Haematology and Blood Transfusion Vol. 28
Modern Trends in Human Leukemia V
Edited by Neth, Gallo, Greaves, Moore, Winkler

Determination of Virulence Properties of Leukemia Viruses*

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Understanding the basis of virulence of leukemia viruses is a major unsolved problem. Under natural and experimental conditions, retroviruses may replicate freely in the host with no profound consequence. Such is typically the case for vertically transmitted viruses that express early in life, e.g., the endogeneous baboon and murine Akv virus [1]. We call such viruses avirulent. Horizontally transmitted retroviruses usually induce disease, but typically only after a lengthy latent period and then in only a small fraction of infected individuals [2]. Viruses of moderate virulence include feline, bovine, and gibbon ape leukemia viruses. In these cases, rampant viremia is usually followed by immune suppression of the infection. Onset of frank leukemia is delayed by months or years and is a low probability event. The virus-associated with adult T-cell lymphoma in humans, HTLV, probably falls into this class of virus (Gallo et al., this volume). Some experimentally manipulated leukemia viruses are highly virulent. For example, the Moloney strain of murine leukemia virus induces a rapid (3 months to death) disease in a high proportion of injected animals.

To approach the problem of a murine leukemia virus, we selected avirulent and highly virulent strains for intensive study. Our initial goal was to isolate strains of murine leukemia virus that were isogenic except for the virulence phenotype. This criterion was met by isoloation of the SL3-3 virus from a lymphoid cell line derived from a thymus of a diseased AKR mouse. This virus induces terminal thymic leukemia in close to 100% of injected newborns of the low-incidence leukemia strain, C3H/f, NSF/N, CBA, and SJL, within 60–100 days. the Akv virus is avirulent in similar tests. The biological properties of this virus resemble closely those of the avirulent virus Akv. The viruses are N-tropic, ecotropic strains that form XC plaques. Both grow to similar titers on NIH 3T3 fibroblast cultures, and replicate to similar titers upon injection of newborn mice.

Analysis of the genomic RNA of Akv and SL3-3 viruses shows them to be closely related [3]. Oligonucleotide maps of several AKR viruses are presented in Fig. 1. Using this method, we estimate that the SL3-3 and the Akv virus differ by no more than 50–70 point mutations.

To further characterize the structure of these viruses, we obtained an infectious clone of the provirus of the SL3-3 strain. Comparative studies of the proviruses were done using an infectious clone of the Akv provirus provided to us by Doug Lowy [4]. Heteroduplex and restriction enzyme analyses confirm the close structural relationship between these strains [5]. Viruses produced by transfection of NIH 3T3 cells with these proviral DNAs have the same biological and virulence properties as the parental strains [5].

Our present strategy for further study of these involves:

Determination of the entire nucleotide sequence of both proviral DNAs. This information will provide definitive in-

* This work was supported by a grant from the National Cancer Institute. W. Haseltine is the recipient of the American Cancer Society Faculty Research Award No. FRA-166

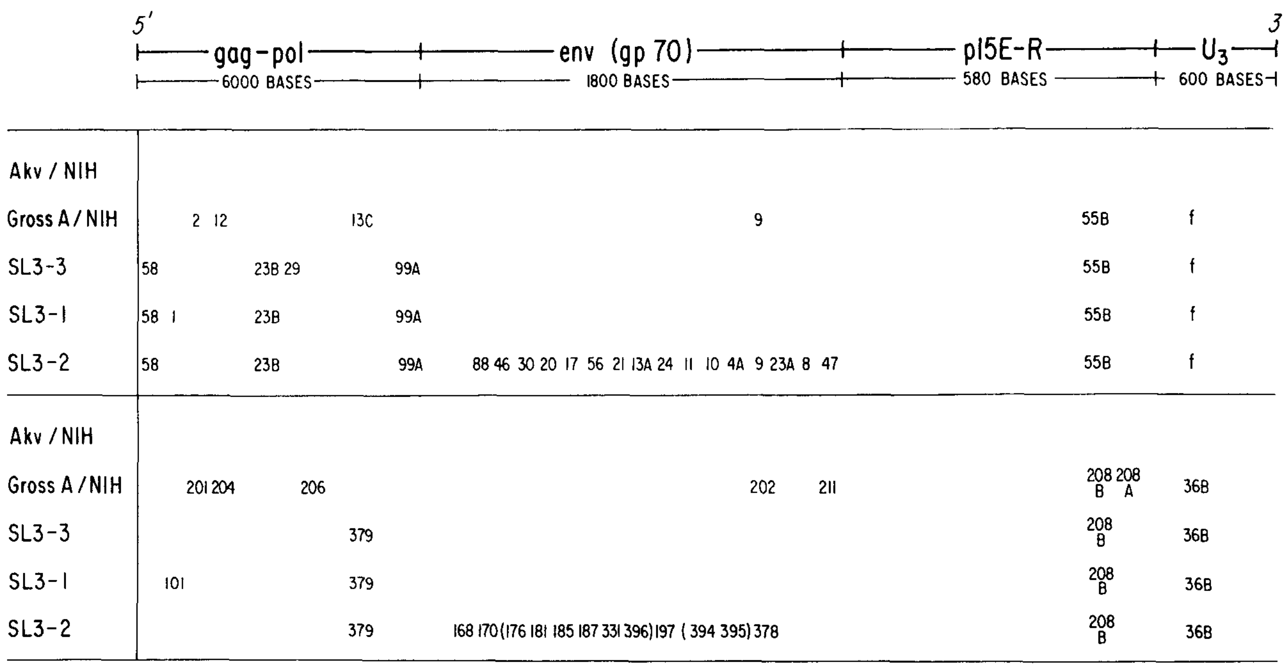

Fig. 1. Oligonucleotide maps of the AKR viruses. The *upper panel* represents oligonucleotides present in Akv which are missing from other viruses. The *lower panel* represents the positions of oligonucleotides which are not present in Akv. For the Akv genome, there are a total of 34 oligonucleotides within the *gag-pol* region, 16 within gp70, 9 within p15E-R, and 6 within U_3

formation regarding the structural differences in the viral genomes.

Construction of recombinant viruses, using proviral DNA intermediates, to localize virulence determinants.

Progress to date includes:

1. Determination of the sequence of the gp70-p15E region encoded by the Akv virus [6]. Analysis of this sequence permits construction of a speculative model for association of this protein with the viral membrane (Fig. 2A, B). Comparison of the sequence of the Akv virus with that of the SL3-3 virus and other vertically transmitted and other virulent strains isolated from AKR thymoma cell lines show a common divergence in the p15E region. The arg-leu to his-met change indicated in Fig. 2B occurs at a site at which the postulated "anchor" of the p15E protein enters the membrane. This change may have a physiological consequence.

Another change in sequence of the virulent versus avirulent strains was detected in the noncoding LTR region of the virus. A single point mutation from A to G located near the 5′ end of the LTR (58 to f, Fig. 1) is characteristic of the virulent strains.

2. Construction of a virulent recombinant virus. A recombinant virus produced by reassortment of fragments of the Akv and the SL3-3 proviruses was used to infect newborn mice. The structure of this recombinant is pictured in Fig. 3. The virus induced thymic leukemia in most of the injected animals. The latent period of disease onset was similar to that of the SL3-3 strain itself. In this recombinant, most of the LTR (all but the 5′ 36 base pairs), the entire *gag* region as well as half of the *pol* region of this virus is of SL3-3 origin. The 3′ half of the *pol* gene and the entire gp70-p15E, as well as the noncoding region including 36 bases of the 5′ end of the LTR, is of Akv origin.

The simplest explanation for these data is that changes in the gp70-p15E region do not effect the virulent phenotype, nor do changes in the 3′ region of *pol*. Changes in the LTR, *gag*, and 5′ region of *pol* are, therefore, implicated in the virulent phenotype. However, a firm conclusion is somewhat premature as the properties of the reciprocal recombinant are still not known. Nonetheless, the results of such recombinant constructions should permit

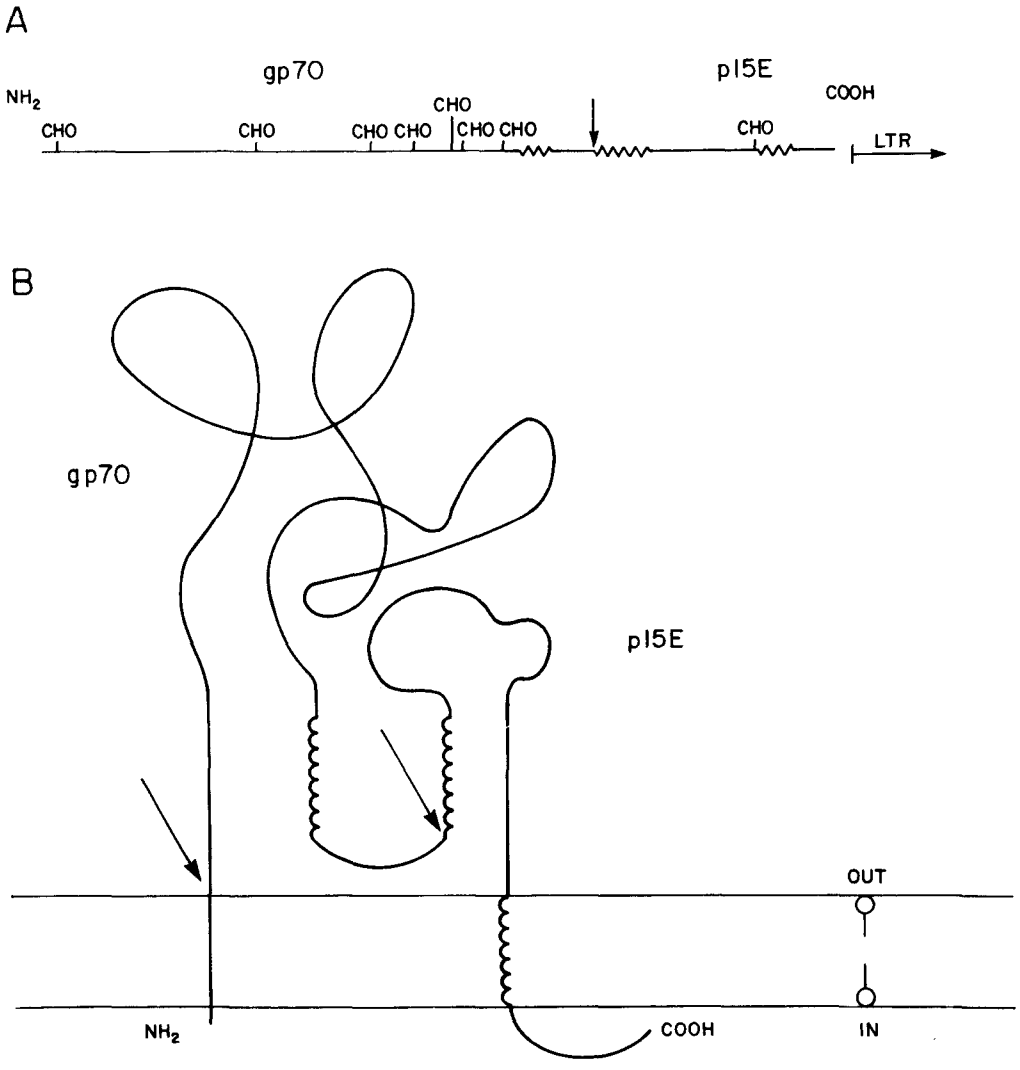

Fig. 2 A, B. **A** Linear representation of the *env* precursor from the amino terminus of gp70 ot the carboxyl terminus of the precursor. The amino terminus of pl5E is indicated by an *arrow,* as is the position of the boundary of the LTR. Potential glycosylation sites (Asn-X-Thr or Ser) are indicated by CHO. Regions of uncharged, hydrophobic amino acids are indicated by *wavy lines.* Three of these regions are present, one near the carboxyl terminus of pl5E. **B** Figurative representation of the potential structure of gp70 and pl5E relative to the lipid portion of the membrane. Four potential membrane traversal regions exist. These are the leader signal sequence at the amino terminus and the three regions described above. The latter three are depicted by *short loops.* According to this structure, only the hydrophobic region near the carboxyl terminus of pl5E is located in the lipid bilayer in mature peptides. The relatively hydrophilic regions which form most of the peptides are indicated by the *curved lines.* The *arrows* indicate the amino terminus of gp70 after removal of the leader sequence and the amino terminus of pl5E. No implication of secondary or higher order structure is intended, except that the three potential membrane traversal regions are sufficiently long to be capable of crossing the membrane in a helical configuration

definitive localization of virulence determinants.

Structural analysis of genomic determinants of virulence will be of value, provided they shed some light on the biological aspects of viral induction of leukemia. In this regard, the multistep nature of the leukemia process must be remembered. Probable steps include:

Initial infection of the animal
Viremia
Immune response to viremia
Infection of critical target issues
Preleukemia (proliferation of target tissues)
Frank leukemia (fixation of the malignant tumor cell)

The Akv and SL3-3 viruses seem equally proficent with regard to the first two steps.

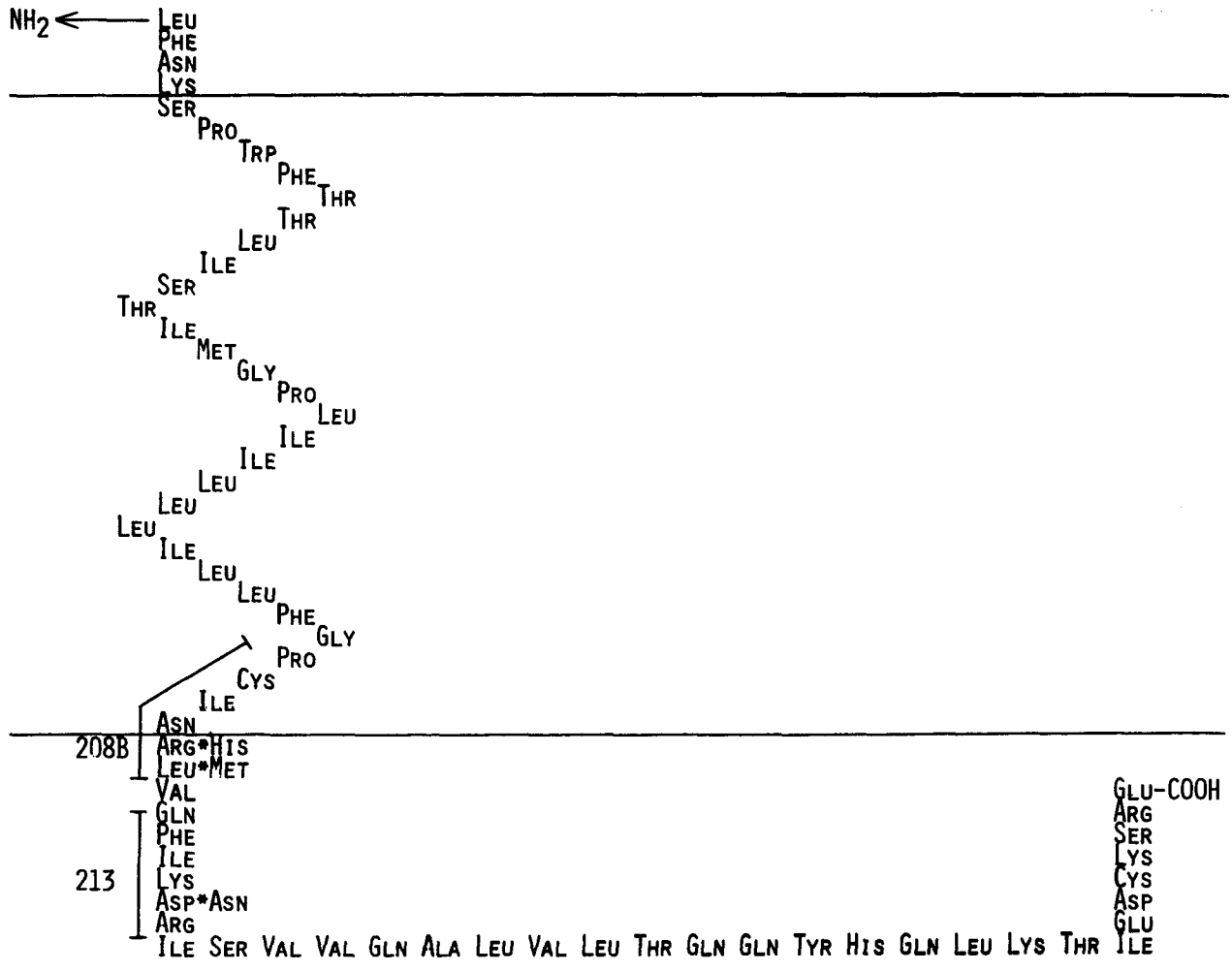

Fig. 2C. Localization of changes in the ecotropic, leukemogenic virus relative to the Akv genome in the anchor region of the pl5E proteins. The Akv sequences presented show the membrane traversal region near the carboxyl end of pl5E. The positions and amino acid changes of the Gross A/NIH oligonucleotides relative to Akv are shown. Oligonucleotide 208 b is shared by Gross A/NIH, SL3-3, and SL3-3. This oligonucleotide is also found in viruses produced by a number of other thymoma cell lines established from spontaneous tumors of AKR mice

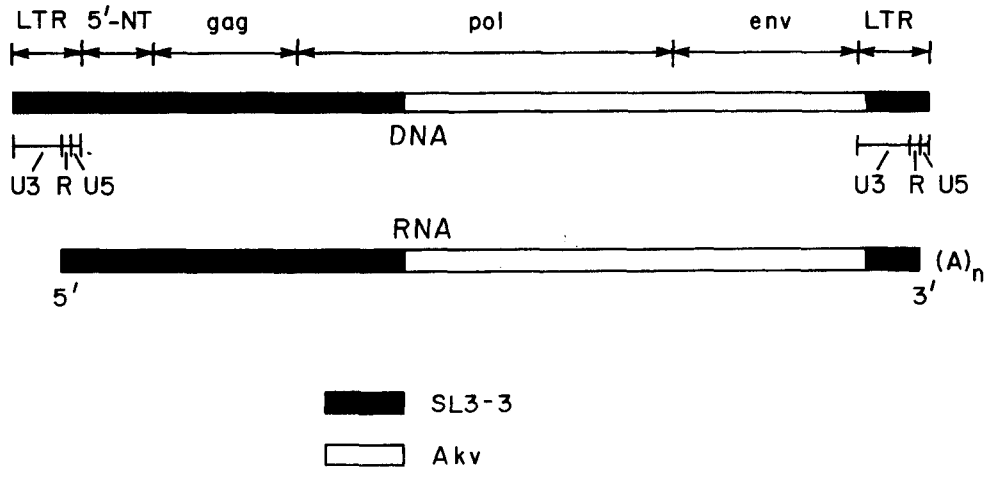

Fig. 3. Construction of a recombinant DNA provirus from infectious Akv and SL3-3 proviral DNAs. The recombinant provirus was constructed and the location of the SL3-3 and Akv components verified by restriction enzyme mapping. DNA from this virus was used to transfect NIH 3T3 cells so as to produce infectious virus. This virus accelerates the onset of disease in AKR mice, with a latent period similar to that of the SL3-3 virus. The virus also induces disease in CBA mice with a short latent period at high incidence

The immune response to the viruses is as yet incompletely characterized. There may be important differences in infection of the target tissue. Preliminary evidence suggests that even though both viruses may infect T-cell cultures in vitro the virulent strain replicates much more efficiently in such cells than does the avirulent Akv virus (Haseltine, unpublished results). Restriction of replication in the target tissue might be a sufficient explanation of the virulent phenotype.

Events that trigger cell proliferation in the preleukemic phase of the disease are poorly characterized. Typically, preleukemic cells are not transplantable and do not establish themselves in cell cultures. Evidence from the Friend leukemia system suggests that the envelope glycoprotein determinants may play a direct role in preleukemic proliferation [7] – a proliferation of lethal consequence in the case of Friend virus. Subtle alterations in *env* gene structure may affect this phase of disease for the AKR viruses as well.

There is mounting evidence that the long latent period and low disease incidence of virally induced leukemias can be attributed to the stochastic nature of events that occur subsequent to infection of the target cell population. Such events may include activation of cellular genes such as *myc, erb,* and *sis* by insertion of proviral DNA in nearby cellular sequences [8]. Chromosomal rearrangements or duplications (for example, trisomy 15 in the case of murine T-cell leukemias) may also represent late events in this process [9]. Changes that do not involve either mutations or genetic rearrangements in cellular DNA, but do affect, in a metastable way, the cellular program, may also be involved in fixation of the malignant cell phenotype. A sequence of such events might also be necessary before a fully malignant phenotype develops. It does seem likely that a precondition for the late events is creation of preleukemic proliferative population within the target cell pool. Such a population of preleukemic cells may result either from viral infection, or possibly from chemical exposure. The role of the virus in the later stages of the disease has yet to be fully established.

The murine system has several advantages for approaching these problems. A series of well-defined, isogenic viral reagents can be constructed, and the consequence of each change determined. Moreover, the entire process, from infection to frank leukemia, occurs within a 2-month period, in almost 100% of the injected animals. It is our hope that insights obtained in this system will be applicable to understanding the disease process in such cases as the adult T-cell leukemia of humans that arise under natural circumstances.

Note in Added Proof

Complete leukemogenicity data is now available for the recombinant genome shown in Fig. 3. Viruses with this genomic structure induced disease in 10 of 11 AKR mice and 3 of 10 CBA mice, showing that the SL3-3 segment of the recombinant genome contains the virulence determinants. Viruses were also derived by transfection of recombinant genomes with the reciprocal structure of the genome shown in Fig. 3. Viruses of the opposite structure were nonleukemogenic (0 of 22 AKR mice, and 0 of 10 CBA mice). Since these viruses contained the 3′ half of the SL3-3 *pol* gene and the entire SL3-3 *env* gene, this result clearly demonstrates that this segment of the genome does not encode the virulence determinants.

References

1. Rowe WP (1973) Genetic factors in natural history of murine leukemia virus infection. G.H.A. Clowes Memorial Lecture. Cancer Res 33:657–667
2. Nowinski RC, Hayes EF (1978) Oncogenecity of AKR endogeneous leukemia viruses. J Virol 27:13–18
3. Pedersen FS, Crowther RL, Tenney DY, Reimold AM, Haseltine WA (1981) Novel leukemogenic retroviruses isolated from cell line derived from spontaneous AKR tumor. Nature 292:167–170
4. Lowy DR, Rands E, Chattopadhyay SK, Garen C, Hager GL (1980) Molecular cloning of infectious, integrated murine leukemia virus DNA from infected mouse cells. Proc Nat Acad Sci USA 77:614–618
5. Lenz J, Crowther R, Klimenko S, Haseltine WA (in press) Molecular cloning of a highly leukemogenic, ecotropic retrovirus from an AKR mouse. J Virol

6. Lenz J, Crowther R, Straceksi A, Haseltine WA (1982) Nucleotide sequence of the Akv *env* Gene. J Virol 42:519–529
7. Oliff A, Linemeyer D, Ruscetti S, Lowe R, Lowy DR, Scolnick E (1980) Subgenomic fragments of molecularly cloned friend leukemia virus DNA contains the genes responsible for friend murine leukemia virus-induced disease. J Virol 35:294–936
8. Hayward WS, Neel BG, Astrin SM (1981) Activation of a cellular *onc* gene by promoter insertion of ALV-induced lymphoid leukosis. Nature 290:475–480
9. Spira J, Weiner F, Ohno S, Klein G (1979) Trisomy cause or consequence of murine T cell leukemia development? Studies on robetsonian friends location mice. Proc Natl Acad Sci USA 76:6619–6621