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Endogenous Retrovirus and Radiation-Induced
Leukemia in the RFM Mouse

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Introduction

Recombination and/or reinsertion of retroviral sequences have recently emerged as important mechanisms in oncogenesis (1). Recombination may involve viral sequences with normal cellular (c-onc) genes (2), or other endogenous virus genes (3). Reinsertion of viral sequences adjacent to c-onc sequences may induce RNA transcription under control of a viral promoter sequence (4). Both virus-cell and virus-virus recombinants have been identified involving ecotropic host range viruses, and reinsertions by ecotropic viruses have been recognized in somatic cells and in germ lines (2, 5). Such events have most likely arisen via exogenous spread of the virus from spontaneously or exogenously induced cells to uninfected cells.

The induction of myeloid leukemia in irradiated RFM/Un mice has been associated with retrovirus infection (6). However, two characteristics of this strain complicate efforts to define the role of the virus. This strain possesses only one inducible host range class of endogenous virus and a unique gene, in addition to the Fv-1ⁿ locus, which specifically restricts exogenous infection by endogenous viruses (7). These characteristics possibly account for absence of recombinant viruses in this strain, even though virus is amply expressed during most of the animal's life span. We have examined further the distribution of retrovirus sequences and the chromosomal locus of the inducible virus in this strain. This report describes evidence for additional viral sequences in cells of a radiation-induced myeloid leukemia line and discusses the possible origin of these added copies.

Endogenous ecotropic murine leukemia virus locus of RFM/Un mice

Retrovirus is expressed in hematopoietic tissues of RFM mice throughout their life span. However, it is possible to isolate cells in culture which do not produce virus unless they are exposed to 5-iododeoxyuridine or other specific chemicals. The focus of these studies is an isolate of virus from induced cells, called RFV, which appears to be similar to the endogenous X-tropic virus induced from BALB/c cells. We have constructed a restriction endonuclease map of the unintegrated linear DNA isolated ⁸47 hr after infection of SC-1 cells with RFV. Based on the cleavage sites, shown in Figure 1, this virus and the endogenous ecotropic MuLV of BALB/c mice are indistinguishable.

F-1

The endogenous provirus composition of RFM/Un mouse cell DNA was analyzed by EcoRI restriction endonuclease digestion and compared to several other mouse strains. Ecotropic MuLVs, including RFV, do not contain a cleavage site for EcoRI and each provirus is contained in a single fragment which includes flanking cellular sequences; the size depends on where the EcoRI site is in the flanking regions. The majority of proviruses in mouse cells, however, appear to be non-ecotropic and do contain a cleavage site for EcoRI. Each provirus of this type therefore yields two EcoRI fragments resulting in a complex pattern of viral specific fragments. Figure 2 is an autoradiograph of a Southern blot of mouse cell DNAs hybridized with a cDNA probe prepared from WN1802B 70S RNA; BALB/c, AKR and C3H all appear to share some proviral loci with each other and RFM, and each also has unique fragments. The RFM 3T3 spleen, liver and embryo DNA all display the same fragments, suggesting that no major gene rearrangements occurred in these

F-2

tissues which would alter the provirus pattern. Since the ecotropic virus does not have an EcoRI site, the inducible provirus locus (rfv-1) must be one of the 10 or so bands larger than 8.8 Kbp. We have taken two different approaches to determine which fragment contains the RFV ecotropic provirus.

Hamster-mouse hybrids

We have correlated virus induction, chromosomal segregation and physical mapping of the inducible endogenous virus. Hamster-mouse somatic cell hybrids were obtained by fusing normal RFM mouse spleen or bone marrow cells with Chinese hamster E36 cells deficient in hypoxanthine phosphoribosyl-transferase (EPRT⁻) and selection in HAT medium (8). Mouse chromosomes are selectively lost in such hybrids, and mouse and hamster enzymes were distinguished by electrophoresis in starch, cellulose acetate or isoelectric focusing, followed by specific enzyme staining (9). Individual hybrid clones were analyzed for release of infectious virus following induction with 5-iodo-2'-deoxyuridine (IdUrd) (10), for virus specific EcoRI fragments by electrophoresis-hybridization, and for chromosome complement.

Of eight clones analyzed for virus induction with IdUrd, clones RFS-8, RFS-12 and RFS-15 were positive for viral antigens by immunofluorescence and also by cocultivation with SC-1 cells. RFS-16 had a low inducibility (10%) and the others were negative. The mouse chromosome complement of these hybrids was determined and is shown in Table 1. Only the presence of chromosome 5 was concordant with the inducibility of virus.

T-1

In order to correlate the physically detectable viral genome with induction and chromosome complement, the DNA of each clone was digested with

EcoRI and hybridized with a probe prepared by nick translation of molecularly cloned Gross leukemia virus (pG100). The band pattern was found to be less complex than parental RFM DNA due to loss of some mouse chromosomes (the hamster parent cell is free of detectable MuLV related sequences). Several clones contained the same fragments although no two clones were identical. Assignment of each band to a specific chromosome might be possible with a much larger sample population. However, correlation of a single band with the inducibility of RFV was readily apparent. The three clones, S15, B12, and S8, which were capable of IdUrd induction of infectious virus shared a 20 Kbp viral specific fragment which was absent from the other clones. There was no other common fragment which was also absent from all non-inducible clones. These data strongly suggest that the 20 Kbp band contains the inducible ecotropic provirus and that it is present on chromosome 5.

Molecular cloning of the endogenous RFV genome

The second approach was to generate a specific hybridization reagent which recognized only ecotropic proviral DNA. In order to prepare specific molecular probes, and to facilitate more detailed analysis of the RFV genome, we molecularly cloned the unintegrated covalently closed circular viral DNA as follows. SC-1 cells were infected with RFV at an MOI of 1 and lysed by a modified Hirt procedure (11) 48 hr after infection. The supernatant DNA was banded in a CsCl-propidium iodide gradient and the covalently closed circular DNA isolated and digested with Hind III which cleaves once in the viral genome, approximately 3 Kbp from the left end of the linear map (see Fig. 1). This DNA was initially cloned using the Charon 9 bacteriophage

~~Lambda~~ vector, and the insert fragment of some clones was transferred to the plasmid vector pBR322 to facilitate analysis. The restriction enzyme map of this cloned genome is identical to the unintegrated RFV DNA (see Fig. 1) except for the permutation caused by cloning a circular molecule (11).

For a hybridization reagent specific for ecotropic viral DNA, we subcloned a small region of the RFV env gene. This is based on the findings of Chan et al. (13) and Chattopadhyay et al. (14) that ^a small segment of the ecotropic MuLV env gene did not hybridize to xenotropic or other endogenous virus related sequences. The DNA of pRFV105, an infectious plasmid subclone of RFV which has two LTRs, was digested with Ava I and ligated to Ava I cut pBR322. Recombinant clones were screened by hybridization and gel analysis. Many of the clones had multiple inserts. A 310 bp fragment which appeared in several clones contains a sequence identical to the 400 bp Sma I fragment described by Chattopadhyay et al. (14) as ecotropic type-specific. This fragment was subcloned as a separate insert, nick translated, and determined to be specific for ecotropic MuLV. An LTR fragment subclone was similarly constructed from pRFV105 using Pst I, and used as an LTR specific probe.

The results of hybridizing these two probes to RFM liver DNA is shown in Figure 3. It appears that the pattern with the LTR probe is the same as the total genome probe, suggesting that all viral specific fragments contain an LTR. The Eco specific probe yielded a single band at 20 Kbp. This is in agreement with the results from the hamster-mouse hybrid cell clone experiments.

Ecotropic proviral sequences in a radiogenic myeloid leukemia line

Having established that there is a single fragment which contains the endogenous ecotropic provirus, we examined ^d normal and leukemic tissues of the RFL mouse to determine if there was any evidence for specific chromosomal rearrangements of endogenous virus genes. DNA from normal spleen and a cultured myeloid leukemia cell line were digested with EcoRI, subjected to electrophoresis and Southern transfer, and hybridized with total genomic probe. It was found that the ^e myeloid cell line demonstrated several additional fragments which hybridize to the viral probe (indicated by a dot in the margin of the figure). This suggests that the leukemic cells, or their precursors, are sensitive to reinfection or transposition of a retrovirus genome or that viral sequences are amplified in these cells. In order to determine that these fragments are ecotropic provirus we hybridized the myeloid leukemia DNA and normal spleen DNA with the "eco" specific probe and an LTR specific probe. The results are shown in Figure 4 and indicate that additional fragments are detected by the "eco" specific probe.

F-4

The "eco" specific and LTR specific hybridization analysis of EcoRI digested DNA indicated that in normal cells the ecotropic provirus was present at one copy per haploid genome, whereas the remainder of retroviral sequences comprised greater than 10 copies. An alternative way to demonstrate additional copies of ecotropic provirus in normal and neoplastic cells is to compare the relative ^b and intensities of the ecotropic and non ecotropic genomes. Advantage is taken of the fact that Pst I cuts in the LTR of both proviral classes. The non ecotropic proviruses are composed of two major classes, one of which has an additional Pst I site in the coding

portion of the genome. Digestion of RFM DNA with these two enzymes yields a 3.2 Kbp band from the ecotropic provirus, and a 6.6 Kbp band from one class of the non-ecotropic genome, and the relative intensity reflects the difference in copy number of these two classes of endogenous proviruses. Such analyses performed with DNA preparations from the myeloid leukemia and normal RFM/Un mouse spleens revealed that there was an increase in the relative copy number of ecotropic genomes in the myeloid cells (Fig. 5). Exact quantitation is difficult from these kinds of data; however, together with the EcoRI digest pattern it is very clear that the myeloid leukemia cells possess additional copies of the ecotropic provirus.

F-5

Concluding remarks

We believe that the radiogenic myeloid leukemia in RFM/Un mice is a potentially valuable model system in which to study specific chromosomal rearrangements of retrovirus genes. One of the key aspects of this system is that infection by the endogenous virus is strongly restricted and therefore extracellular spread of the virus is not likely to play a role in the formation of additional integrated viral genomes as is the case in other well studied murine retrovirus models. The role RFV plays in radiogenic myeloid leukemia is uncertain. As with other endogenous ecotropic retroviruses the virus does not transform cells in culture and does not appear to accelerate leukemogenesis in vivo. The recently described downstream promotion model for leukemogenesis by avian leukosis virus, supported by the work of Neel et al. (1), does provide an attractive hypothesis for the involvement of RFV in the myeloid leukemia.

Our present studies indicate that there is a single locus for the inducible ecotropic provirus which resides on chromosome 5. The myeloid leukemic cell line clearly has additional copies of the ecotropic provirus. This is particularly interesting in view of the strong restriction referred to earlier. Does this represent true genetic transposition which is causally related to the myeloid leukemia? We hope to answer the second part of this question by analysis of the integration pattern in a large number of primary myeloid leukemias. Association with a particular downstream oncogene, as in the avian leukosis virus system, will be taken as evidence for a role of this endogenous virus in the disease. The finding of increased copies of the viral genome in myeloid leukemia cells is consistent with this mechanism and encourages us to investigate further.

The mechanism of acquiring these additional proviral genomes is more difficult to experimentally determine. The mechanism of RFV restriction of exogenous infection has not been elucidated. This restriction may not operate in hematopoietic cells and thus, conventional retroviral infection could be responsible for the new integrated sequences. The idea that retroviruses may relocate via a transposon mechanism is based on its structural similarity to known transposable elements (15). It is very important to establish whether retrovirus can use this alternate pathway.

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Figure Legends

- Figure 1. Schematic map of unintegrated DNA intermediate of RFV. Map positions were determined by single and double digests followed by electrophoresis and Southern blot hybridization with ^{32}P labeled cDNA prepared from WN1802B 70S RNA. Enlarged region represents the long terminal repeat (LTR).
- Figure 2. EcoRI digested DNA from RFM and other mice. DNA was digested with EcoRI, subjected to electrophoresis and transferred to DBM paper. Hybridization was with ^{32}P labeled cDNA prepared from WN1802B 70S RNA. Lane (A) BALB/c 3T3; (B) AKR 3T3; (C) RFM 3T3; (D) RFM spleen; (E) RFM liver; (F) C₃H 3T3; (G) RFM embryo. Molecular size markers are Hind III fragments of λ DNA.
- Figure 3. Comparison of LTR-specific and "Eco" specific probes hybridized to normal RFM liver DNA. EcoRI digested DNA was subjected to electrophoresis and transfer to DBM paper. Hybridization was with ^{32}P labeled nick-translated LTR or "Eco" specific subcloned DNA. Molecular size markers are Hind III fragments of λ DNA.
- Figure 4. "Eco" specific and LTR specific hybridization analysis of normal and myeloid leukemia cell DNA. DNA from (A) radiogenic myeloid leukemic cells and (B) normal RFM spleen, was digested with EcoRI and subjected to electrophoresis and transferred to DBM paper. ^{32}P labeled hybridization probes and molecular size markers as in Fig. 3.

Figure 5. Relative intensity of "Eco" and "non-eco" proviral DNA fragments. DNA was digested with EcoRI and Bam H I and analyzed by gel electrophoresis and blot hybridization. (A) Normal liver, (B) radiogenic myeloid leukemia cells (in vitro culture) (C) liver of tumor-bearing mouse, (D) normal spleen, (E) thymoma. Probe was ³²P labeled nick-translated Gross leukemia virus clone PG100. Schematic map indicates cleavage sites in the major classes of endogenous provirus.

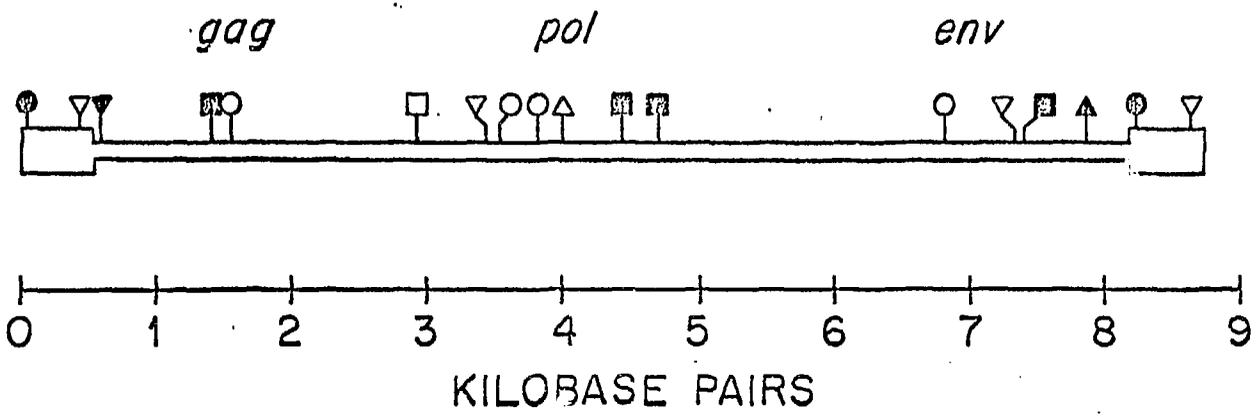
Segregation of Virus Inducibility, Virus Sequences and
Mouse Chromosomes in RFM x Chinese Hamster Cell Hybrids

Chromosome	Marker Enzyme	Concordant	Discordant
1	PEP-3	7	1
2	AK-1/SODH/ACP-2	3	5
3	*	3	5
4	PGD/PGM-2	4	4
5	PEP-7/PGM-1	8	0
6	TPI	7	1
7	LDH-1/GPI/PEP-4	5	3
8	GR/APRT	5	3
9	ME/MPI	5	3
10	PEP-2/HK-1	7	1
11	GLK	5	3
12	ACP-1	7	1
13	*	4	4
14	ES-10	6	2
15	*	3	5
16	SOD-1	6	2
17	GLO	6	2
18	PEP-1	1	7
19	GOT	5	3
X	HPRT	3	5

Legend for Table

The gene nomenclature for the enzyme markers, their chromosome assignments and the electrophoretic procedures used to separate the Chinese hamster and mouse enzymes have been described (). Viral sequence, enzyme and chromosome analyses were performed on parallel cultures for each hybrid clone so that all data were correlated. The presence or absence of mouse enzyme markers agreed with the presence or absence, respectively, of the particular mouse chromosome. The concordant column gives the number of clones in the viral sequences and a particular enzyme marker or chromosome were present or absent together; the discordant column gives the number of clones in which only the viral sequences or the enzyme marker (mouse chromosome) was present.

*Enzyme markers have not been assigned to chromosomes 3, 13, and 15; their concordance, discordance was based on karyotypic analysis alone.



- *Bam*HI □ *Hind*III △ *Sal*I ▽ *Kpn*I
- *Pst*I ■ *Bgl*I ▲ *Xba*I ▾ *Pvu*I

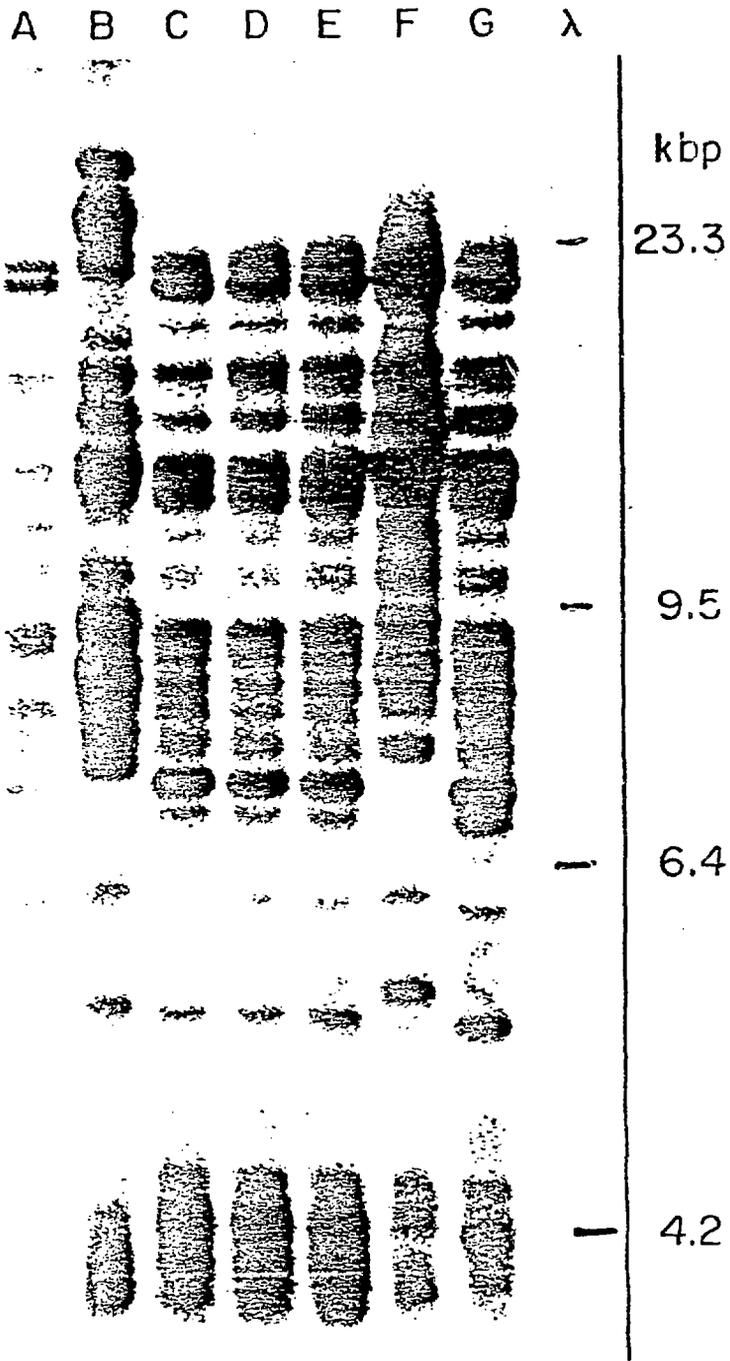
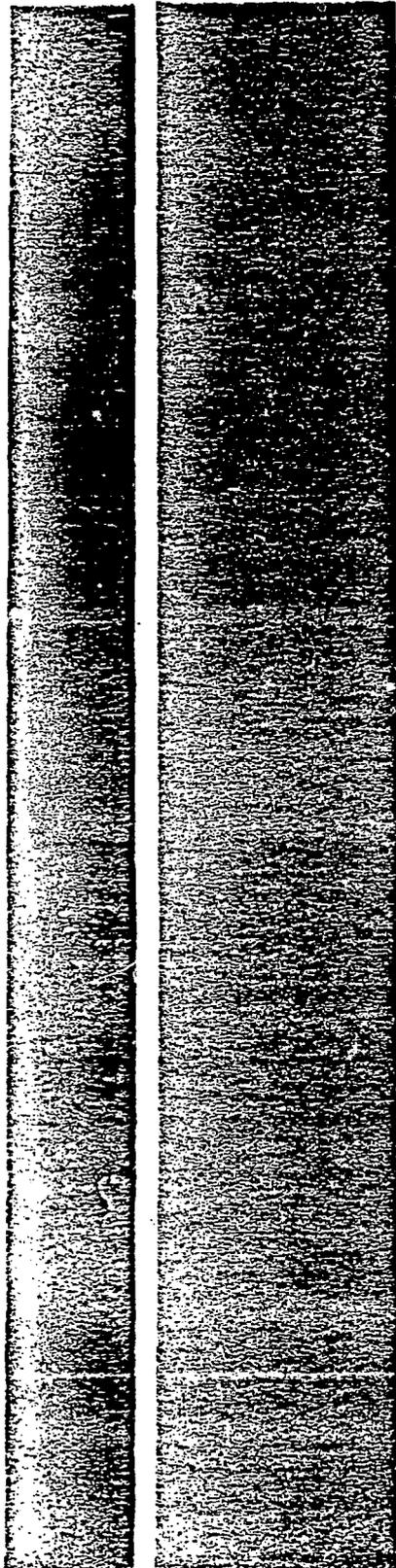


Fig 2

L T R

E c o

λ



-2 3.3

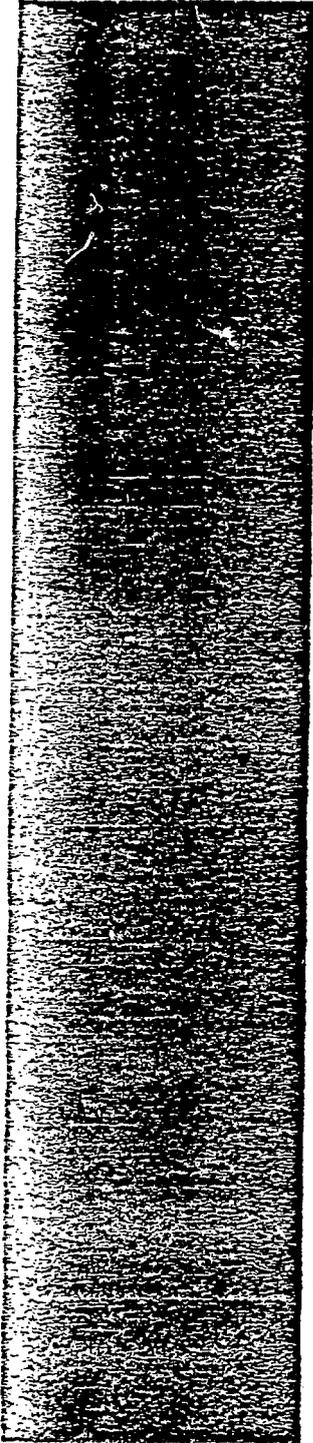
- 9.5

- 6.4

- 4.2

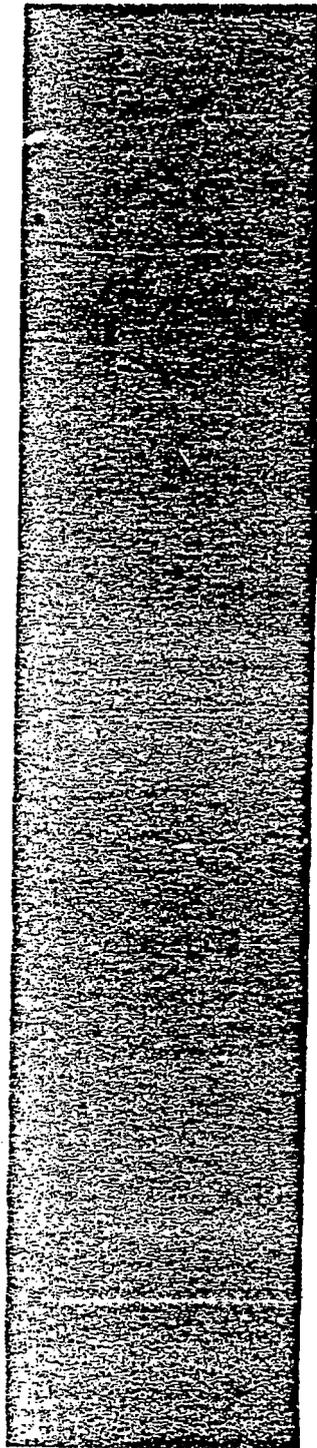
LTR PROBE

A B λ



"ECO" PROBE

A B λ



kbp

— 23.3

— 9.5

— 6.4

— 4.2

