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Avian Lymphoid Leukosis is Correlated with the Appearance of Discrete New RNAs Containing Viral and Cellular Genetic Information

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Lymphoid leukosis (LL) is a B-cell lymphoma of birds which is caused by a class of RNA tumor viruses called avian leukosis viruses (ALVs). These viruses also occasionally cause other neoplasms such as sarcomas and nephroblastomas. ALVs induce tumors in infected animals only after a latent period of 4–12 months and do not transform cells at detectable frequency in tissue culture (Hanafusa 1977). Despite intensive efforts, no transforming gene has been identified in these viruses. This suggests that the mechanism involved in neoplastic transformation by ALV is fundamentally different from that of the avian acute transforming viruses (sarcoma viruses and acute leukemia viruses). The acute viruses induce tumor formation within about 2 weeks, transform appropriate target cells in tissue culture, and code for transforming proteins (Bister et al. 1979; Erikson 1980; Graf und Beug 1978; Hanafusa 1977; Hanafusa et al. 1980; Hayman et al. 1979; Lee et al. 1980).

We have analyzed the virus-related RNA and DNA from more than 20 ALV-induced tumors. The data suggest that integration of the provirus of ALV induces increased expression of one or more normal cellular genes by providing a strong upstream promoter. We propose that enhanced expression of these cellular genes causes neoplastic transformation.

A. Results

I. Incidence of Tumor Induction

ALV ($\sim 10^7$ I.U./bird) was injected intravenously into 2–7-day-old chicks. Four virus strains were used: RAV-1, RAV-2, td103, and td107A. The latter two viruses are deletion mutants of RSV, lacking most or all of the *src* gene (Halpern et al. 1979; Kawai et al. 1977). No strain-specific differences were observed in the experiments described below.

Lymphomas were first detected at 4 months, and by 6 months (when all surviving birds were sacrificed) approximately 40% of the birds had developed visible tumors. The most commonly involved tissues were bursa, liver, kidney, and spleen. Of 23 tumors used for RNA analyses, 22 were diagnosed as lymphomas and one (#16L) was a fibrosarcoma in the liver. We also analyzed a continuous cell line, RP9, derived from a RAV-2-induced lymphoma (kindly provided by W. Okazaki).

II. Virus-Related RNAs in Tumor Cells

Tumor cell RNAs were analyzed by a modification (Brian Seed, personal communication) of the Northern transfer technique (Alwine et al. 1977). Virus-related RNA was identified by hybridization to ^{32}P-labeled "strong stop" DNA ($cDNA_{ss}$), which corresponds to the 5′ 101 nucleotides of the viral RNA (Haseltine et al. 1977).

The normal 35S and 21S RNAs of ALV (Hayward 1977; Weiss et al. 1977) were present in many, though not all, of the lymphomas analyzed. In addition, 19 out of 22 lymphomas (from 14 of 17 birds) contained new RNA species not found in uninfected cells or in nontransformed infected cells. These tumor-specific RNAs fell into three size classes of 2.5, 2.9, and 5.45 kb (designated classes I–III, respectively; examples are shown in Fig. 1, top). Other new RNAs were detected in some samples (e.g., RP9 and 19K), but these RNAs were not found in more than one tumor. In all cases where more than one tumor from

the same animal were examined (e.g., 7L, 7K, 10B, 10L, 11B, 11L, 9B, 9K) the same new tumor-specific RNA was found in each tumor, suggesting that metastases were derived from the same initial clonal event.

RNAs of classes I and II were not detectable with $cDNA_{rep}$, a probe representing the entire viral genome (Fig. 1, bottom). Thus these RNAs contain little, if any, viral information other than the 5′ sequences and presumably contain cellular sequences. The absence of viral sequences (other than 5′) in these RNAs was confirmed by both liquid and Northern gel hybridization using probes specific for different regions of the viral genome (data not shown). RNA of class III could be detected with $cDNA_{rep}$, but the intensity of the bands suggested that only a small portion of the information in these RNAs (not enough to account for their size) could be virus-specific. Analyses with other probes indicated that class III RNAs contain some gag-specific information, but no *pol*- or *env*-specific sequences.

RNA from several tumors was analyzed by liquid hybridization to determine the relative abundance of the virus-related RNAs. Three of the tumors selected (7K, 23L, and 9B) contained low or undetectable amounts of 35S and 21S RNAs in the Northern analysis. In each of these tumors the level of RNA detected with $cDNA_{ss}$ was at least 20-fold higher than that detected with $cDNA_{rep}$ (Table 1). The ss-containing RNA, presumably representing the new tumor-specific RNAs, was present at levels ranging from 100–1300 copies per cell. In other tumors the contribution of tumor-specific RNAs was obscured by the high levels of 35S and 21S RNAs.

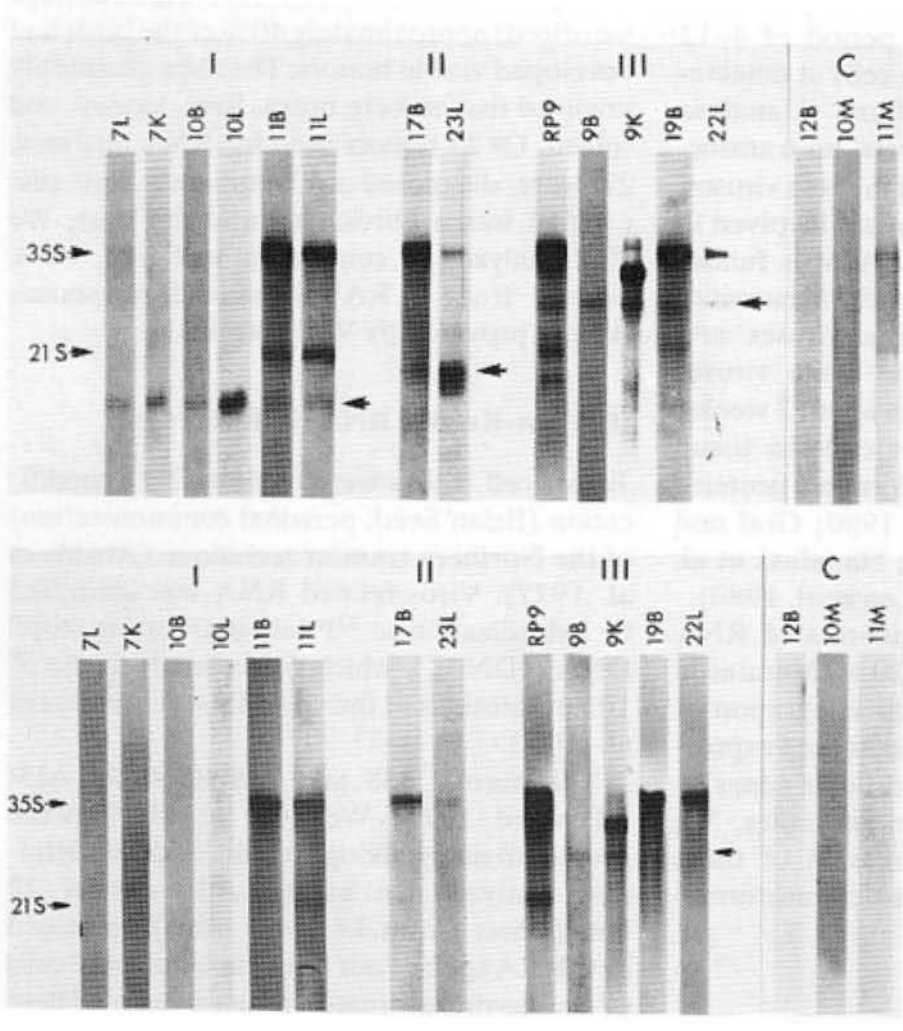

Fig. 1. Virus-related RNAs in lymphoma cells. Poly (A)-containing RNA from normal and tumor tissues were analyzed by the Northern gel transfer technique (Alwine et al. 1977). *Top panels*, RNAs detected with a probe ($cDNA_{ss}$) representing the 5′ 101 nucleotides of RAV-2. *Bottom panels*, RNAs detected with a probe ($cDNA_{rep}$) representing the entire RAV-2 genome. Sample designations indicate the animal number and the tissue involved: L, liver, K, kidney, B, bursa, M, muscle. Control RNAs *(C)* were from a normal bursa from an uninfected bird *(12B)* or from nonneoplastic tissues of infected birds *(10M, 11M)*. Tumor-specific RNAs of classes I, II, and III are indicated by arrows

Table 1. Viral and transformation-specific RNAs in tumor tissues

Tissue	RNA size (kb)[a]	RNA (copies/cell)[c] s.s.	rep	*src*	*erb*	*myb*	*fps*
12B (uninfected)	–	<1	<1	3	2	3	1
31L (uninfected)	–	<1	<1	2	4	1	1
7K (lymphoma)	2.5[b]	100	2	4	6	5	1
11B (lymphoma)	8.4, 3.3, 2.5[b]	2500	2000	3	2	4	2
23L (lymphoma)	8.4, 2.9[b]	100	5	5	2	3	1
9B (lymphoma)	5.45[b]	1300	1000	5	4	2	1
22L (lymphoma)	8.4, 5.45[b], 3.3, 2.5	300	300	4	N.D.[d]	5	2
RP 9 (cell line)	8.4, 7.2[b], 5.45[b], 4.0[b], 3.3, 2.9[b]	4000	1500	3	3	40	1
16L (sarcoma)	8.4, 6.7[b], 3.3	2000	1500	4	5	2	300

[a] Sizes were estimated by Northern gel analysis of glyoxalated RNA (see Fig. 1) using RAV-2 35S and chicken 27S and 18S RNAs as markers. [b] New RNA species not present in normal ALV-infected cells
[c] RNA concentrations were determined by liquid hybridization as described previously (Hayward 1977). cDNA probes used were: "strong-stop" (s.s.), corresponding to the 5′ 101 nucleotides of RAV-2 RNA; "rep," containing a relatively uniform distribution of all RAV-2 sequences; unique (presumably transformation-specific) sequences of RSV *(src)*, AEV *(erb)*, AMV *(myb)*, and Fujinami sarcoma virus *(fps)*
[d] Not determined

III. Expression of Cellular Transformation-Specific Sequences

Normal cells contain genes closely related to (and probably progenitors of) the putative transforming genes of the acute avian viruses (Chen, to be published; Erikson 1980; Hanafusa et al. 1977; Sheiness and Bishop 1979; Stehelin et al. 1976; to be published; Wang et al. 1979). To test whether these cellular genes are encoded in the new tumor-specific RNAs, we prepared probes corresponding to the unique sequences of RSV (termed *src*), AEV *(erb)*, AMV *(myb)*, and Fujinami sarcoma virus *(fps)*. None of the lymphoma tissues contained elevated levels of these RNA sequences (Table 1). However, the lymphoma-derived cell line, RP9, contained a higher level of *myb*-specific RNA (40 copies/cell vs 1–3 copies in control tissues). This RNA comigrated with a 4.0 kb RNA detected with $cDNA_{ss}$, but we do not yet know whether the ss and *myb* sequences are covalently linked.

An elevated level of *fps*-specific RNA was found in tumor 16L (the only fibrosarcoma found in our ALV-infected birds). Preliminary evidence indicates that this tumor was induced by a defective virus related to, but not identical to, Fujinami sarcoma virus. The origin of the virus in the fibrosarcoma is unknown, but one possibility is that it was derived by recombination between the infecting ALV and endogenous *fps*-specific sequences. We can find no evidence for participation of a defective transforming virus in any of the lymphomas examined.

IV. Restriction Analysis of Tumor DNAs

Tumor cell DNA was digested with the restriction endonuclease Eco R-1, size fractionated on agarose gels, and transferred to nitrocellulose paper by the technique of Southern (1975). Virus-specific restriction fragments were detected by hybridization with $cDNA_{ss}$. This combination of enzyme and probe identifies two fragments for each integrated provirus: an internal fragment from the left end and a virus-cell junction fragment from the right end.

Figure 2 compares the restriction patterns of DNA from normal and neoplastic tissues from three different birds. Restriction fragments from endogenous proviruses were detected in both normal (left panels) and tumor DNAs. The most frequent were the 8.4 and 18-kb fragments of ev-1, which were present in all birds used (Astrin 1978). In each case, however, new tumor-specific restriction fragments (indicated by arrows) were present in lympho-

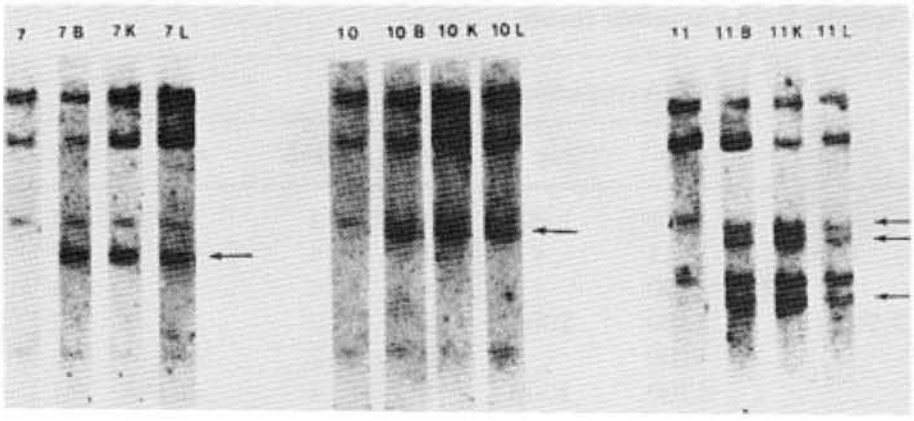

Fig. 2. Restriction analysis of DNAs from metastatic tumors. DNA was isolated from lymphomas of bursa *(B)*, kidney *(K)*, and liver *(L)* and from nonneoplastic muscle tissue *(M)* from three different birds. DNAs were digested with Eco R-1 and analyzed by the gel transfer technique of Southern (1975). Restriction fragments encoding 5′ sequences of ALV were identified by hybridization to ^{32}p-cDNA$_{ss}$. Tumor-specific junction fragments are indicated by *arrows*

ma DNAs. These fragments contain both viral and cellular sequences ("junction fragments") as they are not detected with cDNA$_{rep}$ (data not shown). The fact that distinct junction fragments could be detected argues strongly that all or most of the cells in each tumor are derived from a single infected cell. (Distinct junction fragments are not detected in randomly infected cell populations, since integration occurs at many different sites.) All tumors from a single bird contained the same tumor-specific junction fragments (Fig. 2); thus metastatic tumors are clonally derived. Similar observations have been reported by others (Neiman et al. 1980; G. Payne, J. M. Bishop and H. Varmus, personal communication; H.-J. Kung, personal communication).

The integrated proviruses in tumors from birds 7 and 10 are apparently defective, since these DNAs lack the 2.6-kb internal fragment (which would be below the tumor-specific bands indicated by arrows). This is consistent with the absence of 35S and 21S viral RNAs in these tumors (Fig. 1).

New tumor-specific junction fragments were found in all tumors analyzed (21 birds). Four size classes of junction fragments, designated class A (3.8 kb), B (3.2 kb), C (3.0 kb), and D (3.5 kb), were found to be common to more than one tumor (Fig. 3). All tumors tested contained one of these new junction fragments. These data are consistent with the interpretation that each tumor contains viral information at one of four common sites, but further analyses of the flanking cellular sequence are needed before any final conclusions can be drawn.

The presence of a specific junction fragment correlated with the presence of a specific new tumor-specific RNA in eight of the ten birds in which both RNA and DNA were analyzed. (The exceptions were birds 7 and 19). In general, class A DNA correlated with class I RNA, class B, with class II, and class C with class III.

B. Discussion

From these data we conclude that expression of viral genes is not required for maintenance of neoplasia, since many tumors (at least 30%) lack complete proviruses and do not express the normal viral 35S and 21S mRNAs. The data suggest that lymphoma induction occurs only when the provirus integrates at one of a limited number of sites on the host cell chromosome. The provirus could induce the expression of an adjacent cellular gene, analogous to the endogenous transformation-related genes, by read-off from the strong viral promoter. We call this the "promoter insertion" model of leukemogenesis.

The promoter for viral RNA synthesis is located at both the left and right ends of the provirus, within the "3′" region of the terminal repeat (Dhar et al. 1980; Sutcliffe et al. 1980; Yamamoto et al. 1980), (see Fig. 4). Read-off from the right end promoter of a normal provirus (Fig. 4, top) would generate an RNA transcript containing viral 5′ sequences plus adjacent cellular sequences. Alternatively, read-off could occur from the left end promoter of a permuted (Fig. 4, bottom) or defective

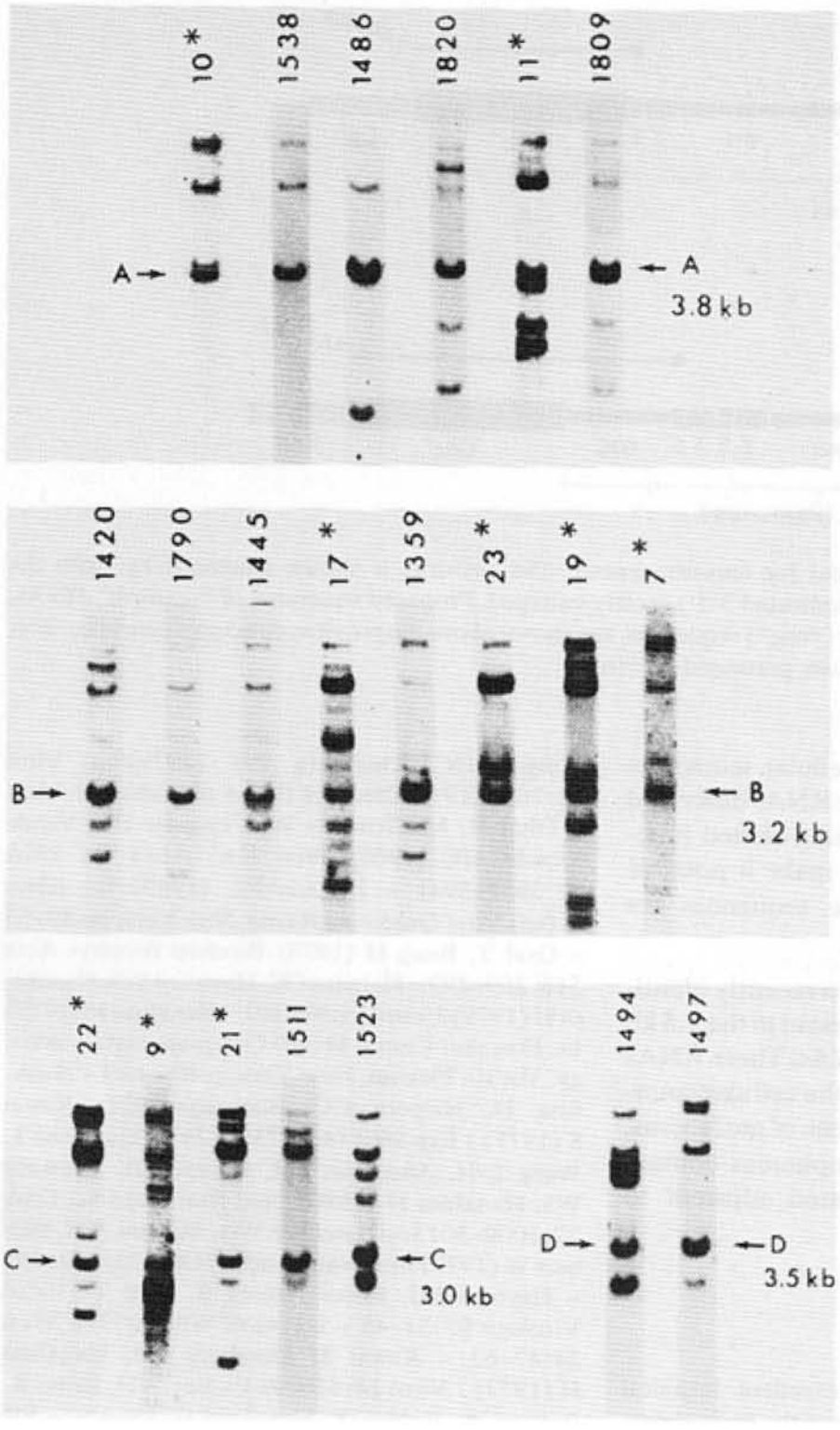

Fig. 3. Restriction analysis of DNAs from ALV-induced lymphomas. Lymphoma DNAs from 21 birds were analyzed as in Fig. 2. Junction fragments common to more than one tumor fell into four size classes *(A–D)* as indicated by *arrows*

(not shown) provirus, generating a transcript containing viral 5′, (possibly) gag, and cellular information. The latter mechanism would explain the presence of gag-specific sequences in class III RNA.

The major assumption of the promoter insertion model is that cell transformation is caused by the elevated expression of a normal cellular gene. If this model is correct, it seems likely that oncogenesis by other, nonviral agents could occur by similar mechanisms. Expression of one of these cellular genes could be altered by mutation or structural rearrangements induced by external agents such as radiation or chemical carcinogens rather than by insertion of the viral promoter. While we

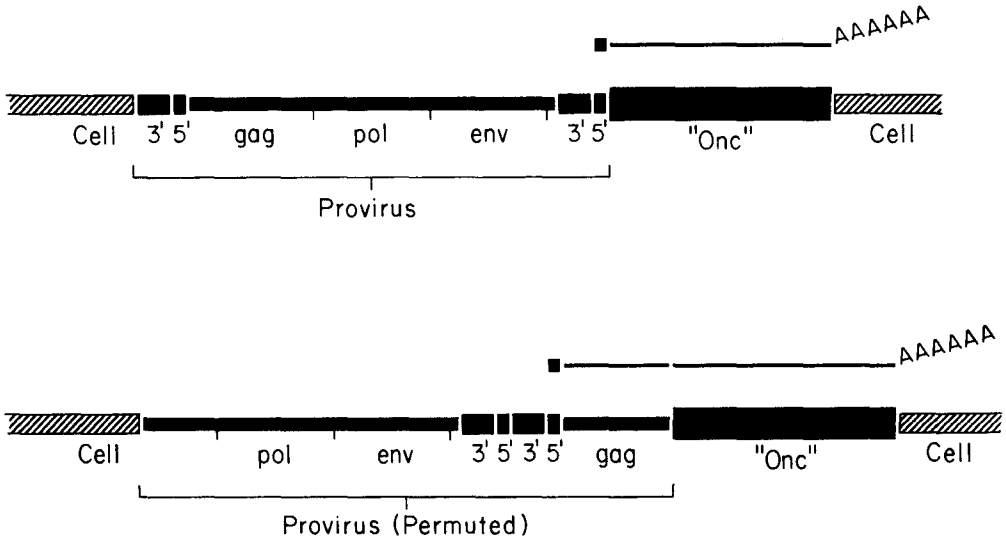

Fig. 4. Promoter insertion model for leukemogenesis. The provirus is shown schematically, with the terminally repeated sequences (indicated 3'5') greatly enlarged. Proposed structures of "read-off" RNAs, containing both viral and cellular ("onc") sequences, are shown above the provirus structures (see text). *Top,* normal integrated provirus. *Bottom,* permuted provirus

have not yet identified the cellular sequences encoded in the tumor-specific RNAs described above, studies in progress using cloned junction-fragment DNAs should make it possible to determine whether these sequences are involved in nonviral cancers.

Note added in proof: We have recently identified the cellular sequences present in the 2.5 kb and 2.9 kb tumor-specific RNAs. These RNAs are transcribed from c-*myc,* the cellular counterpart of the transforming gene of mc2a virus. Nearly all ALV-induced lymphomas contain proviral information integrated adjacent to *c-myc.*

Acknowledgments

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