## *Brief Definitive Report*

# THYMIC LYMPHOMA INDUCTION BY THE AKT8 MURINE RETROVIRUS

BY STEPHEN P. STAAL AND JANET W. HARTLEY

*From the Johns Hopkins Oncology Center, Baltimore, Maryland 21205; and the Laboratory of Immunopathology, National Institute of Allergy & Infectious Diseases, National Institutes of Health Bethesda, Maryland 20892*

The AKT8 virus was isolated from a spontaneous thymic lymphoma of an AKR mouse (1). It produces foci of malignant transformation in vitro and is defective, requiring helper virus for propagation. The AKT8 proviral DNA was cloned and shown to contain cell-derived sequences termed *akt*, substituting for a portion of the virus genome (2). *akt* was designated an oncogene based on the ability of the AKT8 virus to transform cells in vitro and the finding of a tumor-specific amplification of the *AKT1* gene (an homologous allele identified in man) in a human gastric adenocarcinoma. We have now examined the ability of the AKT8 virus to produce tumors in mice, and find that it induces thymic lymphomas in two and possibly the third of the three strains tested. The AKT8 virus is thus tumorigenic in vivo, further confirming the oncogenic potential of the *akt* gene.

## Material and Methods

*Cells and Virus.* The AKT8-transformed, nonproducer mink(AKT8)cll cell line, a subclone of mink(AKT8) (1), was used to prepare pseudotypes of AKT8 virus by infection with either the 1504A or 4070A strains of amphotropic murine leukemia virus (MuLV) (3, 4). The preparation of virus stocks, tissue extract, and titration of transforming and helper virus were as previously described (5–7). Pools of AKT8(1504A) and AKT8(4070A) titered $10^4$ focus forming units per milliliter on mink lung cells (MiLu, ATCC CCL 64); helper virus titers were 30–100-fold higher.

*Tumorigenicity Assay.* AKR/J mice were obtained from The Jackson Laboratory, Bar Harbor, ME, and AKR/N and NFS mice from the National Institutes of Health animal facility. [NFS × NS.C58V1]$F_1$ mice were the progeny of NFS females and males of an NFS strain congenic for an endogenous ecotropic MuLV of the C58/Lw mouse strain (8). AKR, NFS, and [NFS × NS.C58vl]$F_1$ mice were inoculated when 1–2 d old with 0.04 ml of virus divided intrathymically and intraperitoneally and observed for a minimum of 6 mo (AKR) or 9 mo (NFS and [NFS × NS.C58V1]$F_1$). Animals that appeared ill were autopsied to confirm the presence of thymic lymphoma grossly and, in selected cases, microscopically.

*DNA Analysis.* Extraction of DNA from tissues and Southern blot analysis were as previously described (2). To detect the AKT8 virus we used the pvakt/1 probe (2) which contains cell-derived *akt* sequences but does not hybridize with any endogenous retrovirus genes in the mouse.

This work was supported by an American Cancer Society grant to S.P.Staal and by contract NO1-AI-22673 to Microbiological Associates Inc., Bethesda, MD.

TABLE I
*Tumor Induction by AKT8 Virus*

| Virus | Mouse | Lymphoma/total | Positive | Days to disease | AKT8 sequences present in tumor |
|---|---|---|---|---|---|
| | | | % | | |
| AKT8 (1504A) | AKR | 13/13 | 100 | 120 | 9/9 |
| 1504A | | 1/16 | 6 | 192 | |
| AKT8 (4070A) | | 3/3 | 100 | 120 | 1/1 |
| 4070A | | 2/7 | 29 | 154 | 0/1 |
| NFS TL EXTR. | | 3/6 | 50 | 130 | 3/3 |
| AKT8 (1504A) | [NFS × NS.C58V1]V1 | 6/10 | 60 | 139 | 4/4 |
| 1504A | | 0/6 | 0 | | |
| AKT8 (4070A) | | 2/7 | 29 | 238 | |
| 4070A | | 0/8 | 0 | | |
| NFS TL EXTR. | | 7/7 | 100 | 88 | 3/3 |
| AKT8 (4070A) | NFS | 1*/6 | 17 | 141 | 1/1 |
| 4070A | | 1/6 | 17 | 165 | 0/1 |
| NFS TL EXTR. | | 1/4 | 25 | 84 | 0/1 |

* Mouse from which the NFS thymic lymphoma extract (NFS TL EXTR.) was prepared.

## Results

Initial attempts to establish the in vivo oncogenicity of the AKT8 virus (1) were limited by several factors: (*a*) the use of a MCF helper virus which, due to the production of cytopathic foci on MiLu cells, obscured the transforming titer of AKT8; (*b*) the ability of MCF alone to accelerate thymic lymphomas in AKR mice (5, 7); and (*c*) the deletional inactivation of the AKT8 provirus in the original mink (AKT8) cell clone (2) resulting in reduced titers of AKT8. We have now reexamined the in vivo pathogenicity of the AKT8 virus with improved reagents, using helper viruses and mouse strains selected to overcome possible masking of the biological effect of the AKT8 virus.

To minimize tumor induction by helper virus, and to allow assay of transforming virus titers without interference by cytopathic foci on MiLu cells, the poorly leukemogenic amphotropic MuLV strains 1504A and 4070A were chosen to rescue the AKT8 genome. Mink(AKT8)cll cells, which yield 10-fold higher titers of AKT8 than mink(AKT8), were used to prepare the virus pools. To provide the high endogenous ecotropic MuLV background found to be required for tumor induction by certain oncogenic MuLVs (9), we selected for inoculation the high-virus strains AKR, in which thymic lymphoma occurs at high frequency, and [NFS × NS.C58V1]$F_1$, in which spontaneous lymphomas and leukemias are rare before 1 yr of age (10). Ecotropic $MuLV^-$ NFS mice, a strain with a low incidence of spontaneous neoplasms, were also inoculated.

The incidence of tumors in the inoculated mice is shown in Table 1. All the AKR mice inoculated with AKT8(1504A) developed tumors with an average delay of 120 d (range 80–178 d). Only one of the control mice developed a tumor at 192 d. Since AKR mice only begin to develop thymic lymphomas at ~6 mo of age, inoculation with AKT8(1054A) resulted in at least a 2-mo acceleration of the disease. Tumors developed in 60% of the [NFS × NS.C58V1] $F_1$ mice with a latency of 139 d (range 84–236 d), whereas no tumors were

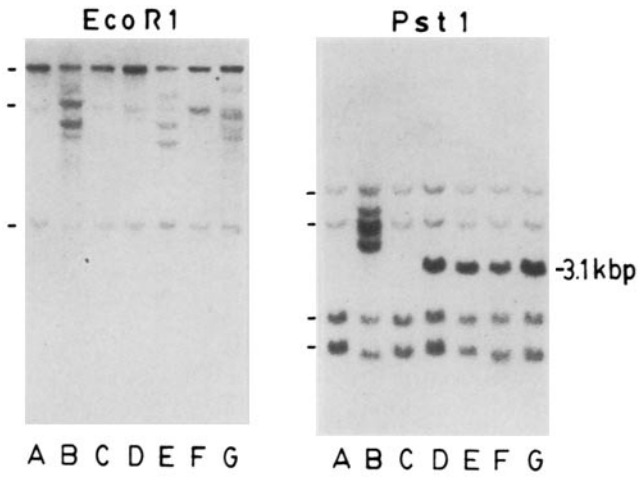


FIGURE 1. Southern blotting analysis of some AKT8-induced tumors. The marks to the left of the blots indicate restriction enzyme fragments recognized because of cross-reactivity between the viral *akt* probe and the cellular *akt* proto-oncogene(s). The 3.1-kbp marker shows the position of the internal fragment characteristic of the AKT8 virus. The mouse/virus combinations are as follows: (Lane *A*) NFS/4070A, (lanes *B*, *F*, and G) AKR/NFS thymic lymphoma extract, (*C*) NFS/NFS tl extr., (*D* and *E*) [NFS × NS.C58V1]$F_1$/NFS tl extr. The other 16 AKT8-induced tumors similarly had the characteristic 3.1-kbp Pst I AKT8 fragment and a variable number of extra Eco RI bands. The possible reason for the altered mobility of the bands in lane *B* of the Pst I analysis are explained in the text.

observed in the control group. Spontaneous thymic lymphomas occur rarely, if ever, in these mice (10 and Hartley, J. W., unpublished data). The results with AKT8(4070A) virus were generally consistent with those obtained with AKT8(1504A), but suggest that this pseudotype was less efficient in inducing or accelerating neoplasia.

Southern blot analysis of some of the tumors is presented in Fig. 1 and reported in total in Table I. The 3.1-kbp Pst I fragment characteristic of the AKT8 provirus was seen in all of the tested tumors, but not in nontumor tissue, developing in AKT8(1504) and AKT8(4070A) inoculated mice. The two tumors examined that developed in mice inoculated with helper virus alone lacked any fragments other than those due to hybridization with the *akt* proto-oncogene(s). Analysis with Eco RI demonstrated from one to five unique integrations of the AKT8 provirus in each tumor DNA. Since Eco RI cuts in the flanking cellular sequences but not in the AKT8 provirus, this result also demonstrates, given the randomness of retrovirus integration into chromosomes, the clonal, or possible oligoclonal, origin of these tumors.

Only a single tumor was observed in the six NFS mice inoculated with AKT8(4070A). Analysis of the DNA from this tumor, however, revealed the clonal presence of the AKT8 genome. An extract of this tumor was prepared and inoculated into both AKR and [NFS × NS.C58V1]$F_1$ mice. As shown in Table I, this extract was highly leukemogenic in [NFS × NS.C58V1]$F_1$ mice, producing tumors in all of the seven animals inoculated with a latency of 88 d (range 51–160 d). All three of the tumors developing in the AKR mice, and three of the three [NFS × NS.C58V1]$F_1$ tumors tested, contained the AKT8 genome. The tumor extract contained MCF virus; it is likely that the presence

of this leukemogenic helper virus allowed the more rapid entry of the AKT8 genome into thymic tissue, and the shortened latency to tumor development, in the [NFS × NS.C58V1]$F_1$ mice. MCF virus alone induces tumors in these mice. In a separate study, 67% of mice inoculated with AKR MCF 247 MuLV developed tumors at a mean of 85 d (Hartley, J. W., unpublished observations). The clonal presence of the AKT8 genome in the current tumors, however, demonstrates a direct role for the AKT8 virus in their induction.

One of the tumors developing in an AKR mouse lacked the 3.1-kbp Pst I fragment, but contained three extra fragments of differing mobility (Fig. 1, Pst I, lane *B*). These variant fragments are probably the result of recombination within the envelope gene between AKT8 and other retrovirus genomes that remove or introduce Pst I restriction enzyme sites into the AKT8 provirus.

## Discussion

The more rapid and frequent induction of thymic lymphomas in susceptible mouse strains by the AKT8 virus, in combination with the exact correlation between inoculation with the AKT8 virus and the presence of AKT8 provirus in the DNA of the subsequent tumor, provides in vivo evidence of the oncogenicity of this virus and its oncogene, *akt*. These data also support the potential of the cellular *akt* proto-oncogene to contribute to malignant transformation. Whether the AKT8 virus alone is capable of malignant transformation of thymocytes is unclear from these experiments; its transforming activity could be further analyzed by attempts to transform thymocytes or other primary cells in vitro, or by the creation of a transgenic mouse strain carrying the tumorigenic *akt* gene. Although the AKT8 virus can directly transform a mink lung epithelial cell line in vitro, further genetic changes affecting other cellular genes, or additional retrovirus interactions with the cell, are likely to be necessary for final malignant transformation of normal tissue.

The thymic lymphoma that develops in AKR mice (11, 12) results from a perinatal and persistent viremia (13) consequent to the activation of one or more of the ecotropic MuLV loci inherited as Mendelian elements in this strain (14). During the 6 mo before tumors begin to appear there is recombination between the poorly leukemogenic ecotropic MuLV and other endogenous retrovirus genes present in these mice (5, 15, 16), which generates highly leukemogenic MCF or polytropic viruses. These recombinant viruses may be, in part, more leukemogenic due to an increased ability to infect thymocytes (17). Several groups have demonstrated chromosomal insertion of the MCF provirus in the vicinity of the *myc* oncogene in a significant percentage of AKR thymic lymphomas (18–22). Repeated virus integration in different chromosomal locations has also been documented in other retrovirus-induced thymic lymphomas. The loci in the vicinity of these integrations are thought to be potentially oncogenic and have been designated *pim-1* (23), *Mlvi-1*, *Mlv-2*, *Mlv-3* (24), *mis-1/pvt-1* (25), and *Dsi-1* (26). The *akt* gene is unrelated to *myc* and other viral oncogenes as determined by crosshybridization of DNA (2), and the chromosome location of the *akt* proto-oncogene does not match that of any of the above putative oncogenes (Staal, S., and C. Kozak, unpublished data). Thus, the *akt* proto-oncogene may not be involved in the pathogenesis of most spontaneous AKR

thymic lymphomas, or it may be involved by less easily detected point mutation, altered regulation, or virus integration events occurring at a distance. It should now be possible, using molecular clones of *akt*, to determine whether alteration of the *akt* proto-oncogene is a factor in the development of the spontaneous AKR thymic lymphoma, as well as other murine tumors.

## Summary

The directly transforming murine retrovirus, AKT8, was isolated from a spontaneous AKR thymoma and carries the cell-derived viral oncogene, *akt*. We have now shown that this virus produces thymic lymphomas after inoculation of susceptible mouse strains. The presence of the AKT8 genome in the DNA of the virus-induced tumors was demonstrated by Southern blotting using an *akt*-specific probe. These results establish the in vivo pathogencity of the AKT8 virus and its *akt* oncogene, and imply a potential role for the cellular *akt* proto-oncogene in tumor development.

Svetlana Khodarakovsky provided excellent technical assistance.

*Received for publication 9 November 1987 and in revised form 21 December 1987*

## References

1. Staal, S. P., J. W. Hartley, and W. P. Rowe. 1977. Isolation of transforming murine leukemia viruses from mice with a high incidence of spontaneous lymphoma. *Proc. Natl. Acad. Sci. USA.* 74:3065.
2. Staal, S. P. 1987. Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: amplification of AKT1 in a primary human gastric adenocarcinoma. *Proc. Natl. Acad. Sci. USA.* 84:5034.
3. Hartley, J. W., and W. P. Rowe. 1976. Naturally occurring murine leukemia viruses in wild mice: characterization of a new "amphotropic" class. *J. Virol.* 19:19.
4. Rasheed, S., M. B. Gardner, and E. Chan. 1976. Amphotropic host range of naturally occurring wild mouse leukemia viruses. *J. Virol.* 19:13.
5. Hartley, J. W., N. K. Wolford, L. J. Old, and W. P. Rowe. 1977. A new class of murine leukemia virus associated with development of spontaneous lymphomas. *Proc. Natl. Acad. Sci. USA.* 74:789.
6. Peebles, P. T. 1975. An in vitro focus-induction assay for xenotropic murine leukemia viruses. *Virology.* 65:128.
7. Cloyd, M. W., J. W. Hartley, and W. P. Rowe. 1980. Lymphomagenicity of recombinant mink cell focus-inducing murine leukemia viruses. *J. Exp. Med.* 151:542.
8. Kozak, C. A., and W. P. Rowe. 1982. Genetic mapping of ecotropic murine leukemia virus-inducing loci in six inbred strains. *J. Exp. Med.* 155:524.
9. Cloyd, M. W., J. W. Hartley, and W. P. Rowe. 1981. Genetic study of lymphoma induction by AKR mink cell focus-inducing virus in AKR × NFS crosses. *J. Exp. Med.* 154:450.
10. Fredrickson, T. N., H. C. Morse III, and W. P. Rowe. 1984. Spontaneous tumors of NFS mice congenic for ecotropic murine leukemia virus induction loci. *J. Natl. Cancer Inst.* 73:521.
11. Furth, J., H. R. Seibold, and R. R. Rathbone. 1933. Experimental studies on lymphomatosis of mice. *Am. J. Cancer* 19:521.

12. Lynch, C. J. 1954. The r.I.L. strain of mice: its relation to the leukemic aK strain and aKR substrains. *J. Natl. Cancer Inst.* 15:161.
13. Rowe, W. P., and T. Pincus. 1972. Quantitative studies of naturally occurring murine leukemia virus infection of AKR mice. *J. Exp. Med.* 135:429.
14. Rowe, W. P. 1978. Leukemia virus genomes in the chromosomal DNA of the mouse. *Harvey Lect.* 71:173.
15. Elder, J. H., J. W. Gautsch, F. C. Jensen, R. A. Lerner, J. W. Hartley, and W. P. Rowe. 1977. Biochemical evidence that MCF murine leukemia viruses are envelope (*env*) gene recombinants. *Proc. Natl. Acad. Sci. USA.* 74:4676.
16. Chattopadhyay, S. K., M. W. Cloyd, D. L. Linemeyer, M. R. Lander, E. Rands, and D. R. Lowy. 1982. Cellular origin and role of mink cell focus-forming viruses in murine thymic lymphomas. *Nature (Lond.)* 295:25.
17. Cloyd, M. W. 1983. Characterization of target cells for MCF viruses in AKR mice. *Cell.* 32:217.
18. Li, Y., C. A. Holland, J. W. Hartley, and N. Hopkins. 1984. Viral integration near c-myc in 10–20% of mcf 247-induced AKR lymphomas. *Proc. Natl. Acad. Sci. USA.* 81:6808.
19. Steffen, D. 1984. Proviruses are adjacent to c-myc in some murine leukemia virus-induced lymphoma. *Proc. Natl. Acad. Sci. USA.* 81:2097.
20. Corcoran, L. M., J. M. Adams, A. R. Dunn, and S. Cory. 1984. Murine T lymphomas in which the cellular *myc* oncogene has been activated by retroviral insertion. *Cell.* 37:113.
21. Selten, G., H. T. Cuypers, M. Zijlstra, C. Melief, and A. Berns. 1984. Involvement of c-*myc* in MuLV-induced T cell lymphomas in mice: frequency and mechanisms of activation. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:3215.
22. ODonnell, P. V., E. Fleissner, H. Lonial, C. F. Koehne, and A. Reicin. 1985. Early clonality and high-frequency proviral integration into the c-*myc* locus in AKR leukemias. *J. Virol.* 55:500.
23. Selten, G., H. T. Cuypers, and A. Berns. 1985. Proviral activation of the putative oncogene Pim-1 in MuLV induced T-cell lymphomas. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:1793.
24. Tsichlis, P. N. 1987. Oncogenesis by Moloney murine leukemia virus. *Anticancer Res.* 7:171.
25. Villeneuve, L., E. Rassart, P. Jolicoeur, M. Graham, and J. M. Adams. 1986. Proviral integration site Mis-1 in rat thymomas corresponds to the pvt-1 translocation breakpoint in murine plasmacytomas. *Mol. Cell. Biol.* 6:1834.
26. Vijaya, S., D. L. Steffen, C. Kozak, and H. L. Robinson. 1987. Dsi-1, a region with frequent proviral insertions in Moloney murine leukemia virus-induced rat thymomas. *J. Virol.* 61:1164.