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A retroviral promoter is sufficient to convert proto-*src* to a transforming gene that is distinct from the *src* gene of Rous sarcoma virus

(proto-*src* recombinant virus/mutation enhances transforming function/recombinant cancer gene hypothesis)

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ABSTRACT **The *src* genes of four natural isolates of avian sarcoma viruses differ from cellular proto-*src* in two genetic substitutions: the promoter of the cellular gene is replaced by a retroviral counterpart, and at least six codons from the 3′ terminus are replaced by retroviral or heterologous cell-derived elements. Since virus constructs with a complete proto-*src* coding region failed to transform avian cells but acquired transforming function by point mutations of various codons, it has been proposed that point mutation is sufficient to convert proto-*src* to a transforming gene. However, promoter substitution is sufficient to convert two other proto-*onc* genes, proto-*ras* and proto-*myc*, to retroviral transforming genes. In view of this, we have reexamined whether promoter substitution, point mutation, or both are necessary to convert proto-*src* into a retroviral transforming gene. It was found that a recombinant virus (RpSV), in which the *src* gene of Rous sarcoma virus (RSV) was replaced by the complete coding region of proto-*src*, transformed quail and chicken embryo cells. The oncogene of RpSV differs from the *src* gene of RSV in three genetic properties: (*i*) it is weaker—e.g., transformed cells are flatter; (*ii*) it is slower—e.g., focus formation takes 9 to 12 days compared to 4 days for RSV; and (*iii*) its host range is narrower than that of RSV—e.g., only subsets of heterogeneous embryo cells are transformed by RpSV even after weeks or months. Replacement of the proto-*src* 3′ terminus of RpSV by that of *src* from RSV generates a recombinant virus (RpvSV) that equals RSV in transforming function. It is concluded that a retroviral promoter, naturally substituted via illegitimate recombination with retroviruses, is sufficient to convert at least three proto-onc genes, *src*, *myc*, and *ras*, to retroviral transforming genes.**

Four independent isolates of avian sarcoma viruses carry transforming *src* genes: Rous sarcoma virus (RSV) (1–3), avian sarcoma viruses S1 and S2 (4, 5), and avian sarcoma virus PR 2257 (6). The *src* genes of these avian sarcoma viruses differ from their cellular progenitor proto-*src* (7–9) in two genetic substitutions: (*i*) the promoter and other upstream regulatory elements of proto-*src* are replaced by a retroviral promoter; and (*ii*) at least six codons from the 3′ terminus of proto-*src* are replaced by retroviral elements (5), by an alternative proto-*src* reading frame (6), or by a combination of cell-derived (9) or retrovirus-derived (10, 11) elements. This C-terminal substitution always replaces or deletes the tyrosine 527 codon of proto-*src* (4–6), which is located six codons from the 3′ terminus (12). Since all four viral *src* genes lack the native 3′ terminus of proto-*src*, and since virus constructs with complete proto-*src* coding regions prepared by others failed to transform primary avian cells (13–15) unless the C-terminal tyrosine or some other codons had been point mutated (16–19), it was proposed that point mutation is sufficient to convert proto-*src* into a viral transforming or oncogene (16–19).

The concept that a point mutation may convert a normal cellular gene to a cancer gene derives from the precedent that proto-*ras* genes acquire transforming function for the aneuploid murine NIH 3T3 cell line from one of several point mutations (20–22). This was originally observed with a proto-*ras* gene that had been isolated from a human bladder cancer cell line (20–22). The proto-*ras* gene is the cellular precursor of the murine Harvey sarcoma virus (12). However, subsequent experiments showed that this observation was limited to some morphologically unstable, aneuploid rodent cell lines; i.e., primary rodent and human cells could not be transformed by proto-*ras* genes with point mutations (23, 24).

Instead of point mutation, replacement of the cellular promoter by that of a retrovirus was found to be sufficient to convert two proto-*onc* genes, proto-*ras* and proto-*myc*, to retroviral transforming genes equivalent to those of authentic murine sarcoma viruses, such as Harvey sarcoma virus (25, 26), and avian carcinoma viruses, such as MC29 and MH2 (27, 28). In view of this, we have reexamined the question of whether promoter substitution, point mutation, or both are necessary to convert proto-*src* to the *src* genes of avian sarcoma viruses. For this purpose the transforming function of a recombinant virus in which the *src* coding region of RSV had been replaced by that of proto-*src* was studied in primary quail and chicken cells.

MATERIALS AND METHODS

Construction of Recombinant Virus RpSV. A recombinant provirus with a complete, spliced chicken proto-*src* coding region was constructed from an infectious provirus of Prague-RSV of subgroup A, termed pJD100 (29, 30), and from a cDNA clone of proto-*src*, termed c-*src* pgem (SP6), that had been derived from pc-*src* *neo* Moloney virus (17, 31) by subcloning in the *Sal* I site of pGEM-4 (Promega). The Rous sarcoma provirus contains two complete long terminal repeats (LTRs) each bordered by a *Hin*dIII recognition site, inserted into the *Hin*dIII site of pBR322. The proto-*src* clone contains the complete proto-*src* coding region, from an *Nco* I site at the initiation codon to a *Sac* I site downstream of the stop codon, within a 1.6-kilobase (kb) proto-*src* cDNA sequence (Fig. 1). The *src* coding region of pJD100, from the *Nco* I site at the ATG initiation codon of viral *src* to a *Pvu* II site 36 base pairs (bp) upstream of the stop codon was replaced by the 1609-bp proto-*src* sequence from c-*src* pgem, which extends from the common 5′ *Nco* I site at the ATG initiation codon to a *Sac* I site 10 bp downstream of the TAG stop codon of proto-*src* (12, 31) (Fig. 1). To fit the *Nco* I- and *Sac* I-bordered proto-*src* region of c-*src* pgem into the *Nco* I

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Abbreviations: RSV, Rous sarcoma virus; LTR, long terminal repeat.

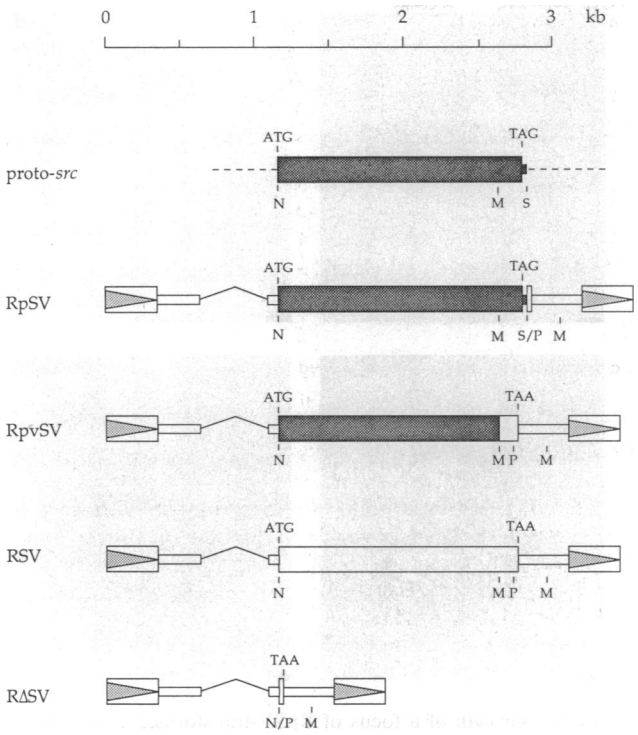

FIG. 1. The genetic structures of the coding region of chicken proto-*src*, the proto-*src* hybrid gene of recombinant virus RpSV, the proto-*src* hybrid gene of recombinant virus RpvSV, the *src* gene of RSV, and the *src* deletion mutant RΔSV of RSV. The boxes with stippled arrowheads represent the retroviral LTRs. The arrowhead points in the transcriptional orientation of the LTR-promoters. Shaded boxes represent proto-*src* and white boxes represent viral *src* coding elements. The narrow boxes are noncoding elements. The bent lines connect proviral splice sites of the 5′ noncoding with the 3′ coding region of the viral *src* genes. The triplets above the boxes identify start and stop codons. The letters below the boxes identify restriction enzyme sites: M, *Mst* II (*Sau* I); N, *Nco* I; P, *Pvu* II; and S, *Sac* I.

and *Pvu* II sites that flank the 1544-bp *src* region of pJD100, c-*src* pgem was first digested with *Sac* I (Fig. 1). The *Sac* I sites were then blunted with T4 DNA polymerase to fit the *Pvu* II site of pJD100. Thereafter, the DNA was digested with *Nco* I and the 1613-bp proto-*src* fragment (Fig. 1) was isolated by preparative agarose gel electrophoresis following published procedures (32). The following modifications of pJD100 were necessary to replace its *src* coding region by that of proto-*src*. Since the provirus of RSV contains five *Nco* I sites in *gag*, *pol*, and *env*, in addition to that at the 5′ end of *src* (12), a 6610-bp *Sac* I-flanked segment of pJD100 that includes these five *Nco* I sites was first deleted from pJD100 by digestion with *Sac* I and subsequent intramolecular ligation. This generated a 7.4-kb plasmid termed pJD100ΔSac. In addition, the *Pvu* II sites in v-*src* and the site from pBR322 of pJD100 had to be eliminated prior to the insertion of the proto-*src* coding region into the *Nco* I and *Pvu* II sites flanking *src* of pJD100ΔSac. For this purpose pJD100ΔSac was digested with *Pvu* II and *Eco*RV. The 3750-bp fragment that includes the origin of replication as well as the ampicillin resistance gene of pBR322 and the 5′ region of pJD100ΔSac up to the 5′-most *Pvu* II site of *src*, and the 877-bp fragment of pJD100ΔSac from the 3′-most *Pvu* II site of *src* to the *Eco*RV site of pBR322, were prepared electrophoretically and then ligated. A 4.6-kb plasmid with the correct orientation of the fragments was selected and termed pJD100ΔSEP. This plasmid was then digested with *Nco* I and *Pvu* II and its truncated *Nco* I–*Pvu* II *src* region was separated from the major 3953-bp fragment by electrophoresis. The 3953-bp fragment of pJD100ΔSEP was then ligated to the 1609-bp proto-*src* sequence prepared as described above (Fig. 1). The resulting 5562-bp plasmid was termed pJD100ΔSEP-p*src*. A complete provirus was regenerated from pJD100ΔSEP-p*src* by reinserting the previously deleted 6610-bp *Sac* I region of pJD100. For this purpose pJD100ΔSEP-p*src* was digested with *Sac* I and the digest was mixed with pJD100 DNA that had been digested with *Sac* I, as well as with *Cla* I and *Pvu* I, to fragment the pBR322 vector of pJD100. After ligation of the fragments, a recombinant plasmid was selected that carried a Rous sarcoma provirus with a complete proto-*src* coding region. This recombinant virus was termed RpSV (Fig. 1).

Construction of RpvSV. A recombinant virus with a 5′ proto-*src*/3′ viral *src* hybrid gene was constructed from RpSV by replacing an *Mst* II (*Sau* I)-bordered 3′-terminal 312-bp domain of RpSV by its 249-bp counterpart from RSV (Fig. 1). Since there are only two *Mst* II sites in pRpSV, the plasmid was digested exhaustively with *Mst* II. The 312-bp *Mst* II fragment was eliminated from the digest by preparative electrophoresis. In parallel, a 249-bp *Mst* II-bordered viral *src* counterpart was prepared from pJD100ΔSac. The large RpSV-derived fragment was then ligated to the 249-bp fragment from RSV and a provirus with a proto-*src*/viral *src* hybrid gene in the correct orientation was selected, based on the *Pvu* II coordinate in the RSV-derived *Mst* II-resistant fragment (Fig. 1). This recombinant virus was termed RpvSV.

Construction of RΔSV. A *src* deletion mutation of RSV was constructed by deleting the *src* domain between a 5′ *Nco* I site and a 3′ *Pvu* II site from pJD100ΔSEP. The ends were then ligated after converting the *Nco* I site to a blunt end with T4 DNA polymerase. A provirus with complete *gag*, *pol*, and *env* genes was then regenerated from the pJD100ΔSEP provirus with the *src* deletion, by reinserting the previously deleted 6610-bp *Sac* I region as described above.

RESULTS

A Recombinant Virus with a Native Proto-*src* Coding Sequence Transforms Primary Cells. To determine whether mutation of specific codons or substitution of the 3′ terminus of proto-*src* is necessary for transforming function of viral *src* genes, a recombinant provirus was constructed in which the entire coding region of the *src* gene of Prague RSV was replaced by that of proto-*src* (*Materials and Methods*). In preparing this recombinant virus special care was taken not to alter anything but the coding region of *src*. This would ensure that all effects of the substitution were due to the proto-*src* coding region alone. The resulting recombinant virus was termed RpSV (Fig. 1). The provirus of Prague RSV was derived from an infectious bacterial plasmid, termed pJD100 (29, 30). The proto-*src* coding region was derived from a chicken proto-*src* cDNA sequence also cloned in a bacterial plasmid (31). Under the direction of the promoter of a murine retrovirus the coding region of this proto-*src* clone produced authentic p60 src protein with kinase activity (17, 31). However, the clone failed to transform mouse NIH 3T3 cells unless the C-terminal tyrosine codon was mutated (17).

Transforming function of the cloned provirus of RpSV was tested after transfection into 70–90% confluent cultures of primary quail or chicken embryo cells (27, 28). In parallel, cells were transfected either with the plasmid pJD100 carrying the provirus of RSV or with a plasmid carrying a *src*-deletion mutant of RSV, RΔSV (Fig. 1). Two days after transfection, cells were transferred and diluted 1:4 and overlaid with 0.5% soft agar to prevent virus spread (33). Between 4 and 6 days after transfection over 10^5 foci of transformed cells appeared per pmol of pJD100 DNA and 9 to 12 days after transfection about 10^4 foci appeared per pmol of pRpSV DNA (Table 1) (Fig. 2). No transformation was observed in cells transfected by pRΔSV (Table 1). (The culture looked like

Table 1. Transforming functions

Cloned proviral DNA	Foci per pmol of proviral DNA	Days until focus formation
pRSV (pJD100)	4.4×10^5	4–6
pRpSV	2.9×10^4	9–12
pRpvSV	6.6×10^5	5–6
pRΔSV	0	—

Transfection and maintenance of primary chicken embryo fibroblasts were as described (27, 28). Focus formation was under an overlay of 0.6% agar (33). Focus-forming units reported reflect single experiments that are representative of independent results.

untransfected embryo cells, as for example the untransformed regions shown in Fig. 3.)

The morphology of RpSV-transformed cells was clearly distinguishable from that of RSV-transformed cells. Quail embryo cells transformed by RpSV were typically flatter, morphologically more heterogeneous, and more attached to the culture dish (Fig. 2*a*) than cells transformed by wild-type RSV (Fig. 2*b*). Moreover, only cells on top of others were as rounded and refractile as typical RSV-transformed cells (Fig. 2). It would appear that the transforming gene of the recombinant virus with a native proto-*src* coding region, RpSV, is distinct from that of RSV.

Kinetics and Transforming Host Range of RpSV Compared with Those of RSV. Within 4 to 6 days after transfection with a proviral plasmid of RSV (pJD100), foci of RSV-transformed quail cells appeared, and in the absence of an agar overlay, 2 to 4 days later all cells in the culture dish became transformed. By contrast, foci of RpSV-transformed quail cells appeared only 9 to 12 days after transfection and then spread more slowly than RSV foci, while cells between foci remained untransformed. The growth of a typical RpSV-focus at 12, 14, 17, and 19 days after transfection is shown in Fig. 3. Many cells of an RpSV-transfected culture remained untransformed even for 2 months after the first foci had appeared in the culture. When quail cells were infected with RpSV, instead of proviral DNA, at a multiplicity of infection of about 0.001, focal areas of transformed cells appeared about 11 days later. As with plasmid-transfected cultures, not all cells were transformed for at least 3 to 4 weeks. RpSV also displayed slow and selective transforming function, compared to RSV, in primary chicken embryo fibroblasts (data not shown).

The progression of virus production with time after transfection in an RSV-transfected and an RpSV-transfected culture is shown in Fig. 4. Within 2 weeks after transfection RpSV had reached a titer of 10^3 focus-forming units per ml of cell culture medium (33), while a culture of RSV-transformed cells had reached a titer of 10^4 to 10^5 focus-forming units per ml within 4 to 6 days after transfection (Fig. 4, Table 1). This

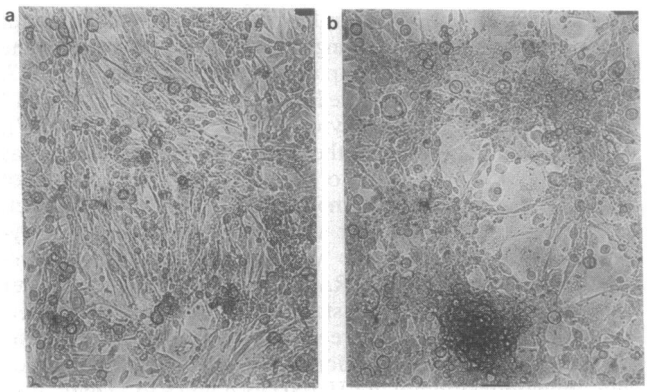

FIG. 2. Quail (*Coturnix japonica*) embryo cells transformed by plasmid-cloned proviral DNA of RpSV (*a*) and RSV (*b*). Cells were photographed 3 weeks after transfection. (×55.)

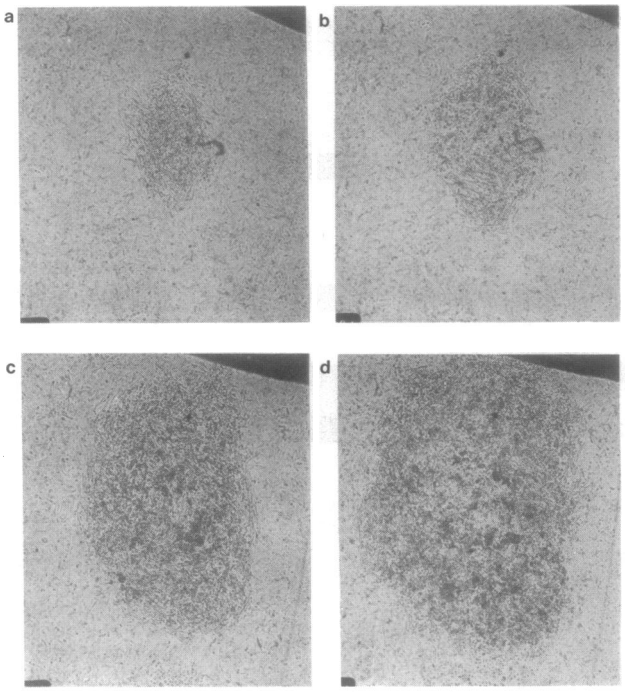

FIG. 3. Growth of a focus of RpSV-transformed quail embryo cells as a function of time. Quail embryo cells were transfected with a plasmid-cloned provirus of RpSV as described in the text. The growth of a typical focus, which was first detectable 10 days after transfection, is shown at 12, 14, 17, and 19 days (*a–d*) after transfection. (×16.)

indicates that, compared to RSV, the transforming host range of RpSV is narrower for heterogeneous primary embryo cells and that transformation by RpSV is slower.

It is concluded that the oncogene of RpSV differs from that of RSV in three genetic properties: (*i*) it is weaker, because RpSV-transformed cells are typically flatter, morphologically more heterogeneous, and more attached to the culture dish than cells transformed by wild-type RSV; (*ii*) it is about half as fast, because it takes 9 to 12 days compared to 4 to 6

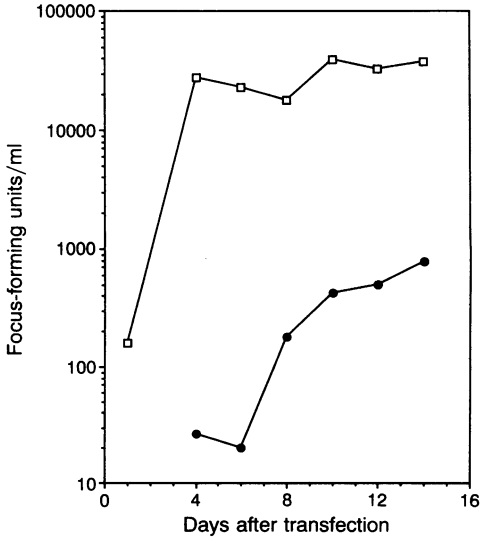

FIG. 4. The progression of virus production, measured as focus-forming units per ml of medium, by quail cell cultures transfected with cloned proviruses of RSV (□) and RpSV (●) (see Fig. 1). Primary quail embryo cells were transfected and 2 days after transfection were transferred 1:4 as described (27, 28). The virus titers in culture media were then measured (33) at various days for 2 weeks as described for Table 1.

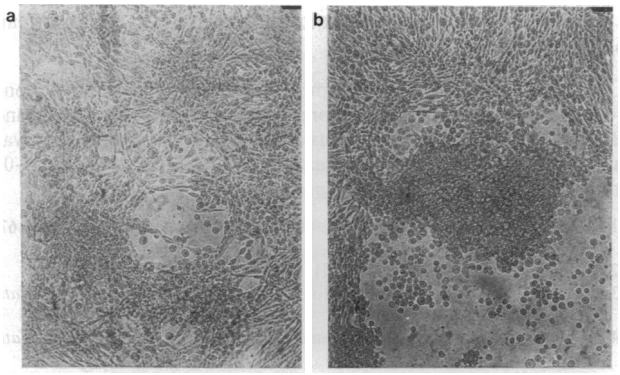

FIG. 5. Chicken embryo cells transformed by plasmid-cloned proviral DNA of RpvSV (*a*) and RSV (*b*). Cells were photographed 1 week after transfection. (×55.)

days for wild-type RSV; and (*iii*) its host range is narrower than that of RSV, because even within weeks or months RpSV transforms only subsets of primary cells. Further, it is concluded that these distinct genetic properties of RpSV are stable for at least two rounds of infection.

Replacement of 3′-Terminal Codons of Proto-*src* by Viral *src* Counterparts Confers RSV-Like Transforming Function to RpSV. Since the transforming function of the recombinant RpSV with a complete proto-*src* coding region is less than that of wild-type RSV, we tested whether the RSV-specific 3′-terminal codons of *src* may enhance transforming function by RpSV. For this purpose the 3′-terminal proto-*src* sequence of a provirus of RpSV was replaced by its counterpart from viral *src*, starting at a common *Mst* II site (Fig. 1). This replacement included the tyrosine 527 codon, which is located six codons upstream of the stop codon. The resulting proviral plasmid carrying the recombinant virus, termed RpvSV (Fig. 1), was then transfected into primary chicken cells. In parallel the pJD100 plasmid, which carries a provirus of RSV, was transfected into chicken cells. About 5 to 6 days after transfection 10^5 foci appeared per pmol of pRpvSV DNA, approximately the same number as for proviral RSV DNA (Table 1). Likewise, the morphology of RpvSV-transformed cells 7 days after transfection (Fig. 5*a*) was very similar to that of RSV-transformed cells (Fig. 5*b*). The kinetics of cell transformation by RpvSV were also very similar to those of wild-type RSV (Table 1).

DISCUSSION

Distinction Between the Transforming Genes of RpSV, of Proto-*src* Recombinant Viruses Prepared by Others, and of RSV. We have found that replacement of the native proto-*src* promoter by that of a retrovirus is sufficient to impart transforming function on proto-*src*. This conclusion is at variance with previous studies reporting that recombinant *src* genes, consisting of retroviral or simian virus 40 promoters linked to proto-*src* coding regions, failed to transform fibroblasts and established cell lines (13–19). It is possible that the discrepancy between our results and those of others reflects the structures of the proto-*src* recombinant viruses studied. Our recombinant differed from wild-type RSV only in the coding region of the *src* gene (Fig. 1), while those of others also differed from RSV in noncoding elements (13–19).

"The potential to cause a low level of transformation" by some recombinant viruses with proto-*src* coding regions has been observed by other investigators (13, 34, 35). In contrast to our RpSV, these transformants were reported to be equivalent to wild-type RSV in transforming function (13, 34, 35). It was proposed that these transformants reflected spontaneous proto-*src* point mutations, rather than intrinsic transforming function of the unmutated proto-*src* recombinant genes.

Several observations argue that the distinctive transforming function of our RpSV is the genuine property of its recombinant proto-*src* gene rather than being due to spontaneous mutations: (*i*) Cells transformed by RpSV were consistently flatter and more firmly attached to the substrate than RSV-transformed cells. (*ii*) Transformation by RpSV was consistently half as fast as that by wild-type RSV. (*iii*) The transforming host range of RpSV for primary avian cells was consistently narrower than that of wild-type RSV. (*iv*) Replacement of the C-terminal proto-*src* coding region by that of the *src* gene of RSV enhances transforming function of RpSV to equal that of RSV. (*v*) The specific transforming activity per picomole of molecularly cloned RpSV DNA was far too high to be the result of spontaneous point mutations. As can be seen in Table 1, 1 pmol of RpSV DNA generated 2.9×10^4 foci of transformed cells, whereas 1 pmol of RSV DNA generated 4×10^5. Since the mutation frequency of retroviruses is 1 in 10^4 nucleotides per replicative cycle (36, 37), about 10 (4×10^5 divided by 10^4) or several times 10 if several mutant codons generate transforming function but not 2.9×10^4, focus-forming units per pmol of RpSV DNA would have resulted from spontaneous mutation. Therefore we conclude that the transforming function of our recombinant proto-*src* virus, RpSV, is genuine, rather than derivative due to spontaneous mutation.

In agreement with us, Parsons and collaborators (30) have reported that a virus with a recombinant *src* gene that consisted of a 5′ viral *src* domain linked to a proto-*src*-derived 3′ domain was able to transform fibroblasts slowly. Like our RpSV, this recombinant *src* gene transformed primary cells 7 to 10 days later than RSV (30).

Retroviral *onc* Genes Generated from Nontransforming Genes by Illegitimate Recombination. Each of the *src* genes of four different natural avian sarcoma viruses differs from cellular proto-*src* in two major genetic substitutions. The native proto-*src* promoter is replaced by a retroviral counterpart, and at least six 3′-terminal proto-*src* codons, including the tyrosine 527 codon, are replaced by heterologous retrovirus- or cell-derived termini. By using recombinant viruses with either one or both proto-*src* substitutions, we were able to dissociate the functional roles of each substitution in the generation of the sarcoma viruses into two complementary events: (*i*) replacement of the native promoter of proto-*src* by that of a retrovirus, which is sufficient to convert proto-*src* to a relatively weak oncogene capable of transforming primary cells; and (*ii*) replacement of the C-terminal coding domain of proto-*src* by heterologous termini, which enhances the transforming function of this oncogene to the level of natural viral *src* genes.

Since each of these substitutions has involved rare illegitimate recombination between normal proto-*src* and a nontransforming retrovirus, our results are compatible with the recombinant cancer gene hypothesis. This hypothesis is that certain cellular genes, termed proto-*onc* genes, may be converted to cancer genes by illegitimate recombination with heterologous viral and possibly cellular genes (23, 24). For example, replacements of the native regulatory elements of proto-*ras*, proto-*myc*, and now proto-*src* by retroviral counterparts were each shown to be sufficient to convert these proto-*onc* genes to recombinant genes capable of transforming primary cells.

Role of Point Mutations in the Generation of Cancer Genes. Replacement of the C-terminal codons of proto-*src* by a counterpart derived from viral *src* converted the slow-transforming RpSV to the fast-transforming RpvSV, equaling wild-type avian sarcoma viruses (Table 1). According to several studies, this substitution is functionally similar to a point mutation of the tyrosine 527 codon of proto-*src* in recombinant viruses with a complete proto-*src* coding region (16–19). In the generation of the natural avian sarcoma

viruses, this point mutation of proto-*src* was achieved either by illegitimate recombination with heterologous virus- or cell-derived termini or by frameshift mutation of proto-*src* (5, 6, 9, 10). In addition, other point mutations of viral *src* genes, compared with proto-*src*, have been shown to enhance transforming function of viral *src* genes (19). Enhancements of transforming function of retrovirus-promoted proto-*ras* recombinant genes have also been noted (25, 26). However, no effects of point mutations on the transforming functions of retrovirus-promoted proto-*myc* recombinant genes were observed (27, 28). Thus, point mutations appear to modulate transforming function of oncogenes generated by illegitimate recombination.

However, the hypothesis that a point mutation without other alterations, even a specific one (20–22), may convert a cellular gene to a cancer gene is implausible in view of the enormous, $>10^8$-fold, greater probability of point mutations than of cancer. The probability of point mutation per nucleotide per cell division in both eukaryotes and prokaryotes is about 10^{-9} (23, 24). But the probability that a human cell becomes a cancer cell per mitosis is only about 10^{-17} (23, 24). This estimate is based on the facts that nearly all cancers are clonal, deriving from a single autonomous cancer cell (38–40), and that humans consist of about 10^{14} cells that go through an average of at least 100 mitoses per lifetime (23, 24, 40). Since about every third of us develops cancer in a lifetime (23, 40), and a lifetime corresponds to about 10^{16} mitoses, only 1 in about 3×10^{17} mitoses generates an autonomous cancer cell from which a clonal tumor can emerge (23, 24). Thus, the probability that an autonomous cancer cell is generated per mitosis is about 10^{-8} the probability of a point mutation at any specific site.

Since cellular genes with point mutations have been found in many cancer cells, but none of these genes has yet been found capable of transforming a diploid cell to a cancer cell, it has more recently been postulated that additional mutations are necessary for carcinogenesis (41–43). Because additional mutations would have to take place in the same cell, this proposal can be numerically reconciled with the rare occurrence of a cancer. Indeed, hypothetical helper genes that complement point-mutated genes in carcinogenesis have been named, but as yet not one has been functionally or genetically confirmed (42, 43). Moreover, it is arbitrary to assume that point mutated cellular genes that are related to retroviral *onc* genes, like proto-*ras* genes, are necessary for carcinogenesis, because retroviral *onc* genes are sufficient for carcinogenesis and thus are not models for helper-dependent cancer genes (23, 24).

In view of this, it is unlikely that proto-*onc* genes exist that can be converted to cancer genes by single point mutations, as has been postulated (20–22). It appears more likely that cancer genes are generated *de novo* by rare, illegitimate recombinations, like those that have generated the retroviral *onc* genes (23, 24). Point mutations or equivalent substitutions may then enhance transforming function of recombinant cancer genes as secondary events, as demonstrated for the viral *src* genes compared to proto-*src*.

This proposal provides an answer to the question with which Hunter recently closed a review on the generation of viral *src* genes: "Given the frequency with which activating point mutations are detected in c-*ras* genes isolated from tumors, one might have expected that c-*src* genes oncogenically activated by point mutations would also be common. The fact that no example of an activated c-*src* has been found in tumors suggests that other events are needed in the animal for oncogenic conversion of the c-*src* gene." (19). Our results suggest that replacement of the native proto-*src* promoter by a retroviral or perhaps heterologous cellular counterpart can be such an "event."

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