenes by viruses carrying only replicative genes, such as the avian lymphomatosis viruses (ALV). Most generally, the transduction of oncogenes sacrificed a large part of the retroviral replicative genes so that the resulting transforming viruses are replication defective and need helper associated viruses. In this respect, nondefective strains of Rous sarcoma virus (RSV) are exceptional. Although it was first hypothesized (60) that the original isolate of RSV possessed all the genetic elements found in the present replication-competent strains of RSV (Schmidt-Ruppin [SR] Prague [PR]), there are now several lines of evidence suggesting that the ancestral virus was probably replication defective, as are the Bryan and Harris strains (24, 54), and that nondefective forms evolved through further recombination with ALV genomes during long passage histories in different laboratories (33).

The mechanism of cellular *src* gene transduction by ALV leading to the generation of nondefective strains of RSV is largely unknown. Recombinations between c-*src* and viral genomes are frequently observed only when at least a portion of the 3′ end of the v-*src* gene is retained in transformation-defective (*td*) mutants of RSV (73). When infected with ALV or full *td* mutants of the SR strain of RSV, chickens most often developed B-cell lymphomas or erythroblastosis after several months (1, 68). After a longer latency (about 1 year), *td* mutants of RSV and ALV also induce a broader spectrum of diseases including sarcomas, nephroblastomas, and osteopetrosis (52, 57, 70).

Recently, two new avian sarcoma viruses (ASV), S1 and S2, carrying the *src* gene were isolated from tumors induced by a subgroup A ALV strain (29). Molecular cloning and sequencing of these viruses has shown that, like most acutely transforming retroviruses, they have lost substantial parts of the replicative genes (32). Recombination with an ALV genome within the 3′ end of c-*src* modifies the C-terminal part of their *src*-encoded protein, a structural alteration which seems to be essential for the activation of the oncogenic potential of this gene (33).

We have already reported that tumors were obtained with low incidence and after a long latency in chicken embryos injected with *td* mutants of the PR-C strain of RSV (65). Among the four tumors studied, two produced low titers of replication-defective transforming viruses. Restriction enzyme analysis of the proviral DNA of one of these viruses, PR2257, revealed that it contains essentially long terminal repeat (LTR) and *src* gene sequences (J. Nehyba, J. Svoboda, I. Karakoz, A. V. Rynditch, and J. Geryk, Folia Biol. [Prague], in press). The absence of *src* sequence in the *td daPR-C* parental mutant was verified by molecular cloning and sequencing (A. V. Rynditch, unpublished data), excluding the possibility that PR2257 gained *src* sequences by recombination of the c-*src* gene with a partially deleted v-*src* gene as in recovered viruses (23). We report here the molecular cloning and complete nucleotide sequence of the provirus obtained from the first passage of the original PR2257 tumor in vivo. As in RSV $p60^{v\text{-}src}$- and S1 and S2 *src*-encoded proteins, a modification of the transduced c-*src* near the 3′ end of its coding region is responsible for the synthesis of a protein with a new C terminus, which was designated $p66^{src}$.

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MATERIALS AND METHODS

Cell cultures and viruses. Embryonic fibroblasts derived from avian leukosis virus-free 10-day-old Brown Leghorn chicken embryos, phenotype C/E, and from 8-day-old Japanese quail embryos, phenotype Q/BD, were prepared by standard procedures.

Isolation of the C7 line of quail embryo fibroblasts containing one proviral copy of PR2257 virus has been described elsewhere (Nehyba et al., in press). Briefly, fragments of the chicken tumor 2257 passaged once in vivo and kept at −70°C without cryoprotective agents were thawed and seeded onto secondary quail embryo fibroblast cultures. The next day, the medium was removed and the cultures were overlaid with agar medium. After 2 weeks, foci of transformed cells were isolated and subcultured. Cells derived from one focus, designated C7, were used for further study.

Replication-defective virus PR2257 was obtained from a tumor induced by intraembryonal inoculation of *td da*PR-C into Brown Leghorn chickens (65). The virus replicates to low titer, reaching about 100 focus-forming units per ml, and contains a large excess of the *td* mutant. The biological characterization of virus PR2257 and the origin of other viruses used (PR-C, *da*PR-C, *td* PR-C, *td da*PR-C) have been described previously (65).

Analysis of cellular RNA. Total cellular RNA was isolated by the guanidium thiocyanate-cesium chloride method (3). The RNAs were denatured at 60°C in a formamide-formaldehyde mixture (45), fractionated by electrophoresis in 1% agarose–2.2 M formaldehyde gels (42), transferred onto nitrocellulose filters in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (69), and hybridized to ^{32}P-labeled probes.

Detection of proviral sequences in cellular DNA. High-molecular-weight DNA was isolated from cells by standard procedures (20). Digestion with restriction enzymes was done under conditions specified by the suppliers (New England BioLabs, Inc., Beverly, Mass.). Digested DNA was electrophoresed in 0.7% agarose gels and transferred to nitrocellulose filters (63). Hybridization was performed by the method of Wahl et al. (72) with specific probes labeled by nick translation (56). The following probes were used. The *src* probe is the *Pvu*II E fragment derived from the C-terminal part of *src* gene, and the LTR probe is an *Eco*RI fragment containing a complete LTR sequence, both subcloned in pBR322 (17).

Molecular cloning and DNA sequencing. High-molecular-weight-DNA isolated from the C7 cell line was digested to completion with *Eco*RI and fractionated by centrifugation through a sucrose gradient (20). Fractions of 4 to 5 kilobases (kb) were ligated to purified *Eco*RI arms of λgt11 bacteriophage vector (Promega Biotec, Madison, Wis.) and packaged in vitro (45). Recombinant clones containing proviral sequences were selected by plaque hybridization with the ^{32}P-labeled v-*src*-specific probe. One of the positive recombinants was purified by three rounds of plaque purification. DNA was isolated and digested with *Eco*RI, and the 4.1-kb proviral unit was subcloned into the *Eco*RI site of phage M13tg130 (Amersham Corp., Arlington Heights, Ill.) in both orientations. An ordered set of deletions was generated by digestion with exonuclease III (27) after protection of the M13tg130 *Sal*I fragment with 5-[α-thio]ATP and digestion with *Eco*RV (53). The nucleotide sequence was determined by the dideoxy-chain termination method of Sanger (59).

Radioactive labeling of cells. Cells seeded in 35-mm dishes were labeled with L-[^{35}S]methionine (150 μCi/ml; specific activity, 1,000 to 2,000 Ci/mmol; Dupont, NEN Research Products; Boston, Mass.) or carrier-free $^{32}P_i$ (1 to 1.5 mCi/ml; Commissariat à l'Energie Atomique, Saclay, France). Cell extracts were prepared 5 to 8 h later, using modified RIPA buffer to lyse the cells as previously described (49).

Immunoprecipitation and protein analysis. Antiserum directed against p60^{src} synthesized in *Escherichia coli* (19) was kindly supplied by R. L. Erikson. Samples of cell extracts were incubated with an excess of antibodies, and the immune complexes were bound to protein A-Sepharose and washed with RIPA buffer as previously described (49). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (39) and treatment of immunoprecipitates (49) were done as described previously.

In vitro protein kinase assay. v-*src* gene products complexed to specific antibodies were assayed for autophosphorylation and phosphorylation of exogenous substrate by incubation with [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham), and reaction products were analyzed by polyacrylamide electrophoresis as already described (7).

Partial hydrolysis of proteins with protease V8 of *Staphylococcus aureus*. Protein samples in gel slices were partially digested with *S. aureus* V8 protease (Miles Laboratories, Inc., Elkhart, Ind.), and peptide maps were generated as described previously (44). Gel slices containing v-*src* proteins were cut out, incubated three times for 15 min in 10% methanol at room temperature, lyophilized, and applied to an SDS- 12% polyacrylamide gels. Slots were filled with protease V8 solution (2 μg/ml in 0.625 M Tris hydrochloride [pH 5.8], 10% sucrose, 0.15% SDS). Gels were run at 5 mA constant current.

Phosphoamino acid analysis of peptides. Gel slices containing the peptides were washed in methanol, treated with performic acid, and trypsinized as described previously (41). Lyophilized peptides were hydrolyzed in 200 μl of 6 N HCl at 100°C for 2 h. After two cycles of washes with 100 μl of H_2O and lyophilization, the samples were dissolved in a marker mixture containing phosphoserine, phosphothreonine, and phosphotyrosine, each at 1 mg/ml. The hydrolysates were analyzed by two-dimensional electrophoresis on thin-layer cellulose plates. Phosphoamino acid markers were stained with ninhydrin, and ^{32}P-labeled spots were detected by autoradiography.

Measurement of amount of acid-stable phosphoamino acids in total cellular protein. Preparation of proteins from $^{32}P_i$-labeled cells for acid hydrolysis was performed as previously described (50). Acid hydrolysis and electrophoresis of samples was as described above. After exposure of plates for 24 h, colored spots corresponding to each phosphoamino acid were scraped off and radioactivity was evaluated by liquid scintillation counting. Blanks were prepared by using areas of equal surfaces in regions where no radioactivity was detected by autoradiography.

RESULTS

Molecular cloning of PR2257 provirus. Previous restriction enzyme analysis of PR2257-transformed cell DNA showed that the complete provirus has a size of about 4.4 kb and contains *src* and *env* sequences flanked by LTRs (Nehyba et al., in press). This was confirmed by an analysis of viral mRNAs in the C7 cell line. Both *src* and LTR probes detected a 4.3-kb mRNA, which corresponds to the size expected for the polyadenylated transcript. No other v-*src*-containing mRNA was found in these cells (Fig. 1).

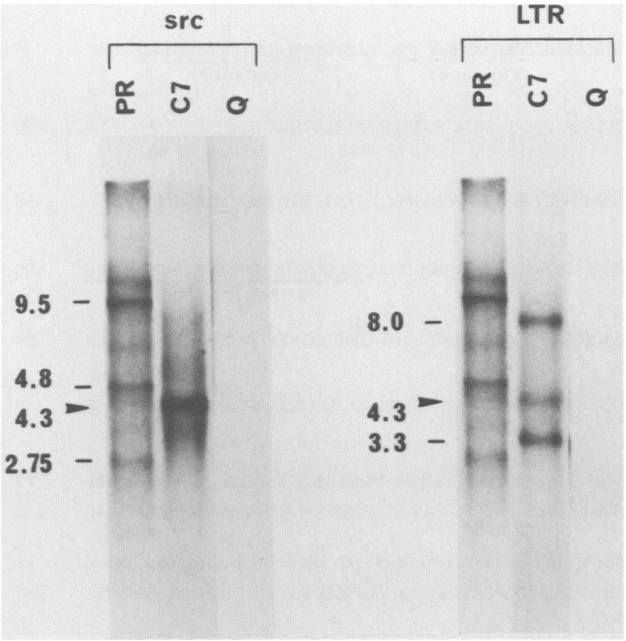


FIG. 1. PR2257 viral mRNA in C7 cell line. RNAs from C7 cells (C7) and from PR-C-transformed (PR) or uninfected (Q) quail fibroblasts were electrophoresed on a formaldehyde-agarose gel and transferred to nitrocellulose membrane as described in Materials and Methods. Hybridization with *src* probe revealed only one 4.3-kbp band, whereas the 9.5-kbp genomic RSV RNA, the 4.8-kbp *env-src*, and the 2.75-kbp *src* viral mRNAs are revealed in PR-C-transformed cells. LTR probe revealed in C7 cells the 8.0-kbp genomic RNA and 3.3-kbp *env* mRNA of the helper virus (*td da*PR-C).

Therefore, DNA fractions of around 4 kb obtained after complete digestion of C7 cellular DNA by *Eco*RI were cloned into the λgt11 phage vector. Screening of recombinants with a *src* probe gave several positive clones. The 4.1-kb insert from one of these clones was subcloned in M13tg130 and sequenced by the Sanger (59) dideoxy-chain termination method.

Nucleotide sequence of PR2257. PR2257 provirus with two LTRs is 4,413 base pairs (bp) long. Its complete nucleotide sequence is shown in Fig. 2. Alignments with PR-C sequence (60) and c-*src* sequence (67) demonstrated that this virus is composed of the following segments: 5′ LTR-5′ untranslated region-c-*src*-unknown DNA-delta *env*-3′ untranslated region-3′ LTR. Each of these segments is described in detail in the next sections.

LTR and 5′ untranslated region. The LTR of PR2257 proviral DNA comprises 335 nucleotides. Alignment of this part of the sequence with the sequence of PR-C showed that there are six single-nucleotide differences between both viruses, all localized in the R and U5 domains (Fig. 3A). Comparison with LTR sequences of other avian retroviruses revealed a perfect homology of the PR2257 U5 region with Rous-associated virus type 0 (RAV-0) sequence (30). They differ at the same four positions of the sequence of closely related exogenous viruses: SR and PR strains of RSV and RAV-2. This suggests that at least part of U5 in PR2257 has been acquired from endogenous virus, whereas U3 is derived from the PR-C genome. All the signals required for the efficient transcription of proviral DNA, which have been mapped in the U3 of exogenous avian retroviruses, are therefore present in PR2257 (Fig. 2).

The untranslated region following the LTR (up to the ATG start codon of *gag* gene) differs at only one position from the sequence of PR-C, whereas several differences are observed when compared with the sequence of RAV-0, implying that this part of PR2257 probably originated from the *td da*PR-C virus. The change of A to G at position 354, just at the border of the $tRNA^{Trp}$ primer-binding site, introduces a new initiation codon with a G at position 4 and recurrence of G at positions −3 and −6. This sequence corresponds to a strong codon for the initiation of translation in eucaryotes (38). Except for this new ATG codon, the 5′ untranslated region of PR2257 is identical to that of PR-C and thus contains all the signals important for viral replication mapping in this part of the genome (Fig. 2).

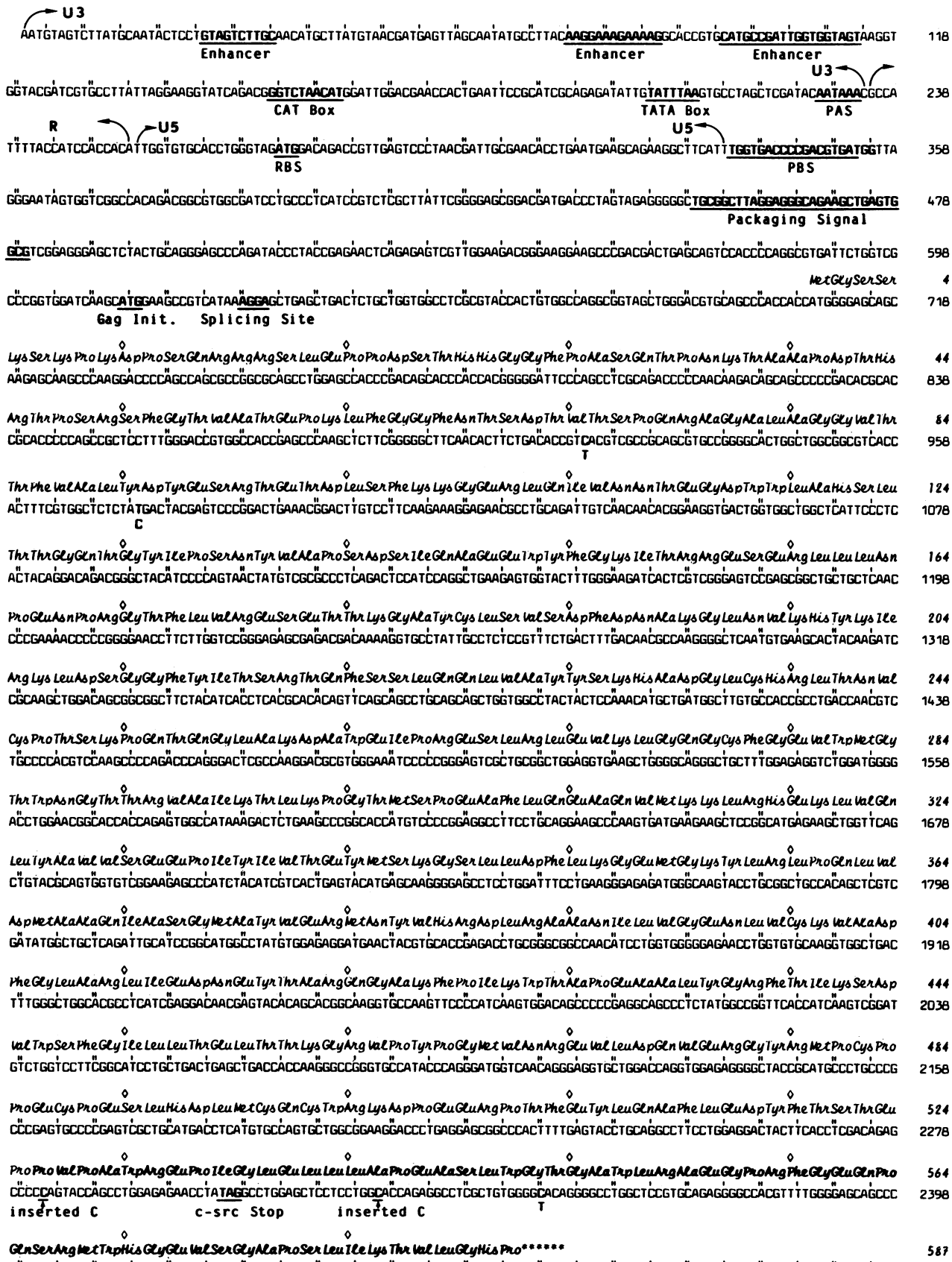
***src* coding region and adjacent unknown DNA.** Downstream of the 5′ untranslated sequence, we found in PR2257 a region of homology with the v-*src* gene, spanning approximately nucleotide 630 to nucleotide 2250, followed by a stretch of about 1,000 bp displaying no homology with any viral sequence. Then the homology with PR-C sequence resumes within the end of *env* gene, in the gp37-coding region.

The organization of PR2257 genome upstream of the *src* coding region is identical to the structure of the v-*src* subgenomic mRNA. The splice donor site located after the *gag* gene start codon (22) is linked to the splice acceptor site in the first untranslated c-*src* exon (67) at nucleotides 631 and 632 (Fig. 3B).

Alignment of the PR2257 *src* coding sequence with the v-*src* sequence of PR-C showed 29 single-nucleotide differences up to position 2248, where the C termini of viral and cellular *src* proteins are divergent. By contrast, the alignment with chicken c-*src* sequence displayed only five single-nucleotide differences until the end of the known c-*src* DNA sequence (up to nucleotide 2356 of PR2257). Two of these differences, located in the N-terminal part of the protein (C instead of T at position 916 and T instead of C at position 976), do not change the amino acid sequence of the protein. Three other mutations are in the vicinity of the C-terminal end of c-*src*: two C residues are inserted at positions 2283 and 2328, and we found a T instead of C at position 2350.

The C insertion at position 2283 profoundly modifies the C terminus of PR2257 *src* protein. This insertion shifts the reading frame and extends the 3′ coding part of the gene up to nucleotide 2467. The PR2257-encoded *src* protein should therefore contain 587 instead of 533 amino acids for c-*src* and should have a theoretical molecular mass of 65.7 kilodaltons (kDa). Interestingly, the tyrosine residue at position 527 of the cellular protein is replaced by a valine residue (Fig. 4).

The 956-bp DNA region of unknown origin between the c-*src* stop codon and the beginning of *env* gene homologous sequence (nucleotides 2310 to 3266 on PR2257 sequence) begins with a sequence of 46 nucleotides which is, except for two nucleotides, identical to the published DNA sequence following the c-*src* coding region (64). We found that it is also highly homologous to the preliminary sequence data of a portion of about 1,100 bp of cellular DNA downstream of the c-*src* coding region (T. Takeya, personal communication). At 677 bp after the c-*src* stop codon, it contains the 39-bp sequence that is utilized for reshaping the 3′ end of v-*src*. This result shows that the originally unidentified DNA region of PR2257 corresponds to cellular DNA immediately downstream of the c-*src* coding region, which has been transduced together with this cellular oncogene in the PR2257 viral genome. Recent findings (74) showed that about 2,000 bp of DNA sequences 3′ to the c-*src* stop codon


U3
Enhancer
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TATA Box
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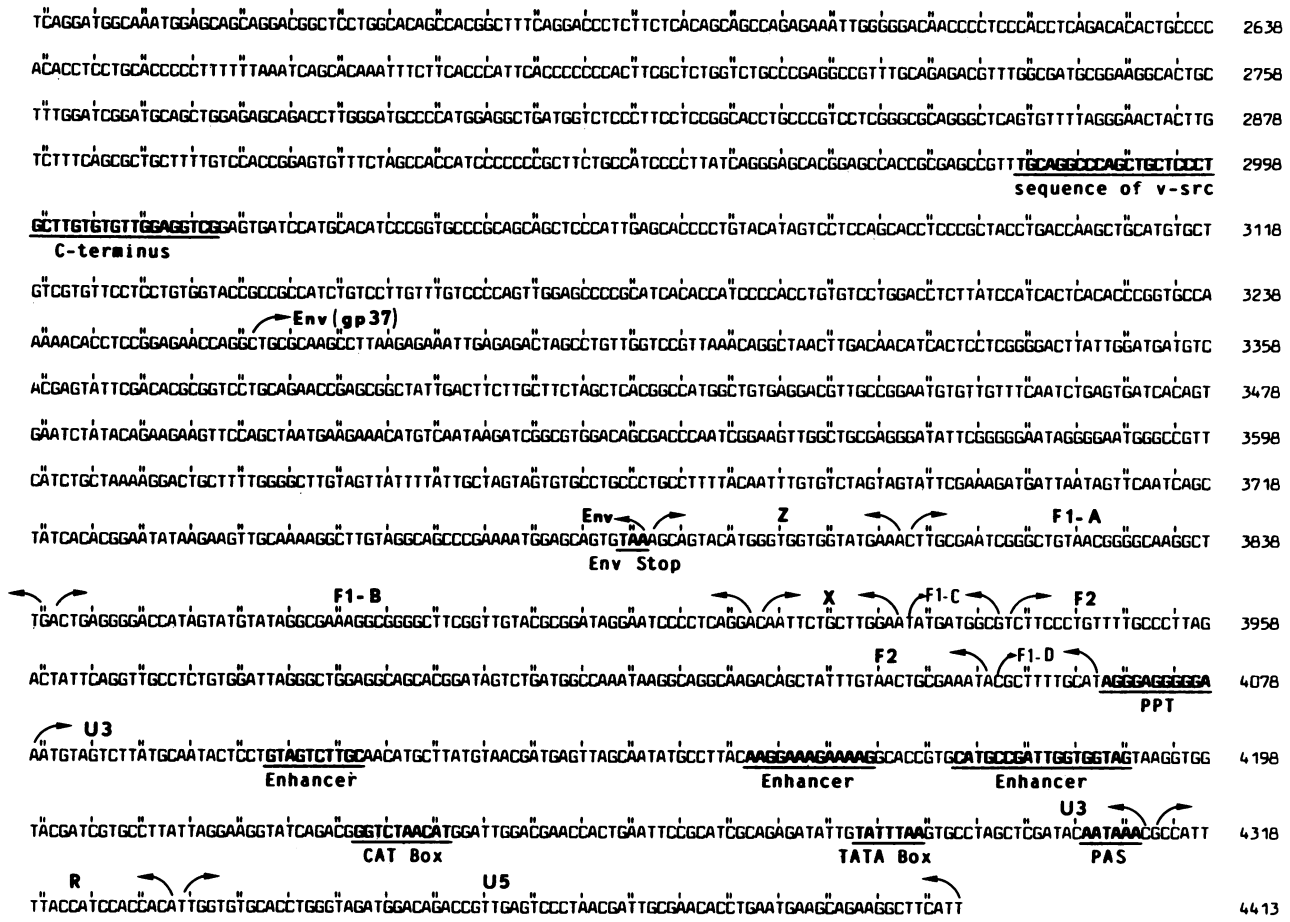

FIG. 2. Nucleotide sequence of PR2257. The DNA sequence presented corresponds to the coding DNA strand of the virus. Since the sequenced proviral DNA spans from the *Eco*RI site in the 5′ LTR to the *Eco*RI site in the 3′ LTR, the complete sequence with two LTRs that is reported here was reconstructed by adding to each side the sequence of the missing part of the LTR found on the opposite end. Differences with c-*src* sequence in the *src* coding region of PR2257 are shown in bold-face letters below the line, and the amino acid sequence of PR2257 *src* protein is shown above. The modified C terminus is indicated. Regions of the sequence that are important for the regulation of viral replication and expression have been reported previously: TATA and CAT boxes (18); 5′ packaging signal (35); U3 enhancer (13, 40); PPT (polypurine tract) (14); RBS (ribosome-binding site) (16); R (terminal redundancy of genomic RNA) (26); PAS (polyadenylation signal) (51); PBS ($tRNA^{Trp}$ primer-binding site) (25); splicing site (junction of the leader mRNA splice donor site [24] to the *src* gene splice acceptor site [67]). The elements of the 3′ untranslated region of the genome, Z, F1-A to F1-D, F2, and X, are as described by Lerner and Hanafusa (43).

are transcribed and conserved in the 4-kb spliced mRNA of c-*src*.

Delta *env* gene and 3′ untranslated region. Recombination between 3′ c-*src* DNA and the viral *env* gene probably occurred because of a region of ca. 10 nucleotides where both DNAs are highly homologous (Fig. 3C). This region of *env* gene is located about 90 nucleotides after the beginning of the gp37-coding sequence.

Whereas the homology of PR2257 with the PR-C sequence is almost complete up to nucleotide 3716, the end of the *env* gene and the subsequent noncoding region of PR2257 up to nucleotide 3894 showed only scattered homology with this virus. Here again, a comparison with the RAV-0 sequence revealed a high degree of homology (Fig. 3D). After nucleotide 3894, the homology with RAV-0 ends and a nearly perfect homology with the PR-C sequence is found until the end of the genome (U3). According to the dissection of the 3′ untranslated region of avian retroviruses made by Lerner and Hanafusa (43), the corresponding region of PR2257 comprises the Z and F1-A sequences, probably derived from the RAV-0 genome. After a recombination with the *td da*-PR-C genome, which probably took place within the F1-B region (the direct repeat flanking *src* gene in SR and PR strains of RSV), the succession of X, F1-C, F2, F1-D, polypurine tract (PPT), and U3 sequences characteristic of the PR-C strain was identified in the PR2257 genome (Fig. 3D).

A detailed analysis of the PR2257 sequence demonstrates that, as was found for the 5′ untranslated region, the genesis of the 3′ untranslated region involved several recombination events between endogenous virus and the parental *td da*PR-C.

Characterization of PR2257-encoded *src* protein. To identify the *src* protein coded for by PR2257, we labeled C7 cells and in vitro-cultured tumor fragments from the first passage in vivo of the original 2257 tumor either with [^{35}S]methionine or with [$^{32}P_i$]. Uninfected and PR-C-transformed quail fibroblasts were used as controls. Whereas sera raised in tumor-bearing rabbits were not efficient in precipitating the PR2257 *src* protein, the serum directed against $p60^{v\text{-}src}$ synthesized

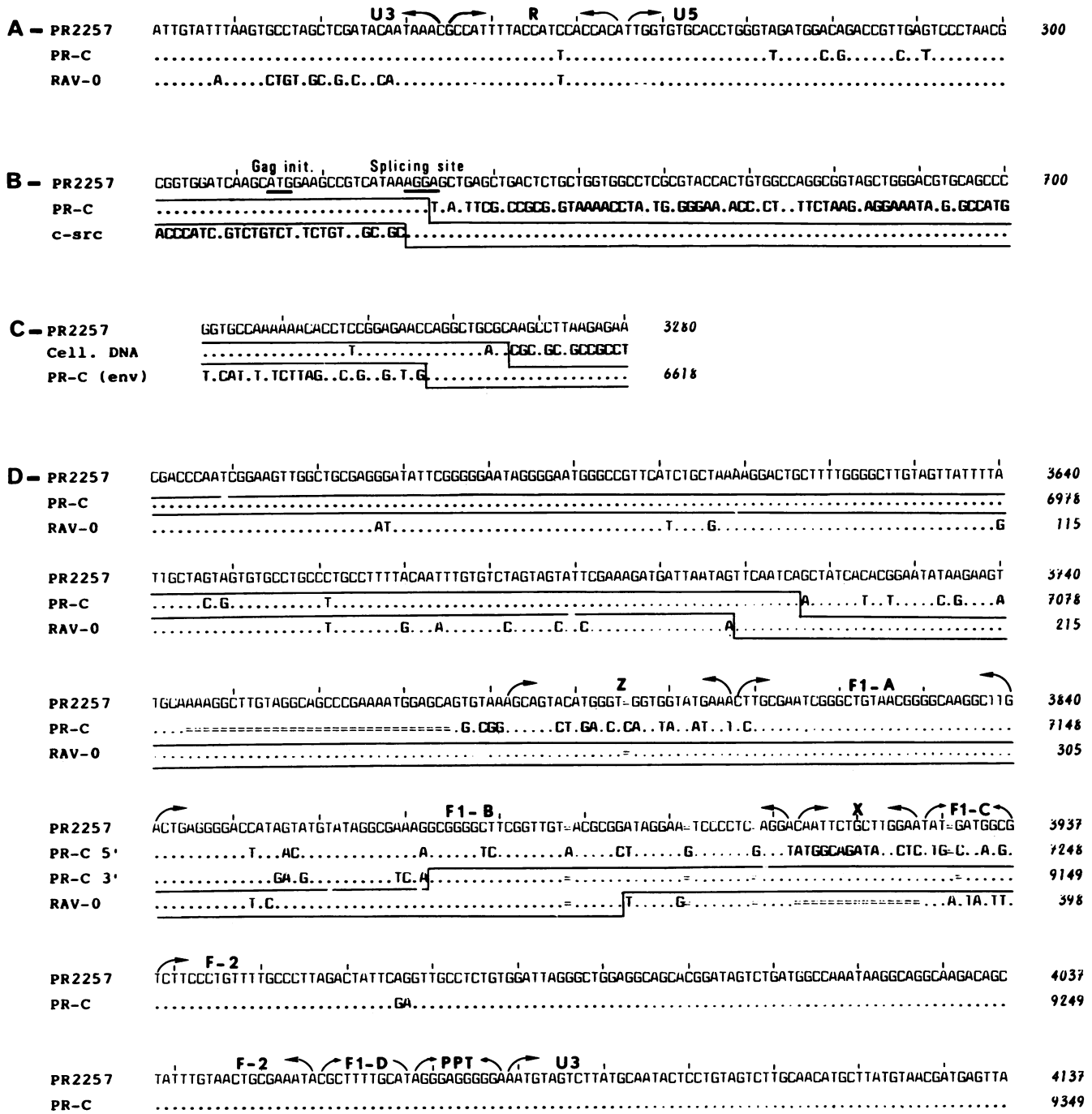

FIG. 3. Comparison of PR2257 untranslated regions with sequences of PR-C (60), RAV-0 (30), and c-*src* gene (67). Nucleotide numbers of PR-C and RAV-0 sequences are as in Genbank (version 50, May 1987, mnemonics ALRCG and ALELTR0). The viral genome displaying the best homology with PR2257 sequence is emphasized by two solid lines, and regions of probable recombination appear as rectangular boxes. Equal signs (=) indicate nucleotides not present. PR-C 5′, Region upstream of *src* gene in PR-C; PR-C 3′, region downstream of *src* gene in PR-C. For explanation of U3, R, U5, PBS, Z, F1-A, F1-B, X, F1-C, F2, F1-D, and PPT, see the legend to Fig. 2. (A) Comparison of U5 regions of PR2257, PR-C, and RAV-0; among the five single-nucleotide differences between RAV-0 and PR-C sequences, four (positions 273, 279, 281, and 291) are also found in closely related exogenous viruses RSV strain SR and RAV-2. (B) Splice junction of viral leader mRNA with c-*src* exon 1. (C) Recombination site of cellular DNA downstream c-*src* (T. Takeya and H. Hanafusa, unpublished data) and viral *env* gene. (D) Comparison of 3′ untranslated regions of PR2257 with PR-C and RAV-0 sequences.

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          510        520        530        540        550        560        570        580
c-src     ETLQAF[L]EDYFTSTEPQ[Y]QPGENL  '          '          '          '          '          533 AA (59,1 kDa)
PR-C      K****Q[*]LPACVLQVAE[ ]'          '          '          '          '          '          526 AA (59.1 kDa)
PR2257    ******[*]*********P[V]PAWREPIGLELLLAPEASLWGTGAWLRAEGPRFGEGPQSRMWHGEVSGAPSLIKTVLGHP  587 AA (65.7 kDa)
S1        ******[*]*********T[A]VHMATDPGLYYTTAHKSRARVSSWTAVARPAAPVRVLLKPSV'          '          568 AA (63.3kDa)
S2        ******[*]***LGYLAWT[P]WEDKQEGPRGETASWKQERPGQDTLAAES  '          '          '          556 AA (62.5 kDa)
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FIG. 4. Comparison of carboxy-terminal regions of PR-C, c-*src*, PR2257, and S1 and S2 *src* proteins. In PR2257, the last 8 amino acids (AA) of c-*src* are replaced by 62 new amino acids. Boxed amino acids depict the conserved leucine at position 516 (75) and the substitution or absence of c-*src* tyrosine 527 in all the virus-coded proteins.

in *E. coli* (19) precipitated a protein with an apparent molecular size of 62 kDa in PR2257-transformed cells, compared with the 59 kDa measured for p60$^{v\text{-}src}$ of PR-C-transformed cells (Fig. 5). This apparent molecular mass is lower than the expected theoretical molecular mass of 65.7 kDa. The ^{32}P-labeled PR2257 *src* protein appeared to be phosphorylated to about the same extent as the wild-type virus-encoded p60$^{v\text{-}src}$ (Fig. 5).

After labeling of C7 cells with $^{32}P_i$, the *src* protein was immunoprecipitated and isolated on a preparative SDS-polyacrylamide gel. Partial hydrolysis of p60$^{v\text{-}src}$ by the *S. aureus* V8 protease yields four peptides: V1 (34 kDa), which represents the amino-terminal part of the protein; V2 (26 kDa), which is the carboxy-terminal part; and two peptides resulting from further cleavages of V1, peptides V3 (18 kDa) and V4 (16 kDa) (8). Peptides V1, V3, and V4 are phosphorylated on a serine residue, whereas V2 is phosphorylated on a tyrosine residue in wild-type RSV p60$^{v\text{-}src}$ (31). The PR2257 *src* protein gave also four peptides (Fig. 6) of 34 kDa (V1), 31 kDa (V2), 18 kDa (V3), and 16 kDa (V4). As expected, the V2 peptide, i.e., the C-terminal part of the protein, is larger than the corresponding peptide of p60$^{v\text{-}src}$. Summing the molecular mass of PR2257 V1 and V2 fragments yielded a value of 65 kDa, which is close to that predicted by PR2257 sequence. The 62-kDa value based on the electrophoretic mobility of the undigested protein is most probably underestimated, perhaps because of tertiary structure in PR2257 *src* protein persisting in SDS-polyacrylamide gels. Therefore, we designate the PR2257 v-*src* product as p66^{src}.

The qualitative determination of phosphoamino acids in each V8 peptide was achieved by two-dimensional electrophoresis after partial hydrochloric acid hydrolysis. V1, V3, and V4 are phosphorylated on a serine residue, as other viral and cellular *src* proteins. In contrast, V2, which is usually phosphorylated only on a tyrosine residue, appeared to be phosphorylated on both tyrosine and serine residues (Fig. 6). Thus, an additional serine phosphorylation site distinguishes the PR2257 *src* protein from other v-*src* proteins.

p66^{src} protein kinase activity. Protein kinase activity of immunoprecipitated PR2257 p66^{src} was determined by the phosphorylation of immunoglobulin G heavy chain (7) or by

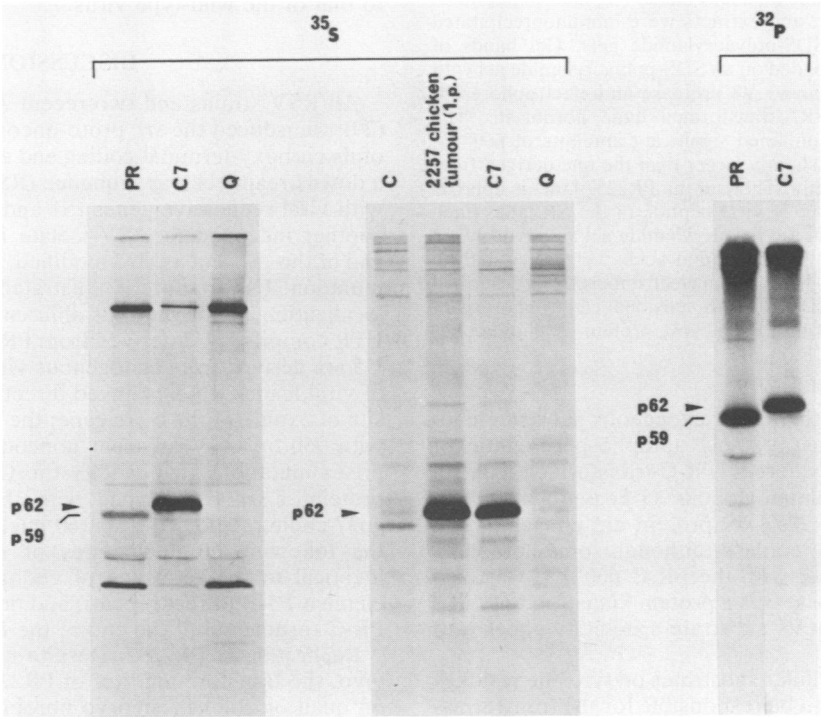

FIG. 5. Immunoprecipitation of PR2257 *src* protein. Cells and in vitro-cultured fragments of 2257 chicken tumor (first passage) were labeled either with [^{35}S]methionine or with $^{32}P_i$. After lysis, *src* proteins were immunoprecipitated and electrophoresed in an SDS-polyacrylamide gel. PR, PR-C-transformed quail fibroblasts; C7, C7 cells; Q, uninfected quail fibroblasts; C, uninfected chicken fibroblasts.

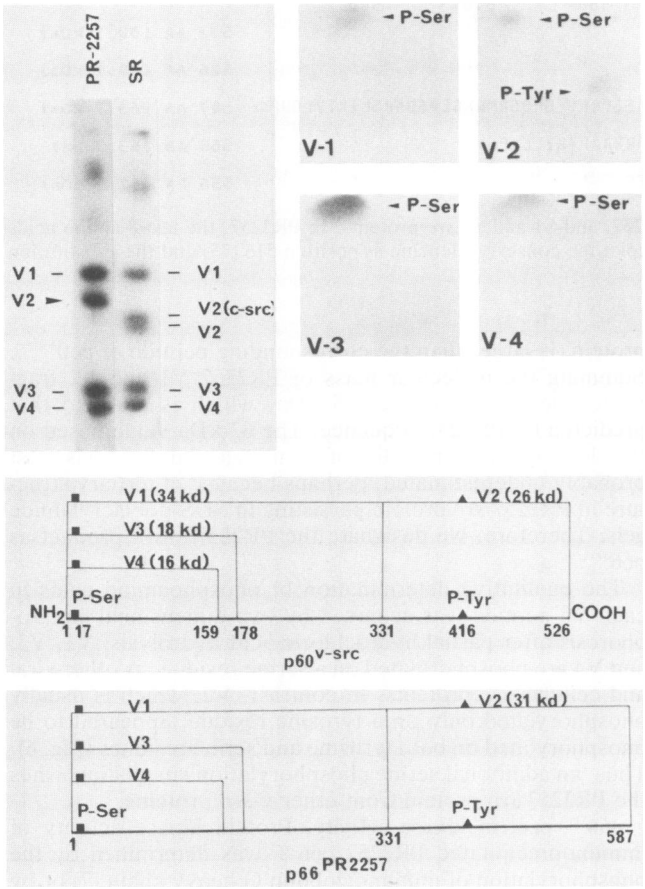

FIG. 6. *S. aureus* V8 protease partial digest pattern of PR2257 *src* protein and phosphoamino acid analysis of $^{32}P_i$-labeled peptides. Cells were labeled for 8 h, and extracts were immunoprecipitated and electrophoresed on SDS-polyacrylamide gels. Gel bands of labeled *src* proteins were loaded on an SDS-polyacrylamide gel with a 2-μg/ml solution of *S. aureus* V8 protease and electrophoresed. PR2257, C7 cells; SR, SRA-transformed quail fibroblasts. The immunoprecipitates also contained significant amounts of $p60^{c\text{-}src}$, which yields a V2 peptide slightly larger than the one derived from $p60^{v\text{-}src}$ (9). This band, faintly visible in the PR2257 lane, is approximately equal to the amount of viral peptide in the SR lane. Each peptide was recovered from the polyacrylamide gel and hydrolyzed in 6 N hydrochloric acid. Phosphoamino acids were separated on thin-layer cellulose by two-dimensional electrophoresis as described in Materials and Methods. Only two-dimensional electrophoresis of V8 peptides generated from PR2257 *src* protein is shown. kd, Kilodaltons.

the phosphorylation of the added exogenous substrate enolase (10). In the first assay, only autophosphorylation of $p66^{src}$ was observed, whereas PR-C-transformed control cells gave the usual immunoglobulin G heavy-chain phosphorylation (Fig. 7). PR2257 *src* protein did phosphorylate the exogenous substrate enolase, although some autophosphorylation, not detected with the PR-C $p60^{v\text{-}src}$, was also noticed. Thus, $p66^{src}$ possesses a protein kinase activity like other v-*src* proteins, but its substrate specificity appears to be different in vitro.

Phosphorylation of cellular substrates on tyrosine residues by $p60^{v\text{-}src}$ is presumed to be responsible for the transformation of RSV-infected cells. The level of phosphotyrosine is increased 10-fold in RSV-transformed chicken fibroblasts (31) and neuroretinal cells (49). Therefore, we measured in

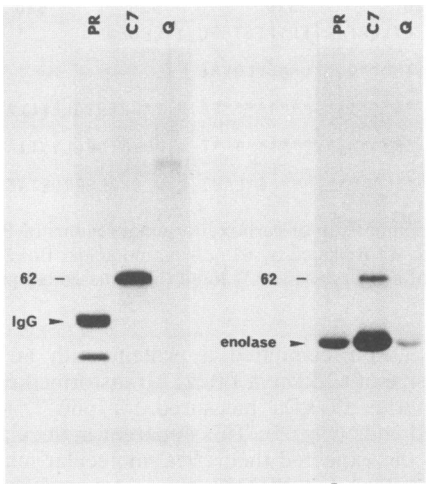

FIG. 7. In vitro protein kinase activity of PR2257 *src* protein. Extracts of C7 cells (C7), PR-C-transformed cells (PR), and uninfected quail fibroblasts (Q) were immunoprecipitated and assayed for protein kinase activity as described in the text, either without exogenous substrate (left lanes) or with added enolase (right lanes). IgG, Immunoglobulin G; 62, 62-kDa protein.

C7 cells the degree of tyrosine, serine, and threonine phosphorylation and compared it with the respective values found in normal and PR-C-transformed quail fibroblasts. The amount of phosphotyrosine in normal quail fibroblasts is substantially higher (0.32% instead of 0.10%) than the amount previously determined in chicken fibroblasts (50). The degree of phosphorylation was increased about five times in C7 cells (1.77%) and was slightly higher than the value found in PR-C-transformed cells (1.33%). Thus, the in vivo phosphorylating activity of PR2257 *src* protein is similar to that of the wild-type virus.

DISCUSSION

All RSV strains and two recent ASV isolates, S1 and S2 (29), transduced the *src* proto-oncogene with a modification of its carboxy-terminal-coding end either by transposition of a downstream cellular sequence (RSV) or by recombination with viral replicative genes (S1 and S2). PR2257 represents another independent ASV isolate in which the C-terminal end of the *src* gene is also modified, this time by a frameshift mutation. Its structure is illustrated in Fig. 8, in which the localization and origin of its different sections are shown. Its LTR comprises U3 derived from PR-C virus, whereas R and U5 are derived from endogenous virus; the splice donor site of viral leader RNA is linked directly to the splice acceptor site of exon 1 of the c-*src* gene; the c-*src* gene is transduced with 950 bp of 3′ adjacent noncoding sequence and comprises mutations that modify the C terminus of the coded protein; c-*src* recombined with the *env* gene within the gp37-coding region; the last 60 nucleotides of this gene and the following 60 nucleotides of untranslated region are identical to the sequence of endogenous virus, up to the element F1-B (direct repeat); and finally, the homology with PR-C resumes until the end of the PR2257 sequence.

Replication of PR2257. Despite its high tumorigenicity in vivo, the transforming titer of PR2257 is low when assayed on quail or chicken embryo fibroblasts (65). This could be due to a defect in replication or to a low transforming potential of PR2257 or both. The presence of an additional strong ribosome-binding site at the 3′ limit of the primer-

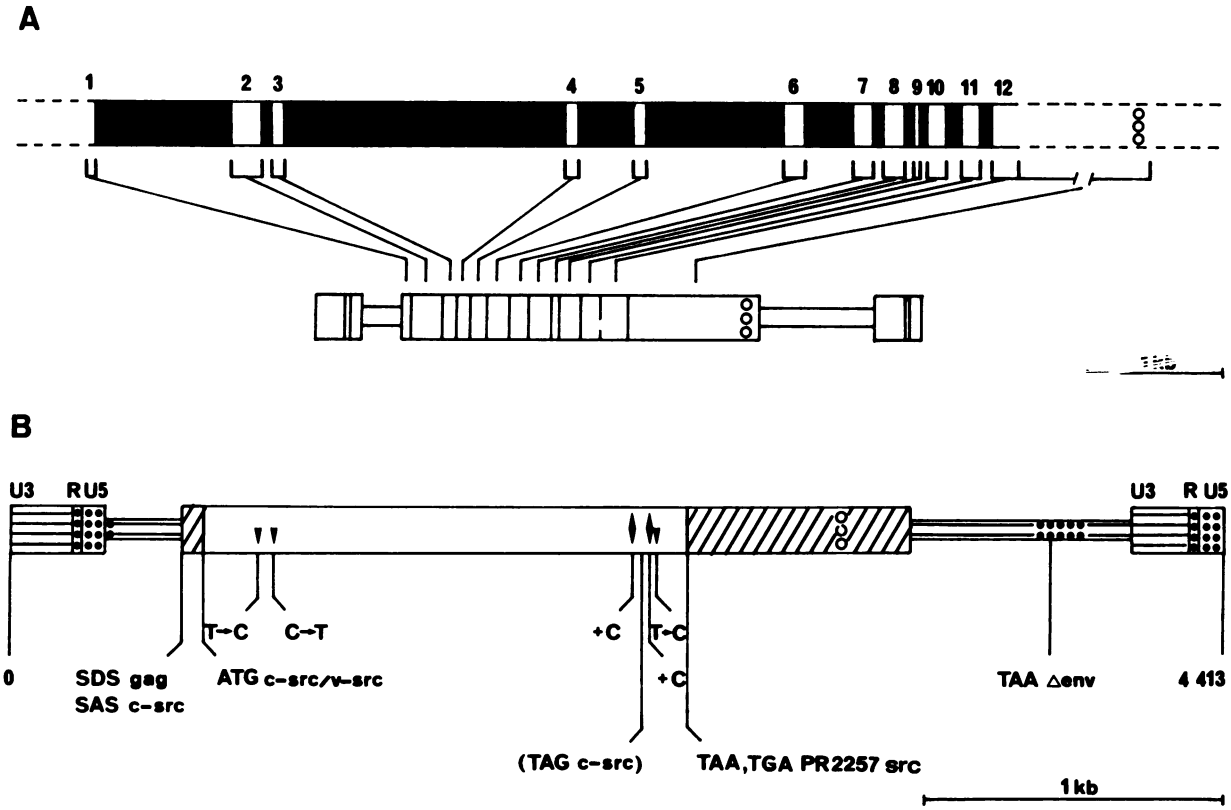

FIG. 8. (A) Origin of sequences transduced by PR2257. The upper part summarizes the topography of the c-*src* gene as given by Takeya and Hanafusa (67). Open boxes indicate exons, and solid boxes indicate introns. The boundaries of the 5′ end of exon 1 and the 3′ end of exon 12 have not been identified. The broken lines indicate regions where exon (or intron) structures are not clear. Open circles represent a stretch of 39 nucleotides utilized for reshaping the 3′ end of RSV v-*src*. The lower part schematically illustrates regions of c-*src* utilized for generation of PR2257. The length and organization of the c-*src* region to the right of exon 12 are not known exactly. Therefore, this region incorporated in PR2257 might have been modified by deletion or splicing and is indicated by a line interrupted with two vertical bars. The boxes at both ends of the PR2257 provirus represent LTRs. (B) Structural organization of PR2257 provirus. U3, Sequence unique for the 3′ end of viral RNA; R, sequence repeated terminally; U5, sequence unique for the 5′ end of viral RNA; SDS *gag*, leader mRNA splice donor site; SAS c-*src*, *src* gene splice acceptor site; ATG c-*src*, initiation codon of the c-*src* gene; T → C, transition of thymine to cytosine; C → T, transition of cytosine to thymine; +C, insertion of cytosine nucleotide; TAG c-*src*, termination codon of the c-*src* gene; TAA, TGA PR2257 *src*, termination codons of PR2257 viral *src* gene; TAA *env*, termination codon of the *env* gene. Dotted and horizontally barred regions represents sequences derived from chicken endogenous virus and parental *td da*PR-C virus, respectively. Dashed areas in the c-*src*-derived sequences indicate noncoding regions.

binding site could be responsible for the low replication of PR2257 in newly infected cells by destabilizing the secondary structure of viral RNA required for efficient reverse transcription (4, 15).

PR2257 *src*-encoded protein. A very characteristic modification at the 3′ end of the c-*src* last coding exon took place in the PR2257 genome. In this respect, PR2257 virus resembles all naturally generated ASV strains. The insertion of a cytosine 24 bases upstream of the c-*src* termination codon changed the last eight terminal amino acids because a new reading frame was generated, which was further modified by the insertion of another cytosine 19 nucleotides downstream of the c-*src* termination codon. These 3′-end modifications resulted in the expansion of the coded protein by a total of 54 amino acids.

The most critical change likely to be responsible for activation of the PR2257 v-*src* protein seems to be replacement of tyrosine by valine at position 527. The phosphorylation of this residue was demonstrated to regulate the kinase activity and transforming properties of c-*src* (11, 12). When contained in the c-*src* gene, a substitution similar to that encountered in PR2257 (phenylalanine substituted for tyrosine at position 527) is sufficient to confer transforming properties to $p60^{c\text{-}src}$. Chicken fibroblasts (55) or NIH 3T3 cells (2, 36, 48) are readily transformed by such altered c-*src* genes in vitro, and the transformed NIH 3T3 cells were shown to be tumorigenic in nude mice (36). However, the significance of tyrosine 527 substitution for tumorigenicity in chickens has been proved so far only with ASV strains S2 and PR2257, in which c-*src* sequence is modified uniquely at the C-terminal end. Extensions of the carboxy ends by 36 and 23 amino acids, respectively, are also observed in $p62^{S1\text{-}src}$ and $p62^{S2\text{-}src}$. Comparison of the amino acid sequences of the C termini of these three proteins showed that their amino acid compositions are completely different and that there are no sequence homologies among them (Fig. 4). It is likely, therefore, that the length and composition of the C termini that are substituted for the c-*src* normal end do not play an important role in the activation of its transforming properties, provided that these substitutions alter tyrosine 527.

Besides the structural modification of the carboxy end, the PR2257 transforming protein has an extra phosphoserine in its V2 fragment. It is possible that this new phosphoamino

acid located in or near the kinase catalytic domain (38) has some regulatory function.

Recombinations with endogenous virus genome. In the genome of PR2257, two regions originate from endogenous virus: (i) the U5 domains of the LTR and possibly a part of the downstream untranslated region and (ii) the end of the *env* gene and a part of the adjacent 3′ untranslated region (Fig. 8B). Recombinations with endogenous virus in Brown Leghorn chickens may occur repeatedly since they carry RAV-0 sequences in several loci (*ev-3*, *ev-6*, *ev-22*, *ev-23*, *ev-24*, *ev-25*, *ev-26*) and three of them (*ev-3*, *ev-6*, *ev-22*) are transcribed (21).

The significance of endogenous virus sequences acquired by PR2257 is not clear. They could be purely adventitious events, but it is also possible that some recombinations that increase the tumorigenic properties of the *td da*PR-C have been selected for. RAV-0-derived sequences in the U5 domain of the LTR are frequently found in recombinants between RAV-0 and exogenous viruses (6, 57, 58) and in Fujinami sarcoma virus (62), in which they seem to have little or no influence on virus replication and the expression of viral genes. In contrast, endogenous virus-related sequences located in the 3′ region of PR2257 might have some biological significance since they confer to this virus a 3′ end identical to that of NTRE7, a recombinant of PR-B with RAV-0 that induces sarcomas and other neoplasms rather than lymphomas or erythroblastosis (57, 70).

Mechanism of c-*src* transduction in PR2257. The sequence data demonstrate that PR2257 virus arose by a different mechanism than the recovered ASV strains obtained with *td* mutants retaining part of the v-*src* sequence (47, 73). In the *td da*PR-C mutant, the *src* gene has been completely excised. Hence, PR2257 represents the first ASV generated from a complete *td* mutant.

On its 5′ end, PR2257 was generated by joining the regular splice donor site 18 nucleotides into *gag* with the splice acceptor site in the first untranslated exon of c-*src*. In this way, it is identical to the 5′ end of ASV S1 described by Ikawa et al. (32). It is generally assumed that transduction of cellular oncogenes by retroviruses proceeds by the fortuitous integration of the provirus upstream of the c-*onc* gene, followed by deletion of 3′ viral sequences (notably the 3′ LTR), allowing a cotranscription of viral and c-*onc* sequences (66). The cotranscript can be formed even without deletion of the 3′ proviral domain, as shown by Hermann and Coffin (28). Normal splicing of this cotranscript would eventually lead to the junction of 5′ viral leader mRNA to exon 1 of c-*src* as observed in PR2257 and S1. However, for the *src* gene, the integration of an ALV provirus upstream of the c-*src* gene has yet to be demonstrated. This raises the possibility that another mechanism could be used to generate this junction.

trans splicing of the 5′ leader region of viral mRNA to exon 1 of c-*src* mRNA is another possible mechanism. *trans* splicing has been now demonstrated in vitro (37) and in vivo in several species (reviewed in reference 61) and is noticeably involved in the junction of common 5′ leaders to the coding regions of different mRNAs. Recently, it was shown that chicken neuroretinal cells infected with RAV-1 are induced to proliferate, and several viruses in which the c-*mil* gene has been transduced have been molecularly cloned and characterized (46). One of these viruses, IC2, showed also a direct splice junction of the leader region of RAV-1 to the v-*mil*-homologous exon 2 of the c-*mil* gene. With PR2257, S1, and IC2 viruses, there are now three examples consistent with the hypothesis that the first step in the capture of cellular oncogenes by ALVs might be a *trans* splicing of viral leader mRNA to an exon of a c-*onc* mRNA.

In both cases (*cis* splicing of a cotranscript of viral sequences and c-*src* sequences or *trans* splicing of viral leader RNA to c-*src* mRNA), the following step would be the packaging of the resulting spliced RNA in virions together with the *td da*PR-C genomic RNA. Then recombination with viral genes might occur either by the copy choice mechanism (5, 71) or by the displacement-assimilation mechanism of recombination (34) to generate the junction of the 3′ region of c-*src* gene with the gp37 coding region of *td da*PR-C.

Because it has been demonstrated that v-*src* mRNA can be efficiently packaged into and transmitted by virions of a helper virus (64), it is conceivable, as a third possible mechanism, that a c-*src* transcript could be included in the virions of *td da*PR-C and then recombine with the viral genome by one of the processes already mentioned. More experimental work, including the isolation and characterization of the integrated DNA intermediates that are hypothesized in the models discussed above, is necessary to demonstrate which one reflects the mechanism of c-*src* capture and activation by avian retroviruses that are not acutely oncogenic.

ACKNOWLEDGMENTS

We thank Richard Jove for critical reading of the manuscript and Danielle Laugier and Maria Marx for help and advice in the *src* protein and RNA experiments. We also thank F. Arnouilh for assistance in the preparation of the manuscript. Sequence data treatment was performed with the computer facilities at CITI2 on a PDP8 computer with the help of the French Ministère de la Recherche.

This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale (CRE 85 2007), the Centre National de la Recherche Scientifique, and the Fondation pour la Recherche Médicale. J.G. was the recipient of a long-term fellowship from the International Agency for Research on Cancer.

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