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Perspectives in Cancer Research

Evolution of Cancer Genes as a Mutation-driven Process[1]

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Cancers are the result of somatic heritable changes in certain genes. It is generally accepted, although there are some dissenters, that we are beginning to isolate and characterize some of these genes, both dominant oncogenes and recessive oncogenes (also called antioncogenes and suppressor genes) (1–6). In addition, some of the genetic processes involved in oncogenesis are beginning to be specified, in particular the role of chromosomal translocations and gene amplifications.

It is also generally accepted that cancer is the result of an accumulation of several genetic changes (7, 8), although oncogenesis by highly oncogenic retroviruses appears to be an exception. I have previously argued that the difference in kinetics between oncogenesis by highly oncogenic retroviruses (single hit) and oncogenesis by other carcinogenic agents (multihit) is apparent rather than real (9, 10) and that oncogenesis by highly oncogenic retroviruses is pseudo-single hit, not really single hit, because there has been an accumulation of several genetic changes during the evolution of a highly oncogenic retrovirus. Thus, the single step of infection of a normal cell by a highly oncogenic retrovirus results in a cell incorporating a virus genome that contains several genetic changes.

In this article I describe the evolution of a particular highly oncogenic retrovirus, highlighting the different genetic changes involved in this evolution to test whether oncogenesis is wholly selection-driven or partially mutation-driven. I propose that the stages in the evolution of highly oncogenic retroviruses are analogous to stages in oncogenesis by other carcinogens. Since cancer is primarily a heritable somatic disease, I am concerned with asexual replication rather than sexual reproduction in discussing carcinogenesis.

Selection-driven and Mutation-driven Processes

The usual way to look at an evolutionary process is to propose that there is natural selection among spontaneous variants appearing in a population which results in an increase in the number of the fittest progeny. The rate of mutations resulting in the appearance of new variants is low. Thus, each variant differs in only one gene from its parent. In a selection-driven process there is selection among individuals in a population in which variants differ by one mutation from the parents.

In a mutation-driven process, by contrast, the rate of mutation is higher such that a variant cell or organism can accumulate multiple mutations before a change in fitness is achieved. Thus, selection is operative on cells or organisms with more than one mutation (Fig. 1).

If oncogenesis is a purely selection-driven process, then each step in oncogenesis confers a selective advantage on the precancerous cells (Fig. 2). For example, the precancerous cells may be able to multiply more than cells that are not precancerous or the precancerous cells may be less susceptible to differentiation. By contrast, if oncogenesis is a purely mutation-driven process in which each precancerous mutation does not result in a selective advantage of the precancerous cells, then only a high rate of mutation will allow accumulation of several precancerous mutations in one cell (Fig. 1).

Transformation by Highly Oncogenic Retroviruses

Highly oncogenic retroviruses are the most rapid and efficient oncogenic agents known (11). Thus, they provide a good experimental system to study oncogenesis. In addition, the presence of activated oncogenes in some human tumors suggests that there are common mechanisms for oncogenesis by highly oncogenic retroviruses and for oncogenesis by other carcinogens (4).

Highly oncogenic retroviruses are rare representatives of the oncovirus subfamily of retroviruses (12). Retroviruses are RNA-containing viruses that replicate through a DNA intermediate. Replication-competent retroviruses contain controlling sequences at the ends of their genomes and coding sequences for the viral proteins *gag*, *pol*, and *env* in the internal portions of their genomes. Highly oncogenic retroviruses differ from other retroviruses in containing other coding sequences substituted for (or, in the case of Rous sarcoma virus, in addition to) *gag*, *pol*, and *env*. Thus, they usually are defective. These other coding sequences are derived from normal cellular genes, protooncogenes. The other sequences in highly oncogenic retroviruses, which are often found fused to viral coding sequences, are called oncogenes.

Oncogenes code for proteins that transform susceptible cells. Thus, transformation of cells by highly oncogenic retroviruses is the result of the introduction into a susceptible cell by virus infection of a gene coding for a transforming protein and driven by retrovirus control sequences (Fig. 3).

Highly oncogenic retroviruses share nucleotide sequences both with related replication-competent retroviruses and with cellular protooncogenes. Comparison of the nucleotide sequence of a highly oncogenic retrovirus with the nucleotide sequences of its two progenitors allows definition of the changes that occurred during the evolution of the highly oncogenic retrovirus. For illustration, I shall describe these changes for a virus being studied in my laboratory, reticuloendotheliosis virus strain T. However, similar descriptions could be given for other highly oncogenic retroviruses.

Evolution of a Highly Oncogenic Retrovirus

Reticuloendotheliosis virus strain T is a highly oncogenic retrovirus originally isolated from a turkey and able to cause rapid lethal lymphomas in young chickens. Comparing Rev-T[3] to the related replication-competent reticuloendotheliosis virus strain A, we see that Rev-T has a large deletion and a large

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[3] The abbreviations used are: Rev-T, reticuloendotheliosis virus strain T; Rev-A, reticuloendotheliosis virus strain A.

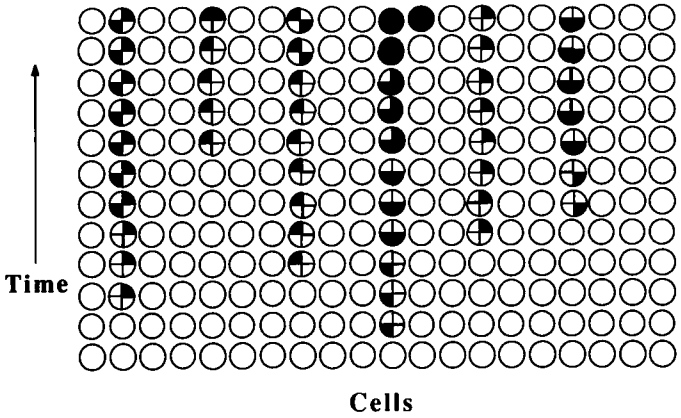

Fig. 1. Mutation-driven process. A cohort of cells is shown moving through time. (For clarity, no increase in cell number is shown.) Mutant cells have filled in quadrants. A cancer cell has completely filled in quadrants. In this figure it is assumed that only the cell with completely filled in quadrants has a growth advantage and that the mutation rate is higher than in Fig. 2.

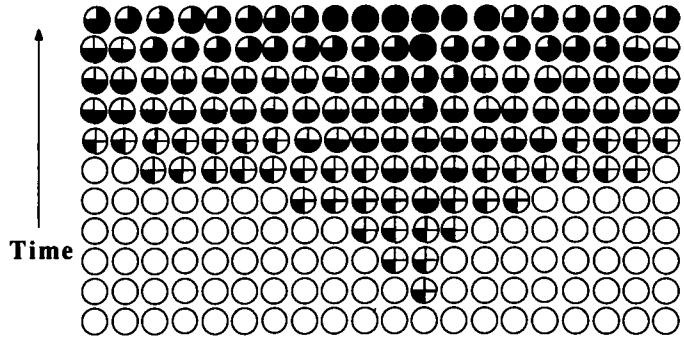

Fig. 2. Selection-driven process. A cohort of cells is shown moving through time. (For clarity no increase in cell number is shown.) Mutant cells have filled in quadrants. A cancer cell has completely filled in quadrants. Each mutant cell has a growth advantage over the less mutated cells. Thus, mutant cells increase in relative frequency and are more likely to suffer further mutations. For clarity only a single lineage is shown, and it is symmetrically placed in the figure.

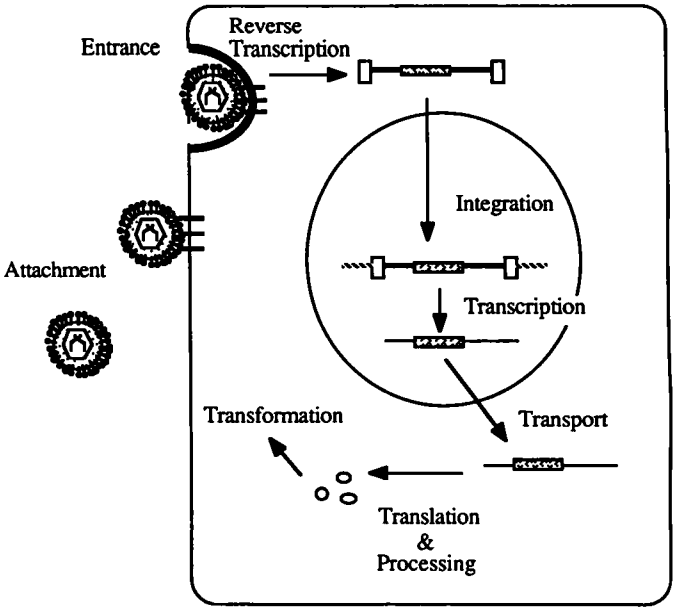

Fig. 3. Transformation by a highly oncogenic retrovirus. The life cycle of a highly oncogenic retrovirus in one cell is shown. The virus is defective for replication. The virion attaches to specific receptors (*three lines* in cell). After reverse transcription, unintegrated viral DNA is formed. *Open boxes*, long terminal repeats; *heavy lines*, internal virus sequences; *speckled bar*, oncogene sequences. The viral DNA is integrated into cellular DNA (*dashed lines* attached to viral DNA). Viral DNA is transcribed to viral RNA (*thinner line*) which is translated to form oncoproteins (open ellipses) which mediate transformation.

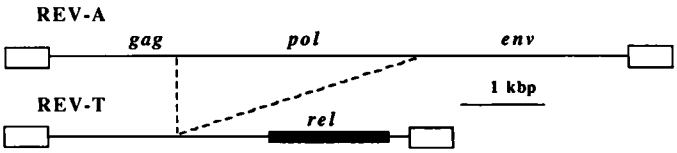

Fig. 4. Maps of Rev-A and Rev-T. DNA genomes of Rev-A and Rev-T are shown. *Open boxes*, long terminal repeats; *filled box*, *rel* oncogene; *dashed lines* delimit deletion of sequences in Rev-T; *gag*, *pol*, and *env* are coding sequences of Rev-A. *kbp*, kilobase pair.

substitution (Fig. 4) (13). Nucleotide sequence analysis indicates that the deletion is the result of two separate deletion events and that the substitution resulted in an open reading frame composed of the amino terminus of *env* with three mutated codons, the central portion of the turkey c-*rel* protooncogene with 12 mutant codons, and some out-of-frame *env* sequences (14).

Recombinational analyses were carried out to determine which of these genetic differences are important to the transforming ability of Rev-T. To this end various recombinants were made between the *rel* oncogene in a retrovirus vector and c-*rel* sequences, and the resulting proteins were tested for their transforming abilities (15). The results of these experiments indicated that the amino-terminal *env*-c-*rel* fusion was necessary for the transforming ability of Rev-T. In contrast, the carboxyl-terminal gene fusion was only important in allowing virus replication, because a protein with c-*rel* carboxyl coding sequences was transforming. Further 3′ c-*rel* noncoding sequences prevented viral replication. Thus, a recombinant virus expressing a *rel* gene with carboxyl terminal c-*rel* coding sequences was able to transform spleen cells. Other experiments indicated that some of the mutations in the internal c-*rel* sequences were also necessary for the transforming ability of the rel protein.

In addition, recombinants between Rev-T and Rev-A established that the *gag-pol* deletions in Rev-T were also necessary for its transforming ability (16). In this case the deletions were necessary to obtain the high expression of the rel product needed for transformation (17). Thus, both qualitative and quantitative changes in Rev-A and c-*rel* were necessary to form the transforming virus, Rev-T.

Other experiments demonstrated that high levels of expression of the rel protein in most cells did not result in transformation (18). Only when the rel protein was expressed at a sufficiently high level in susceptible lymphoid stem cells did transformation occur.

With this information we can propose possible evolutionary pathways for the formation of a transforming Rev-T (Fig. 5). The requirement for the *gag-pol* deletion suggests that these sequences were deleted in the original parental virus. (Otherwise there would not be enough expression of the rel protein to cause transformation.) Integration of a deleted virus 5′ of c-*rel* and in the same orientation as c-*rel* transcription would have occurred by chance at some low frequency. Readthrough transcription would have occurred. However, only an abnormally spliced RNA derived from readthrough transcription would have formed a chimeric rel protein. Such an abnormally spliced mRNA would then have to be packaged in viral proteins and recombined with wild-type virus to form an RNA with viral 3′ sequences necessary for integration and reverse transcription. The resulting provirus would then be a doubly recombinant genome that could express the rel protein at a high level.

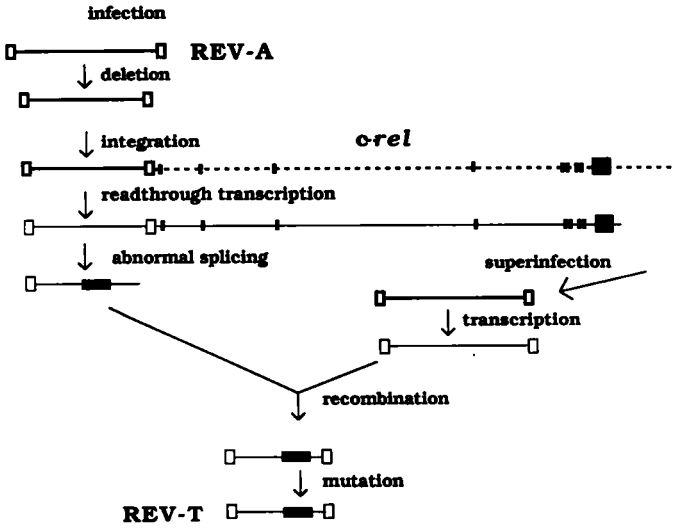

Fig. 5. Possible pathway for the evolution of Rev-T from a replication-competent retrovirus and c-*rel*. The different steps postulated to occur in the evolution of Rev-T are shown. *Dotted line* with *small filled boxes* across it, c-*rel* sequences; *small filled boxes*, c-*rel* exons. Other *symbols* are as described in the legends of Figs. 3 and 4. Since Rev-T is defective for replication, it would be selected only if it was transforming.

However, such a chimeric rel protein would only be transforming after the occurrence of some of the internal amino acid mutations found in v-*rel* and when expressed in a lymphoid stem cell.

Thus, only at the end of all of these processes would a transforming virus have appeared, and then transformation could only have occurred in rare cells. The processes involved in this evolutionary pathway were deletion, integration in a specific region, readthrough transcription, abnormal splicing, packaging of a chimeric viral-cellular mRNA, recombination with a superinfecting virus, and base pair mutation followed by infection of a primitive lymphoid stem cell. (The alternative of mutation of a provirus in an infected primitive lymphoid cell is considered unlikely, because of the low rate of mutation of cellular genes.) Since the intermediate infected cells or viruses were not transforming, Rev-T would have evolved only if the rate of occurrence of all of these processes was very high.

Rate of Variation in Retrovirus Replication

Integration of a retrovirus 5′ of a particular cellular gene and in the same orientation would be expected by chance in approximately 1 per 10^5 infections assuming random integrations. The rate of integration in a specific region would be higher if integration was not random and if the integration site was one of the preferred ones, *i.e.*, had an open chromatin configuration (19, 20).

Herman and Coffin (21) reported that readthrough transcripts comprise 16% of the viral RNA of wild-type Rous sarcoma virus. In avian leukosis virus-induced erythroblastosis, an abnormally spliced mRNA involving viral and c-*erb*B sequences is frequently present (22). However, the probability of formation of such an abnormally spliced RNA is not known. It probably is low. Packaging of such a chimeric mRNA in a retrovirus particle would be efficient when the RNA contains encapsidation sequences and the chimeric RNA is not over 10 kilobases (23, 24). Since the deleted virus does not encode all virion proteins, packaging of the chimeric RNA depends upon the presence of viral proteins produced after superinfection by a replication-competent retrovirus. If the original animal was viremic, such superinfection might have occurred at a high frequency since the provirus in the cell is defective; therefore, there may not have been interference with superinfection. Heterozygous particles would be formed with the chimeric viral-cellular mRNA and wild type viral RNA. Recombination of genomes in a heterozygous retrovirus particle occurs at high frequency (25). Thus, none of these steps appears to have been limiting for the evolution of Rev-T.

Rate of Mutation in Retrovirus Replication

To measure the rates of deletions and of base pair mutations, the other steps in Rev-T evolution, a protocol was developed using retrovirus vectors and helper cells to isolate a single round of retrovirus replication (Fig. 6) (26). A single round of retrovirus replication can be represented as either virus-provirus-virus or provirus-virus-provirus. The same nucleic acid polymerization steps occur in both formulations. In the single round of retrovirus replication protocol, stocks of a helper virus-free retrovirus vector are used to infect helper cells, and clones of helper cells containing parental test proviruses are selected. Virus from these clones (helper virus free) is then used to infect test cells where the total number of viruses and the number of mutant viruses can be determined. Since there has been only a single round of virus replication, the frequency of mutant viruses over the total number of viruses gives the mutation rate.

A mutational analysis was carried out with a splicing vector with two genes, only one of which could be expressed (Fig. 7, *top*). The expression of the other gene was prevented because steady state accumulation of spliced viral RNA was very low, probably because of an inhibition of splicing. Mutant virus was defined as being able to express the blocked gene. The rate of this mutation was 5×10^{-3} per replication cycle (26). The mutations included both large deletions in the intron and small deletions or insertions near the 3′ splice site. [This rate is much higher than even the high rate of deletions seen in the immunoglobulin gene (27).] Thus, approximately 0.5% of proviruses formed in infected cells would have a deletion or another mutation allowing the high levels of mRNA needed for a transforming virus.

To determine the rate of base pair substitutions, a splicing vector able to express two genes at equal efficiencies was constructed where one gene had a stop codon preventing formation of an active protein (Fig. 7, *bottom*). In addition, the presence

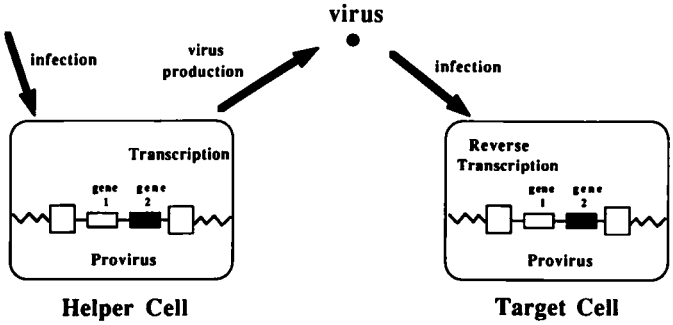

Fig. 6. Protocol for measurement of retrovirus mutation rates. A retrovirus helper cell supplies viral proteins in *trans* but cannot by itself produce virus. When such a helper cell is infected by a helper-virus-free vector (produced from another helper cell), a provirus is formed. RNA transcripts from that provirus will be packaged in viral proteins, and the resulting virus can infect and form a provirus in a test cell. The vector used to measure the rate of deletions expresses only gene 1 unless a mutation allows expression of gene 2 (Fig. 7, *top*). The vector used to measure the rate of base pair substitutions can express both genes 1 and 2, but gene 1 has an amber codon in its coding sequence (Fig. 7, *bottom*).

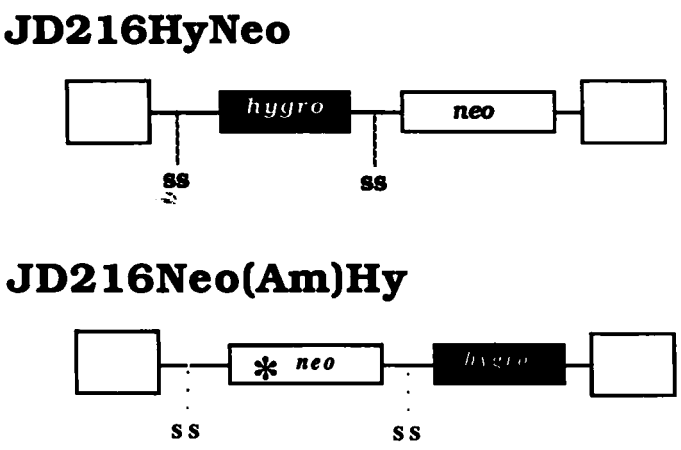

Fig. 7. Retrovirus vectors used for determination of mutation rates. The vector on the top contains active coding sequences for hygromycin resistance and for neomycin resistance. However, the neomycin resistance is not expressed. The vector on the bottom contains an active hygromycin resistance gene and a neomycin resistance gene containing an amber codon in its coding sequence (*) (28). *Symbols* are as described above.

or absence of this amber codon could be determined directly by a restriction enzyme cleavage site polymorphism (28). The rate of mutation of this amber codon was 2×10^{-5} per site per round of replication.[4] [This rate is much higher than that for cellular genes, which is estimated to be less than 10^{-9} per site per generation, but it is similar to the rate of hypermutation found in immunoglobulin genes (29).] Since a retrovirus has about 10^4 nucleotides in its genome, approximately 50% of the virus genomes will have one base pair substitution. Thus, in the postulated evolution of Rev-T, mutant transforming virus would have appeared soon after unselected replication of a deleted virus expressing a chimeric protein.

Therefore, it appears that the high rate of retrovirus mutation allows accumulation of several mutations in an unselected virus, resulting in the formation of a highly oncogenic retrovirus with the several genetic changes necessary for transformation. In other words, this evolution was mutation-driven.

Other Highly Oncogenic Retroviruses

Most other highly oncogenic retroviruses differ in several respects from their replication-competent virus and homologous protooncogene parents, suggesting that their evolution was also mutation-driven. For example, it has been proposed that Rous sarcoma virus was originally formed as a replication-defective virus with several changes in *src* (30, 31). Similarly murine sarcoma viruses with *fos* require several amino acid changes in the *fos* coding sequences and a large deletion of virus sequences to make a transforming virus (32).

Generality of Mutation-driven Models of Oncogenesis

Other Retrovirus-induced Cancers. There are several mechanisms other than introduction of an active oncogene by which retroviruses induce cancer: addition of an altered envelope protein followed by further change(s) in the infected cells (Friend murine leukemia virus) (11); insertional activation of a protooncogene followed by other change(s) in the infected cells (weakly transforming retroviruses) (11); transactivation of a cellular protooncogene followed by other changes in the infected cells (human T-cell leukemia virus 1 and bovine leukemia virus) (33, 34); destruction of the host immune system followed by carcinogenic changes in other cells (human immunodeficiency virus) (35); and stimulation of the host immune system followed by carcinogenic changes in other cells (possibly human T-cell leukemia virus 1 and murine leukemia virus) (36).

In some of these mechanisms there is selection for intermediate precancerous cells. For example, Friend murine leukemia virus-infected cells become hyperplastic and form spleen foci. However, the further changes in all of these mechanisms include steps similar to those involved in nonretroviral cancers.

Nonretroviral Cancers. The hypothesis of mutation-driven oncogenesis requires that there be a high mutation rate in precancerous cells and that some precancerous cells have no selective advantage over their parental cells (37). It is clear that most chemical and radiation carcinogens are mutagenic (38, 39). Therefore, the presence of these agents increases the mutation rate in precancerous cells and the frequency of cancer, indicating that the frequency of cancer is limited by mutation rate. An increased mutation rate in precancerous cells would be perpetuated if some mutations resulted in the cells having a higher mutation rate. Examples of possible genetic changes resulting in a higher mutation rate are formation of an error-prone DNA polymerase or a mutant repair system, increase in efficiency of activation of mutagens, and decrease in efficiency of inactivation of mutagens (40).

Is There Selection for Precancerous Cells? It is clear that at least some precancerous cells do not have a selective advantage over cells with a parental genome. One line of evidence comes from people carrying genes for susceptibility to cancer (hereditary retinoblastoma or Wilms' tumor mutation carriers) and mice transgenic for activated oncogenes (41–46). Although cancer appears more frequently in organisms carrying these genes than in wild type organisms, the mutant retinoblastoma or Wilms' tumor gene carriers and the transgenic mice develop normally, except for a higher probability of developing cancer. Thus, although all of their cells contain precancerous changes, these cells do not appear to behave differently than normal cells. In these cases and in the case of cells infected with precursors of highly oncogenic retroviruses, there does not appear to be selection for precancerous cells.

Summary and Conclusions

Cancer is primarily a somatic genetic disease resulting from the accumulation of several precancerous mutations in a cell lineage. The evolution of highly oncogenic retroviruses has been used as a model for the evolution of a cancer cell. The properties of intermediates between one set of replication-competent retrovirus and protooncogene progenitors and the homologous highly oncogenic retrovirus were analyzed to differentiate between selection-driven and mutation-driven models of this evolution. In this case and in some other cases where sufficient data are available, it appears that the intermediates in the evolution of highly oncogenic retroviruses are not transforming, indicating that they were not formed in a purely selection-driven process. Furthermore, analysis of retrovirus mutation rates indicates that there is a high rate of mutation in retrovirus replication such that the evolution of highly oncogenic retroviruses could be mutation-driven. Other evidence is mentioned suggesting that oncogenesis in general is at least partially mutation-driven, although mutational mechanisms are involved that are different from those involved in the evolution of highly oncogenic retroviruses.

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[4] J. P. Dougherty and H. M. Temin, manuscript submitted for publication.

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