Genes Responsible for Transformation by Avian RNA Tumor Viruses

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Summary

Avian sarcoma viruses probably contain 4 genes. One of these, src, controls the process of transformation of the infected cell and has been located near the 3' end of the viral genome. The remaining 3 genes direct the synthesis of virion proteins: env (envelope proteins), pol (polymerase), and gag (internal proteins). They are probably in the order gag-poly-env-src. A scheme for the replication of these viruses is proposed, and several possible mechanisms for their origin and variability are discussed.

Introduction

A major research effort in recent years has led to significant advances in our understanding of the structure, genetics, and mechanisms of replication of tumor viruses. Nevertheless, the mechanisms by which viruses cause tumors in animals and transform cells in culture remain obscure. Study of the phenotype of transformed cells has turned up an embarrassingly large number of changes, including: ability to form tumors, altered morphology, altered response to growth factors, loss of anchorage dependence for growth, increased agglutinability by lectins, loss of cell surface glycoproteins, production of plasminogen activator, increased sugar uptake, appearance of new (tumor) antigens, and appearance of embryonic antigens. None of these has yet been identified as the primary event of transformation, i.e., that specified directly by a viral gene. Another approach is to study the molecular biology of the virus itself in the hope of learning enough about virus genes coding for cell transformation (their structure, origin, and relationship to viral replication) to be able to make an educated guess as to what the viral gene product responsible for cell transformation might be.

Work along these latter lines with papovaviruses has shown that transformation is probably a side effect of an aborted replication cycle in a nonpermissive cell. In such cells late viral functions are not expressed (14), and the continued presence of an early gene product in cells with an integrated viral genome, perhaps the one responsible for the induction of cell DNA synthesis (25), is believed to lead to cell transformation.

As we shall see later, the situation with RNA tumor viruses is rather different. In the remainder of this paper I shall discuss genes for transformation in avian RNA tumor viruses and, largely for lack of recent data of my own, advance some suggestions as to how these viruses might replicate and how this replication scheme might be involved in the origin of genes for transformation.

Types of Genes for Transformation

Elaborating on a classification proposed by Temin (21) a few years ago, we can divide avian RNA tumor viruses into 4 groups, depending on their transforming interactions with host cells in culture and the types of tumors they induce in animals (Table 1). The strongly transforming viruses include all strains of RSV, both those derived from Rous's original isolate and relatively recent isolates. All of these viruses transform fibroblast in culture and cause sarcomas in animals. These viruses are the best known of all RNA tumor viruses on a molecular level, and most of the remaining discussion will be devoted to them. The 2nd group consists of viruses that rapidly (within 2 weeks) induce leukemias and similar diseases. Some of these (such as MC29 virus) also transform fibroblasts in culture; others (such as avian myeloblastosis virus) do not transform fibroblasts but do transform cultured yolk sac cells (16). The 3rd group consists of viruses that are not known to transform any type of cell in culture but induce leukemias with a latent period of about 2 years (6). This group contains the RAV of Subgroups A to D and, possibly, transformation-defective strains of RSV (3). Comparison of the weakly transforming and nontransforming groups suggests a qualitative difference in the nature of the transforming event. It is intriguing to speculate that the weakly transforming viruses contain a specific gene responsible for transformation and leukogenesis and that the nontransforming viruses do not; however, genetic evidence to support this idea is lacking. The 4th group neither transforms cells in culture nor causes disease in chickens (8, 17). It contains only 1 known member, designated RAV-0. RAV-0 is probably the only endogenous virus of chickens. It is produced spontaneously by some lines of chickens (24) and can be induced by bromodoxyuridine from other lines (18). All chickens apparently carry the complete information for this virus in their DNA (27).

There are 3 lines of evidence which support the conclusion that strongly transforming viruses contain a gene
specifically responsible for transforming cells in culture. All the evidence is based on mutations that affect the transforming ability of RSV but not the replication of the virus. Their properties are outlined in Table 2. For reasons which will become apparent in the next section, the transformation-defective mutants are particularly useful. These mutants arise so frequently from some strains of RSV that it is often quite difficult to grow stocks of RSV containing non-defective virus as the majority component. Studies by polyacrylamide-gel electrophoresis show that the RNA of transformation-defective mutants is 10 to 15% shorter than that of nondefective viruses (23), indicating that these mutations arise by a deletion of some of the RNA of the parent.

The transformation-defective and temperature-sensitive mutants shown in Table 2 define a region of the genome (loosely, a gene) which has been called, most recently, src (pronounced “sarc”) and, formerly, onc (1). Since none of the many src mutations so far isolated complement one another (28), there is probably only 1 gene product involved. This gene product controls all the characteristics of transformed cells listed in the Introduction, so that cells infected with transformation-defective or temperature-sensitive mutants at the nonpermissive temperature behave like uninfected cells in all respects, except that they produce infectious virus.

Structure of the RSV Genome

The existence of deletion and temperature-sensitive mutants has made it possible to develop a partial map, physically locating specific coding regions on the RSV genome. The detailed procedures for physical mapping of tumor RNA's have been described elsewhere (7). The procedure is based on the use of RNase T1 (which cleaves RNA next to G residues) to generate a set of fragments from the virus genome. Since the genome is 10,000 nucleotides long (2, 4) and about 25% G, there will be 2,500 oligonucleotide products. A few of these will, by chance, be very long and should appear only once in the genome. They can thus be used as physical markers for a region. These unique oligonucleotides can be separated from the rest by 2-dimensional gel electrophoresis (4). Fig. 1 shows fingerprints of Pr-RSV-B and a transformation-defective mutant of Pr-RSV-B generated by this procedure. It can readily be seen that the pattern of large oligonucleotides (the isolated spots toward the bottom) is identical in these 2 viruses with the exception of Spots 5 and 6 (Fig. 1A, arrows). These 2 spots thus contain 3 oligonucleotides (6 is a mixture of 2) present within the region deleted in the transformation-defective variant. Clearly, physical mapping of these spots should locate the src gene on the genome.

Such mapping is accomplished as outlined in Chart 1. 3H-labeled virus RNA is partially fragmented and passed through polyuridylate (poly(U))-Sephadex. The bound material is eluted, mixed with complete 32P-labeled RNA, and fingerprinted following digestion with RNase T1. The yield of 3P and 3H in each large oligonucleotide relative to a control mixture of unfragmented RNA is determined and given the relative position of each along the genome.

Table 1

<table>
<thead>
<tr>
<th>Type</th>
<th>Transformation in vitro</th>
<th>Disease</th>
<th>&quot;Transforming&quot; gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Strongly transforming</td>
<td>+</td>
<td>Sarcoma</td>
<td>+</td>
</tr>
<tr>
<td>2. Weakly transforming</td>
<td>±</td>
<td>Leukemia (rapid)</td>
<td>+</td>
</tr>
<tr>
<td>3. Nontransforming</td>
<td>−</td>
<td>Leukemia (slow)</td>
<td>−</td>
</tr>
<tr>
<td>4. Endogenous</td>
<td>−</td>
<td>None</td>
<td>−</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Description</th>
<th>Characterization</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transformation-type variants</td>
<td>Round or fusiform</td>
<td>Mutations in both</td>
</tr>
<tr>
<td></td>
<td>transformed cells</td>
<td>directions common;</td>
</tr>
<tr>
<td>(20)</td>
<td></td>
<td>replication unaffected</td>
</tr>
<tr>
<td>Temperature-sensitive mutants</td>
<td>Cells transformed at</td>
<td>Replicate equally</td>
</tr>
<tr>
<td>(15)</td>
<td>35°, not at 41°</td>
<td>well</td>
</tr>
<tr>
<td>Deletion mutants</td>
<td>Do not transform</td>
<td>RNA 10-15% shorter;</td>
</tr>
<tr>
<td>(23)</td>
<td></td>
<td>cause leukemias;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>replication unaffected</td>
</tr>
</tbody>
</table>

Chart 1. Method of mapping large T1 oligonucleotides on a poly(A)-containing RNA. 3H-labeled RNA is partially fragmented with alkali and passed through polyuridylate (poly(U))-Sephadex. The bound material is eluted, mixed with complete 32P-labeled RNA, and fingerprinted following digestion with RNase T1. The yield of 3P and 3H in each large oligonucleotide relative to a control mixture of unfragmented RNA is determined and given the relative position of each along the genome.
end having the highest yields. Chart 2 shows the results of such an experiment. The oligonucleotides are arranged in their most probable order. The order is probably not exact; some of the differences in yield between adjacent oligonucleotides are very small. This map locates oligonucleotides 5 and 6 and, therefore, src, nearest the 3' end of the RNA.

Besides src, there are probably 3 other genes in the RSV genome: env, which codes for virion surface glycoproteins and specifies the host range of the virus; pol, which codes for the virion RNA-directed DNA polymerase; and gag, which codes for the internal structural proteins ("group-specific antigens") of the virion. These have been partially mapped by fingerprint analysis of recombinants involving host range (12) or temperature-sensitive pol mutations (13). Env maps in the region of oligonucleotides 1 to 20 and temperature-sensitive pol mutations between 24 and 3. Gag has not yet been mapped but is presumed to lie near the 5' end.

A model for the structure of the RSV genome is shown in Chart 3. The genetic regions are shown in their most probable positions. Salient physical features include: the poly(A) at the 3' end; a "cap" consisting of 2 methylated nucleotides in 5' to 5'-triphosphate linkage at the 5' end (10); the tRNA molecule near the 5' end (19) which serves as a primer for virus DNA synthesis shortly after infection; and a postulated terminal sequence (abc) which is identical at both ends. The significance of these to the life-style of the virus will be discussed in the next section.

Replication of RNA Tumor Viruses

That RNA tumor viruses replicate through a DNA intermediate which is formed immediately after infection, integrated into the cell genome, and transcribed by host RNA polymerase to give progeny viral RNA (21) is by now well established. The molecular details of this process remain unclear. A plausible model for the early steps in replication is shown in (Chart 4). The model proposes a terminal redundancy in the genome which allows the DNA molecule to form circles, either by digestion of the short 5' end of the template RNA with RNase H and then circularization by hydrogen binding, as shown, or by synthesis of double-stranded DNA containing the redundancy and circularization by recombination. In either case, the final double-stranded DNA circle contains the redundant sequence only once. To regain it in the progeny, it is necessary to integrate the viral DNA into the identical sequence in the host genome. Transcription of both host- and virus-derived information then gives back a molecule with the redundant sequence intact. Preliminary experiments suggest a terminally redundant sequence in murine leukemia virus (W.}

Chart 2. Mapping of the large T, oligonucleotides of RSV. Pr-RSV-B 36S RNA labeled with 3H was chromatographed on polyuridylicate-Sephadex after no treatment (A) or after treatment with alkali sufficient to give 1.8 (B) or 3.6 (C) breaks/molecule. The material bound to the columns was eluted, mixed with 32P-labeled Pr-RSV-B RNA, and fingerprinted after digestion with RNase T, a, b, and c, yields of each large oligonucleotide arranged in the most probable order along the genome; d, theoretical yields of each oligonucleotide as a function of its distance from the 3' end for molecules with an average of 0, 1.8, and 3.6 breaks. Reprinted from Coffin and Billeter (7) by permission of the Journal of Molecular Biology.

Chart 3. Structure of the avian tumor virus genome. Physical features are shown above the line representing the RNA, and genetic features are shown below. abc, postulated terminal redundancy.
Cell Transformation by Avian RNA Tumor Viruses

Table 3
Possible genetic constitution of various avian RNA tumor viruses

<table>
<thead>
<tr>
<th>Type</th>
<th>Example</th>
<th>Possible genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endogenous</td>
<td>RAV-0</td>
<td>gag-pol-env^</td>
</tr>
<tr>
<td>Nontransforming</td>
<td>RAV-1</td>
<td>gag-pol-env^</td>
</tr>
<tr>
<td>Weakly transforming (defective)</td>
<td>Avian myeloblastosis virus</td>
<td>gag-pol-(en)-(luk)^6</td>
</tr>
<tr>
<td>Strongly transforming</td>
<td>RSV-</td>
<td>gag-pol-(en)-src</td>
</tr>
<tr>
<td>Replication defective</td>
<td>Pr-RSV-A</td>
<td>gag-pol-env^-src</td>
</tr>
<tr>
<td>Nondefective Transformation</td>
<td>td-Pr RSV-A</td>
<td>gag-pol-env^-</td>
</tr>
</tbody>
</table>

^A RAV of subgroup A.
^b The genes in parentheses are postulated. (en) denotes a deletion of part or all of env.

Hazeltine and J. Coffin, unpublished observation) and in RSV (M. Collett, J. Coffin, and A. Faras, unpublished observation). These results support the 1st part of this model.

Avian Tumor Virus Evolution

The viruses listed in Table 1 can also be classified by their genetic constitution. Table 3 shows such a classification with the addition of several subgroups, the defective viruses. These include the transformation-defective viruses discussed above, as well as replication-defective viruses, which are apparently deletions in all or part of the env gene (or, less likely, in a gene required for the synthesis of env protein). Viruses of this latter group appear only very rarely as spontaneous mutations of nondefective sarcoma viruses but are relatively widely found in nature. All strains of murine sarcoma virus are of the replication-defective type (although they may be lacking genes other than env), as well as weakly transforming viruses such as avian myeloblastosis virus and others, and probably the original isolates of RSV as well. R. Weiss (personal communication) has proposed that all “spontaneous” virus-induced tumors contain

Chart 4. A possible mechanism of the replication of RNA tumor virus nucleic acid. ABC, terminally redundant sequences of the tumor virus genome; A'B'C', similar sequences in the host cell genome. P', sequences on the RNA genome complementary to the primer site (“antiprimer” sequences). DNA synthesis begins at the primer and proceeds to the 5' end of the RNA genome synthesizing a DNA transcript (abc) complementary to the redundant region (ABC). The RNA complementary to the DNA transcript is removed by RNase H hydrolysis. The complex forms a circle by base pairing of the ABC region of the DNA transcript at the 5' end of the genome with the ABC region adjacent to the poly(A) sequence at the 3' end. Transcription of the RNA genome is completed starting from the 3' end of the redundant sequences. The opposite (+) DNA strand is synthesized as RNase H hydrolyzes the RNA genome from the 5' end, and the antiprimer sequence is copied from the (−) strand DNA. At this point the entire genome has been transcribed into circular double-stranded DNA with the exception of 1 of the redundant sequences and the poly(A) sequence. The completed circular DNA molecule aligns with a region containing the homologous sequence A'B'C' in the host chromosome. The provirus is integrated into this sequence by a mechanism such that the region derived from the terminal redundancy is split and joined to the broken ends of the corresponding host sequences. The virus-specific sequences and a portion of the host sequences (region between the asterisks) are transcribed to regain the terminally redundant sequences. Alternatively, a larger portion of the host sequences is transcribed into a precursor RNA which is subsequently cleaved to the proper genome size. The RNA is matured by the addition of the tRNA primer and the poly(A) sequence at the 3' end. ——, viral RNA genome; ——, RNA primer; ——, proviral DNA; ——, host cell genome.
a mixture of this type of virus and a RAV and that the appearance of nondefective sarcoma virus is due to some poorly understood aspect of laboratory manipulation.

Studies by fingerprinting (13), nucleic acid hybridization (27), and immunology (5) show that all the viruses of Tables 1 and 3 are very closely related and are probably relatively recently derived from a common ancestor. In fact, it is quite possible, especially with the strongly transforming viruses which are transmitted horizontally very poorly, if at all, that such a virus “evolution” might take place within a single animal. The most likely candidate for this process is, of course, the endogenous viral DNA sequences of the host cell. In the remainder of this paper I shall try to describe how such an evolution might proceed.

Starting with the endogenous sequences, one can imagine 2 different series of events that would give all of the viruses of Table 3. Both would start by induction of the viral genome to give a RAV-0-type virus. One series would lead to a nontransforming (RAV-type) virus and could proceed simply by introducing a few mutations into the env gene to change the protein to another type. This could be diagrammed

\[ \text{gag-pol-env} \rightarrow \text{gag-pol-env}^4 \]

Additional changes might occur elsewhere to yield a virus with the ability to replicate to higher titers.

To evolve to a strongly (or weakly) transforming virus it seems likely that the changes occur in the sequence

\[ \text{gag-pol-env}^x \rightarrow \text{gag-pol-(en)-src} \rightarrow \text{gag-pol-env}^y\text{-src} \]

There are 2 reasonable possibilities for the origin of the src information. These are shown in Chart 5. The 1st is that src could be derived by mutations in all or part of env, rendering the env protein nonfunctional by replacing it with src protein. This path is shown by the arrows on the right.

The 2nd route to src is by “recombination” with the host genome. One way such a recombination could occur is shown in Chart 5, left. Instead of integrating into the abc sequence, the circular provirus could integrate into another site somewhere near it, perhaps through a chance homology. Transcription of this integrated provirus between the abc sequences would then yield a virus with some of env replaced by information derived from the host cell. The number of different possible sequences which could be obtained in this way would depend on the frequency of the abc sequence in the cell genome. Probably, further steps involving mutational events would be necessary to arrive at a src (or luk) gene.

Replication-defective transforming viruses derived by either of these 2 lines would also be defective in recombination, in the sense that any recombinational event between 1 of these viruses and a nontransforming virus will transfer the defect to the nontransforming virus along with src. Such defectiveness in recombination is in fact observed with replication-defective viruses of this type (28). To obtain a nondefective transforming virus would require an unequal recombination with a RAV. Such an event would probably be quite rare.

Evidence to distinguish these possibilities is lacking. Experiments which show no homology between endogenous viral information and src (27) were performed under conditions of high stringency and might not have detected the small degree of homology remaining after the accumulation of a number of mutations. Experiments with DNA probes complementary to src indicate that uninfected cells contain related, but not identical, information (20) but do not say whether this information is within the endogenous viral sequences.

Conclusions

The above suggestions follow somewhat along the lines of the “protovirus” hypothesis of Temin (22), in that transforming viruses arise by evolution from endogenous information. Note that there is no involvement of “oncogenes” (11) in the processes suggested, since mutational events would be sufficient to change the function of the gene products to something completely different.

The nature of the src gene product itself remains a mystery. If src is derived from cell information, it could be anything. If it is derived from viral envelope glycoproteins, then one might suspect the involvement of a similar protein in cell transformation.
References

Fig. 1. Fingerprints of RSV RNA. $^{32}$P-labeled Pr-RSV-B (A) and transformation-defective Pr-RSV-B (B) were digested with RNase T$_1$ and fingerprinted by 2-dimensional gel electrophoresis as previously described (4). C, a drawing of the gels. Numbers, location of large, unique, T$_1$ oligonucleotides, D$_1$, and D$_2$, positions of the xylene cyanol FF and bromphenol blue dye markers, respectively. Arrows, 2 spots (5 and 6) missing in the transformation-defective variant. Reprinted from Coffin and Billeter (7) by permission of the Journal of Molecular Biology.
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*Cancer Res* 1976;36:4282-4288.

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