Unmutated proto-src coding region is tumorigenic if expressed from the promoter of Rous sarcoma virus: Implications for the gene-mutation hypothesis of cancer

(retroviral oncogenicity from transcriptional activation/mutated genes and cancer)

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ABSTRACT  The transforming (onc) genes of oncogenic retroviruses share most or all of their coding sequences with normal cellular genes termed proto-onc genes. The viral genes differ from proto-onc genes in virus-specific promoters and in various point mutations and substitutions of cell-derived coding regions. In view of the structural similarities between viral oncogenes and cellular proto-onc genes, the hypothesis has been advanced that proto-onc genes become cellular cancer genes if they have suffered mutations. Indeed, point mutations and substitutions have been observed in the proto-onc genes of some cancers. However, the hypothesis has been difficult to prove because mutated proto-onc genes from tumors do not transform diploid cells. Moreover, owing to the popularity of this hypothesis, even viral oncogenes are thought to derive transforming function from mutations of this cell-derived coding region. A competing hypothesis proposes that enhanced expression from retroviral promoters is necessary and sufficient for oncogenic function of proto-onc genes. To distinguish between these hypotheses we have tested tumorigenicity of RpSV, a synthetic retrovirus with the normal proto-src coding region in a vector derived from Rous sarcoma virus (RSV). In addition, we have tested the role of RSV-specific src point mutations on the tumorigenicity of RpSV. It was found that RpSV with an unmutated proto-src coding region is tumorigenic in chickens and that tumorigenicity is enhanced by RSV-specific src point mutations. It is concluded that retroviral promoters are essential for the transforming function of viral oncogenes and that certain point mutations merely supplement their transforming function. Thus retroviral onc genes are not models for the hypothesis that mutated, but transcriptionally normal, proto-onc genes of certain tumors are cancer genes.

Most or all of the coding regions of retroviral transforming (onc) genes are derived from cellular genes termed proto-onc genes (1). The viral genes differ from proto-onc genes in virus-specific promoters and various point mutations, deletions, and substitutions of their coding sequences (2). For example, the transforming src gene of Rous sarcoma virus (RSV) and of three other strains of avian sarcoma viruses differs from the proto-src gene of normal cells in (i) virus-specific promoters, (ii) a virus-specific carboxy terminus that replaces the six carboxy-terminal amino acids, including Tyr codon 527, of proto-src by heterologous virus- or cell-derived sequences, and (iii) scattered, virus strain-specific point mutations within the 514 codons that viral src and proto-src have in common (1, 3).

In view of the structural similarities between retroviral oncogenes and cellular proto-onc genes, the hypothesis has been advanced that proto-onc genes become cellular cancer genes if they have suffered mutations (1, 4–9). Indeed, mutated proto-onc genes have been found in some tumors. However, the hypothesis has been difficult to prove, because mutated proto-onc genes from tumors do not transform diploid cells upon transfection (10) or introduction into the germ line of transgenic animals (11). An apparent exception is the evidence that point-mutated proto-ras genes from certain cancers would transform the aneuploid 3T3 mouse cell line (5, 6).

By contrast, retroviral oncogenes are inevitably oncogenic in diploid cells or animals (1, 2). Due to the popularity of the proto-onc gene-mutation hypothesis, even the transforming function of viral onc genes is thought to derive from mutations that set apart the coding regions of viral onc genes from corresponding proto-onc genes (8, 9, 12, 13). For example, the transforming function of viral src genes (13–15) and viral ras genes (1, 7, 12) has been interpreted as the result of point mutations that set apart the proteins encoded by the viral and corresponding cellular genes.

However, transforming function of typical retroviral onc genes, including src, myc, and ras genes, was recently shown to depend on retroviral promoters rather than on the mutations that set apart the coding regions of viral and proto-onc genes (3, 16–21). These promoters increase expression about 100-fold compared to the corresponding proto-onc genes (11, 21, 22). In addition, transformation of 3T3 cells by point-mutated proto-ras genes from tumors was recently shown to reflect expression artifacts generated by the transfection assay, rather than a genuine function of native, point-mutated proto-ras genes from tumors. These artifacts include substitution of native cellular proto-ras control elements and of the native proto-ras promoter by heterologous counterparts and concatenization of proto-ras genes during transfection (11, 21).

In view of this, we and others have advanced the hypothesis that transforming function of retroviral onc genes is due to the 100-fold transcriptional activation of proto-onc-derived coding sequences by retroviral promoters (2, 11, 18, 20, 21). Certain virus-specific or cellular mutations in the coding sequence merely enhance this transforming function derived from heterologous promoters but would not be able to create it on their own.

To put our hypothesis to a further test, we ask here whether the native proto-src coding region artificially introduced into a retrovirus vector would be tumorigenic in animals and whether virus-specific point mutations would enhance the transforming function of such a virus. Previously, we had found that RpSV, a synthetic virus with a native proto-src coding region (see Fig. 1), is sufficient to transform primary

Abbreviations: RSV, Rous sarcoma virus; RpSV, proto-onc sarcoma virus.
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avian fibroblasts in vitro but is slower than RSV, requiring 9–12 days compared to 4–5 days, in cell transformation (3). Here we show that RpSV is tumorigenic in chickens after 12–15 days, compared to 4–5 days for RSV, and that tumorigenicity is enhanced by point mutation. Further, we have proved that RpSV tumorigenicity is due to the input virus rather than to spontaneous mutation of the virus in the animal.

MATERIALS AND METHODS

Construction of Recombinant Viruses. RpSV has been constructed from a DNA provirus of RSV, pJD100 (23), by substituting the coding region of src from an Neo I site at the AUG start codon to a Pvu II site 36 base pairs (bp) upstream of the TAA stop codon by a sequence that includes the complete coding region of proto-src (Fig. 1). The proto-src DNA extended from the Neo I site at AUG to a Sac I site 10 bases downstream of the TAG stop codon of proto-src (3). The resulting recombinant virus contains the complete coding region of proto-src (Fig. 1). RpSV with a RSV-derived src mutation in src codon 95, termed RpSV 95, was constructed by replacing a 2906-bp Kpn I/Mlu I fragment of RpSV that includes the Arg codon 95 of proto-src (Fig. 1) with the corresponding fragment from RSV that encodes Trp in codon 95 (1). For this purpose, RpSV was digested with Kpn I and Mlu I and the resulting 9266-bp fragment was ligated with the Kpn I/Mlu I fragment from the RSV clone pJD100 (3). RpSV 338, with a RSV-specific point mutation in src codon 338, was derived from pJD100 in two steps: First, a RSV-specific Mst II site 5' of Mlu I was eliminated by replacing the 2906-bp Kpn-Mlu sequence by a 765-bp counterpart from λ phage. Next the 249-bp Mst II-bordered viral src carboxyl terminus was replaced by the 312-bp equivalent of RpSV (Fig. 1). Subsequently, the λ phase-derived Kpn-Mlu sequence was replaced by the original 2906-bp Kpn-Mlu sequence of RpSV. RpSV 95–338 with RSV-specific src codons 95 and 338 was made from the immediate precursor construct of RpSV 338 by replacing the λ-derived Kpn-Mlu sequence with the original 2906-bp sequence of RSV.

Reverse Transcription of Virus RNA to cDNA in Vitro. About 0.2 \( A_{260} \) unit (measured in 0.1% SDS) of purified virus were incubated at 40°C for 24 hr in a solution containing the four dNTPs each at 10 μM, 50 mM NaCl, 10 mM Tris-HCl at pH 7.9, 10 mM MgCl₂, 1 mM dithiothreitol, 2.5 μg of oligo(dT) per ml, and 0.25% Nonidet P-40 as described (24). The cDNA was isolated from the reaction mixture by phenol extraction and ethanol precipitation. In some experiments the viral RNA was eliminated from the cDNA by treatment for 4 hr at 40°C in 0.3 M NaOH.

Specific Amplification of cDNA by the Polymerase Chain Reaction (PCR). The cDNA from about 0.04 \( A_{260} \) unit of purified RpSV or RSV virus was incubated in 100 μl of 100 μM each of the four dNTPs, 10 mM Tris-HCl at pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, and 1.0 μM each of two specific primers as recommended by the manufacturer (Perkin-Elmer/Cetus). One of the primers was a 25-mer of the src sequence of RSV from position 8598 to position 8622 just 5' of a Mst II site at position 8635; the other was a 27-mer complementary to a sequence of RSV 3' of src from position 8899 to position 8925 (Fig. 1) (1). After 2 min at 94°C, 2.5 units of Taq DNA polymerase were added. The solution was carried through 45 cycles of denaturation for 1 min at 94°C, annealing for 1 min during a temperature shift from 94°C to 55°C and by 2 min at 55°C, followed by polymerization for 3 min at 72°C. Finally, the mixture was incubated at 72°C for 10 min to complete any incomplete DNA products (25, 26). The PCR products were analyzed by electrophoresis in 1.7% agarose in 40 mM Tris-acetate/1 mM EDTA containing 0.5 μg of ethidium bromide per ml (25).

RESULTS

Transformation of Cells in Culture by Synthetic Viruses Carrying Proto-src Coding Regions with RSV-Specific Point Mutations. To determine whether RSV-specific src point mutations other than those affecting the carboxyl terminus of proto-src—e.g., the RSV-specific src codons 95 and 338—play a role in transforming function, such mutations were introduced singly or together into the proto-src coding region

![Fig. 1. Genetic structure of the recombinant proto-src gene of RpSV. ATG and TAG mark the proto-src coding region (high box), the arrows mark the proviral long terminal repeats including the viral promoters, and the lower white boxes are noncoding regions (3). The positions of src codons 95 and 338 and of several restriction enzyme sites are indicated. Restriction enzyme sites are abbreviated as follows: K, Kpn I; M, Mst II; Ml, Mlu I; P, Pvu II; Ps, Pst I; S, Sac I. The lower panel shows the 3' terminal proto-src region that was amplified for size and sequence analysis (see text).](image)
of RpSV. For this purpose, the amino-terminal domain of the proto-src coding region of RpSV, including codon 95, and an internal domain, including codon 338, were exchanged for the RSV equivalents (Fig. 1) (Materials and Methods). The resulting proviruses, termed RpSV 95, RpSV 338, and RpSV 95–338, were transfected onto quail embryo fibroblasts as described (3). In parallel, quail cells were transfected with proviruses of RpSV and RSV. RSV began to transform cells on day 4. Foci of RpSV-transformed cells were observed on day 6 for RpSV 95–338, on day 7 for RpSV 95, on day 8 for RpSV 338, and on day 9 for RpSV 95. Cells transformed by the three recombinant RpSVs were all more refractile than those transformed by RpSV. Within 2 weeks most cells of cultures transfected by RpSV 95, RpSV 338, and RpSV 95–338 were transformed. By contrast, only about half the cells in cultures transfected by the provirus of RpSV were transformed, confirming previous observations (3). It is concluded that some RSV-specific src point mutations enhance transforming function of RpSV in vitro.

Tumorigenicity of RpSV, RpSV 95, RpSV 338, and RpSV 95–338 in Newborn Chickens. Next the tumorigenicity of RpSV and of the RpSV mutants, RpSV 95, RpSV 338, and RpSV 95–338 was tested in newborn chickens. For this purpose the viruses were purified by sucrose gradient ultracentrifugation from the growth media of cultures of transformed quail cells (27). The media were collected at daily intervals (28). One-day-old chickens were injected under the wing web with 50–150 μl of purified virus in physiological saline buffer, derived from 10–20 ml of culture medium. Sarcomas were detected on day 7 after injecting RpSV 85–338, on day 9 after injecting RpSV 85 and RpSV 338, and on days 12–15 after injecting RpSV (Fig. 2). Sarcomas were observed on day 4 after injecting RSV. Thus, incubation periods from inoculation of RpSV mutants to tumorigenicity varied with distinct proto-src mutations, and all proto-src viruses were slower in causing tumors than RSV. Since all tumors caused by synthetic viruses carrying proto-src coding regions had appeared later than those caused by wild-type RSV, it may be argued that the longer incubation periods to tumorigenicity reflected time required to generate oncogenic mutants.

Sequence Analyses of RpSVs from Viral Tumors. To determine whether tumorigenicity of proto-src viruses was genuine or due to mutations of proto-src, the proto-src sequences of RpSVs recovered from tumors were analyzed. Because mutation of the proto-src-specific carboxyl terminus is the most effective enhancer of transforming function of a proto-src coding region under a retroviral promoter (3, 13), we investigated whether the sequence of this region had been mutated in viruses recovered from tumors.

Viruses were recovered from the sarcomas of six chickens inoculated with RpSV. The sarcomas were washed with 75% ethanol, excised, and dissected with scalpels. The minced tissue was washed three to five times by vigorous stirring with 10 vol of buffered saline at 0–4°C to eliminate blood cells. Subsequently, the tissue was stirred with trypsin at 36°C and single cells were decanted about three times at 20-min intervals and propagated in 10-cm Petri dishes as described (3). The growth media were then collected for 1–2 weeks and the viruses were purified by sucrose gradient ultracentrifugation (3). The viral RNAs were then transcribed in vitro to cDNA by viral reverse transcriptase (Materials and Methods). A nucleotide sequence of the cDNA from six RpSVs recovered from sarcomas from a position corresponding to nucleotide 8598 in the src gene of RSV to position 8925, which is located downstream of the src coding region of RSV (1), was then amplified by the PCR with specific primers and analyzed for its size and sequence (Materials and Methods). In parallel, the cDNA of wild-type RSV and the cloned DNA of RpSV were amplified with the same primers.

The sequence between our primers is expected to measure 395 nucleotides for intact, unmutated RpSV and 328 nucleotides for RSV (Fig. 1). As can be seen in Fig. 3, all RpSV src DNAs had the same, expected size of about 395 nucleotides as the RpSV control, indicating that their proto-src carboxyl termini were not mutated with regard to their size. The RSV 3′ terminal src sequence also had the predicted size (Fig. 3). Next we asked whether the 3′ terminal proto-src coding region, particularly the carboxyl-terminal Tyr codon 527 (13), had been mutated in any of the tumorigenic RpSVs. For this purpose most of the sequences amplified by the above

![Fig. 2. Sarcoma induced by injecting RpSV into a 1-day-old chicken. The supernatant medium from cultured quail cells transformed by RpSV was collected and virus particles were purified by sucrose gradient ultracentrifugation. About 50–150 μl of purified virus was injected into the wing web of a 1-day-old chicken. The sarcoma shown above became detectable 12 days after injection and was photographed 3 weeks after the injection.](image-url)

![Fig. 3. The 3′ terminal proto-src coding regions of RpSVs recovered from six viral tumors. Viruses from sarcomas of chickens injected with RpSV were purified by sucrose gradient ultracentrifugation. An in vitro reverse transcriptase reaction was carried out to make cDNA. Two primers flanking the 3′ terminal proto-src sequence of RpSVs were used to amplify the sequence in PCR reactions (see text and Fig. 2). The DNA products were analyzed by electrophoresis in a 1.7% agarose gel containing 0.5 μg of ethidium bromide per ml. Lanes 1–6, cDNAs from RpSVs recovered from six separate chicken sarcomas; lane 7, cDNA from RpSV amplified with the same primers as for RpSV (see text); lane 8, DNA from the plasmid containing the provirus of RpSV amplified as above; lane 9, DNA size markers (in nucleotides) of phage φX174 replicative form DNA digested with Hae III.](image-url)
primers—e.g., 292 nucleotides from a Pst I site at position 8662 to a Mst II site at position 8884 (see Fig. 1)—were cloned into the polylinker of a PUC plasmid (Pharmacia pSL1180) and analyzed. As can be seen in Fig. 4, the 3' terminal sequences of three RpSVs from three different tumors were unchanged in their coding regions, each carrying the native Tyr in codons 527. It is concluded that the unmutated proto-src coding region of the cell is tumorigenic in a RSV-derived retroviral vector.

DISCUSSION

Retroviral Promoter Sufficient to Convert Proto-src Genes to Cancer Genes. Our experiments have demonstrated that enhanced expression of the normal proto-src coding region from a RSV-derived retroviral promoter is sufficient for tumorigenicity. Further, we have demonstrated that tumorigenicity of the retrovirus-promoted proto-src protein is enhanced by RSV-specific src point mutations other than that that alters Tyr codon 527. These experiments confirm and extend studies with other proto-onc genes, which have demonstrated that about 100-fold increased expression from retrovirus promoters is sufficient for transforming function of diploid cells and tumorigenicity. Examples include retrovirus-promoted coding regions of proto-ras (3, 16, 18, 20, 21) and proto-myc (17, 19, 29). Thus, our experiments support the hypothesis that retroviral onc genes derive transforming function from strong viral promoters rather than from point mutations that affect the coding sequence of native proto-onc genes (2, 11). Such mutations may merely enhance the transforming function derived from retroviral promoters.

Retrovirus Model Casts Doubts on the Somatic Gene-Mutation Hypothesis of Cancer. Retroviral onc genes provide as yet the only proven examples that mutated cellular genes can cause cancer. Therefore they are considered the most critical support for the hypothesis that mutation of cellular genes causes cancer (8, 30–32). The hypothesis that mutated cellular genes cause cancer is based on two kinds of circumstantial evidence: (i) many carcinogens are mutagens in animals (30) or bacteria (33, 34) and (ii) the coding regions of the proto-onc genes of some cancers sometimes have mutations that are similar to those of the coding regions of related retroviral onc genes (1, 4–9, 35–37). However, our results cast doubt on the hypothesis that mutations do not enhance transcription like retroviral promoters can “activate” proto-onc genes to cancer genes for several reasons.

(i) Since retroviral onc genes derive transforming function from strong retroviral promoters rather than from mutations in the coding region, there are no precedents or models for how mutations that do not enhance transcription would convert proto-onc genes to cancer genes. Indeed, none of the mutated proto-onc genes observed in cancer cells is transcriptionally activated like retroviral onc genes (11).

(ii) The probability of the mutations that are postulated to convert proto-onc genes to cancer genes, such as point mutations and truncations, is much higher than the incidence of cancer. For example, the probability of a point mutation per nucleotide per mitosis is about 1 in 106 (2, 11, 30, 34). Given this point mutation rate and 109 nucleotides per human DNA (38), a cell with any possible point mutation of the human genome would be found in 109 cells. Since the human body consists of more than 1014 cells (2, 30), this corresponds to a minimum of over 100,000 hypothetical cancer cells (10^14 * 10^9 = 10^23) per human body at any given time. Considering that many proto-onc genes are said to be activated by point mutation and some like proto-ras even by >50 different point mutations (7, 16, 39), the number of cancer cells per human body would be correspondingly higher.

It is possible, however, to reconcile numerically the high probability of such mutations with the low probability of cancer by assuming cooperations of multiple mutated proto-onc genes and anti-oncogenes in the same cell. This has been done in the case of colon cancer (10, 35, 40) and some other tumors (10, 11, 36). However, these ad hoc assumptions create new unresolved problems. They assume that mutated proto-onc genes, as, for example, point-mutated proto-ras in colon cancer, cause cancer by second- and higher-order mechanisms of carcinogenesis, although all known retroviral onc genes, including the retroviral ras genes, are first-order carcinogens that are sufficient for carcinogenesis (2, 11).

(iii) Nothing would be evolutionarily more implausible for a multicellular organism than a battery of cellular oncogenes that can each be activated to cancer genes by point mutations and other mutations, because just a single cancer cell is sufficient to initiate a clonal cancer (2, 30). Moreover, it would be unlikely that such common mutations as point mutations and substitutions could activate genes that have been optimized over billions of years of evolution. On the contrary, mutations are typically silent or inactivating (11, 41, 42).

(iv) There is no functional proof for cellular cancer genes. (a) Mutated or “activated” proto-onc genes from tumors do not transform normal diploid cells upon transfection or in transgenic animals (10, 11). According to Stanbridge (10), “...despite intensive efforts to transform normal human fibroblasts or epithelial cells with varying combinations of

![Fig. 4](image_url)

The 3' terminal proto-src sequences of three RpSVs recovered from chicken sarcomas. The sequences of the RpSV DNAs L2, L4, and L6 correspond to the DNAs shown in lanes 2, 4, and 6 in Fig. 3. The sequence from the original RpSV is included for comparison (1). Dots indicate identity. The Tyr codon 527 (TAG) and the stop codon (TAG) of proto-src are capitalized and highlighted. The sequences were determined by Lark Sequencing Technologies (Houston).
activated cellular oncogenes, the results have been uniformly negative." Even combinations of mutated proto-onc genes have failed to transform cells unless linked to viral promoters (11, 21). By contrast, proviral DNA from oncogenic retroviruses transforms diploid cells like retroviruses (1, 11). (b) Mutated proto-onc genes of tumors do not determine the character of a given type of tumor compared to one without such a gene (11, 35, 43). By contrast, retroviral oncogenes determine many characters of the resulting tumors (1, 44).

It appears that the somatic gene-mutation hypothesis of cancer is numerically and evolutionarily implausible and functionally unconfirmed. Similar conclusions were reached by Rous (45, 46) and Ruben (47) after studying oncogenic viruses and cancer for over 50 and 30 years, respectively. Rous concluded, "A favorite explanation has been that oncogenes [Rous' term for carcinogens] cause alterations in the genes of the ordinary cells of the body . . . somatic mutations as these are termed. But numerous facts, when taken together, decisively exclude this supposition" (46) and, "A hypothesis is best known by its fruits. What have been those of the somatic mutation hypothesis? . . . It acts as a tranquilizer on those who believe in it, and this at a time when every worker should feel goaded now and again by his ignorance of what cancer is" (45). Likewise, Cairns (48) . . . suggests that most human cancers are not caused by conventional mutagens . . . ." It is conceivable, therefore, that the gene-mutation hypothesis of cancer is limited to the genesis of retroviral onc genes from the coding regions of cellular proto-onc genes and retroviral promoters. Indeed, retroviruses have evolved dominant promoters to override cellular controls and thus have the real potential to activate genes by promoter substitution via rare illegitimate recombination (2). There is however no evidence for cellular promoters that could activate heterologous cellular genes to retrovirus-like transforming genes. An alternative hypothesis suggests that nonviral cancers are not caused by mutation of singular genes but are the product of gross genetic imbalances of normal genes due to chromosome abnormalities (11), as originally proposed in 1914 by Boveri (49).

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