Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 1225–1229, February 1982
Genetics

At least two regions of the viral genome determine the oncogenic potential of avian leukosis viruses

(*in vivo* oncogenicity test/leukemogenic potential/osteopetrotic potential/viral promoter in oncogenesis/viral sequence in oncogenesis)

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Communicated by Paul Zamecnik, November 5, 1981

ABSTRACT **Recombinants of oncogenic and nononcogenic avian leukosis viruses were tested for their oncogenic potential in chickens. The results indicate that at least two regions of the viral genome determine the oncogenic potential of these viruses. The first region contains sequences that control viral mRNA synthesis. These sequences determine the potential of a virus to induce a low incidence of lymphomas, carcinomas, chondrosarcomas, fibrosarcomas, and osteopetrosis. The second region lies outside the sequences that control viral mRNA synthesis. These sequences determine the ability of a virus to induce a high incidence of lymphomas or osteopetrosis.**

Retroviruses that cause disease after short latent periods (acute viruses) appear to be transducing agents for host genes (for review, see refs. 1–3). The transduced genes, in their viral form, are oncogenes that cause abnormal growth or development or both. Since retroviral oncogenes are typically expressed at 100–1000 times higher levels than their cellular homologs (4, 5), transformation by acute retroviruses has been hypothesized to result from viral encoded overexpression of normal cell genes (6–10).

Retroviruses that cause disease after long latent periods (nonacute viruses) have only genes for replication. Most nonacute retroviruses cause a high frequency of lymphomas. Recent studies have shown that retroviral induced chicken lymphomas contain proviruses integrated adjacent to a specific host gene *c-myc* (*c-myc* is the cellular homolog of the transforming gene of avian myelocytomatosis virus) (11, 12). The proviruses integrated adjacent to *c-myc* increase the transcriptional activity of *c-myc* ≈100-fold (11). Since preleukemic tissue displays proviruses at many sites of integration, selection during lymphomogenesis for proviral insertions that increase the transcriptional activity of a specific gene suggests that nonacute disease, as acute disease, results from viral induced overexpression of host genes.

The present study was undertaken to test whether the lymphomogenic potential of nonacute viruses was indeed encoded by 3′ sequences that control viral mRNA synthesis (see Fig. 1) (13–19). Oncogenic and nononcogenic avian leukosis viruses (ALVs) have characteristic 3′ sequences designated $U3^x$ (x refers to the exogenous origin of oncogenic isolates) and $U3^n$ [*n* refers to the endogenous origin of nononcogenic isolates (4, 5, 20–22)]. $U3^x$ appears to have more efficient transcriptional control elements than $U3^n$. At steady state, a $U3^x$ provirus expresses ≈10,000 copies of viral mRNA per cell while a $U3^n$ provirus expresses ≈1,000 (4, 5). *U3* sequences also encode the growth rates of ALVs (23). $U3^x$ viruses grow to higher titers than $U3^n$ viruses, a phenomenon that has been attributed to the relative efficiencies of their promoters.

The results of our study were both expected and unexpected. As expected, a $U3^x$ recombinant of a nononcogenic ALV was oncogenic. This virus, however, did not cause a high incidence of lymphoma. Rather, it induced a low incidence of a variety of neoplasias.

MATERIALS AND METHODS

Chickens. K28 chickens are a random-bred line of White Leghorns that have been bred for susceptibility to all known subgroups of ALVs, including those with subgroup E envelope antigens characteristic of endogenous viruses (24). K28 has also been bred to contain only one endogenous provirus, the defective provirus that resides at *ev* 1 (25).

Viruses. All viruses were cloned by two cycles of end-point purification. Oligonucleotide compositions were determined as described (22). Viruses were recovered from diseased tissues by cocultivation of minced tumor tissue with susceptible cells (6). In the case of osteopetrosis, virus was recovered from serum or bone marrow of diseased birds.

RESULTS

Isolation and Characterization of NTRE-7, a $U3^x$ Recombinant of the Endogenous Virus RAV-0. In the process of characterizing a series of recombinants of the oncogenic virus PrRSV-B and the nononcogenic virus RAV-0, we had the good fortune to isolate a virus that, except for *U3* oligonucleotides, had the oligonucleotide composition of its nononcogenic parent (Fig. 1) (23, 27). This recombinant was named NTRE-7, nontransforming subgroup E virus, isolate number 7. NTRE-7 appears to have been generated by a single recombinational event in the noncoding region between the 3′ and of *env* and the beginning of *U3*. Sequence analyses indicate that the crossover occurred ≈100 bases 3′ of *env* and ≈200 bases 5′ of *U3* (unpublished observations). NTRE-7 has the growth potential of $U3^x$ viruses (ref. 28; unpublished).

Oncogenic Potential of NTRE-7. To examine the oncogenic potential of NTRE-7, 5×10^6 infectious units of RAV-0 or NTRE-7 were inoculated into the leg veins of 1-day-old K28 chickens. NTRE-7 and uninfected control birds were maintained in separate quarantines and observed for disease for 1 year.

One month after infection, sera from infected birds were harvested and analyzed for virus by assaying for particulate RNA-directed DNA polymerase. The median titer of virus in sera of NTRE-7-infected birds (5×10^6 units/ml) was comparable with that previously observed for RAV-60-infected K28

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Abbreviation: ALV, avian leukosis virus.

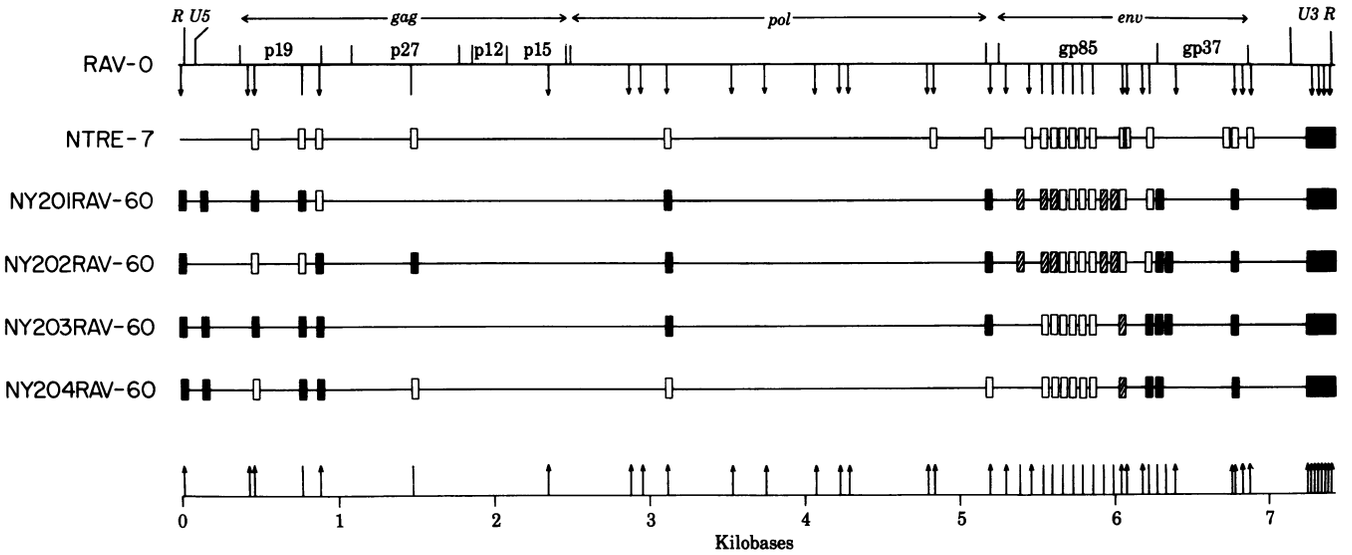

FIG. 1. Genomes of viruses used in oncogenicity tests. RAV-60s are subgroup E recombinants of subgroup A or B ALVs with *ev* 3 (NY201, NY202) or *ev* 9 (NY203, NY204) (6). Viruses were characterized for oligonucleotide compositions and electrophoretic mobility of structural proteins. The genetic organization of viral RNA is indicated at the top. *R*, short terminal repeat; *U5*, unique 5′ sequences; *gag*, *pol*, and *env*, structural genes for the internal proteins (p19, p27, p12, and p15), RNA-directed DNA polymerase, and envelope proteins (gp85 and gp37), respectively; *U3*, unique 3′ sequences. Integrated proviral DNA has the structure 5′-*U3RU5*, *gag*, *pol*, *env*, *U3RU5*-3′ (the *U3RU5* long terminal repeats are designated LTR). *U3* encodes transcriptional controls for viral mRNA synthesis. *R* and *U5* encode 5′ noncoding sequences of viral mRNAs. ↓, Markers positioned by using sequence data for PrRSV-C and *ev* 1 (D. Schwartz, personal communication; ref. 18), |, markers positioned by using oligonucleotide maps (refs. 6 and 22; unpublished results) and peptide maps (26). Markers present in the RAV-0 genome are indicated on the line representing RAV-0. All markers present in the viruses under study are indicated on the kilobase line. Only markers characteristic of endogenous or exogenous viruses are indicated for the genomes of NTRE-7 and RAV-60s. □, Markers characteristic of RAV-0; ■, markers characteristic of an exogenous parent; ▨, markers characteristic of *ev* 3 or *ev* 9. Large T_1 oligonucleotides provide sequence markers for ≈10% of the genome of ALVs. These markers are more frequent in *env* and *U3*, in which sequences present in large T_1 oligonucleotides comprise up to 25% of the genome. Genetic maps that include oligonucleotide numbers are available from H.L.R. or J.M.C.

chickens (2.5×10^6 units/ml) (29) [RAV-60s are subgroup E viruses that induce a high incidence of B-cell lymphomas (6, 30)]. These titers were ≈10 times those observed for RAV-0-infected birds (4×10^5 units/ml) (29).

The results of these analyses indicated that we had successfully infected K28 chickens with RAV-0 and NTRE-7. They also suggest that populations of 1-month-old RAV-0-, NTRE-7-, and RAV-60-infected chickens have comparable numbers of infected cells. Cultures of RAV-60- and NTRE-7-infected cells release 5- to 10-fold higher titers of virus per cell than cultures of RAV-0-infected cells (unpublished observations).

The primary targets for ALV-induced lymphomas are B-dependent lymphoid cells. In chickens, B cells undergo differentiation in the bursa of Fabricius. To compare the bursal tropism of RAV-0, NTRE-7, and RAV-60, bursas from relevant 1-month-old chickens were harvested, homogenized, and analyzed for the internal antigens of ALVs. The median titers of viral antigens in bursas of infected birds were similar (Fig. 2). This result indicates that NTRE-7, RAV-0, and RAV-60s are all bursal tropic.

During the year of the experiment, none of 58 RAV-0-infected birds became moribund with virus-induced diseases, 3 of 75 NTRE-7-infected birds died from lymphomas (Table 1 and Fig. 3), and 41 of 87 RAV-60-infected K28 birds developed lymphomas (6).

At 1 year of age, survivors were sacrificed and autopsied. No disease was found in RAV-0-infected birds. Eight cases of disease were found in 63 NTRE-7 survivors: three cases of osteopetrosis, two fibrosarcomas, one chondrosarcoma, one adenocarcinoma, and one nephroblastoma (Table 2 and Fig. 3). This incidence of disease was not unlike that observed in survivors of RAV-60 infection. Evaluation of 35 RAV-60 survivors showed two cases of osteopetrosis, one fibrosarcoma, and one case of anemia.

To demonstrate that the low incidence of disease in NTRE-7 birds was not due to contaminating viruses, viruses were recovered from seven diseased birds. Recovered viruses had the interference pattern and the oligonucleotide composition of NTRE-7 (data not shown).

Statistical analysis of the incidence of disease in the RAV-0-, NTRE-7-, and RAV-60-infected chickens indicates that the population of NTRE-7-infected birds had a significantly higher incidence of neoplasms than the population of RAV-0-infected

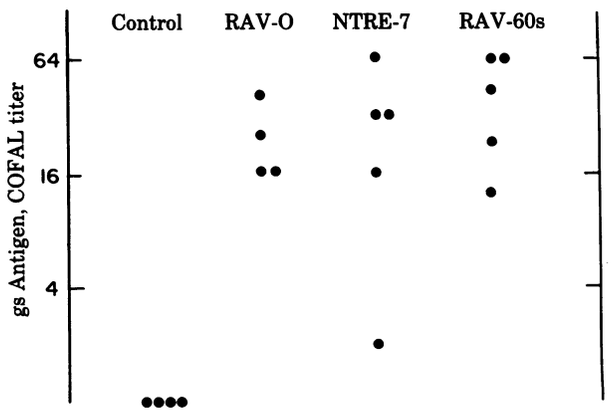

FIG. 2. Bursal tropism of RAV-0, NTRE-7, and RAV-60. Twenty percent (wt/vol) homogenates were prepared from bursas of 4- to 7-week-old birds. The COFAL titer is the highest dilution of the homogenate that gave a strong positive reaction in a complement-fixation assay for ALV internal proteins. Homogenates in which these antigens constitute ≈0.01% of total cell protein titer at 4 or 8.

Table 1. Disease in RAV-0-, NTRE-7-, and RAV-60-infected chickens

Virus	Chickens,* no.	Lymphoma, %	Other disease,† %	Miscellaneous losses,‡ %
RAV-0	58	0	0	17
NTRE-7	75	4	12	13
RAV-60	87	47	16	6
Uninfected	67	0	0	7

* In group at 3 months of age.
† See Table 2.
‡ Miscellaneous deaths include birds that died of no apparent cause or were sacrificed because they had suffered trauma, developed hip dysplasia, contracted bacterial infections, or appeared to have viral related disease. The higher incidence of non-virus-related deaths in RAV-0- and NTRE-7-infected birds was due to the immediate sacrifice of any bird that exhibited signs of lymphoma: diarrhea, lethargy, pale comb. Despite this approach, the three NTRE-7 birds that developed lymphomas were morbid at the time of workup.

birds ($P = 0.0002$) and that RAV-60-infected chickens had a significantly higher incidence of lymphomas than NTRE-7-infected birds ($P < 0.00001$). Differences in the incidence of osteopetrosis, fibrosarcoma, chondrosarcoma, adenocarcinoma, and nephroblastoma in NTRE-7- and RAV-60-infected chickens were not statistically significant.

DISCUSSION

Our results indicate that at least two regions of the ALV genome determine the potential of virus to induce tumors by promoter insertions. First, 3′ sequences that control viral mRNA synthesis encode a broad oncogenic potential. Second, "other sequences" target viruses for a high incidence of lymphoma.

Sequences that Control Viral mRNA Synthesis Determine the Broad Oncogenic Potential of ALVs. Evidence that 3′ sequences determine the broad oncogenic potential of ALVs is provided by the 13% incidence of neoplasias in NTRE-7-infected birds compared with the 0% incidence of disease in RAV-0-infected birds (Table 1 and Fig. 3). NTRE-7 induced a variety of diseases that occurred independently, late in infection. These diseases included lymphomas, osteopetrosis, fibrosarcomas, a chondrosarcoma, an adenocarcinoma, and a nephroblastoma (Table 2). Outside of *U3* and the nonprotein coding sequences immediately 5′ of *U3*, NTRE-7 and RAV-0 have highly similar genomes. Twenty markers distinguish the *U5*, *gag*, *pol*, and *env* regions of the nononcogenic (RAV-0) and oncogenic (PrRSV-B) parents of NTRE-7. In NTRE-7, all 20 of these markers are of RAV-0 origin (Fig. 1).

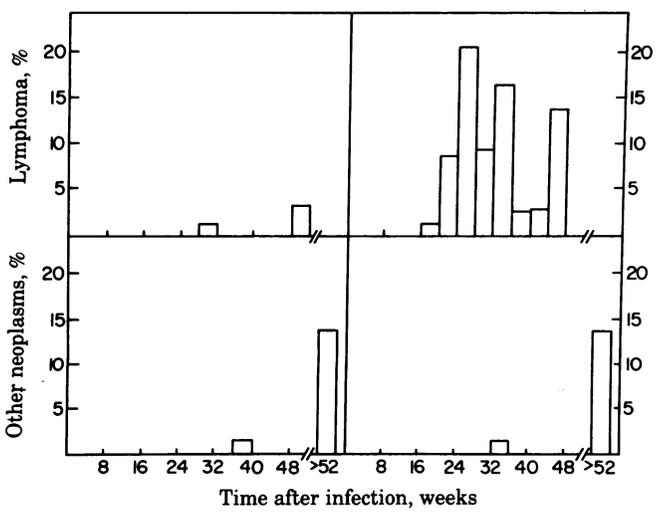

FIG. 3. Incidence of disease in NTRE-7 (*Left*)- and RAV-60 (*Right*)-infected chickens. Other diseases included osteopetrosis, fibrosarcoma, carcinoma, chondrosarcoma, and anemia. Percent of birds with disease was calculated using the number of live birds at the beginning of each time point as the total group size for that time point. Data for RAV-60-infected birds is from oncogenicity tests with NY201, NY202, NY203, and NY204 RAV-60 (6).

Table 2. Disease induced by NTRE-7 and RAV-60

Tissue	Disease	Cases, no. NTRE-7	Cases, no. RAV-60s
Mesenchyme	Fibrosarcoma	2	1
	Chondrosarcoma	1	0
	Osteopetrosis	3	2
Hemopoietic tissue	Anemia	0	2
	Lymphoma	3	41
Kidney	Adenocarcinoma	1	0
	Nephroblastoma	1	0
Unknown	Wasting*	0	10

* Wasted birds are birds that became emaciated for no apparent cause. Wasting was not observed in RAV-0- and NTRE-7-infected birds. The occurrence of wasting in RAV-60-infected birds paralleled that of lymphoma. Populations of wasted and nonwasted birds had similar levels of viremia and incidence of neutralizing activity to virus. Viruses recovered from wasted birds belonged to subgroup E. Sera of wasted birds did not contain antibodies to chicken erythrocytes or antigens present in acetone-fixed chicken livers (unpublished studies).

Quite interestingly, not all retroviruses are oncogenic. Our tests suggest that only retroviruses that have exceptionally efficient transcriptional control elements (such as those found in $U3^x$) or transcriptional control elements with specific adjacent sequences (or both) have the potential for causing cancer.

U3 sequences determine the growth potential as well as the oncogenic potential of ALVs. We do not think that differences in growth potential account for the marked differences in oncogenic potential of RAV-0 and NTRE-7. Differences in growth potential affect the rate of virus spread, not the number of cells that eventually become infected. By 1 month after infection, RAV-0- and NTRE-7-infected birds appear to have comparable numbers of infected cells (Fig. 2). K28 chickens inoculated at 1 month of age with the oncogenic virus RAV-1 develop a high incidence of lymphoma (unpublished observation). Thus, target cells for lymphomogenesis should have been present in birds in which RAV-0 had undergone extensive spread. Furthermore, chickens that develop spontaneous RAV-0 infections in early embryonic life do not develop disease (31).

"Other Sequences" Target ALVs for a High Incidence of Specific Forms of Nonacute Disease. Most ALVs that cause nonacute disease induce a relatively high incidence of a specific form of disease and a low incidence of a variety of neoplasias. In our oncogenicity tests, high-incidence disease occurs 3–9 months after infection while low incidence disease occurs ≈1 year after infection (Fig. 3; ref. 6; unpublished results). Quite interestingly, different ALVs cause different forms of high-incidence disease. RAV-1, RAV-2, and RAV-60s cause a high incidence of B-cell lymphomas (6) while tdPr-B and NTRE-2 cause a high incidence of osteopetrosis (unpublished results). TdPr-B is a transformation-defective (*src* deletion) mutant of the oncogenic parent of NTRE-7 and NTRE-2 is another nontransforming subgroup E recombinant of PrRSV-B and RAV-0 (27). RAV-1, RAV-60s, and tdPr-B also induce a low incidence of a variety of neoplasias that occur at ≈1 year after infection. Thus, NTRE-7 appears to be like other nonacute viruses in that it can induce a low incidence of a variety of diseases late in infection but unlike other nonacute viruses in that it does not cause a high

incidence of a specific disease relatively early in infection (Fig. 3, and Table 2). For convenience, we refer to the potential to induce a high incidence of a specific disease as targeting.

We herewith list the facts we know about targeting. (*i*) The ability to target is inherited. Recombinants between oncogenic and nononcogenic ALVs either target for the same disease as the oncogenic parent or do not target. RAV-1 and RAV-2 are the oncogenic parents of NY201, NY202, NY203, and NY204 RAV-60. All of these viruses cause a high incidence of lymphoma (6). TdPr-B is the oncogenic parent of NTRE-2. Both of these viruses target for osteopetrosis (unpublished results). (*ii*) The ability to target appears to be independent of the envelope antigens of a virus. Viruses that have indistinguishable host ranges and interference patterns target for a high incidence of lymphoma (RAV-60s), a high incidence of osteopetrosis (NTRE-2), or are untargeted (NTRE-7). (*iii*) Targeting does not appear to be encoded by *U3* sequences that control viral mRNA synthesis. NTRE-7 does not cause a high incidence of a specific form of nonacute disease yet has a *U3*-encoded growth potential comparable with that of viruses that target for specific forms of diseases (refs. 23 and 27; unpublished results; Table 2). (*iv*) Viruses that target do so only in certain pedigrees of chickens. RAV-1 causes a high incidence of erythroblastosis and lymphoma in line 15_1 chickens but only a high incidence of lymphoma in K28 chickens (6, 32).

Classically, targeting of viruses for specific forms of disease has been encoded by envelope proteins that determine whether or not a virus enters a cell. We have been unable to obtain evidence for envelope antigens playing a role in the targeting of ALVs for different forms of disease. The vast majority of bursal cells are infected by both nonlymphomogenic and lymphomogenic isolates of ALVs (Table 2; unpublished observations). *In vivo* interference assays do not reveal populations of cells that are infected by RAV-60s but not NTRE-7 (unpublished observations). NTRE-2, a targeted virus, has *env* oligonucleotides that are identical to those of NTRE-7, an untargeted virus (27). The *env* gene is an oligonucleotide-rich region of the genome (Fig. 1). The chance that oligonucleotide mapping would not have detected a recombinational event in this region is unlikely. In short, we have searched for evidence for *env*-encoded targeting but found none.

Speculation on the Molecular Basis of Targeting. Most ALV-induced lymphomas result from proviral control of *c-myc* expression (11, 12). Thus high-frequency lymphomas result from (*i*) a provirus integrating adjacent to *c-myc* and (*ii*) the provirus gaining transcriptional control of *c-myc*. Given that *env*-encoded tissue tropisms do not play a role in targeting (see above), we speculate that the molecular basis of targeting will reflect the frequency or efficiency of one of these two events.

Retroviruses integrate at many sites in their host genome (33–35). Lymphomogenicity tests, however, select for integration in the *c-myc* region and thus may reveal heretofore undetected regional specificities in proviral integration. Our first speculation is that lymphomogenic ALVs encode an integration factor that recognizes the *c-myc* region of the host genome and that nonlymphomogenic ALVs encode integration factors that do not. According to this hypothesis, viruses that induce a high incidence of other forms of disease should have integration factors that recognize regions of the genome that have the potential for causing these diseases, and viruses, such as NTRE-7, that cause only low incidence disease should have integration factors that do not preferentially recognize potentially oncogenic regions of the host genome. Differences in the susceptibility of inbred lines of chickens to ALV-induced erythroblastosis is consistent with this model. Polymorphisms in the host gene, *c-erb*, could determine whether or not a particular *c-erb* were a preferred region for proviral integrations. Thus, this model is consistent with both host and viral genetic elements affecting targeting. Oligonucleotide maps of targeted and untargeted ALVs are consistent with either the 5′ end of *gag* or the 3′ end of *pol* encoding a potential integration factor (Fig. 1; refs. 6 and 27; unpublished results). The 5′ end of *gag* encodes p19, a phosphoprotein that binds to ALV RNAs with high affinity (36). The 3′ end of *pol* encodes a 32,000-dalton protein that has been demonstrated to have *in vitro* endonucleolytic activity (37).

To effect a "promoter insertion," a virus not only has to integrate adjacent to a potentially oncogenic gene but also has to gain transcriptional control of that gene. Most promoter insertions result in the 3′ long terminal repeat gaining control of the expression of a host gene (12). This raises the possibility that sequences adjacent to the 3′ long terminal repeat play an important role in promoter insertions. The second speculation we offer is that sequences adjacent to the 3′ long terminal repeat have the potential of responding to tissue-specific transcriptional signals. According to this speculation, sequences adjacent to the 3′ long terminal repeats of lymphomogenic ALVs would have the potential of being "promoter active" in B cells but not in other cell types, and sequences adjacent to 3′ long terminal repeats of ALVs that induce osteopetrosis would be promoter active in osteocytes but not other cell types. Sequences adjacent to the 3′ long terminal repeats of untargeted viruses would not be uniquely promoter active in any cell type. This model is not easily rationalized with differences in susceptibility of different lines of chickens to specific forms of disease since tissue-specific transcriptional control signals would not be anticipated to vary among sublines of White Leghorns. Sequence data for NTRE-7 and oligonucleotide maps of targeted and untargeted viruses are consistent with such hypothetical tissue-specific transcriptional control elements residing in an ≈100-nucleotide sequence that lies immediately 3′ of oligonucleotide 03. Oligonucleotide 03 is the most 3′ oligonucleotide in the coding region for glycoprotein 37 (Fig. 1). An analogous region of the murine leukemia virus genome may be important in distinguishing leukemogenic from nonleukemogenic recombinants of AKR virus (38–41).

Relationship of These Studies to Studies on the Lymphomogenic Potential of Murine Leukemia Viruses. One of the nice features of our oncogenicity tests in K28 chickens is that genetic changes that have dramatically influenced the lymphomogenic potential of a virus have not affected the bursal tropism of the virus. Most of the genetic factors that influence the lymphomogenic potential of murine leukemia viruses affect the thymotropism of a virus (38). Thus, these factors may have determined whether a virus could enter a cell rather than whether a virus could transform a cell. Nevertheless, lymphomogenic murine viruses consistently have characteristic oligonucleotides in the *U3* regions of the genomes (39–41). The consistent presence of these oligonucleotides raises the possibility that these viruses, as ALVs, will cause disease by promoter insertions. If this is so, then our work with ALVs suggests that lymphomogenic murine leukemia viruses may also have non-*U3* sequences that target them for specific promoter insertions.

We thank S. Herman for oligonucleotide maps of recovered viruses, S. Bloom for assistance with statistical analyses, and S. Bloom and D. Steffen for comments on the manuscript. H.L.R. thanks the directors of the Worcester Foundation for Experimental Biology for making facilities available for *in vivo* oncogenicity tests and L. Crittenden for many informative and provocative discussions. We also thank D. Schwartz for providing us with his unpublished sequence of PrRSV-C. This research was supported by National Institutes of Health Grants CA

27223, CA 10467, CA 17659, and P01 CA 24530 and Core Grant P30-12708 and by the Mimi Aaron Greenberg Research Fund. J.M.C. was a recipient of a Faculty Research Award from the American Cancer Society.

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