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Genetics

Proviral deletions and oncogene base-substitutions in insertionally mutagenized c-*myc* alleles may contribute to the progression of avian bursal tumors

(somatic mutation/homologous recombination/transposable elements)

DAVID WESTAWAY, GREGORY PAYNE*, AND HAROLD E. VARMUS

Department of Microbiology and Immunology, University of California, San Francisco, CA 94143

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ABSTRACT **Bursal lymphomas induced in chickens by avian leukosis viruses (ALVs) harbor proviral insertions that augment expression of an adjacent cellular oncogene, c-*myc*. To analyze such insertionally mutagenized c-*myc* genes in greater detail, we isolated molecular clones from two independent tumors. Precise proviral integration has occurred within the transcribed region of the c-*myc* gene in both mutant alleles. The proviruses bear different internal deletions that preclude the expression of the *gag*, *pol*, and *env* genes. The c-*myc* gene from bursal lymphoma LL4 contains a single copy of an ALV long terminal repeat (LTR), presumably the product of homologous recombination between LTRs at the ends of a normal provirus; the "solo" LTR is positioned in the correct orientation to act as a promoter for the c-*myc* gene. Bursal lymphoma LL3 contains an ALV provirus positioned upstream in the opposite transcriptional orientation to the coding exons of c-*myc* and deleted from a site within the leader region into the *gag* gene. In addition, the nucleotide sequence of the c-*myc* gene from tumor LL3 differs from the published sequence of the normal c-*myc* coding region at 3 positions of 180 determined. One of these changes, a silent nucleotide transition, is documented as a somatic mutation by restriction endonuclease mapping. It is flanked by two other candidate tumor-specific point mutations, one of which predicts an amino acid replacement, Pro → Thr at position 63. Thus, additional lesions that may affect the expression of viral genes and the quantity and nature of the putative c-*myc* gene product occur in provirally mutated c-*myc* alleles and may contribute to tumor progression.**

Avian leukosis viruses (ALVs) are a family of weakly oncogenic, replication-competent retroviruses with a generalized proviral structure 5′. . .LTR-*gag-pol-env*-LTR. . .3′, in which LTR denotes long terminal repeat. The viruses do not contain transforming genes, but they induce tumors, predominantly bursal lymphomas, after a long latency period. Histopathological and epidemiological analysis indicates that lymphoma formation is a multi-stage phenomenon (1, 2).

Recent experiments support the notion that ALVs contribute to this process by acting as insertional mutagens (3–8). The majority of ALV-induced lymphomas exhibit proviral integrations adjacent to the c-*myc* gene, the cellular homologue of the putative transforming gene (v-*myc*) of the acute leukemia virus MC29 (5–8). The tumors contain elevated levels of c-*myc*-specific transcripts, irrespective of the relative configurations of the c-*myc* and proviral transcriptional units (5, 6). Potent transcriptional promoting and enhancing activities located within the proviral LTRs are implicated in the over-expression of the c-*myc* gene. Accordingly, the deletions frequently exhibited by proviruses linked to c-*myc* always spare at least one LTR (3, 4, 7).

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The high frequency of c-*myc*-linked integrations is not thought to reflect an affinity for c-*myc* sequences as an insertional substrate; most experimental findings are consistent with random retroviral integrations into the host genome (9). Integrations within the c-*myc* locus probably bestow a selective advantage that would account for the clonality of bursal lymphomas. However, the nature as well as the quantity of the putative c-*myc* gene product may figure in the outgrowth of tumor cells. For example, the avian bursal lymphoma cell lines RP9, B1, H1, and BK25 encode *myc*-related proteins that differ in size (10).

To understand the mechanism and repercussions of c-*myc* activation, we have generated molecular clones of activated c-*myc* alleles suitable for both structural and functional analysis. These clones were derived from previously described bursal lymphomas (3, 6) induced by a plaque-purified stock of the ALV, Rous-associated virus-2 (RAV-2). Partial sequence analysis of two such alleles reveals that a number of changes in addition to the primary insertion have occurred. These changes may confer selective advantages and contribute to multistep progression towards the malignant phenotype.

METHODS

Molecular Cloning. Tumor LL4 DNA was digested to completion with *Bam*HI and enriched for c-*myc* sequences by rate zonal sedimentation. Approximately 3 μg of the fractionated DNA was ligated to 7 μg of *Bam*HI-digested bacteriophage Charon 30 "arms" (11), prepared by the method of Maniatis *et al.* (12). The ligation products were packaged *in vitro* (13). Recombinant phage were plated on *Escherichia coli* KH802 on square 24-cm plates (14). Plaque hybridization yielded one phage, λLL4, which hybridized with a radiolabeled *Pst* I restriction fragment extending from nucleotide 158 to nucleotide 1676 of v-*myc* (15) and also with a cDNA probe specific for the *U5* domain of the Rous sarcoma virus LTR (3). The 10-kilobase (kb) *Bam*HI insert fragment of λLL4 was subcloned into a derivative of pBR322 (16) lacking the *Eco*RI restriction site (a gift from W. DeLorbe) to yield the plasmid pLL4. Three subclones were derived from the recombinant bacteriophage that span the provirally linked c-*myc* allele in tumor LL3 (6). Both pLL3Ab and pLL3Eb contain *Bgl* II fragments inserted into the *Bam*HI site of pBR322. pLL3Ab contains a 9-kb *Bgl* II fragment that includes 5′ c-*myc* flanking sequences and the 3′ half of the ALV provirus. pLL3Eb contains a 7-kb *Bgl* II fragment that includes the 5′ half of the provirus and c-*myc* sequences homologous to v-*myc*. A 0.9-kb *Pst* I fragment was excised from pLL3Eb and inserted into the plasmid pUC8 (17).

Abbreviations: LTR, long terminal repeat; kb, kilobase; ALV, avian leukosis virus; RAV-2, Rous-associated virus 2; v- and c-*myc*, homologous viral and cellular *myc* gene; bp, base pairs.

*Present address: Department of Biochemistry, University of California, Berkeley, CA 94720.

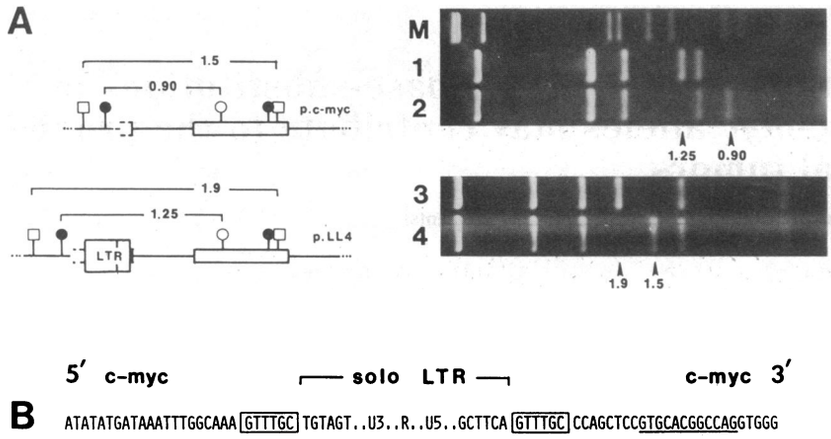

FIG. 1. Structure of the provirally activated c-*myc* gene from bursal lymphoma LL4. (*A*) Sizing the proviral insert. (*A Left*) Partial restriction maps of pc-*myc* and pLL4. Transcription of c-*myc* and the LTR proceeds from left to right. ⍑, *Bgl* I; ⚲, *Sac* I; ↟, *Sac* II. c-*myc* introns are indicated by single lines, and the first large c-*myc* exon homologous to v-*myc* is indicated by a narrow box. A putative c-*myc* exon of uncertain size is indicated by a dashed narrow box (see text). Restriction enzymes that do not cleave within RAV-2-type LTRs (ref. 22; Fig. 3) were used to define the size of the proviral insertion. Fragments spanning the insertion site are bracketed. (*A Right*) Digests fractionated on a 0.8% agarose gel. Lanes: M, λ phage DNA cleaved with *Eco*RI and *Hin*dIII; 1 and 3, pLL4; 2 and 4, pc-*myc*. Lanes 1 and 2 show digests with *Bam*HI, *Sac* I, and *Sac* II. Lanes 3 and 4 show digests with *Bam*HI and *Bgl* I. (*B*) Sequences spanning the LTR/host DNA junctions. A hexanucleotide direct repeat flanking the solo LTR is boxed and 12 nucleotides homologous to the 5′ extremity of v-*myc* are underlined (see text).

Sequence Analysis. DNA sequence analysis was performed as described by Maxam and Gilbert (18). Dephosphorylated 5′ termini were radiolabeled with [γ-^{32}P]ATP and polynucleotide kinase. The 3′ termini of *Eco*RI sites were radiolabeled by a "fill-in" reaction with [α-^{32}P]dATP and the Klenow fragment of DNA polymerase I (19). Products from the piperidine cleavage reactions were recovered by precipitation with ethanol (20).

RESULTS

Identification of a "Solo" ALV LTR in the Activated c-*myc* Locus from Tumor LL4. Previous analysis of tumor LL4 DNA and RNA has defined an extensively deleted RAV-2 provirus that retains at least a portion of one LTR and is positioned on the 5′ side of the c-*myc* gene in the same transcriptional orientation (3, 6). The tumor contains abundant levels of mosaic transcripts initiated within the LTR and extending into c-*myc* (6). Restriction mapping of genomic DNA from tumor LL4 indicated that the activated c-*myc* locus was contained within a 10-kb *Bam*HI fragment (unpublished data). This fragment was cloned into the bacteriophage λ vector Charon 30 and subcloned to yield the plasmid pLL4. The proviral insertion in pLL4 was documented by a comparison to pc-*myc* (21), an analogous subclone derived from a wild-type c-*myc* gene (Fig. 1*A*). Digests of pLL4 and pc-*myc* were electrophoresed in parallel. In each pair of digests (lanes 1/2 and 3/4), a restriction fragment present in pc-*myc* was replaced by a larger fragment derived from pLL4. The increase in fragment size, 0.35–0.40 kb, is in accord with tumor LL4 containing a solo LTR.

This prediction was verified by nucleotide sequence determination. The pLL4 LTR is missing the terminal dinucleotides A-A and T-T of the unintegrated retroviral genome and is bracketed by a 6-base-pair (bp) direct repeat of cellular DNA, features characteristic of a normal proviral integration event (Fig. 1*B*). Furthermore, the normal c-*myc* sequence contains only one copy of the hexanucleotide G-T-T-T-G-C, which flanks the LL4 LTR (M. Linial, C.-K. Shih, and W. Hayward, personal communication). The LTR is inserted into c-*myc* in the same transcriptional direction, slightly upstream of 12 nucleotides, G-T-G-C-A-C-G-G-C-C-A-G, present in v-*myc* but absent from the two known c-*myc* exons (23). These nucleotides are flanked on their 3′ side by a consensus splice donor sequence and could define the 3′ boundary of a third c-*myc* exon ≈450 bp upstream from the first of the two known exons. In any event, the LL4 insertion appears to lie within the transcribed portion of the c-*myc* gene.

Sequence Analysis of a c-*myc*-Linked ALV Provirus from Tumor LL3. Previous analysis of bursal lymphoma LL3 established that it harbors a defective RAV-2 provirus upstream and in the opposite transcriptional orientation from the two coding c-*myc* exons (6). This arrangement has been found in three other tumors and was correlated in at least some instances with an estimated 20-fold increase of c-*myc*-specific transcripts (3, 6). Expression of c-*myc* in these tumors is thought to be potentiated by a transcription-enhancing activity present in the proviral DNA.

Sequence analysis of the LL3 provirus defined an internal deletion anticipated from restriction mapping data. The deletion starts between 15 and 17 bp downstream of the 5′ LTR boundary and extends approximately 1100 bp to a position within the coding sequence for the *gag* polypeptide p27 (ref. 24; Fig. 2). The deletion appears to have occurred by means of a crossover within a dinucleotide shared between the transcribed leader and the p27 sequence. The deletion removes three signal sequences from the proviral genome: (*i*) the ATG initiation codon for the *gag* and *gag-pol* polyproteins (24); (*ii*) the splice donor site used in the biogenesis of *env* mRNA (24, 25); and (*iii*) a signal for the encapsidation of viral genomes into virions (26, 27). The deletion also removes one nucleotide from the $tRNA^{Trp}$ primer binding site (24, 28).

Sequences spanning the LL3 provirus/host cell junction and extending into the v-*myc* homology region confirm the inverted orientation of the provirus with respect to the direction of c-*myc* transcription (Fig. 2; unpublished results). The provirus is inserted into an intron 148 bp 5′ of the first large c-*myc* exon homologous to v-*myc*. The proviral termini lack the LTR dinucleotides A-A and T-T and are flanked by a 6-base-pair direct repeat of cell DNA, A-T-A-T-A-T.

LTR Sequences. The 5′ LTR of LL3 and the solo LTR of LL4 have an identical nucleotide sequence (Fig. 3); 20 of 350 positions vary between the sequence of the LL3/LL4 RAV-2 LTR and one previously determined RAV-2 LTR (22). These differences probably reflect the independent origins (29, 30) of the two RAV-2 isolates used by the laboratories involved. The LL3/LL4 RAV-2 sequence is closely related to that of a Rous sarcoma virus LTR clone used

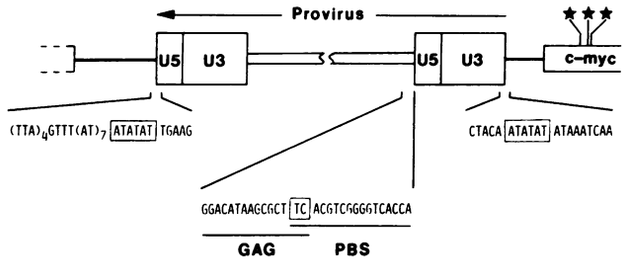

FIG. 2. Structure of the provirally linked c-*myc* gene from bursal lymphoma LL3. Transcription proceeds from left to right for c-*myc* and from right to left for the RAV-2 provirus. Nucleotide sequences are presented in the coding sense for c-*myc* and in the anticoding sense for the RAV-2 provirus. Sequences spanning the provirus/host cell DNA junction are shown on the left and right sides of the figure. A hexanucleotide direct repeat of chicken DNA bracketing the provirus is boxed. The nucleotides in the center of the figure define a deletion that fuses the proviral primer binding site (PBS) to the p27 region of the *gag* gene. The exact breakpoint cannot be determined because the boxed dinucleotide is present both in the *gag* gene and the primer binding site. The *gag* sequences presented here correspond to those from positions 1219–1231 of the published nucleotide sequence of the Prague strain of Rous sarcoma virus (24). c-*myc* introns and exons are as denoted in Fig. 1*A*. The positions of three deviations from the c-*myc* nucleotide sequence (23) are denoted by stars (see Figs. 5 and 6). The 6.7-kb defective provirus, with large boxes indicating *U3* and *U5* domains of the LTRs and broken double lines indicating the internal region, is not drawn to scale.

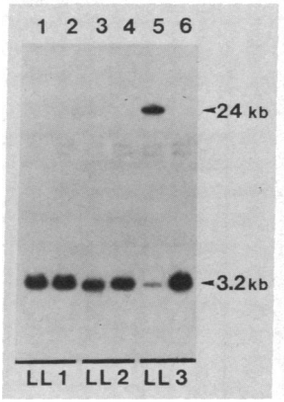

FIG. 4. Southern transfer analysis of c-*myc* from three bursal lymphomas. DNA (5 μg) from the bursal tumors LL1, LL2, and LL3 (ref. 3) (lanes 1, 3, and 5) and unaffected spleens (lanes 2, 4, and 6) of the same three birds was digested to completion with *Sac* I, electrophoresed on 0.6% agarose gel, transferred to a nitrocellulose filter (32), and hybridized with a radiolabeled 1.5-kb *Pst* I fragment derived from the v-*myc* region of MC29 DNA (15). The faint doublet at 11 and 13 kb corresponds to endogenous viral sequences that are detected by 90 nucleotides of *env* sequences included in the probe fragment. A 24-kb tumor-specific c-*myc* *Sac* I fragment is indicated by one arrow, the other indicates the position of a 3.2-kb fragment derived from the normal c-*myc* locus.

to define proviral "enhancing" activity (28, 31).

Candidate Somatic Mutations Within the Provirally Linked c-*myc* Gene from Tumor LL3. Restriction mapping of the mutant c-*myc* allele from tumor LL3 previously revealed the loss of an *Sac* I cleavage site normally present in the 5′ coding exon of c-*myc* at position 190 (6, 23). Figure 4 shows a Southern transfer analysis (32) of *Sac* I digests of normal and tumor DNAs isolated from three birds and probed with a *myc*-specific hybridization reagent. All the samples show a 3.2-kb c-*myc* fragment whose termini are defined by *Sac* I sites at position 190 and downstream of the c-*myc* polyadenylylation site. However, digestion of the LL3 tumor DNA, but not LL3 spleen DNA, also yielded a 24-kb c-*myc* fragment. We conclude that the loss of the *Sac* I site in the LL3 c-*myc* allele cannot be ascribed to allelic polymorphism and is due to a somatic mutation.

Partial sequence analysis of the mutant c-*myc* allele reveals a silent G → A transition in a glutamine codon (number 57), which destroys the *Sac* I restriction site (Fig. 5). Two other deviations from the normal c-*myc* sequence (23) flank the change in codon 57. The first is a silent C → A replacement in the third position of a proline codon at position 46. The second is a conservative C → A replacement, which converts the proline codon at position 63 to a threonine codon. No known restriction enzyme recognition sites span either codon 46 or 63, precluding a convenient assessment of whether the base changes represent germinal or somatic sequence variation. Indirect evidence that these base variants represent somatic events comes from the comparison of *myc* DNA sequences from different sources (15, 23, 33, 34). The three base replacements lie in a domain of the c-*myc* gene that is highly conserved in v-*myc* and in chicken, mouse, and human c-*myc* (Fig. 6). Thus, an amino acid replacement in this domain may affect the behavior of the c-*myc* gene product and participate in tumor pathogenesis.

DISCUSSION

We have analyzed molecular clones of two mutant c-*myc* genes derived from ALV-induced bursal lymphomas and defined deletion and point mutations, in addition to the insertion mutations, that affect these alleles.

Proviral Insertion Sites. Sequence analysis of molecular clones derived from the bursal tumors LL3 and LL4 confirms the position and orientation of the proviral insertions

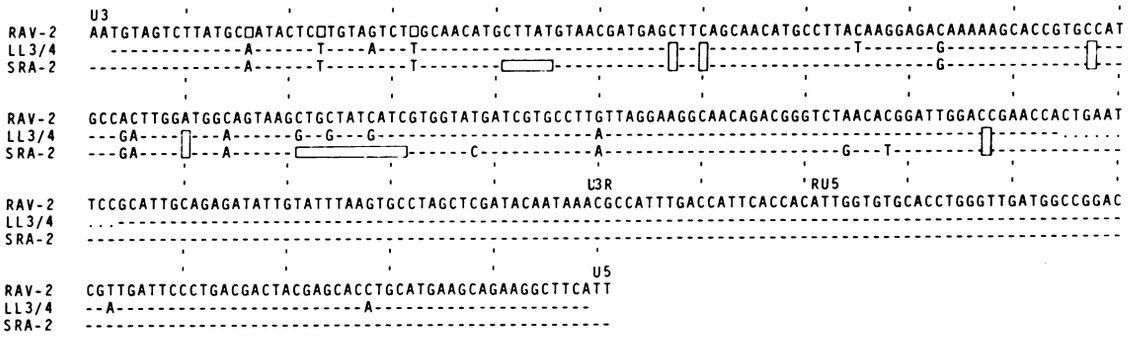

FIG. 3. Comparison of the LTR sequences from RAV-2, the LL3/LL4 proviruses, and the Rous sarcoma virus DNA clone SRA-2. LL3 and LL4 LTR sequences were derived by the Maxam–Gilbert technique after radiolabeling the proviruses at the *Eco*RI sites within the LTRs; hence, the sequence of the *Eco*RI site and a few adjacent nucleotides were not directly determined, as denoted by dots. Nucleotide gaps generated during the alignment of homologous sequences are indicated by open boxes. The terminal dinucleotides are missing from the LL3 and LL4 proviruses, presumably as a result of integration (9). Dashes indicate identity. The sequences of unintegrated RAV-2 and SRA-2 genomes are from Ju and Skalka (22) and Swanstrom *et al.* (28).

FIG. 5. Nucleotide sequences of the c-*myc* gene from bursal lymphoma LL3. The autoradiogram shows base-specific cleavage products derived from the anti coding strand of a pUC8/c-*myc* subclone displayed on an 8% sequencing gel. Deviations from the "normal" c-*myc* sequence are indicated by stars. The sequence encompassing a *Sac* I site in a normal allele is bracketed. Codons are numbered from the ATG codon at nucleotides 16–18 of the published chicken c-*myc* sequence (23).

previously deduced from restriction mapping. Integrated proviruses of ALV and Rous sarcoma virus are characterized by the deletion of the LTR terminal dinucleotides A-A and T-T and are bracketed by 6-bp direct repeats of cell DNA (ref. 9; Figs. 1*B* and 2). The hexanucleotide sequence bracketing the integration site in tumor LL3, A-T-A-T-A-T, is intriguing in that it also flanks three independent insertions of the retrovirus-like elements 297 and 17.6 in the *Drosophila melanogaster* histone gene cluster (35, 36) and flanks a chicken syncytial virus provirus at a different position in chicken c-*myc* (R. A. Swift and H.-J. Kung, personal communication).

Both the LL3 and LL4 insertion sites appear to lie within the transcribed region of c-*myc*. In tumor LL4, efficient transcription of c-*myc* is initiated at the LTR cap site and extends through the *U5* domain into c-*myc*. Candidate c-*myc* splice donor and acceptor sites flanking the possible 0.5-kb intron shown in Fig. 1*A* are available for the maturation of this transcript. The LL3 provirus is located within this putative c-*myc* intron. Unfortunately, we were unable to exam-

	45				50				55				60				65			
	Gln	Pro	Pro	Ala	Pro	Ser	Glu	Asp	Ile	Trp	Lys	Lys	Phe	Glu	Leu	Leu	Pro	Thr	Pro	Pro	Leu	Ser	Pro	Ser
Chicken C-Myc	CAG	CCT	CCC	GCC	CCG	TCC	GAG	GAC	ATC	TGG	AAG	AAG	TTT	GAG	CTC	CTG	CCC	ACG	CCG	CCC	CTC	TCG	CCC	AGC
Chicken C-Myc/LL3			A												A					A*				
V-Myc																		T*						
Mouse C-Myc		G		G		C	AGT		T			A		C		G	AA*		C			G		C	G
Human C-Myc		C	G	G		C	AG		T			A		C		G			C			G		C	T

FIG. 6. Comparison of *myc* DNA sequences. Chicken c-*myc* codons are numbered as in Fig. 5. Other *myc* sequences have been aligned underneath; the v-*myc* sequence is from ref. 15, mouse c-*myc* from ref. 33, and human c-*myc* from ref. 34. Variant positions are indicated with the appropriate nucleotide. Nonsilent changes are indicated by an asterisk. The affected codons, numbered with respect to the chicken sequence, are: LL3 c-*myc*, Pro → Thr at codon 63; mouse c-*myc*, Leu → Gln at codon 59; and v-*myc*, Thr → Met at codon 61.

ine c-*myc* RNA in tumor LL3. Abundant levels of c-*myc* RNAs were found in a hepatic metastasis of tumor LL7, which has a similarly disposed proviral integration (6). These RNA-species did not contain virus-specific sequences, suggesting that the LL3/LL7 proviruses may activate a cryptic promoter in the c-*myc* intron upstream of the first large exon or may enhance transcriptional initiation from the normal c-*myc* promoter(s). In the latter case, viral sequences would be eliminated by splicing.

Proviral Deletions. Proviral deletions are a common feature of ALV-induced lymphomas (3, 4, 7). The deletions we define here are internal and preclude the expression of viral replicative genes. The breakpoints of the LL3 deletion are in the primer binding site within the leader (L) region and in the *gag* gene. The deletion removes the *gag/gag-pol* ATG codon and the splice donor site used to express the *env* gene. The LL4 deletion removes a whole provirus with the exception of one LTR. Both proviral deletions eliminate sequences necessary for the encapsidation of virion RNA. This packaging signal has been mapped between the splice donor site and the *gag* gene ATG codon in spleen necrosis virus and Moloney murine leukemia virus (37, 38), and a similar area of the genome is implicated in the packaging of Rous sarcoma virus (26, 27). The absence of this signal gives a clue as to the timing of the deletions. The deletions could not have existed in the infecting virus stock and must have occurred within the lymphocytes that underwent clonal expansion to form tumors. The deletions could have arisen during reverse transcription or after establishment of the provirus. The structure of viral DNA in LL4 is more easily reconciled with the latter explanation because the simplest mechanism for the generation of a solo LTR is homologous recombination between the LTRs of an intact provirus. This sequence of events has been documented for murine leukemia proviruses in two experimental contexts (39–41) and for the retrovirus-like *Drosophila* transposon *gypsy* (42) and the *Saccharomyces* transposon Ty*1* (43, 44).

The high frequency of proviral deletions in lymphomas indicates that selective pressures favor defective genomes because proviruses are not intrinsically unstable. [For example, generation of solo LTRs in other settings occurs at a frequency between 10^{-6} and 10^{-7} (39, 41).] Two types of selection have been proposed (3, 4). The first is that deletions prevent the expression of viral gene products presented on the cell surface, allowing the tumor to escape immune surveillance. The second is that the proviral deletions facilitate expression of the linked c-*myc* gene, in turn producing a more malignant lymphocyte. The LL3 and LL4 deletions are clearly compatible with both hypotheses.

Perturbations of proviruses have been correlated with the changes in the expression of linked genes. Thus, the reversion of recessive mutations attributed to murine leukemia virus, *gypsy*, and Ty*1* insertions is frequently associated with the generation of solo terminal repeat structures. Reversion to the wild-type (39–42) or cold-sensitive wild-type (43, 44) phenotype is presumably due to an increment in the level of the "target" gene product; however, this has been verified only in one instance (39). Other experiments concern an RSV-derived provirus linked upstream, and in the same transcriptional orientation, from a preproinsulin gene. Deletion or insertion mutations that decrease transcription initiation from the 5′ LTR facilitate the expression of the preproinsulin gene (G. Ju, personal communication). These analogies suggest that the generation of a solo LTR in tumor LL4 might dictate an increase in c-*myc* gene expression.

Point Mutations in c-*myc*. Analysis of part of the c-*myc* oncogene from tumor LL3 has revealed that at least three nucleotides are different from the c-*myc* gene sequence reported by Watson *et al.* (23) (Figs. 5 and 6). We have proven that one of the changes, a G → A transition in codon 57, is a

tumor-specific somatic mutation and that the c-*myc* allele bearing this mutation predominates in DNA extracted from tumor LL3 (Fig. 4). Features commend the other base variants in codons 47 and 63 as somatic mutations that might contribute to the pathogenesis of the LL3 tumor, specifically their occurrence in a conserved domain of c-*myc* and the prediction of an amino acid replacement at codon 63 (Fig. 6). The clustering of the base variants is notable and can be reconciled with two known mutagenic pathways, one of which may operate specifically in lymphoid cells (45–48). Furthermore, simultaneous mutation at several clustered positions could account for the coselection of silent mutations and those that alter amino acid sequence.

Are sequence variations in the c-*myc* gene a common feature of tumors? The codon 57 mutation can be assessed by restriction mapping and is not shared by tumors LL1, LL2 (Fig. 4), and LL4 (Fig. 1*A*). However, it is noteworthy that a c-*myc* gene activated by the translocation of an IgG gene in a Burkitt lymphoma appears to have sustained 25 point mutations in the first protein coding exon (ref. 49; T. Rabbitts, personal communication). Functional assays for c-*myc* transforming activity are needed to assess the significance of these putative somatic mutations.

Multiple Mutations in ALV c-*myc* Loci. Data presented here define three somatic mutations that have occurred at the c-*myc* locus in ALV-induced bursal lymphomas. These are proviral integration, proviral deletion, and point mutation within the c-*myc* coding region. A fourth variation is the amplification of provirally activated c-*myc* genes in chicken syncytial virus-induced bursal lymphomas (50). An event at a different genetic locus has been reported by Cooper and co-workers: the activation of a transforming gene B-*lym* (51). Although questions about the timing, ubiquity, and pathological correlates of these genetic lesions remain to be answered, their identification supports the contention that tumorigenesis is a multistage phenomenon.

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