Haematology and Blood Transfusion Vol. 26
Modern Trends in Human Leukemia IV
Edited by Neth, Gallo, Graf, Mannweiler, Winkler

The Molecular Basis of Avian Retrovirus-Induced Leukemogenesis

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A. Introduction

There is overwhelming evidence that infection by retroviruses can lead to transformation of the infected cell and to tumor development in the target organ of the host. However, the mechanism by which virus-induced oncogenic transformation occurs is not clearly understood. The viral tumorigenesis involves several steps, including infection by the virus, integration of provirus, transformation of the target cells, tumor development, and in some cases metastasis. In an attempt to gain insight into the molecular mechanism of this process, we have studied the avian leukosis virus [(ALV) e.g., RAV-1] induced lymphoid leukosis (LL) by following the fate of proviral DNA of infecting virus through preleukosis, leukosis, and metastasis stages. The results of these studies presented below have indicated that there are multiple sites in the cellular genome of the target tissue where the proviral DNA of infecting virus can integrate, that there may be a few preferred sites at which integration can lead to tumor formation, that deletions and other structural alterations in the proviral DNA may facilitate tumorigenesis, that origin of LL tumors is clonal, and finally that metastasis arise by migration of a primary clone to the secondary site.

B. Experimental Approach

I. Source of DNA

Sixteen newborn chickens from an inbred line $15I_5 \times 7_2$ of Regional Poultry Research Laboratory, East Lansing, Michigan, United States were used for these experiments and each was infected with 10^5 clone-purified RAV-1 viruses. Bursal specimens at 4 and 8 weeks post infection were obtained by biopsy. Around 16–20 weeks after infection chickens showed signs of leukosis. Bursal tumors could be felt by palpation and were surgically removed. Visual inspection of dissected chickens showed that in several cases tumors had metastasized, and distinct-looking foci from liver and spleen were excised. DNA from all specimens was extracted (Huges et al. 1978) and analyzed by the Southern technique (Southern 1975).

II. Hybridization Reagents

$cDNA_{rep}$: 32p-labeled DNA probe complementary to RAV-1 RNA and representative of the entire genome was synthesized using reverse transcriptase (RT) and calf thymus DNA primer (Hughes et al. 1978; Taylor et al. 1976). $cDNA_{3'}$: A probe complementary to the 3′ terminus of RAV-1 RNA and specific for RAV-1 (Neiman et al. 1977; Coffin et al. 1978) was synthesized using RT and oligo dT_{12-18} primer (Tal et al. 1977). $cDNA_{5'}$: This probe was synthesized using an endogenous reaction (Friedrich et al. 1977) and the 101-nucleotide-long "strong-stop" fragment (Haseltine et al. 1976) was purified by polyacrylamide gel electrophoresis (Friedrich et al. 1977). DNA_{gag}: This probe was prepared by nick translation (Rigby et al. 1977) of a gag containing EcoRI-SacI segment of the cloned proviral DNA (Fig. 1a).

III. Identification of Exogeneous Provirus

We have used restriction endonucleases SacI or EcoRI and viral probes capable of distinguishing between endogenous and RAV-1-speci-

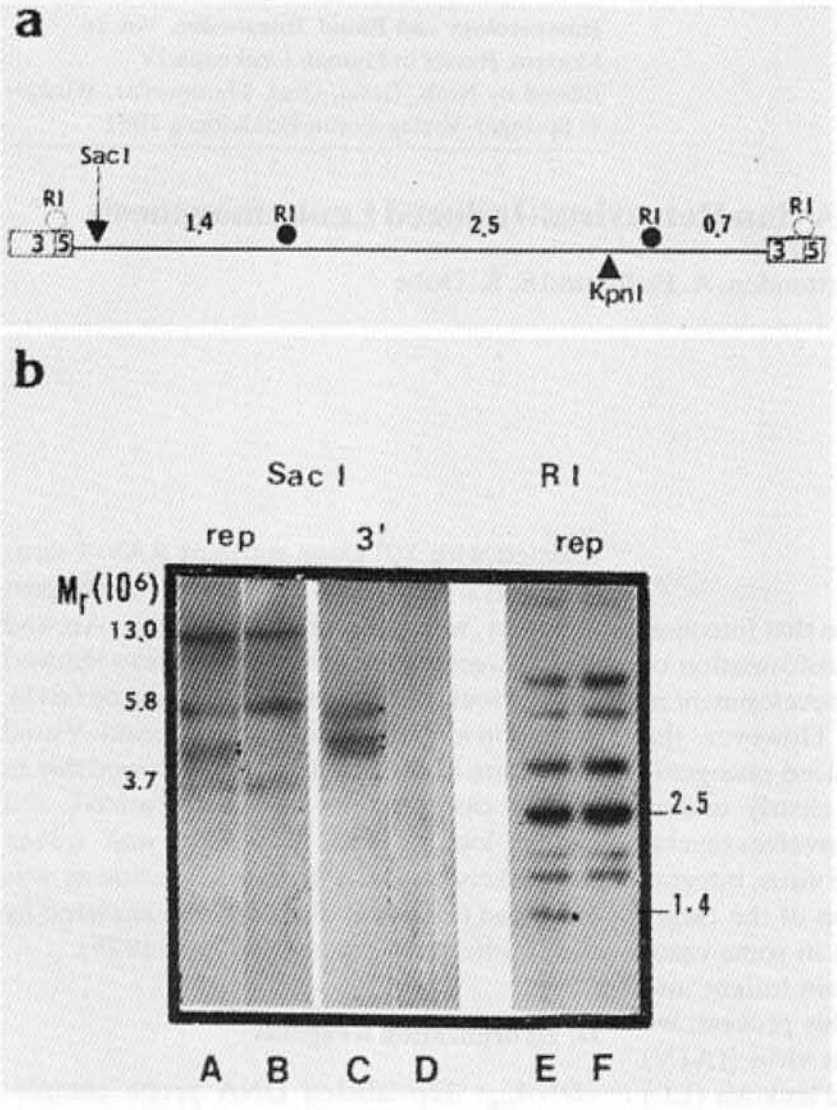

Fig. 1. The restriction-enzyme cleavage maps of RAV-1 DNA and the identification of tumor-specific (TS) proviral DNA. **a** The cleavage maps of restriction enzymes EcoRI, SacI, and KpnI. *Open triangles* indicate EcoRI sites not present in the ev sequences. The *boxed 35* represents the large terminal repeat (LTR), which is located at both termini of the viral DNA and carries the 3′ and 5′ terminal sequences of the RNA genome. **b** Restriction enzyme digestion analysis of proviral DNA. The DNA samples were extracted from bursa tumor #1 *(lane A* and *C)*, from the nontumorous thymus *(lane B* and *D)* of the same bird, and from the in vitro RAV-1-infected *(lane E)* or uninfected *(lane F)* chicken embryo fibroblasts of line $15I_5 \times 7_2$. They were digested with SacI or EcoRI, analyzed on 0.8% agarose gel and by Southern blotting hybridizations with $cDNA_{rep}$ or $cDNA_3$

fic sequences. The rationale of the method is outlined below.

The $15I_5 \times 7_2$ has three ev loci (Astrin 1978) which are readily revealed as three bands of molecular weight 13 md (ev-6), 5.8 md (ev-1), and 3.7 md (ev-2) upon cleavage of genomic DNA with SacI and hybridization with $cDNA_{rep}$. In the example shown in Fig. 1b both nontumor and tumor tissue DNA display these three bands (lane A,B). However, tumor DNA has two additional bands. Since they are present only in tumor DNA, we refer to them as tumor-specific or TS bands. Their exogenous origin was established by hybridization with $cDNA_3$, (lane C,D), since the 3′ terminal region of the RAV-1 genome does not share homology with any endogenous virus sequence (Neiman et al. 1977; Coffin et al. 1978). The specificity of this probe is demonstrated by the complete absence of ev-related fragments in lane D. The tumor DNA shows three distinct bands; two of these are identical to TS bands detected by $cDNA_{rep}$, and the third was presumably obscured by ev-1 in the $cDNA_{rep}$ hybridization.

Since SacI has a single site in proviral DNA, the fragment size is determined not only by the location of this site in viral genome but also by the nearest enzyme cleavage site in the flanking cellular sequence. Therefore, SacI can provide information concerning the integration site of proviral DNA. On the other hand, there are several cleavage sites for EcoRI in the viral genome allowing for the probing of internal structural arrangement of proviral DNA (Fig. 1a). In addition, the ev sequences lack the two outer sites (indicated by open circles) which are present in the exogenous proviral DNA. Consequently, either the 1.4-md or 0.7-md fragment can be used to demonstrate the integration of infecting virus. As shown in lane E and F, the 1.4-md fragment is present in infected but not in uninfected cells.

C.Results

I. Preleukosis

Analysis of SacI-cleaved DNA from bursal specimens obtained at 4 weeks post infection showed a complete absence of TS bands (Fig. 2a). This was not due to inefficient infection of target tissue because the RAV-1 specific 1.4-md EcoRI fragment could be clearly identified at this stage in the inoculated (I) samples (Fig. 2b). Since the TS bands contain viral-cell junction sequences, their absence indicates that proviral DNA integrates in multiple sites in cellular genome. The data in Fig. 2b also provide an estimate of the extent of infection in the target organ at this early stage; based on the relative intensities of the RAV-1 (1.4-md fragment) and ev fragments, at least 25% of the bursal tissue had been infected at the 4 week stage. The analysis of bursal DNA from specimens 8 weeks after infection gave similar results (data not shown) except that the extent of infection was greater.

II. Leukosis

In contrast, SacI-cleaved tumor DNAs from all 16 chickens showed new bands. The results are summarized in Table 1 and gel patterns of representative tumor samples are shown in Fig. 3. In some instances (e.g., 3, 4, and 5 in Fig. 3a) the TS bands were very faint as detected by $cDNA_{rep}$. However, they could be readily detected using a highly specific $cDNA_{3'}$ probe (Fig. 3b). These data taken together with the observation in the preceding section indicating multiplicity of integration sites suggest that only a small population of the infected cells develops into a tumor and the origin of tumor, therefore, must be clonal.

The results also show a size variation of TS bands in different tumors suggesting that integration in a number of sites can lead to the development of a tumor. However, the other equally plausible but not mutually exclusive possibility is that deletion within the proviral DNA contributes to size variation. This possibility was examined using a DNA_{gag} probe for hybridization (Fig. 3c). Most striking are the results of tumor DNAs 2 and 5 where DNA_{gag} failed to detect any TS bands, although these bands were readily detectable by $cDNA_{3'}$. This immediately suggests that gag sequences in the proviral DNA of these tumors have been deleted.

Further evidence for the deletion of gag

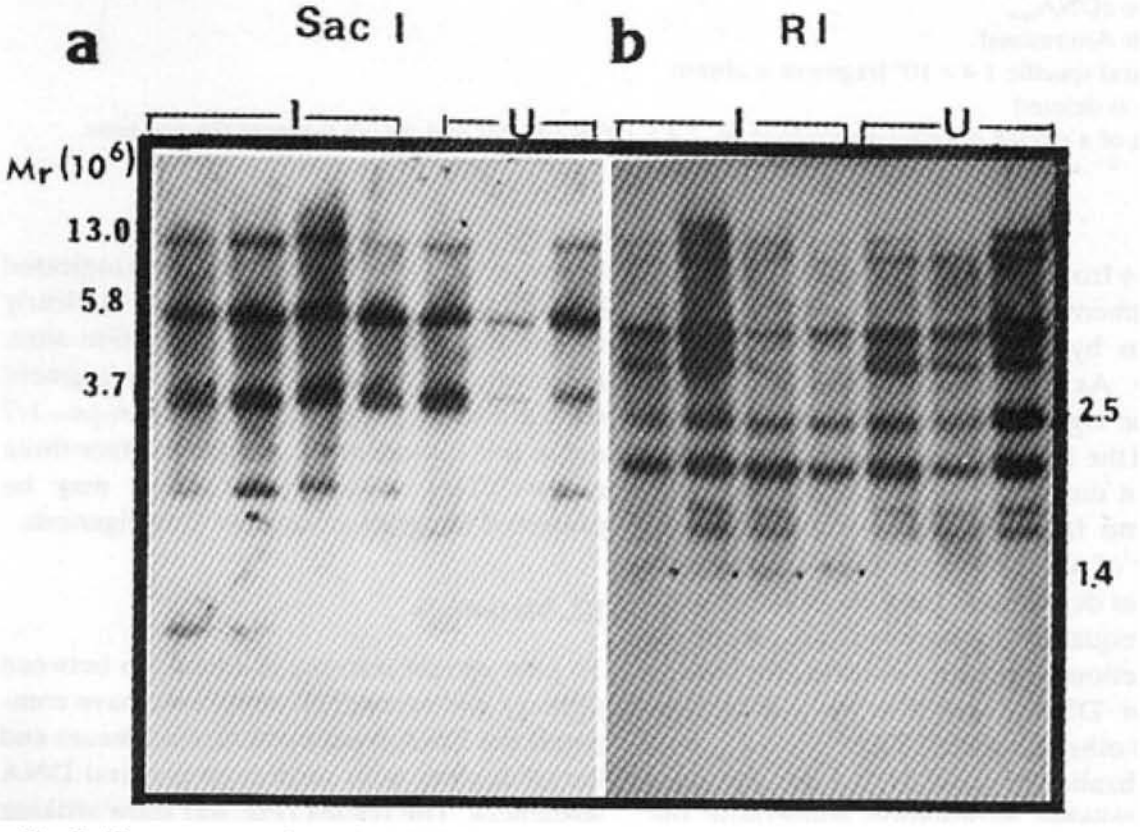

Fig. 2. The structure of proviral DNA in bursa tissues at the preleukosis stage. The DNA samples extracted from the bursae of the uninoculated *(U)* or inoculated *(I)* animals at 4 weeks post inoculation were digested with SacI or EcoRI and analyzed by hybridization with $cDNA_{rep}$

Table 1

Sample	Tissue	TS fragment[a] (SacI/3′)	Right end[b] cell-viral junction (EcoRI/5′)	Deletion and insertion, etc.
1	Bursa	5.8, 4.5, 4.3	1.1, 0.9	–
2	Bursa	8.0, 4.7	ND[c]	–
3	Bursa	8.0	2.3	RI–1.4 (−)[d], gag (Δ)[e]
4	Bursa	5.2, 4.5	1.7	RI–1.4 (−)
5	Bursa	5.3, 4.5, 2.9	2.8, 1.7	RI–1.4 (−), gag (Δ)
6	Bursa	5.8, 5.5	1.7	–
7	Bursa	5.8, 4.8, 4.5 3.0	1.7, 1.58	RI–1.4 (−)
8	Bursa	6.0	0.8	–
9	Bursa	9.0, 5.3	2.1	–
10	Bursa	2.5	2.3, 2.0	RI–1.4 (−)
11	Bursa	8.0, 7.8, 7.5	0.6	–
	Liver	8.0	2.3, 2.0	RI–1.4 (−)
12	Bursa	8.0, 4.0	1.43	RI–1.4 (−)
13	Bursa	5.0	ND	RI–1.4 (−), gag (Δ)
14	Bursa	4.5, 1.2	1.7	–
15	Bursa	8.0, 4.8, 4.2	2.8, 2.6	RI–1.4 (−), Insertion[f]
16	Bursa	–	0.6	RI–1.4 (−)

[a] TS fragment is defined as the SacI fragment which can be detected only in the tumor tissue and is hybridizable to $cDNA_{3'}$. Molecular weight in 10^6 daltons

[b] Right-end cell-viral junction fragment is defined as the EcoRI fragment which hybridizes only to $cDNA_{5'}$ but not to $cDNA_{rep}$

[c] ND = Not determined

[d] EcoRI viral specific 1.4×10^6 fragment is absent

[e] gag gene is deleted

[f] Insertion of a stretch of cellular sequence ca. 2.4×10^6 at the left end, which replaces the gag gene

sequences from these DNAs was provided by an experiment in which EcoRI-cleaved tumor DNA was hybridized with a $cDNA_{5'}$ probe (Fig. 3d). As shown in Fig. 1a, $cDNA_{5'}$ can detect the right end viral-cell junction fragment and the 1.4-md gag-containing fragment. Indeed, in tumor DNAs 2, 3, and 5 (Fig. 3d), the 1.4-md fragment is completely missing. A survey of all 16 chickens (Table 1) demonstrates that deletions in the viral genome occur rather frequently in tumor DNA. Some of these deletions are quite extensive; for instance, tumor DNA 5 contains very little viral sequence other than the LTR.

Hybridization with $cDNA_{5'}$ also provides a more reliable information concerning the right end integration site of proviral DNA, since the results are not influenced by extensive deletions in the viral genome. The size heterogeneity of the end fragments (indicated by dots in Fig. 3d; also see Table 1) clearly argues for a multiplicity of integration sites. However, it is noteworthy that some fragment sizes are more prevalent than others, i.e., 1.7 md in five tumors and 2.3 md in another three tumors. This suggests that there may be preferred integration sites for tumorigenesis.

III. Metastasis

To gain insight into the relationship between primary and secondary tumors we have compared the DNA from metastasized tissues and bursal tumors with respect to proviral DNA sequences. The results (Fig. 4a) show striking similarity in KpnI-derived TS band patterns from liver and bursa, suggesting that primary and secondary tumors share the same clonal

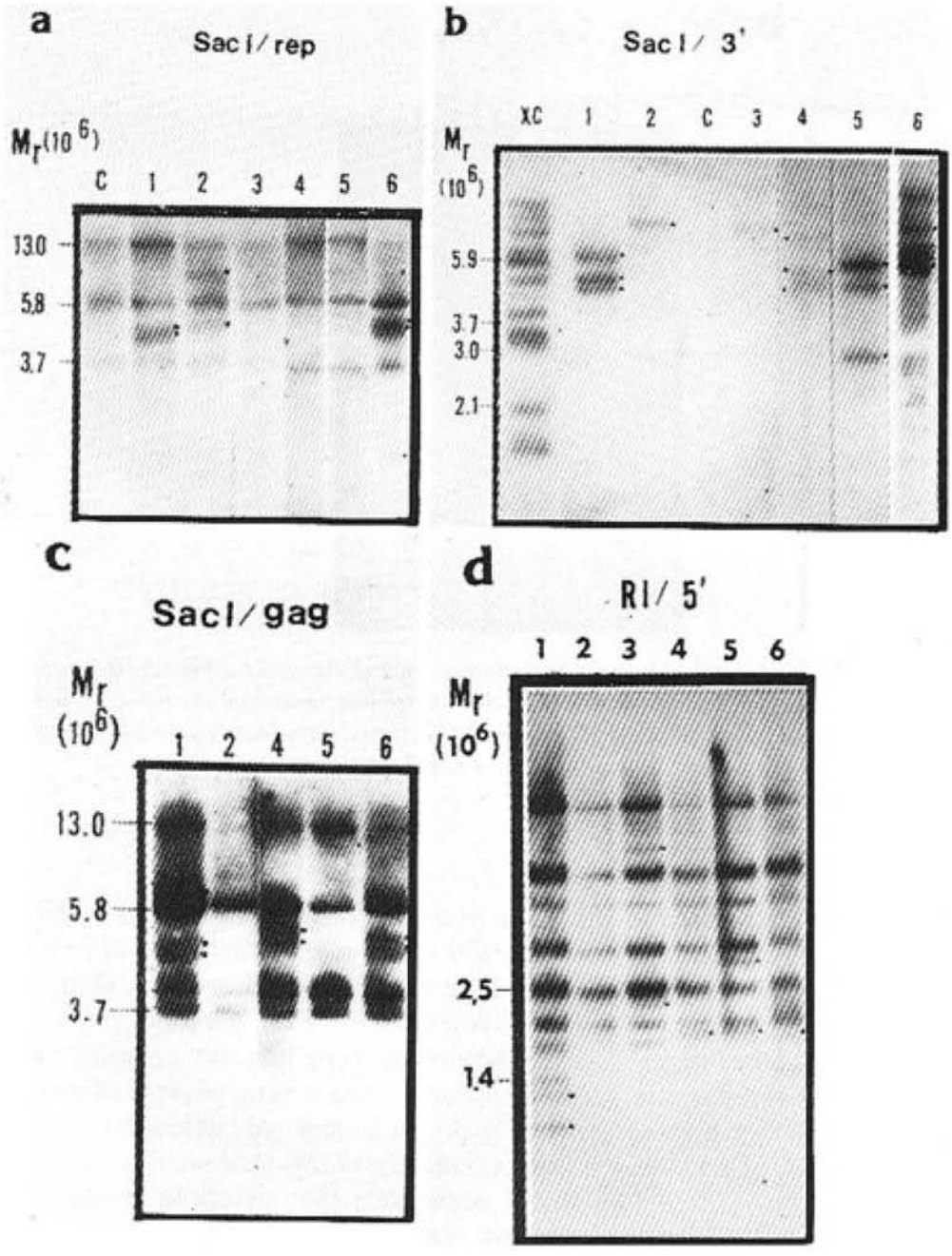

Fig. 3. The structure of proviral DNA in bursa tumor. The DNA samples isolated from the bursal tumors developed in animals Nos. 1 to 6 were digested with SacI or EcoRI and analyzed as described in legend to Fig. 1. The hybridization probes employed are $cDNA_{rep}$ **a),** $cDNA_{3'}$ **(b),** DNA_{gag} **(c),** and $cDNA_{5'}$ **(d).** *Lane C* in either **a** or **b** shows SacI digested thymus DNA isolated from animal No. 2, which was included as a control sample for nontumorous tissue. XC represents EcoRI-cleaved high-molecular-weight DNA from Rous sarcoma virus transformed XC cells, which serves as a molecular size marker

origin. Furthermore, Fig. 4b shows that different liver foci (L1 to L4) from another chicken have identical band patterns which indicates that individual foci derive from a single clonal population. The figure also shows that liver foci have a single SacI-TS band while in bursa there were at least three closely spaced TS bands (Fig. 4b). This suggests that only one of the original multiple tumor clones in bursa was selected for secondary spread.

D. Discussion

As a first step toward characterization of the oncogenes, we have employed EcoRI digestion of tumor DNA in conjunction with $cDNA_{5'}$ hybridization to specifically identify the right end cell-viral junction (i.e., integration sites) of ALV proviruses. Among 16 tumors analyzed, at least ten different size classes could be identified. However, a few size

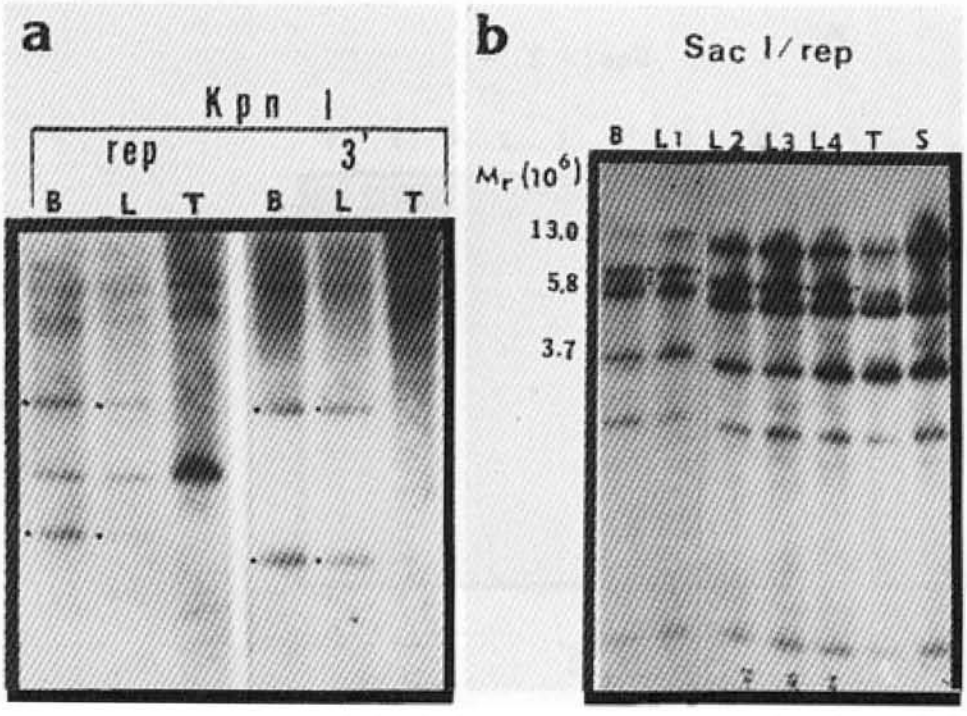

Fig. 4. The structure of proviral DNA in metastic tumors. **a** KpnI digestion analysis of the bursal *(B)* and liver *(L)* tumor DNA of animal No. 8. Included as control is the DNA from nontumorous thymus *(T)*. **b** SacI digestion analysis of the DNA isolated from the bursa *(B)* and four individual liver foci *(L1* to *L4)* of animal No. 12. Nontumorous thymus DNA *(T)* is used as a control. The hybridization probe is $cDNA_{rep}$

classes, e.g., 1.7 md and 2.3 md, appeared to be more prevalent than others. If size variation truly reflects the sequence diversity of the integration sites, our data suggest that all tumors are not the consequence of the integration of provirus into a unique cellular site. Similar conclusions have been drawn by Neiman et al. (1980) from their study of ALV-induced tumors.

Although our sampling is not sufficiently large to give a reliable estimate of the total number of integration sites in the entire genome, the observation that cell-viral junction fragments of identical sizes are present in many tumors does indicate a certain degree of specificity of integration of proviral DNA. Our data, therefore, are consistent with the view that there are a limited number of gene(s) which, upon activation by provirus, could trigger the transformation process. Whether this gene(s) is involved directly in the initiation of transformation process (in a manner similar to the src gene product) or indirectly in the induction of a transforming protein awaits further characterization. To further understand the nature of the downstream sequence, we have recently obtained a clone-purified SacI-TS sequence from two of the tumors, and experiments are underway to characterize their structures.

One of the striking features in our finding is the detection of extensive deletions of proviral DNA in at least 35% of the tumors analyzed. Since deletion of viral genome (with the exception of src gene in avian sarcoma virus) rarely occurs during in vitro passage of viruses, such high frequency of deletions implies a functional role of this process in tumorgenesis. It is possible that deletions in the viral genome (or other means which disrupts the transcriptional program of viral RNA) facilitate the downstream promotion by the right LTR. This possibility is particularly attractive in view of the fact that the two LTRs flanking the viral genome are strong promotors for RNA transcription (Tsichlis and Coffin 1980) and the left end LTR directs the synthesis of viral genomic and messenger RNA (Weiss et al. 1977). Although the functional state of the right LTR remains to be determined, a not unlikely possibility is that it is normally masked by the ongoing RNA synthesis which starts at the left LTR and extends into the 5′ sequence of the right LTR (Yamamoto et al. 1980). However, a disruption of the transcriptional program affected by deletions in the proviral DNA may expose the right LTR, facilitate the RNA polymerase binding, allow efficient transcription of the downstream cellular se-

quence, and activate gene(s) involved in oncogenesis. The detection of novel mRNA species (W. Hayward, see this volume; G. Payne and H. E. Varmus, personal communication) carrying the viral promotor joined with cellular sequences lends further support to this notion.

The deletion of viral sequence may also play a role in the selective growth of the tumor clones. The first sign of lymphocyte transformation after avian leukosis virus inoculation is the appearance of enlarged follicles in the Bursa of Fabricius at 8 weeks of age (Cooper et al. 1968). These enlarged follicles, identified only at the microscopic level, are believed to be the descendents of a single transformed cell and the precursors to the terminal tumors. They number 10 to 100 per infected bursa (Neiman et al. 1979); the terminal tumor follicles, however, are much fewer (i.e., only one or two). This observation led to the suggestion that some of the transformed clones regressed and only a small fraction acquired the ability to develop into tumor. We would like to speculate that deletion of viral genome which stops the synthesis and expression of the viral antigens (especially exogenous virus specific *env* product) on the cell surface would render the cell less immunogenic and furnish it with the ability to cope with the host immunity. Since in this study, due to the reagents and methods employed, most of the deletions are mapped near the gag region, it is likely that more extensive analysis would reveal deletions in other regions as well.

Irrespective of the implication of deletion of provirus in the tumorigenic process, it is evident from our data that the presence of a complete provirus and, hence, virus production is not required at the terminal stage of the tumor. This finding lends further support to the hypothesis that the oncogene(s) involved in the maintenance of cells in the transformed and tumorous state is (are) of cellular rather than of viral origin.

The characteristic proviral DNA structure of each tumor as revealed by SacI digestion provides strong evidence that LL tumors are clonal growth and that primary and secondary tumors share common clonal origin. Furthermore, the metastic tumor consists of a subpopulation of the primary tumor. The factors which dictate the metastatic potential of the primary tumor cells are currently unclear, though deletion of the provirus of the tumor cell may enhance such potential, since we found that the provirus in almost all the metastatic tumors carries extensive deletions.

Acknowledgments

This research was supported in part by a grant from the National Cancer Institute (CA 24798-01) to H.J.K. S.K.D. is a Leukemia Society of America Scholar. We thank Ms. Sue Uselton for excellent technical assistance in the preparation of the manuscript.

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