Genetics of Leukemogenesis by Avian Leukosis Viruses

J. M. Coffin, P. N. Tsichlis, and H. L. Robinson

A. Introduction

Oncoviruses of chickens can be classified into one of three groups, depending on their effects on cells in culture and their pathogenicity and lifestyle in the chicken (Fig. 1). The most intensely studied but rarest of these viruses are the transforming acute leukemia and sarcoma viruses. These viruses cause rapid disease in animals and transform appropriate target cells in culture, properties attributable to an extra gene in place of (or, in the case of nondefective Rous sarcoma virus, in addition to) the three replicative genes. These onc genes are most likely normal genes of the host cell taken under the control of the very efficient sequences that the virus uses for its own expression and therefore expressed at 100- to 1000-fold higher levels than in the uninfected cell (Spector et al. 1978; Stehelin et al. to be published). Since these transforming viruses are very rapidly pathogenic and are transmitted horizontally very poorly, it is improbable that they exist as infectious agents of epidemiological significance. Rather, they probably arise at low frequency by some recombinational process between leukosis virus and host cell information and, if not given a good home in the laboratory, would die out quickly.

The second group of viruses is the more common natural agent of neoplastic disease in many species, for example lymphoid leukosis in the chicken. They do not transform cells in culture and induce disease only after a long latent period. Their genomes do not appear to contain any information in addition to that required for virus replication. There are three types of virus which behave this way: field isolates of lymphoid leukosis virus (LLV); Rous-associated viruses (RAV), which have been isolated as helpers for defective sarcoma viruses; and transformation-defective (td) de-
letion mutants of nondefective sarcoma viruses (Biggs et al. 1973; Vogt 1971). All three types have very similar genome structure.

The third group of avian oncovirus are the endogenous viruses which reside primarily as integrated proviruses in the chromosomal DNA of chickens (for review, see Robinson 1978) and are inherited as though they were usual cellular genes. The endogenous proviruses of chickens have been typed by integration site and phenotype of expression into numerous distinct loci, termed ev-1, 2, etc. (Astrin 1978). The endogenous viruses, when expressed as infectious agents, differ from the former two groups (the exogenous viruses) in several interesting ways: they are nonpathogenic in chickens, even when viremia is present (Motta et al. 1975); they replicate somewhat more poorly in cell culture (Linial and Neiman 1976; Robinson 1976); and they have a distinct host range (subgroup E) (Vogt and Friis 1971). They are, however, very closely related to the exogenous nontransforming viruses with a nearly identical genome structure (Coffin et al. 1978b; Neiman et al. 1977).

In this report, we will describe some experiments in which we exploit the biological differences between endogenous and exogenous avian tumor virus genomes to obtain information regarding the roles of various portions of the exogenous virus genome in pathogenicity, particularly in lymphoid leukosis.

B. Results and Discussion

I. Relationship of Endogenous and Exogenous Virus Genomes

There are numerous field and laboratory strains of avian tumor viruses and there are numerous distinct endogenous viral loci. Thus a detailed survey of nucleic acid sequences of genomes of many members of the two groups should allow us to determine in general terms the historical relationships between them, for example, whether the different endogenous viruses are independently derived from germ line integration of an exogenous virus or represent a distinct lineage of viruses derived only from another endogenous virus. To obtain such information, we have subjected the genomes of various endogenous and exogenous viruses to T1 oligonucleotide mapping (Coffin et al. 1978b), a method sensitive to one base change per 600 nucleotides. Figure 2 shows a selection of different endogenous and exogenous avian tumor virus genomes by comparison with the genome of RAV-0 (a prototype endogenous virus). Note that the absence of a marker oligonucleotide could be due to as little as a single base change or as much as a deletion or a substitution of completely unrelated information. It can be seen that the endogenous viruses are distinguishable from one another by the presence or absence of specific markers. However, no two of these genomes differ from one another by more than 1%. By contrast, all the exogenous viruses examined differ substantially from one another and from the endogenous viruses, with distinctive markers in many places in the genome. We conclude that these viruses represent two distinct but closely related lineages, a conclusion which is consistent with the relationship scheme for the viruses shown in Fig. 1.

II. Role of the \( U_3 \) Region in Growth

Although specific point markers distinguishing endogenous from exogenous viruses are distributed all over the genome, the greatest divergence is in the \( U_3 \) region near the 3' end of the genome (Coffin et al. 1978b; Neiman et al. 1977; Wang et al. 1977). We have termed the exogenous allele of this region \( cx \) and the endogenous type \( cn \) (Tsichlis and Coffin 1980). Major regions of inhomology were also found in a region to the left of \( U_3 \) and in the S (subgroup-coding) region of \( env \) (Coffin et al. 1978b).

To assess the roles of the various regions in growth and pathogenicity of the viruses, we prepared a series of recombinants between RAV-0 and either Pr-B (a nondefective transforming virus) or its \( td \) derivative. Recombinants were selected for their ability to infect turkey (T/BD) cells due to the \( env^E \) gene of RAV-0 and for transformation and/or rapid growth from the Pr-B parent. Selection for transformation and rapid growth led to selection of \( src \), a region immediately to its left, and a short region in \( gag \). Selection for rapid growth only was accompanied in all cases by selection of only the \( U_3^{cx} \) region of Pr-B. The isolation of one particularly useful recombinant is shown in the top portion of Fig. 3. On initial screening one recombinant "clone" (MRE-1) was found to be a mixture of
### Fig. 2. Genomes of endogenous and exogenous avian tumor viruses. The genomes are displayed as oligonucleotide maps by comparison with that of RAV-0 (Coffin et al. 1978b). Regions identical to RAV-0 (by the presence of an identical oligonucleotide) are shown as an open box, regions which differ by a shaded box, and undetermined or deleted regions by a dashed line. The top four lines show RAV-0 and the products of other ev loci [ev-3 is defective, and the map shown was inferred from RAV-60 recombinants (Coffin et al. 1978a)]. The remaining lines show exogenous viruses. Pr-B, Pr-C, and SR-D are nondefective transforming virus. RAV-1 and 2 are lab strains of nontransforming viruses, and Cr-117 is a new field isolate of LILV.

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- **Same as RAV-0**
- **Distinct from RAV-0**

Recombinants, all of which were env<sup>E</sup> - U<sub>3</sub><sup>α</sup>, but which were heterogenous in other portions of the genome. Recloning of this virus by focus formation led to the selection of a virus (TRE-14) which contained all the regions found to accompany transformation. When recloned by infection of QT6 cells (Moscovici et al. 1977), a virus, NTRE-7, was isolated which was entirely derived from RAV-0 except for the 200 to 300 nucleotide U<sub>3</sub><sup>α</sup> region.

The repeated selection of the U<sub>3</sub><sup>α</sup> allele suggested that was responsible for the difference in the growth rate between endogenous and exogenous viruses. NTRE-7 allowed us to test this hypothesis, since it was congenic with RAV-0 except in U<sub>3</sub>. We therefore compared the growth of this virus with RAV-0 and with various RAV-60s, a similar set of recombinants between exogenous and endogenous viruses (Hanafusa et al. 1970; Hayward and Hanafusa 1975), which have various contributions from the exogenous parent in several parts of the genome (Fig. 3, bottom). Parallel cultures of chicken (C/0) cells were infected at various multiplicities of infection and challenged 4 days later with RSV (RAV-60). The extent to which focus formation by the challenge virus was reduced provided a measure of the relative amount of virus replication. As shown in Fig. 4, RAV-0 and the endogenous viruses tested (open symbols) had a virtually identical growth rate, whereas NTRE-7 and the various RAV-60s (closed symbols) had a growth rate 30-fold higher than all the endogenous viruses. Since the only consistent feature distinguishing these recom-
Fig. 3. Recombinants between exogenous and endogenous viruses. The top section shows a cross between Pr-B (shaded bars) and RAV-0 (open bars) leading to a mixture of recombinants, designated MRE-1, which was subcloned to select a transforming (TRE-14) and a nontransforming virus (NTRE-7). Solid lines indicate regions indistinguishable in the two parents, dashed lines the src deletion in RAV-0 and NTRE-7. The lower panel shows 4 RAV-60 strains originally isolated by Hanafusa et al. (1970). Again, regions derived from the endogenous parent are shown by open boxes and from the exogenous by shaded boxes.

Recombinants from the endogenous viruses was the U3α allele, we conclude that this small region of the genome is the major determinant responsible for the more rapid growth of exogenous viruses.

The nucleotide sequence of the U3α region of several different avian tumor virus strains has been determined (Schwartz and Gilbert, personal communication; Czernilofsky et al., to be published; Yamamoto and Pastan 1980), as has that of one U3α region (Hishinuma et al., personal communication), and it is improbable that a protein is encoded by this region. However, this region is reduplicated at the left end of the provirus during DNA synthesis (Hsu et al. 1978; Shank et al. 1978). Since the RNA transcript of the provirus (i.e., the genome) does not contain a copy of the left U3 region, it is likely that U3 contains a promoter for virus RNA synthesis and that the distinction between the two U3 alleles is in the efficiency of the promoters they contain.

III. Role of the U3 Region in Leukemogenesis

The structure of the integrated provirus suggests a possible mechanism for carcinogenesis without a specific “transforming” gene. If the left U3 region carries an efficient promoter, then there must also be one on the right of the provirus where the same combination of sequences is found. Since integration of the provirus into chromosomal DNA is more or less random (Hughes et al. 1978; Sabran et al. 1979), an occasional infected cell might have a provirus integrated to the left of a potentially “transforming” gene. Efficient promotion of transcription of such a region could induce the expression of this gene to oncogenic levels. Compelling evidence for this sort of mechanism has been provided by Hayward et al. (see this book) and by Payne et al. (personal communication).

In its simplest form this model would predict that the nonpathogenicity of endogenous viruses is a consequence of an inefficient U3 promoter sequence. Again, NTRE-7 and the various RAV-60s allowed us to test this hypothesis directly. A full account of the methods used for this experiment can be found in Robinson et al. (to be published). In brief, susceptible chicks were injected with equal amounts of RAV-1, RAV-0, NTRE-7, or the RAV-60s. Figure 5 shows the level of viremia...
**Fig. 4.** Relative growth rates of various viruses. C/0 gp+ chicken embryo-fibroblasts (Robinson et al., to be published) were infected at various multiplicities with either the endogenous viruses RAV-0 (○), C-ILV (○), 15B × K16 ILV (△), 15B E-virus (□), or the recombinant viruses NTRE-7 (●), NY 201 (▲), and NY 203 (■) RAV-60. Four days after infection the cultures were superinfected with B-RSV (RAV-60) and foci were counted 5–7 days later. The extent of interference was determined by dividing the titer of the challenge virus in uninfected control cultures by the titer in the various infected cultures.

**Fig. 5.** Growth of NTRE-7, RAV-0, and RAV-60 in birds. Each point shows the level of serum viremia in C/0 chickens 1 month after infection with 10^6 infectious units of each virus as measured by particulate reverse transcriptase in serum. Circled points show birds which were later diagnosed as having lymphoid leukosis.
in the latter three groups at 1 month of age. Although there was substantial variation from bird to bird, the growth of the viruses in vivo seemed to mimic that in cell culture, with RAV-0 growing to a median value about 10-fold less than NTRE-7 or the RAV-60s.

Figure 6 shows the incidence of leukosis in all the birds tested. The incidence and latent period for the RAV-60 strains were not significantly different from the RAV-1. Thus, the subgroup E host range does not contribute to the nonpathogenicity of endogenous viruses. Similar results were found by Crittenden et al. (1980). As previously reported (Motta et al. 1975), RAV-0 was nonleukemogenic. Although the test of NTRE-7 is not yet complete, the results to date are quite surprising. In the first 34 weeks, there has been no disease whatever, compared with 40% in the RAV-60 infected birds. While we cannot yet conclude that NTRE-7 is completely nonpathogenic, it may well be so, and it is at least significantly slower than the RAV-60s. We must also point out that although td sarcoma viruses have been reported to be leukemogenic (Biggs et al. 1973; Halpern and Hanafusa, personal communication), td Pr-B has not yet been tested.

In any case, this result clearly separates growth rate from leukemogenicity, since RAV-60s and NTRE-7 show identical growth both in vitro and in vivo. This result is inconsistent with the simplest predictions of the downstream promotion model as well as models which invoke pathogenic side effects of the virus gene products. We have additional recombinants available for testing which should provide us with a suggestion of which region of the genome is responsible for these differences. A comparison of the structures of NTRE-7 and the RAV-60s gives a hint of where to look (Fig. 3). The only exogenous virus region besides U\textsubscript{3} consistently inherited by these viruses is immediately to the left of U\textsubscript{3}. This region is at present undefined. There are a number of reasons why such a sequence might have a role in pathogenicity, the most interesting of which is the regulation of downstream promotion by either the sequence itself or its product.

We believe that the nonpathogenicity of the endogenous viruses is an important adaptive feature to their quiescent lifestyle, for which it is quite important that they not harm the host. Experiments such as we have presented here should be useful in identifying and characterizing the regions of the virus genomes relevant to this adaptation and also in exploiting the differences in these regions in different viruses to probe the molecular mechanisms of viral leukemogenesis.

Acknowledgements

We thank M. Champion and L. Lieberman for expert technical assistance. This work was supported by grants CA17659, CA24530, and CA23086 from the National Cancer Institute. J. Coffin was a recipient of a Faculty Research Award from the American Cancer Society, and P. Tsichlis was a postdoctoral fellow of the National Cancer Institute.
References