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Involvement of c-*myc* in MuLV-induced T cell lymphomas in mice: frequency and mechanisms of activation

Gerard Selten, H. Theo Cuypers, Maarten Zijlstra[1], Cees Melief[1] and Anton Berns

Department of Biochemistry, University of Nijmegen, Geert Grooteplein N21, 6525 EZ Nijmegen, and [1]Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, PO Box 9406, 1006 AK Amsterdam, The Netherlands

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In ~45% of the murine leukemia virus (MuLV) induced early developing T cell lymphomas in mice, integration of proviruses occurs near c-*myc*. From the 33 lymphomas with proviral integrations in the c-*myc* domain, 29 insertions were localized upstream of the first exon in a region spanning <2 kbp, and four integrations occurred within the first exon. In 90% of the lymphomas the transcriptional orientation of the proviruses was opposite to the transcriptional direction of c-*myc*. In 20% of the early T cell lymphomas, proviral integrations were detected both near c-*myc* and the *pim*-1 gene. They comprise both lymphomas in which integration near c-*myc* and *pim*-1 occurred in separate tumor cell populations, as well as tumors in which proviral integration near c-*myc* and *pim*-1 occurred in the same cell clone. Proviral integration in the c-*myc* domain is associated with increased *myc* mRNA levels (up to 30-fold). The size and nature of the c-*myc* mRNA precursors and processed transcripts depend on the position and orientation of the integrated proviruses.

Key words: c-*myc*/insertional mutagenesis/murine leukemia virus/*pim*-1/T cell lymphomas

Introduction

c-*Myc* has been reported to be involved in a variety of malignant neoplasms. Translocation, amplification, viral transduction, as well as insertional mutagenesis by integration of retroviruses can cause derangement of normal *myc* expression. The translocation of c-*myc* to the immunoglobulin domain in Burkitt lymphomas (Taub *et al.*, 1982; Dalla-Favera *et al.*, 1983; Erikson *et al.*, 1983; Leder *et al.*, 1983; Croce *et al.*, 1983), and murine plasmacytomas (Crews *et al.*, 1982; Shen-Ong *et al.*, 1982; Stanton *et al.*, 1983; Mushinski *et al.*, 1983; Leder *et al.*, 1983; Adams *et al.*, 1983) is well documented. Amplification of c-*myc* gene sequences has been reported (Collins and Groudine, 1982; Dalla-Favera *et al.*, 1982), and like the frequently observed trisomy of chromosome 15 in mouse T cell lymphomas, has been implicated in enhanced *myc* expression (Klein, 1981). The transduction of c-*myc* by feline leukemia occurs in T cell lymphomas in cats (Neil *et al.*, 1984; Mullins *et al.*, 1984; Levy *et al.*, 1984). Modification of the c-*myc* locus by proviral insertion has been described in avian leukemia virus (ALV)-induced lymphomas (Hayward *et al.*, 1981; Neel *et al.*, 1981; Payne *et al.*, 1982; Westaway *et al.*, 1984), and in MuLV-induced T cell lymphomas in mice (Corcoran *et al.*, 1984), rats (Steffen, 1984), and cats (Neil *et al.*, 1984). A similar mechanism underlies the activation of c-*erb* in ALV-induced erythroblastosis (Fung *et al.*, 1983), the activation of the *int*-1 (Nusse and Varmus, 1982; Nusse *et al.*, 1984) and *int*-2 gene (Peters *et al.*, 1983; Dickson *et al.*, 1984) in mouse mammary tumor virus (MMTV)-induced mammacarcinomas in mice, and the activation of the *pim*-1 gene in MuLV-induced T cell lymphomas in mice (Cuypers *et al.*, 1984). Other common integration regions have been reported in MuLV-induced lymphomas in rats (Tsichlis *et al.*, 1983; Lemay and Jolicoeur, 1984). However, the mechanisms of gene activation by insertional mutagenesis of proviruses appear to differ: in ALV-induced lymphomas, c-*myc* is transcribed predominantly from the promoter of the upstream integrated, frequently deleted, ALV provirus (Payne *et al.*, 1982; Hayward *et al.*, 1981; Neel *et al.*, 1981; Westaway *et al.*, 1984), while in MuLV-induced lymphomas in mice (Corcoran *et al.*, 1984) enhancement of transcription from cellular promoters by mostly intact proviruses seems the predominant mechanism of activation. Also the activation of *int*-1 and *int*-2 in MMTV-induced mammacarcinomas and the activation of *pim*-1 in mouse T cell lymphomas seems to be mediated predominantly through activation of cellular promoters (Nusse *et al.*, 1984; Dickson *et al.*, 1984; Cuypers *et al.*, 1984).

Here we present evidence showing that in MuLV-induced early developing T cell lymphomas in mice, c-*myc* activation is mediated predominantly through proviruses integrated upstream of c-*myc*, which have a transcriptional orientation opposite to c-*myc*. The frequency and mechanisms of activation of c-*myc* is compared with the proviral activation of the *pim*-1 gene in the same group of lymphomas.

Results

Alterations in c-myc locus by proviral insertion

DNA from 130 lymphomas induced by MuLVs in different mouse strains was analyzed for proviral insertions in the c-*myc* domain. Lymphoma DNA was digested with *Eco*RI, *Eco*RV, *Kpn*I and in some instances with *Hind*III, and analyzed by Southern blots (Southern, 1975). Figure 1a shows a map of the c-*myc* region with the positions of the probe and relevant restriction endonuclease sites. Analysis of *Kpn*I and/or *Eco*RI digestions of 130 lymphoma DNAs revealed novel bands in 33 cases (Table I). The sizes of the additional bands in these 33 lymphomas depended on the position and nature of the integrated provirus. Both ecotropic as well as mink cell focus-forming viruses (MCF) were found to be integrated near c-*myc*. MCF viruses, which are generated *in vivo* by recombination between ecotropic MuLV and endogenous xenotropic like sequences (Hartley *et al.*, 1977; van der Putten *et al.*, 1981; Quint *et al.*, 1984) contain an *Eco*RI cleavage site at position 6.9 on the genomic map (Quint *et al.*, 1982; van der Putten *et al.*, 1981), whereas ecotropic viruses lack this site (Verma and McKenneth, 1978; Lowy *et al.*, 1980). Normally sized proviruses (~8.8 kbp) lacking the *Eco*RI site were assumed to be ecotropic. Approximately 50% of the proviruses integrated in the c-*myc* showed an MCF-like structure, whereas 80% of the proviruses in the

Table I. Characteristics of proviral integrations in the c-*myc* locus in independent lymphomas

Tumor #	DNA fragment size (kbp) of additionally hybridizing bands				Provirus
	*Eco*RI	*Eco*RV	*Kpn*I	*Hind*III	
4	30	3.7	8.0	nt	Eco
5	30	nt	6.2	nt	Eco
7	30	3.2	7.8	nt	Eco
8	21	3.5	8.2	nt	MCF
9	21	3.3	7.7	nt	MCF
13	21	nt	8.0	nt	MCF
17	19	nt	8.0	nt	MCF
26	30	3.6	8.0	absent	Eco
27	16	3.2	7.0	absent	MCF
28	21	3.0	7.5	nt	MCF
31	21	2.4	7.0	absent	MCF
32	nt	nt	8.1	nt	nt
33	30	3.3	7.7	nt	Eco
34	21	3.7	8.2	nt	MCF
36	30	2.3	5.8	7.5	Eco
37	30	3.0	7.2	nt	Eco
38	21	3.6	8.2	nt	MCF
39	27	2.9	7.6	nt	Eco
40	21	3.8	8.3	nt	Eco
41	27	2.3	6.0	13.5	Eco
42	30	3.6	7.9	nt	Eco
43	30	4.2	8.8	nt	Eco
44	25	3.1	7.4	nt	Eco
45	21	3.0	7.4	nt	MCF
46	17	nt	7.3	nt	MCF
47	30	nt	7.7	nt	Eco
48	30	nt	7.6	nt	Eco
49	21	nt	9.2	nt	MCF
50	21	nt	8.4	nt	MCF
51	21	nt	8.3	nt	MCF
52	20	nt	7.2	nt	MCF
53	nt	nt	8.7	nt	nt
54	nt	nt	8.5	nt	nt

DNA fragment sizes of normal allele after digestion with *Eco*RI, *Eco*RV, *Kpn*I and *Hind*III are 21, 22+23, 11 and 4.4 kbp respectively. Abbreviations: Eco, ecotropic; MCF, mink cell focus-inducing virus; nt, not tested.

pim-1 domain (Cuypers *et al.*, 1984) were of MCF origin (Table I).

*Eco*RV digestions revealed additional bands in the same tumors which were positive after *Eco*RI analysis (Table I). Since *Eco*RV has a recognition site inside the *myc* probe (Figure 1a), two fragments (22 and 23 kbp) were observed in normal tissues. An *Eco*RV recognition site is also present at 0.14 kbp from the boundary of the 5′ long terminal repeat (LTR) (van Beveren *et al.*, 1980). The site of proviral integration can be estimated quite accurately from the sizes of the additionally hybridizing fragments. Digestions with *Kpn*I confirmed the results and allowed the determination of the orientation of the proviruses after hybridization with U3LTR probes (Cuypers *et al.*, 1984). Since a *Kpn*I site is only present in the U5 portion of the LTR (van Beveren *et al.*, 1980), hybridization of fragments to both the U3LTR and *myc* probe is only seen if the transcriptional orientation of the provirus is directed away from the region corresponding with the *myc* probe. Alternatively, novel *myc*-reactive *Eco*RI fragments should hybridize to the U3LTR probe independently of the orientation of the integrated provirus. An example of the hybridization pattern with the *myc* and MoU3LTR probe on *Kpn*I digests of 14 lymphoma DNAs is presented in Figure 1b and c, respectively. Tumors 39, 42, 44, 26 and 33 show additional bands of reduced size which are recognized both by the c-*myc* as well as the U3LTR probe. A survey of the analyses performed with different mouse strains and various MuLV is presented in Table I. Proviral integration occurred in all cases upstream of the c-*myc* coding sequence in a region of ~2 kbp. More precisely, 29 tumors showed proviral integration in a region ~1.5 kbp upstream from exon 1, whereas in four lymphomas the provirus integrated in the non-coding first exon of c-*myc* (Figure 2). In 24 lymphomas the transcriptional orientation of the proviruses was opposite to the transcriptional direction of c-*myc*, in three lymphomas the same orientation was observed, and in six cases the proviral orientation was not determined. In the lymphomas with integration in exon 1, proviruses were found both upstream (lymphoma 31) and downstream (lymphomas 5, 36 and 41) of promoter 2, in different transcriptional orientations (Figures 2 and 4; Table I).

Frequency of proviral integration near c-myc depends on the latency of lymphoma development

Lymphomas were divided into three groups on the basis of the latency of tumor development (Table II). The first group, which gave rise to lymphomas within 6 months, comprised BALB/c, C57BL and AKR mice, inoculated as newborn with Moloney MuLV and AKR-MCF247, respectively. This group showed an incidence of proviral integration in the c-*myc* domain in ~40% of the lymphomas. Slightly higher percentages of proviral integration near the *pim*-1 gene were observed (Table II). In 15 out of the 66 lymphomas in this group, proviral integration was detected both in the c-*myc* as well as the *pim*-1 domain (see below). In this group a considerable portion of the tumors were oligoclonal as judged by the low hybridizing intensities of the modified allele(s), and the occurrence of different integrations near *pim*-1 or c-*myc* within the same tumor (e.g., Figure 1, tumor 44).

The second group consists of lymphomas of BALB/c and C57BL mice, infected as newborns with MCF1233 (Zijlstra *et al.*, 1983), and spontaneously arising lymphomas in AKR (Hartley *et al.*, 1977) and BALB/Mo mice (Jaenisch, 1976). The incidence of proviral integration near *pim*-1 or c-*myc* in this group, which developed lymphomas after 6–12 months, ranged from 0 to 20%. No proviral integration in both domains were detected within lymphoma tissues of the same animal. Most of these tumors appeared monoclonal.

Group 3 represents lymphomas in BALB/c and C57BL mice, infected as newborns with MCF1130 (Zijlstra *et al.*, 1983) and tumors in C57BL mice which transmit the B-tropic C57BL-MuLV through the milk (Melief *et al*, 1980). No proviral integration near *pim*-1 or c-*myc* were observed here. All lymphomas in group 1 and 2 with proviral insertions near c-*myc* and/or *pim*-1 were identified as genuine T cell lymphomas on the basis of their cell surface markers (Zijlstra *et al.*, unpublished results). Some of the lymphomas in group 2, and a considerable number of the tumors in group 3 were of B- or non-T/non-B cell origin.

Proviral integration near c-myc is associated with enhanced myc mRNA levels

Myc mRNA levels were estimated by dot-blot hybridization of poly(A)$^+$ RNA, isolated from lymphomas and normal tissues. Figure 3 shows the hybridization of poly(A)$^+$ RNA of 12 lymphomas with the *myc* and actin probe. In lymphoma tissues, in which a provirus was integrated near c-*myc* (Figure 3, marked by an asterisk) *myc* RNA levels were in-

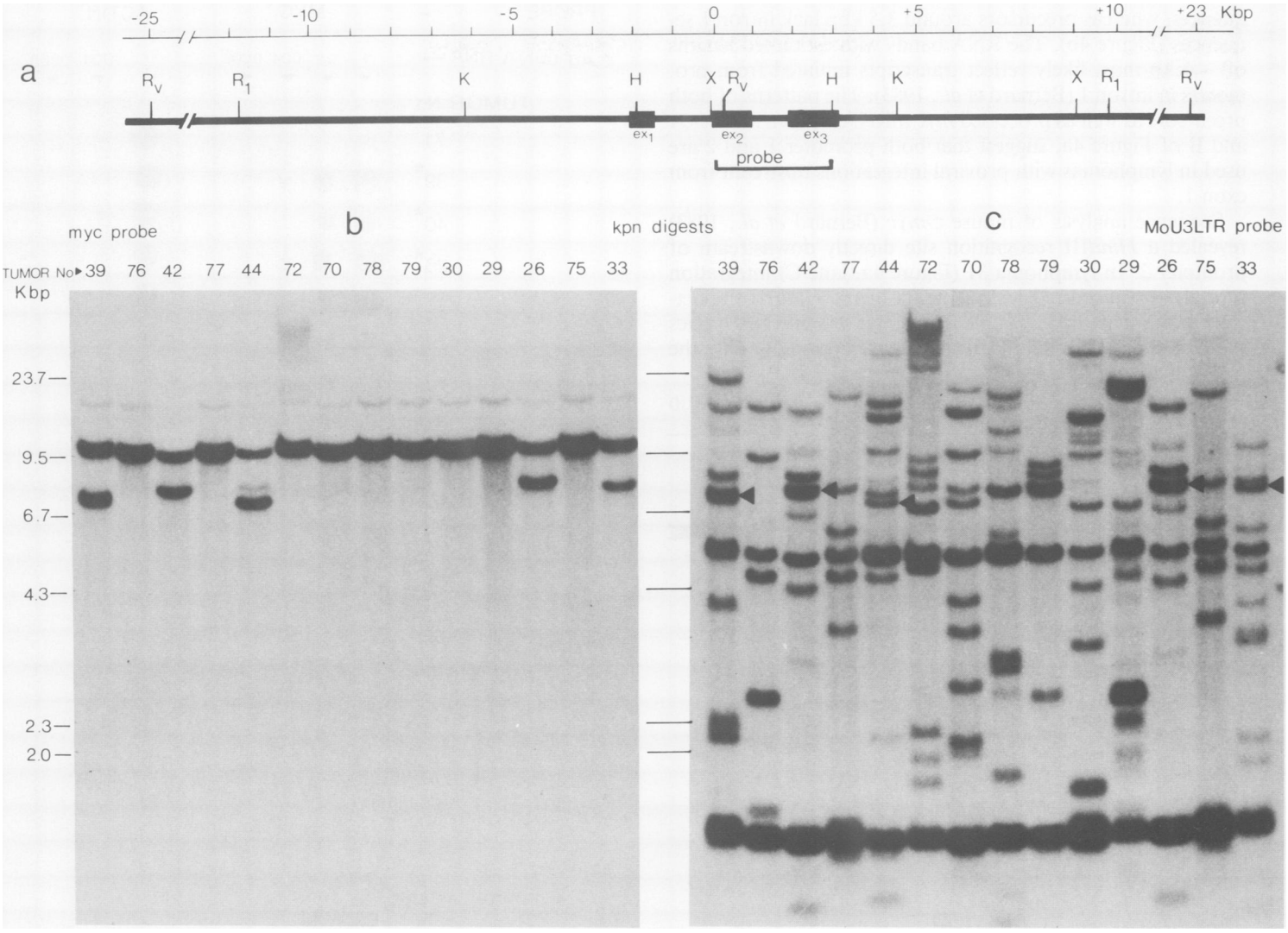

Fig. 1. Characterization of *Kpn*I digests of lymphoma DNAs with c-*myc* and MoU3LTR probes. **(a)** Physical map of the c-*myc* region with the position of the probe (*Xba*I/*Hind*III fragment). DNA samples (10 μg) from 14 different lymphomas were digested with *Kpn*I, separated on 0.6% agarose gels and transferred to nitrocellulose filters. Filters were hybridized with ^{32}P-labeled *myc* probe, washed (0.1 x SSC, 0.1% SDS, 60°C) and exposed to X-ray film **(panel b)**. After treatment with alkali, the filter was hybridized with a ^{32}P-labeled MoU3LTR probe, washed and re-exposed **(panel c)**. Bands marked with an arrow in **(c)**, correspond to the rearranged DNA fragments shown in **(b)**. Numbers correlate to listing in Table I. Abbreviations: R1, *Eco*RI; RV, *Eco*RV; K, *Kpn*I; H, *Hind*III; X, *Xba*I.

creased 5- to 30-fold as compared with *myc* mRNA levels of normal adult tissues. In tumors with proviral integrations near c-*myc* and *pim*-1, increased mRNA levels of both genes were detected (data not shown). However, in lymphoma 29 and 30 increased *myc* mRNA levels were found without concomittant provirus insertion within the vicinity of c-*myc*. Either proviral integration in these tumors occurred outside the region of 45 kbp which was examined for proviral integrations or other mechanisms for c-*myc* activation are operational in these lymphomas. In addition, in lymphomas 15 and 71, which harbor no proviruses near c-*myc*, 10- to 50-fold less *myc* mRNA was detected as seen in normal adult thymus and/or spleen. Over 60% of the early developing T cell lymphomas (29 randomly chosen tumors were tested for RNA expression) showed significantly enhanced *myc* expression, whereas increased *myc* and/or *pim*-1 expression was detected in >80% of the lymphomas. Obviously, the expression of c-*myc* and/or *pim*-1 might be increased in only a subpopulation of the tumor cells, since many of the early T cell lymphomas are oligoclonal.

Size and nature of myc mRNA transcripts depends on position and orientation of the inserted provirus

Northern blot analysis of poly(A)$^+$ RNA from lymphomas with proviral integrations upstream of promoter 1 of c-*myc* (Figure 4, lanes A and B) revealed two *myc* mRNA transcripts of ~2.4 and 2.3 kb in size. These two *myc* mRNA transcripts are probably initiated from promoters 1 and 2, respectively (Bernard *et al.*, 1983; ar-Rushdi *et al.*, 1983; Leder *et al.*, 1983; Corcoran *et al.*, 1984). The use of promoters 1 and 2 for *myc* mRNA synthesis is also reflected in the sizes of the *myc* mRNA precursors. Two bands can be detected in lanes A and B (Figure 4a) at 5.0 kb (reflecting precursors containing both introns, initiated from promoter 1 and 2, respectively) and at 3.5 kb (precursor without intron 1). Hybridization of the same blot with a *myc* intron 1 probe (*Xba*I-*Pvu*II fragment near the border of exon 2) shows that *myc* mRNA precursors around 5.0 kb contain intron 1 se-

quences, whereas precursors around 3.5 kbp lack intron 1 sequences (Figure 4b). The RNA bands with estimated lengths of ~4 kb most likely reflect transcripts initiated from promoters in intron 1 (Bernard *et al.*, 1983). The patterns of both precursors as well as processed *myc* mRNAs shown in lanes A and B of Figure 4a, suggest that both promoter 1 and 2 are used in lymphomas with proviral integrations upstream from exon 1.

Sequence analysis of mouse c-*myc* (Bernard *et al.*, 1983) revealed a *Hind*III recognition site directly downstream of promoter 2. In lymphoma 31 (Figure 4a, lane C) integration of a provirus occurred just upstream of the *Hind*III site between promoter 1 and 2 (Table I). A single *myc* mRNA species of 2.3 kb was detected in this tumor. Accordingly, only the

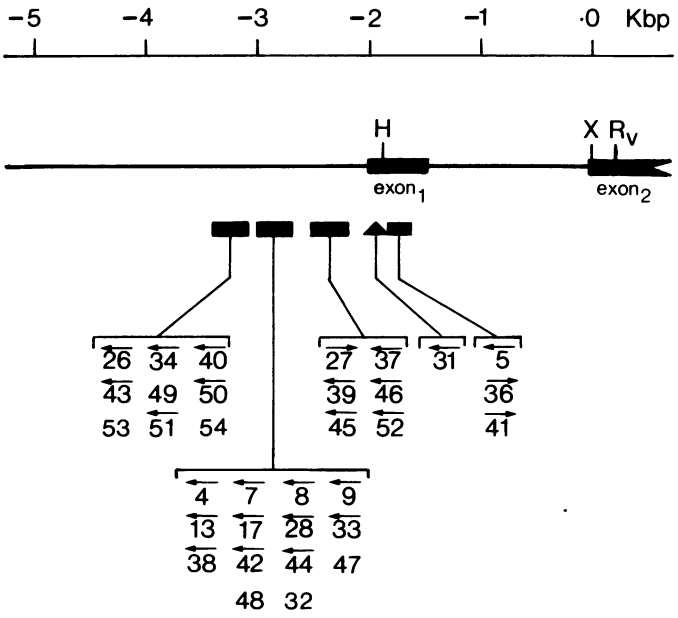

Fig. 2. Position and orientation of integrated proviruses in the c-*myc* region of 33 lymphomas. Localization of integrated proviruses are shown by arrows or blocks. Lymphoma numbers correspond to the listing in Table I. Horizontal arrows above the tumor numbers denote the orientation of the provirus (5′ −3′). The orientation for lymphomas 32, 47, 48, 49, 53 and 54 was not determined. For abbreviations of restriction endonucleases see legend to Figure 1.

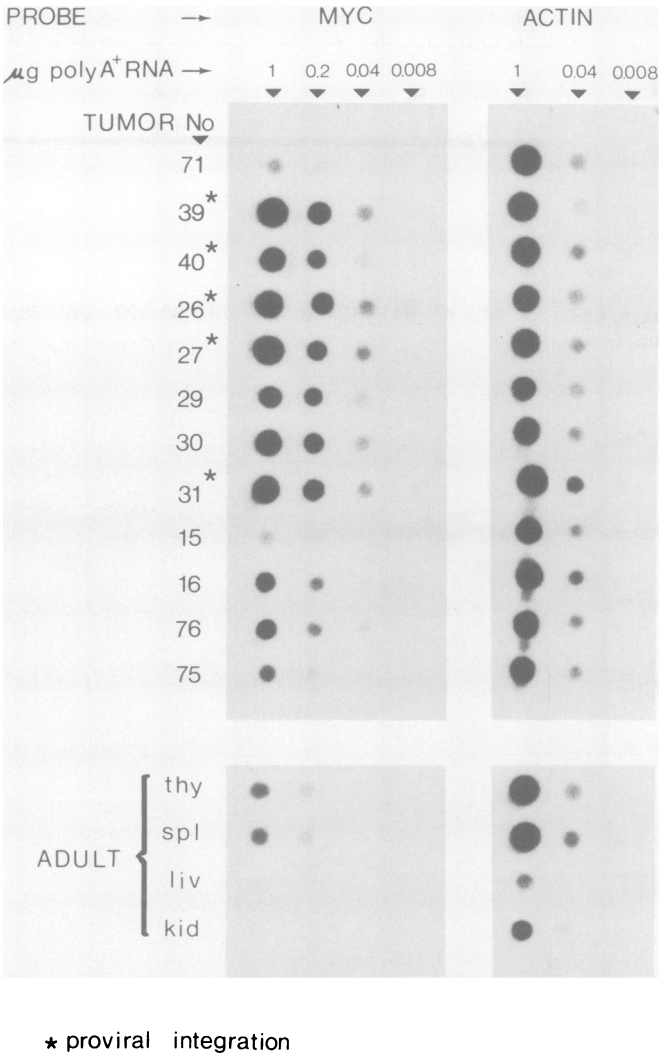

Fig. 3. c-*Myc* mRNA levels in normal and lymphomas tissues. Several dilutions of poly(A)$^+$ RNA, ranging from 1 μg to 8 ng, from 12 lymphomas and from normal adult BALB/c tissues were spotted on nitrocellulose filters and hybridized to ^{32}P-labeled c-*myc* probe or actin probe, which was used as a control for the quantity and integrity of the RNA samples. Lymphomas marked with an asterisk contained a provirus in the c-*myc* region (see also Table I). Abbreviations: Thy, thymus; Spl, spleen; Liv, liver; Kid, kidney.

Table II. Frequency of integration of MuLVs in the c-*myc* and/or *pim*-1 region. Correlation with latency of lymphoma development

Group	Virus strain	Mode inf.[a]	Mouse strain	Latency weeks[b]	Number tested	Number and percentage with proviral integration in: c-*myc*	*pim*-1	c-*myc*+*pim*-1	Total
I	MoMuLV	NBI	BALB/c	13±3	54	27 (50%)	25 (46%)	14 (26%)	38 (70%)
	MoMuLV	NBI	C57BL	24±6	7	1 (14%)	4 (57%)	1 (14%)	4 (57%)
	MCF247	NBI	AKR	18±5	5	0	2 (40%)	0	2 (40%)
II	MCF1233	NBI	C57BL	38±11	11	0	2 (18%)	0	2 (18%)
	MCF1233	NBI	BALB/c	37±8	7	0	1 (14%)	0	1 (14%)
	AKV	G.T.	AKR	37±8	14	2 (14%)	1 (7%)	0	3 (21%)
	MoMuLV	G.T.	BALB/Mo	35±12	16	3 (19%)	1 (6%)	0	4 (25%)
III	MCF1130	NBI	C57BL	62±9	4	0	0	0	0
	MCF1130	NBI	BALB/c	57±5	5	0	0	0	0
	C57-MLV	M.T.	C57BL	67±11	7	0	0	0	0
Total numbers of lymphomas					130	33	36	15	54

[a]NBI, newborn-infected; G.T., germ-line transmitted; M.T., milk-transmitted (Melief *et al.*, 1980).
[b]Latency (mean ± standard deviation) of the total number of lymphomas tested.

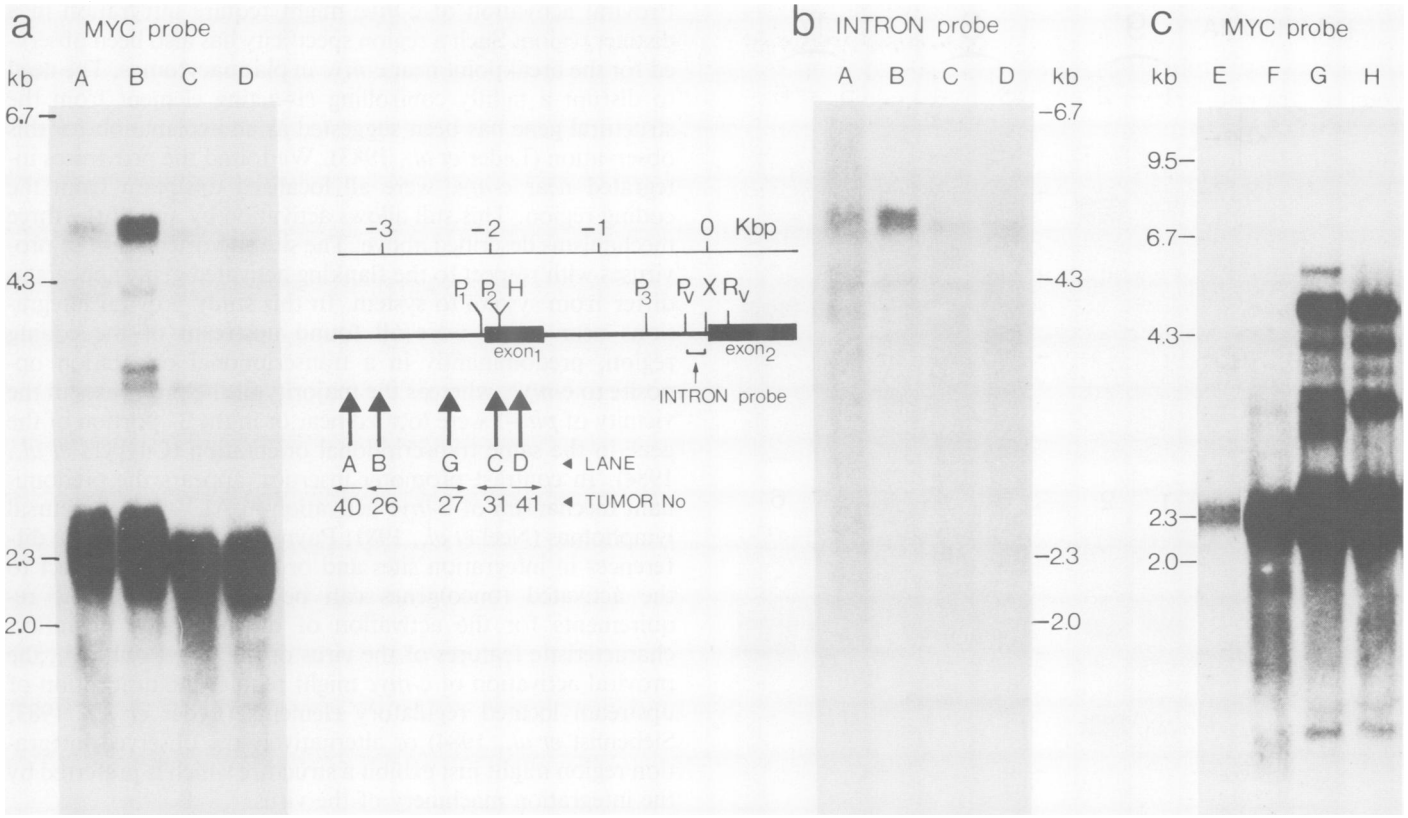

Fig. 4. Northern blot analysis of lymphoma RNAs. Glyoxal-treated poly(A)$^+$ RNA was separated on 1% agarose gels, transferred to nitrocellulose filters and hybridized to c-*myc* probes. **Lanes A,B,C,D** and **G** contain 5 μg poly(A)$^+$ RNA from lymphomas 40,26,31,41 and 27, respectively. **Lane E**, 10 μg poly(A)$^+$ RNA from tumor 15; **lane F**, 10 μg poly(A)$^+$ RNA from newborn BALB/c spleen; **lane H**, 10 μg poly(A)$^+$ from tumor 29. **(a)** and **(c)**, hybridization to the *Xba*I/*Hind*III probe. **(b)** Hybridization of blot **(a)** to *Xba*I/*Pvu*II intron probe. Drawing shows position of c-*myc* promoters 1 and 2 , and intron probe. The position and orientation of integrated proviruses are indicated with vertical and horizontal (5′ −3′) arrows, respectively. Abbreviations: H, *Hind*III; Pv, *Pvu*II; X, *Xba*I; RV, *Eco*RV.

smaller sized precursors were detected. Since the transcriptional orientation of the provirus was opposite to that of c-*myc*, transcription probably initiated from promoter 2 in this lymphoma.

Analysis of RNA from lymphoma 41 (Figure 4a, lane D) revealed a *myc* transcript of ~2.2 kb and concomitant smaller precursors. A provirus, with the same transcriptional orientation as c-*myc*, is integrated to the right of the *Hind*III site, as was evident from Southern blot analysis after cleavage with *Hind*III (Table I). Therefore, *myc* promoter 1 and 2 are no longer available for the initiation of *myc* mRNAs in this tumor. Presumably, c-*myc* transcription in lymphoma 41 is initiated from the viral promoter in the 3′ LTR as seems the case for part of the transcripts in lymphoma 27 (Figure 4c, lane G). The provirus in lymphoma 27 is integrated ~500 bp upstream from the first c-*myc* promoter in the same transcriptional orientation as the c-*myc* gene (Figures 2 and 4). The additional mRNA precursor of 5.7 kb, detected in this tumor (Figure 4c, lane G) probably represents a c-*myc* precursor initiated from the proviral promoter in the 3′ LTR, whereas the additional hybridizing band around 3 kb most likely represents the processed mRNA. Remarkably, also the normal c-*myc* promoters appear to be used, as can be deduced from the presence of normal sized *myc* mRNA precursors.

Figure 4c also shows c-*myc* mRNA and precursors from normal tissue (lane F), and lymphomas without proviral integration near c-*myc*. The latter comprise both lymphomas with an enhanced c-*myc* mRNA level (lane H, tumor 29), as well as lymphomas with a strongly reduced c-*myc* mRNA level (lane E, tumor 15).

Evidence for proviral activation of pim-1 and c-myc within the same tumor cell population

In 23% of the early developing T cell lymphomas, we observed proviral integration both near *pim*-1 and c-*myc* in the same lymphoma DNA. Cells from three different primary lymphomas (8, 9 and 17) with proviral integrations near both genes, were injected i.p. into syngeneic recipient mice. Lymphomas developed after 2−4 weeks. Southern blots containing DNAs from the primary lymphomas and transplanted tumors were hybridized with the *pim*-1 and c-*myc* probes. An example of the hybridization patterns seen with DNAs of the primary and transplanted lymphomas is shown in Figure 5. The original tumors 8, 9 and 17 showed proviral integration in a fraction of the tumor cells both in the *pim*-1 as well as in the c-*myc* domain (lanes 1, 3 and 5). However, upon transplantation of tumor 9 into five different receipients, lymphomas developed which showed the same additional bands as the parental lymphoma with hybridizing intensities which were identical to the intensities of the normal alleles of *pim*-1 and c-*myc* (lanes 2, only one receipient is shown).

In the other transplanted lymphomas (8 and 17) only the rearranged *myc* (tumor 8, lanes 4) or *pim*-1 (tumor 17, lanes 6) was inherited from the original tumor, indicating that the primary tumor was oligoclonal and clones were selected upon transplantation. The transplants of tumor 17 still seemed

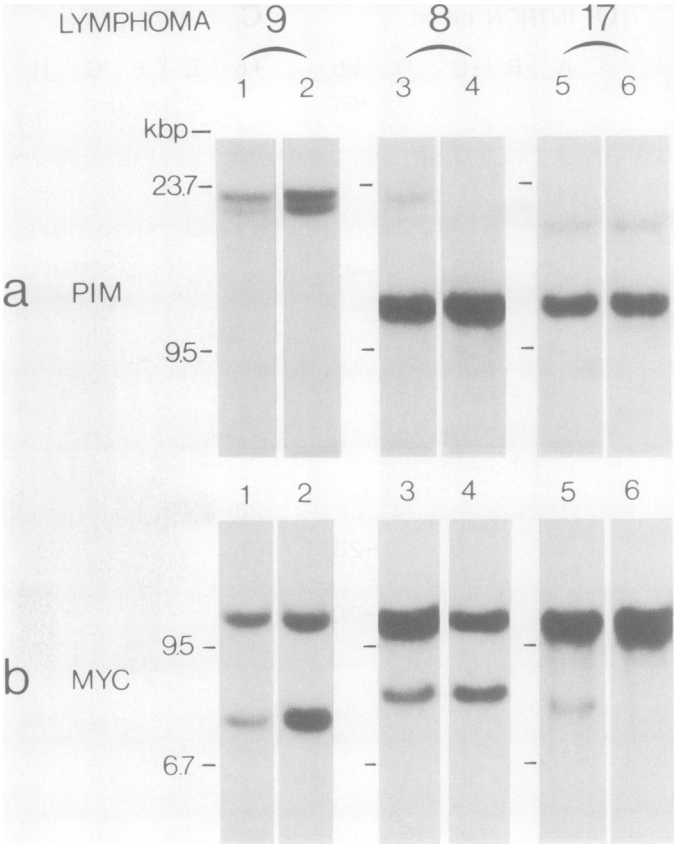

Fig. 5. Segregation of proviral integrations in transplanted lymphomas. *Eco*RV **(panel a, lanes 1** and **2)**, *Eco*RI **(panel a, lanes 3,4,5** and **6)** and *Kpn*I **(panel b)** digests of 10 μg lymphoma DNA from mouse 9,8 and 17 **(lanes 1,3** and **5** respectively), and the corresponding transplanted tumors **(lanes 2,4** and **6**, respectively). **(a)** Hybridization to *pim*-1 probe. **(b)** Hybridization to c-*myc* probe. The normal *pim*-1 allele corresponds to the 22 kbp (*Eco*RV) or the 12 kbp (*Eco*RI) fragment **(panel a)**; the normal c-*myc* allele corresponds to the 11-kbp fragment **(panel b)**.

oligoclonal, although we cannot exclude that in this case, contaminating normal tissue reduced the relative hybridizing intensity of the modified allele. The oligoclonality of many of the primary tumors was also confirmed by comparing the 'multiple proviral integration' pattern between the primary and transplated lymphomas: in several instances the latter harbored a subset of the proviral integration sites, present in the original lymphoma (data not shown).

Discussion

Activation of cellular (onco)genes by insertional mutagenesis is observed in ALV, MuLV and MMTV-induced tumors (Hayward *et al.*, 1981; Neel *et al.*, 1981; Payne *et al.*, 1982; Nusse *et al.*, 1984; Dickson *et al.*, 1984; Fung *et al.*, 1983; Corcoran *et al.*, 1984; Cuypers *et al.*, 1984). Integrated proviruses can activate flanking cellular genes either by transcription from the viral promoter (Neel *et al.*, 1981; Payne *et al.*, 1982), by virtue of the enhancer activity of proviral sequences on adjacent cellular promoters (Nusse *et al.*, 1984; Corcoran *et al.*, 1984; Cuypers *et al.*, 1984; Dickson *et al.*, 1984), or by disruption of *cis*-controlling elements, as suggested for c-*myc* activation upon translocation (Leder *et al.*, 1983).

Obviously, the position and orientation of the integrated provirus with respect to the flanking gene determines to a large extent which activation mechanism can be operational. Proviral activation of c-*myc* might require integration in a distinct region. Such a region specificity has also been observed for the breakpoint near c-*myc* in plasmacytomas. The need to disrupt a tightly controlling *cis*-acting element from the structural gene has been suggested as an explanation for this observation (Leder *et al.*, 1983). We found the proviruses integrated near c-*myc* were all localized upstream from the coding region. This still allows activation by any of the three mechanisms described above. The site and orientation of proviruses with respect to the flanking activated gene, appears to differ from system to system. In this study proviral integrations near c-*myc* were all found upstream of the coding region, predominantly in a transcriptional orientation opposite to c-*myc*, whereas the majority of the proviruses in the vicinity of *pim*-1 were located near or in the 3′ portion of the gene in the same transcriptional orientation (Cuypers *et al.*, 1984). In contrast promoter insertion appears the predominant mechanism of c-*myc* activation in ALV-induced bursal lymphomas (Neel *et al.*, 1981; Payne *et al.*, 1982). These differences in integration sites and orientations with respect to the activated (onco)genes can be caused by specific requirements for the activation of distinct genes or reflect characteristic features of the virus or the target cell, e.g., the proviral activation of c-*myc* might require the dislocation of upstream located regulatory elements (Leder *et al.*, 1983; Siebenlist *et al.*, 1984) or alternatively the observed integration region might just exhibit a structure which is preferred by the integration machinery of the virus.

Frequency of integration near c-myc in T cell lymphomas

Proviral integration near c-*myc* is observed more frequently in early developing T cell lymphomas (28 out of 66) than in lymphomas which develop after a latency between 7 and 12 months (5 out of 48). Similar results were obtained for the *pim*-1 gene (Table II). This difference in incidence could have been caused by the availability of more or different target cells for virus infection in the development of early T cell lymphomas, increasing the chance of proviral integration in transformation-sensitive regions of chromosomal DNA. This could explain the frequently observed oligoclonality of these early T cell lymphomas as compared with the predominantly monoclonal origin of the spontaneously developing tumors in BALB/Mo and AKR mice.

Remarkably, >80% of the proviruses near *pim*-1 showed an MCF-like structure (Cuypers *et al.*, 1984), whereas only 50% of the proviruses near c-*myc* belonged to this class, as judged by the presence or absence of an *Eco*RI cleavage site at map position 6.9 of the proviral genome. MCF viruses show a different target cell specificity than their ecotropic parents (Hartley *et al.*, 1977; Cloyd, 1983; O'Donnel *et al.*, 1984). Interestingly, after infection of newborn mice with MCF viruses, proviral integration seemed to occur near *pim*-1 rather than near c-*myc* (see Table II).

Nature and expression of c-myc mRNA

Proviral integration near c-*myc* is associated with the presence of increased levels (up to 30-fold) of *myc* mRNA. Enhanced *myc* mRNA levels were also found in some lymphomas without proviral integration near c-*myc* (Figure 3). This could be the result of integration of proviruses outside the region we analysed, be caused by other structural changes which escaped detection, or point to alternative mechanisms for *myc* activation (Kelly *et al.*, 1983; Keath *et al.*, 1984). In a few lymphomas without proviral integration near c-*myc*, *myc* mRNA transcripts were hardly detectable (Figure 3, lym-

phoma 15 and 71). The original target cells giving rise to these lymphomas might have produced only minute amounts of *myc* mRNA or the level of *myc* expression was reduced during the progressive growth of the lymphomas. In any event, this observation shows the relative value of normal thymus and spleen, like any other organ, as reference tissues for *myc* expression. Identification of *myc* mRNA precursors on Northern blots clearly shows the involvement of *myc* promoters 1 and 2. When a provirus is inserted in the 'promoter insertion orientation' upstream from promoter 1 (Figure 4, tumor 27), transcription starts at three different sites: at *myc* promoter 1 and 2 and probably at the promoter in the U3LTR of the integrated provirus. In a tumor with a provirus integration between promoter 1 and 2 (lymphoma 31), transcription seemed exclusively to be initiated from promoter 2. In tumors with proviral integrations downstream of promoter 2 (lymphomas 5, 36, 41), transcription most likely starts from a proviral promoter or from promoters in intron 1 (Bernard *et al.*, 1983).

Oligoclonality of virus-induced T cell lymphomas

Analysis of transplanted lymphomas revealed that the primary lymphomas were often oligoclonal, confirming the conclusions drawn from comparison of the hybridization intensities of the rearranged and normal alleles on Southern blots. Injection of cell suspensions from the primary lymphomas 8 and 17, which showed proviral integrations both near *myc* and *pim*-1, resulted in lymphomas in which none or only one of the rearranged *myc* and *pim*-1 alleles were retained, illustrating that the original lymphomas were composed of at least two (tumor 8) or three (tumor 17) different cell clones. Some transplanted lymphomas seemed to remain oligoclonal as revealed by the persistence of the reduced hybridizing intensity of the band corresponding with the rearranged allele in the transplants (Figure 5, lane 6). However, transplantation of lymphoma 9, which also showed proviral integration near c-*myc* and *pim*-1, resulted in tumors which inherited in all instances the rearranged c-*myc* and *pim*-1 allele. Although lymphoma 9 was probably oligoclonal, as judged from the intensities of the additional hybridizing bands, the transplanted tumors appeared monoclonal: the normal and altered allele showed equal hybridizing intensities in five independent transplants (Figure 5, lane 2), suggesting the presence (by proviral insertion) of both a modified c-*myc* and *pim*-1 allele within the same cell clone. It is likely that proviral integration near c-*myc* or *pim*-1 is detectable because of the selective advantage conferred by the activation of these genes. Therefore, proviral integration near both c-*myc* and *pim*-1 within the same tumor cell population suggests a transformation process in which c-*myc* and *pim*-1 might act cooperatively. One could imagine that the first insertion resulted in the massive proliferation of the cell and provided a large target population for the second insertion, which conferred additional growth advantage. We are currently testing whether synergism between c-*myc* and *pim*-1 can also be shown in '*in vitro*' transformation assays.

Materials and methods

Mice, viruses and lymphomas

Lymphomas were induced by injection of newborn BALB/c, C57BL or AKR mice with various MuLV strains as described by Jaenisch *et al.*, (1975). The origin of Moloney MuLV clone 1A, MCF1233, MCF1130, AKR-MCF247, and the milk-transmitted B-tropic MuLV were described previously (Cuypers *et al.*, 1984). Spontaneously developing lymphomas from BALB/Mo and AKR/Fu mice were obtained as reported (van der Putten *et al.*, 1979; Quint *et al.*, 1981). Transplantation of lymphomas was performed by i.p. injection of ~5 x 10^6 viable primary lymphoma cells into syngeneic recipient mice, which developed lymphomas in 2–4 weeks.

DNA isolation and restriction enzyme analysis

Preparation of high mol. wt. DNA was as described (van der Putten *et al.*, 1979). DNA samples (10 μg) were digested under conditions recommended by the suppliers (Boehringer, Amersham, BRL). Gel electrophoresis, transfer to nitrocellulose filter and hybridization to ^{32}P-labeled probes was carried out as described (Quint *et al.*, 1981). As mol. wt. markers *Hind*III-digested phage λ DNA was used.

Preparation of RNA

For RNA isolation, frozen tissues were homogenized with a polytron (2 min, full speed) at 0°C in 3 M LiCl, 6 M urea, and maintained overnight at 4°C as described by Auffray and Rougeon (1980). After centrifugation at 16 000 *g* for 30 min, the supernatant was discarded and the pellets dissolved in 10 mM Tris-HCl, pH 7.4, 0.5% SDS, deproteinized by two successive phenol: chlorophorm:isoamylalcohol (50:48:2) extractions, and precipitated with ethanol. Poly(A)$^+$ RNA was selected by one cycle of oligo(dT)-chromatography, after heating the RNA samples at 65°C for 5 min.

Analysis of polyadenylated RNA

For dot-blot analysis, poly(A)$^+$ RNA was dissolved in water and applied to nitrocellulose filters as described by Muller *et al.* (1982). Glyoxal treatment and Northern blot analysis of poly(A)$^+$RNA was carried out as described (Carmichael and McMaster, 1980; Thomas, 1980). As mol. wt. markers *Hind*III-digested λ-DNA fragments were used. Denaturation was performed as described for RNA.

Hybridization probes

For the detection of *myc* sequences, the *Xba*I/*Hind*III fragment, covering exon 2, intron 2 and the major part of exon 3, was used (Shen-Ong *et al.*, 1982). A probe for intron 1 of c-*myc* was prepared by subcloning a *Pvu*II/*Xba*I DNA fragment, located near the border of exon 2 (see Figure 4). The MoU3LTR probe and the probe for *pim*-1 (probe A) were described previously (Cuypers *et al.*, 1984). The actin cDNA clone was described by Dodemont *et al.* (1982).

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