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Nucleotide Sequence of a Transduced *myc* Gene from a Defective Feline Leukemia Provirus

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The nucleotide sequence of a feline v-*myc* gene and feline leukemia virus (FeLV) flanking regions was determined. Both the nucleotide and predicted amino acid sequences are very similar to the murine and human c-*myc* genes (ca. 90% identity). The entire c-*myc* coding sequence is represented in feline v-*myc* and replaces portions of the *gag* and *env* genes and the entire *pol* gene. The coding sequence is in phase with the *gag* gene reading frame; v-*myc*, therefore, appears to be expressed as a *gag-myc* fusion protein. Viral sequences at the 3′ *myc*-FeLV junction begin with the hexanucleotide CTCCTC, which is also found at the 3′ *fes*-FeLV junction of both Gardner-Arnstein and Snyder-Theilen feline sarcoma viruses. These similarities suggest that some sequence specificity may exist for the transduction of cellular genes by FeLV. Feline v-*myc* lacks a potential phosphorylation site at amino acid 343 in the putative DNA-binding domain, whereas both human and murine c-*myc* have such sites. Avian v-*myc* has lost a potential phosphorylation site which is present in avian c-*myc* five amino acids from the potential mammalian site. If these sites are actually phosphorylated in normal c-*myc* proteins, their loss may alter the DNA-binding affinity of v-*myc* proteins.

The v-*myc* oncogene was first identified as that portion of the avian myelocytomatosis virus MC29 genome responsible for its ability to rapidly induce neoplastic disease (43, 48). As is the case with other retroviral oncogenes, v-*myc* was acquired by viral transduction of a homologous host cellular sequence (c-*myc*) through an as yet undefined mechanism. Retroviral interactions with *myc* produce a broad range of neoplasias, including myelocytomas, endotheliomas, and carcinomas induced by MC29 or other *myc*-containing retroviruses (20) and B-cell lymphomas induced by the insertion of avian leukosis virus near the host c-*myc* locus (25). Interest in *myc* has been further stimulated by its apparent involvement in cancers which do not have a retroviral etiology. These include Burkitt's lymphomas and murine plasmacytomas, where specific chromosomal translocations involving *myc* occur (51), and leukemias, carcinomas, and neuroblastomas, where amplification of the c-*myc* locus or the c-*myc*-related sequence N-*myc* occurs (2, 10, 28, 47). The spectrum of malignancies in which *myc* has been implicated suggests that it may play a very basic role in oncogenesis.

Investigations of *myc* gene products have shown that the mature protein is localized in the nucleus and has DNA-binding activity (14, 23, 38). The c-*myc* gene is expressed in a variety of cell types (49). Levels of expression are low in quiescent cells but elevated during cell proliferation, and they are modulated in a cell cycle-specific manner (27). Expression of c-*myc* can be induced by platelet-derived growth factor, and *myc* proteins may play a role in mediating the mitogenic effects of this growth factor (3). These results suggest that c-*myc* normally functions in the control of cell growth, perhaps by regulating the activity of other genes.

Recently, *myc*-containing feline leukemia virus (*myc*-FeLV) proviruses have been identified in three independent studies of naturally occurring T-cell lymphosarcomas of cats (31, 33, 35). Such recombinant proviruses were found in 8 of 32 T-cell tumors examined in these studies, suggesting that *myc* transduction or horizontal transmission of *myc*-FeLV is a common occurrence. Moreover, FeLV isolates containing at least five other oncogenes (*fms, fes, fgr, sis, and abl*) have been obtained from naturally occurring tumors (5, 6, 13, 19, 34). These findings suggest that transduction of oncogenes by FeLV may play a significant role in the epidemiology of feline cancer.

To more clearly define the molecular mechanism by which *myc*-FeLV proviruses may induce T-cell neoplasia, we determined the nucleotide sequence of the feline v-*myc* gene and flanking FeLV sequences from the *myc*-FeLV molecular clone of Levy et al. (31). In the present study we report that sequence, compare it to previously determined *myc* and FeLV sequences, and comment on several features which may be relevant to oncogenic activation of *myc* genes.

MATERIALS AND METHODS

The recombinant plasmid pBam8, which contains a full-length copy of the *myc*-FeLV provirus isolated by Levy et al. (31), was used for sequence analysis. Transient assays indicate that this provirus is transcribed after DNA transfection of tissue culture cells (M. Braun and J. Casey, unpublished data). Preliminary attempts to rescue the *myc*-FeLV provirus with replication-competent helper FeLV have been unsuccessful, but this may be due in part to the lack of a focus-forming assay in which the *myc* oncogene is active. The entire pBam8 plasmid was sonicated to produce a random assortment of fragments. The fragments were end repaired and cloned in the *Sma*I site of the M13mp8 vector (32) to produce a random or "shotgun" sequencing library (11). Clones containing *myc*-related sequences were selected by their ability to hybridize with the radiolabeled 1.5-kilobase *Pst*I fragment of chicken v-*myc* (1). A total of 26 *myc*-hybridizing clones were isolated and sequenced by the Sanger dideoxy nucleotide method (7, 12, 45) to derive the 1,744-base-pair (bp) feline v-*myc* sequence (plus flanking sequences) shown in Fig. 1. The entire sequence was determined completely on both strands of DNA. The high guanine-plus-cytosine (G+C) content of the *myc* gene caused an

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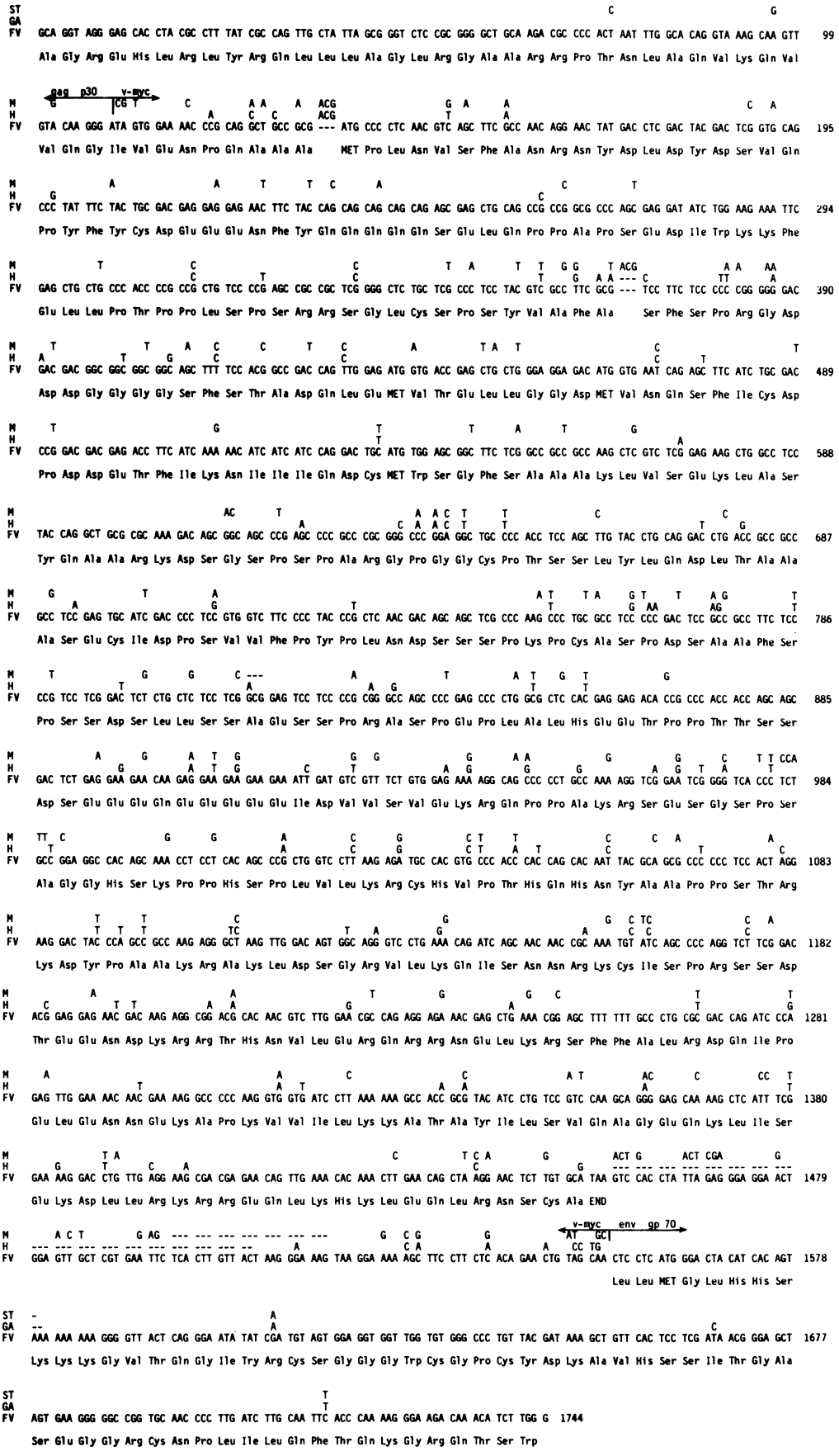
gag p30
v-myc
v-myc env gp 70

inordinate number of compression artifacts on standard 8 M urea sequencing gels. We therefore resequenced a number of clones on gels containing 25% formamide and 7 M urea as denaturants, thereby eliminating most of these artifacts.

Certain restriction enzyme sites predicted from the nucleotide sequence were confirmed in the appropriate M13 single-stranded sequencing clones. First, a radiolabeled second strand was synthesized by using DNA polymerase I (Klenow fragment) and a pentadecameric universal sequencing primer (New England BioLabs, Inc.). The clones were then digested with the restriction enzymes of interest, and the molecular weight of the resulting fragments was determined after electrophoresis in 6% polyacrylamide gels with molecular weight markers by autoradiography.

RESULTS AND DISCUSSION

Organization of the feline v-*myc* gene. The 1,744-bp segment of *myc*-FeLV that we sequenced can be aligned with a number of published FeLV and *myc* sequences (Fig. 1). Based on homology considerations, our sequence consists of five parts: 109 bp of the FeLV group-specific antigen (*gag*) gene, 26 bp of the 5′ *myc* leader sequence, 1,317 bp of the *myc* coding sequence, 102 bp of the 3′ untranslated *myc* trailing sequence, and 190 bp of the FeLV envelope (*env*) gene (Fig. 2). The 5′ junction between *myc* and FeLV occurs at nucleotide 109 (Fig. 1), which corresponds to nucleotide 1592 of the FeLV *gag* gene (30). FeLV sequences coding for 103 amino acids of the *gag* p30 protein, the entire *gag* p10 protein, the entire RNA-dependent DNA polymerase (*pol*) gene, and a portion of the envelope (*env*) gene have been deleted from *myc*-FeLV. Homology to mammalian c-*myc* sequences begins immediately after the *myc*-FeLV junction with a 26-bp segment corresponding to untranslated *myc* leader, which is followed by the c-*myc* coding sequence. Assuming that the splice junctions of feline c-*myc* are the same as those of the human and murine genes, 14 bp of this leader sequence are derived from exon 1, and 12 bp are derived from exon 2 (Fig. 2). The majority of exon 1, which comprises an unusually long untranslated region in mouse and human c-*myc*, has not been transduced into feline v-*myc*. The *myc* reading frame is in phase with that of *gag*, and there are no termination codons in the short *myc* leader; therefore, it is plausible that *v-myc* is expressed as a *gag* fusion protein, a common pattern among defective retroviruses containing oncogenes.

The open reading frame for feline v-*myc* continues until the termination codon beginning at nucleotide 1453. This codon is followed by 99 bp homologous to the 3′ untranslated region of both mouse and human c-*myc* (Fig. 1). The 3′ *myc*-FeLV junction occurs at position 1554 of our sequence, where homology to FeLV resumes within the *env* gene. The first 443 bp of *env* have been deleted. Significantly, no polyadenylation signal (AATAAA) is included in the portion of *myc* which has been transduced. Such a signal occurs in mouse and human c-*myc* genes 200 to 230 bp beyond the 3′ point of *myc*-FeLV fusion (4). Polyadenylation signals are also missing from the v-*myc* gene of MC29 and the v-*onc* genes of several other acutely transforming retroviruses (42). The lack of a polyadenylation signal probably allows transcription of a full-length copy of the defective provirus, which presumably is required for it to be infectious.

Three restriction endonuclease sites (two *Sac*II and one *Pst*I) that did not appear in a previously published map (31) were predicted by the nucleotide sequence. All of these were sufficiently close to other *Sac*II or *Pst*I sites to be below the limits of resolution of the mapping study, and all have been confirmed experimentally (see above).

Comparative sequence analysis of *myc* genes. The portion of feline v-*myc* homologous to the coding sequences of murine and human c-*myc* has been highly conserved during evolution (Fig. 1 and 3). All three coding sequences are 1,317 bp long. The cat and human genes are colinear, whereas the mouse sequence has one 3-bp deletion and one 3-bp insertion. At the nucleotide level, there are 109 substitutions between cat and human (92% identity). The number of substitutions between mouse and cat (153; 88.4% identity) and mouse and human (158; 88% identity) are similar, both being approximately 40% larger than the number of substitutions between cat and human.

Homology among the feline, murine, and human *myc* genes is also extensive at the amino acid level (Fig. 3). There are 30 amino acid replacements between cat and human, 34 between cat and mouse, and 38 between mouse and human for a total of 47 variable positions in the 439-amino-acid sequence. The percent identity in these comparisons ranges from 91 to 93%. Furthermore, many replacements are conservative; only 10 result in charge changes (Fig. 3). It has previously been observed that, when compared to chicken c-*myc*, exon 3 is more conserved in mouse and human c-*myc* than is exon 2 (4), and this observation extends in a general way to the feline v-*myc* sequence. Furthermore, with three mammalian *myc* genes for comparison, it becomes clear that the majority of amino acid replacements among the mammalian genes are confined to five variable regions (Fig. 3). These five regions account for 27 of 47 variable positions (57%) but include only 54 of 439 residues (12%). Two of these regions have been deleted from chicken c-*myc*, and two others show little sequence conservation between avian and mammalian *myc* homologs, suggesting that exact sequence conservation in these regions is not critical to *myc* function. One of the variable regions overlaps the proline-rich area of chicken *myc* (amino acids 156 to 170, Fig. 3). It has been speculated that this proline-rich segment would provide a flexible bridge that might link two functional domains of the *myc* protein (42). Lack of sequence conservation discourages attributing the same function to this region of the mammalian *myc* proteins.

The 26-bp 5′ *myc* leader sequence and 102-bp 3′ *myc* untranslated sequence which appear in *myc*-FeLV can be aligned with comparable portions of murine and human c-*myc* sequences (Fig. 1). The similarity between these sequences is obvious, with 69 to 88% identity in the 5′ leader and 68 to 79% identity in the 3′ untranslated region (deletions not included). Therefore, we assume that these sequences are derived from the corresponding portions of feline c-*myc*. The high degree of sequence conservation in these untrans-

FIG. 1. Nucleotide sequence of feline v-*myc* and FeLV flanking regions. The feline v-*myc* (FV) sequence is compared to the human (H) and murine (M) c-*myc* genes (4, 9, 52, 54). FeLV-derived regions are compared to Gardner-Arnstein (GA) and Snyder-Theilen (ST) FeLV sequences (16, 22, 30, 36). Only nonidentical nucleotides are shown in M, H, GA, and ST. Dashes indicate deleted residues. FeLV sequences from the group-specific antigen p30 coding region are marked *gag* p30; those from the envelope gp70 coding region are marked *env* gp70. The predicted amino acid sequence is shown only for *myc*-FeLV. The longest possible amino acid sequence for *env* gp70 is shown; this becomes in frame with the functional sequence (GA) only after the insertion at nucleotide 1579.

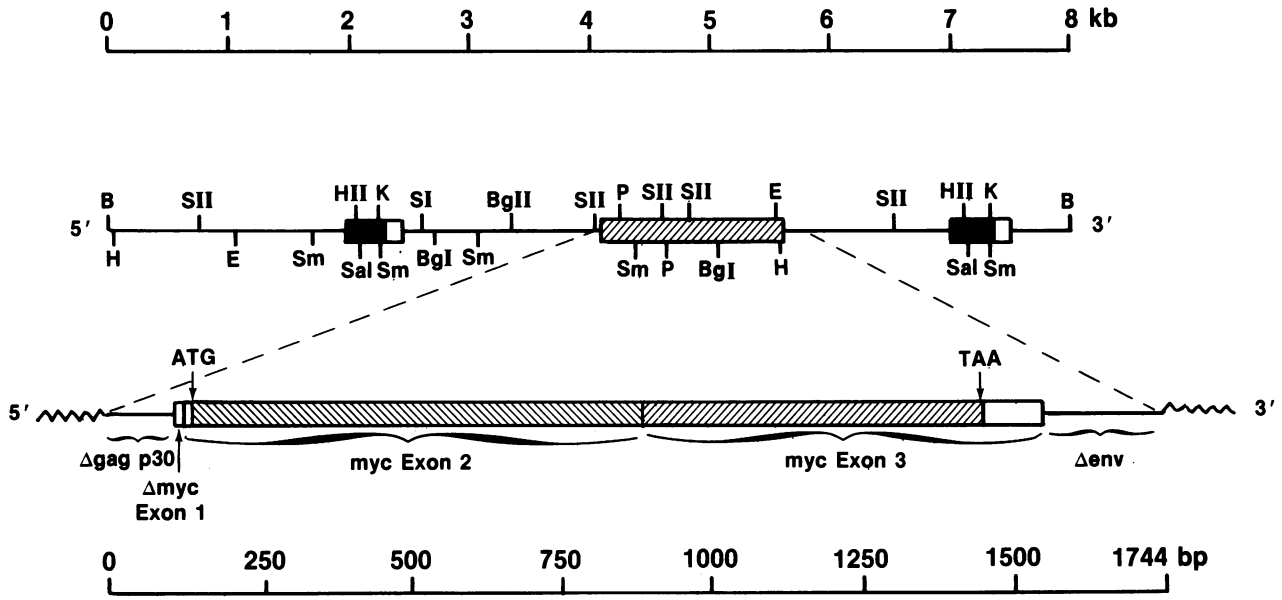

FIG. 2. Sequence organization of *myc*-FeLV. Upper drawing, Restriction enzyme site map of the 8-kilobase (kb) insert of pBam8. Shaded boxes and open boxes represent the U_3 and U_5 regions of the proviral long terminal repeats. Cross-hatched box represents v-*myc*. Abbreviations: B, *Bam*HI; BgI, *BglI*; BgII, *BglII*; E, *Eco*RI; HII, *Hin*dII; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; SI, *Sac*I; SII, *Sac*II; Sal, *Sal*I; Sm, *Sma*I. Lower drawing, Structure of feline v-*myc* gene (boxed) and flanking FeLV regions (straight line) for which the DNA sequence is reported herein (1,744 bp, total). Boxed region includes all transduced cellular sequences. Initiation (ATG) and termination (TAA) codons delimit the segment homologous to the c-*myc* coding sequence (cross hatched).

lated regions reinforces the notion that these sequences may function in the regulation of c-*myc* expression (42).

FeLV sequences. The FeLV-related portions of the *myc*-FeLV sequence are very similar to previously reported FeLV *gag* and *env* gene sequences. The 109-bp *gag*-related segment is identical to a 109-bp sequence in the p30 region of the *gag* gene of Gardner-Arnstein feline sarcoma virus (GA-FeSV), and it has only three nucleotide substitutions

FIG. 3. Amino acid sequences of feline v-*myc* (FV), human c-*myc* (H), murine c-*myc* (M), chicken c-*myc* (C), and chicken v-*myc* (CV). Stars mark residues conserved between chicken and mouse. Residues are shown in the feline and human sequences only when they differ from mouse sequences; chicken v-*myc* residues are shown when they differ from chicken c-*myc*. Arrowheads mark potential phosphorylation sites which are lost in the v-*myc* sequences. Clusters of amino acid replacements among the mammalian genes are boxed. Dots mark amino acid replacements among mammalian sequences which result in charge changes. Dashes and parentheses represent deleted residues. Amino acid sequences shown are predicted from the nucleotide sequences in references 1, 4, 9, 42, 52, 53, and 54 and Fig. 1. The alignment of mammalian and chicken sequences follows Bernard et al. (4).

from the same region of the Snyder-Theilen feline leukemia virus (ST-FeLV), none of which result in amino acid replacement (Fig. 1). In the 190-bp *env*-related segment, there are three nucleotide substitutions and a 2-bp insertion with respect to GA-FeLV *env* and two nucleotide substitutions and a 1-bp insertion with respect to ST-FeLV *env*. Sequence identity between *myc*-FeLV and GA-FeLV is therefore somewhat greater than that between *myc*-FeLV and ST-FeLV in the 300 bp where they can be compared. Taken together with sequence data from the long terminal repeat (Braun and Casey, unpublished data), where *myc*-FeLV is once again more similar to GA-FeLV than ST-FeLV, these data confirm the earlier conclusion based on restriction enzyme site mapping that this *myc*-FeLV isolate was derived from a GA-FeLV-like ancestor (31).

The fact that the two small insertions in the *myc*-FeLV and ST-FeLV *env* genes both occur at the same site is surprising and suggests that this site may be prone to insertion-deletion events. Both insertions involve the addition of adenosine residues to a run of adenosines already seven residues long (Fig. 1). Runs of adenosines have previously been identified as mutational hot spots in other systems, such as the lysozyme gene of bacteriophage T4 (37) and the mitochondrial DNA of *Rattus norvegicus* (G. G. Brown, R. Prussick, and R. J. Desrosiers, Genetics **104**:9–10, 1983). Such runs of a single base are thought to be prone to small insertion-deletion mutations because transient misalignments of the complementary strands can occur during DNA replication (15).

In the present case, it is likely that neither of the two insertions was opposed by natural selection (even though they would cause frameshift and premature termination) because neither the *myc*-FeLV nor the ST-FeLV *env* genes are functional. The ST-FeLV clone from which the *env* sequence was derived is known to be noninfectious (36), and *myc*-FeLV could only replicate with the aid of helper virus. The possibility exists that these insertions could have occurred during cloning. This example suggests that caution should be exercised in interpreting DNA sequence data from molecular clones whose biological function has not been or cannot be tested.

FeLV-oncogene junctions. The 3′ fusion points between FeLV sequences and oncogenes are remarkably similar in several defective FeLV proviruses (13). The restriction maps of GA-FeSV, ST-FeSV, and McDonough FeSV all have *Pst*I and *Xho*I sites in FeLV sequences immediately to the right of the 3′ *onc*-FeLV junction, indicating that the same FeLV sequences may have been involved in all three recombinational events. The possibility that some sequence specificity exists in recombination between FeLV and oncogenes was brought sharply into focus when DNA sequencing revealed that the junctions between the 3′ end of v-*fes* and FeLV are identical in ST-FeSV and GA-FeSV (22). The identity of the junction in these two *fes*-containing isolates could also be explained if they originated from a single recombinational event and subsequently diverged, but two lines of evidence argue against this possibility. First, there are substantial differences in the organization of their *gag-fes* fusion regions (22). Second, the degree of sequence divergence between the *gag* genes of GA-FeSV and ST-FeSV is much greater than that between the *gag* genes of ST-FeSV and its natural helper virus (30), a result that would be unexpected if both defective isolates originated from one transducing event.

In the present case, FeLV sequences immediately 3′ to the *myc*-FeLV junction begin with the same short direct repeat (CTCCTC) that is found 3′ to the *fes*-FeLV junction in both ST-FeSV and GA-FeSV (22). Given the similarity of FeLV sequences at the 3′ *onc*-FeLV junctions discussed above, it is tempting to speculate that this viral hexanucleotide represents a preferred sequence which is in some way involved in the transduction of host sequences by FeLV. Sequence preference might occur if transduction arises as a side product of some normal mechanism such as viral replication or RNA processing. If such a sequence preference exists, however, it is not restricted to a unique viral location, since the 3′ *onc*-FeLV junction in *myc*-FeLV occurs 443 nucleotides within the *env* gene, whereas those of ST-FeSV and GA-FeSV are apparently just 5′ to the *env* gene (13). Furthermore, the CTCCTC hexanucleotide is not an absolute requirement for transduction, since it is not present at the 3′ *fgr*-FeLV junction in the Gardner-Rasheed feline sarcoma virus (34). Nucleotide sequence analysis of other *onc*-FeLV junctions will be required to determine the actual frequency of the CTCCTC sequence at such junctions and its importance in the transduction of host sequences.

With regard to 5′ fusion points between FeLV and oncogenes, little similarity has been evident in published restriction maps (13, 18, 31, 34, 35, 36). However, the nucleotide sequence shows that the 5′ fusion of *myc*-FeLV occurred 17 bp upstream of the 5′ fusion in GA-FeSV (22). Again, full evaluation of this observation awaits comparative sequence data from other *onc*-FeLV junctions.

Mechanisms of *myc* activation. There are a number of viable models for mechanisms by which proto-oncogenes may be converted to oncogenes (8). The most popular models are either quantitative (overexpression of an otherwise normal gene) or qualitative (mutations which alter the function of the gene). The most convincing evidence so far generated is for qualitative activation in the case of *ras*, where each of the known *ras* proto-oncogenes appears to be susceptible to activation by point mutations in codon 12 or 61 (46). In the case of *myc*, quantitative models of activation have received more attention. Cellular *myc* genes come under the control of powerful promoters or enhancers in several types of tumors, and elevated levels of *myc* RNA have been found in some of these (see, for example, references 24 and 25). Transgenic mice that carry a normal *myc* gene directed by control sequences of mouse mammary tumor virus show both elevated levels of *myc* expression in lactating breast tissue and a high propensity to develop breast cancer (50). Since *myc* appears to be an intracellular mediator of cell proliferation (3), a link between *myc* overexpression and oncogenesis seems reasonable. However, other studies have suggested that the elevation of *myc* expression in several tumor cell lines is no greater than that seen in rapidly proliferating normal cells (21, 26). In these cases point mutations, such as those observed in several v-*myc* and tumor-derived c-*myc* genes (39, 53, 55), may contribute to oncogenesis.

We can make a preliminary assessment of the importance of point mutations for *myc* activation by looking for amino acid replacements which are common to several activated *myc* genes. Published sequences of v-*myc* from MC29 and chicken c-*myc* differ at five to seven predicted amino acids (Fig. 3). If point mutations are solely responsible for *myc* activation, one or more of these residues must be involved. Although the feline c-*myc* sequence is not available, a comparison can be made between feline v-*myc* and those residues which are conserved in mouse and human c-*myc*. This comparison assumes that residues critical to normal *myc* function will be conserved in c-*myc* sequences. There

are 16 positions at which feline v-*myc* differs from amino acid residues conserved in mouse and human c-*myc* (Fig. 3). In no case do both feline and avian v-*myc* genes differ from the corresponding c-*myc* genes at homologous amino acid positions. Similar comparisons of feline v-*myc* with altered c-*myc* alleles from Raji cells (39) and bursal lymphoma LL3 (55) also reveal no replacements at homologous positions. We therefore conclude that *myc* activation does not depend upon point mutations at a unique site.

However, the possibility exists that there are multiple sites or regions of the molecule in which point mutations can cause *myc* activation. Especially intriguing is the observation that both feline and avian v-*myc* have lost potential phosphorylation sites of the type Lys/Arg-X-Ser/Thr (56). Two of these sites are within five residues of each other (amino acid 343 of feline v-*myc* in Fig. 3). These potential phosphorylation sites occur within a region that is well conserved between avian and mammalian *myc* genes and shows homology to the E1A protein of adenovirus (40), a protein which is thought to have a function similar to *myc* (29, 44). This region is known to be multiply phosphorylated in $p110^{gag\text{-}myc}$, the transforming protein of MC29 (17, 41). This same region is rich in basic amino acids and is thought to be involved in DNA binding by $p110^{gag\text{-}myc}$ (1). It can be envisioned that phosphorylation might regulate the DNA-binding activity of normal *myc* proteins by neutralizing a positively charged region of the protein. Loss of a potential phosphorylation site might result in a *myc* protein with a constitutively high affinity for DNA, which could alter its normal physiological function.

A second region where point mutations may be involved in *myc* activation is near position 58 of the mammalian amino acid sequence (Fig. 3). Threonine is replaced by asparagine at this position in the altered allele of Raji cells and by methionine in chicken v-*myc* (39). In the avian bursal lymphoma LL3, there is a proline-to-threonine transition two amino acids away (55). Although these replacements could in theory represent normal polymorphisms, the fact that they occur in a region which is perfectly conserved in chicken, mouse, and human c-*myc* and feline v-*myc* makes this possibility unlikely.

The hypothesis that point mutations could cause *myc* activation does not conflict with the overexpression model. It is conceivable that the crucial change during *myc* activation is an overall increase in DNA binding by *myc*. Total DNA-binding activity would increase if either the number of *myc* molecules per cell increased (through overexpression) or the DNA affinity of each *myc* molecule increased (through point mutation). Thus, several distinct molecular mechanisms could cause the activation of the same oncogene.

Although the exact role of *myc* in the transformation process remains unknown, knowledge of the feline v-*myc* sequence will facilitate direct tests of the importance of point mutations, phosphorylation, and overexpression to *myc* activation in the feline system.

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