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Structure, Origin, and Transforming Activity of Feline Leukemia Virus-*myc* Recombinant Provirus FTT

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A *myc*-containing recombinant feline leukemia provirus, designated FTT, was molecularly cloned from the cat T-cell lymphoma line F422. Its transforming activity, as well as the nucleotide sequence of the 3′ 2.7 kilobases of FTT, including v-*myc*, was determined. The predicted v-*myc* protein differs from feline c-*myc* by three amino acid changes and is truncated by two amino acids at the carboxyl terminus. Comparison with feline leukemia virus (FeLV), feline c-*myc*, and other FeLV proviruses indicates that recombination junctions involved in the generation of FeLV-*onc* viruses occur at preferred locations within the virus. They usually follow or occur within the sequence ACCCC at 5′ junctions and may result from homologous recombination between sequences of marked purine-pyrimidine strand bias, especially at 3′ junctions. Some recombination sites also resemble recombinase recognition sequences utilized in immunoglobulin and T-cell receptor variable-region joining. Transfection of primary rat embryo fibroblasts and subsequent in vivo analysis revealed that morphologic and tumorigenic transformation require cotransfection of FTT with human EJ-*ras* DNA; neither gene alone is sufficient. FTT v-*myc* is expressed in these transformed rat cells as a 3.0-kilobase subgenomic RNA; however, in contrast to the depressed level of c-*myc* expression in v-*myc*-involved feline tumors, steady-state levels of rat c-*myc* RNA and protein are apparently unaltered.

Experimental induction of lymphoid tumors by non-oncogene-bearing feline and avian retroviruses involves provirus insertion near the *myc* proto-oncogene in about 80 and 90% of the cases examined, respectively (44, 64). In contrast, about 20% of naturally occurring feline leukemia virus (FeLV)-positive cat T-cell tumors have been shown to involve rearrangements of the *myc* gene, and about half of these possess FeLV-*myc* recombinant proviruses (34, 39, 43). In addition, experimental induction of erythroblastosis by avian leukosis virus in 151 chickens has been shown to involve transduction of the c-*erbB* proto-oncogene (36). These latter findings challenge the prevailing view that oncogene-bearing viruses arise only very rarely in retrovirus infections (6, 64) and allow analysis of multiple instances of transduction of the same proto-oncogene and the viral and target cellular sequences involved, as well as their resultant transforming capability. Such analyses may be expected to yield new insight into the molecular mechanisms of transduction and the requirements for proto-oncogene activation.

F422 is a T-cell line established from a cat thymic lymphosarcoma (52). An FeLV-*myc* recombinant virus is shed from these cells and can be transmitted along with its helper virus, FeLV-Rickard, to mink lung cells without causing obvious morphological alteration (39). Neither the F422-derived virus, which we will refer to as FTT, nor four other FeLV-*myc* viruses morphologically transform feline embryo cells (43) or NIH 3T3 cells (34, 43). In this report, we present evidence which indicates that v-*myc* may transform cells through synergistic actions with other gene products, rather than as the sole causative agent. In addition, structural differences between v-*myc* and c-*myc* are assessed, and data are presented which suggest that some FeLV-*myc* junctions arise via mechanisms similar to homologous recombination and that some junctions may involve recognition sequences similar to those involved in immunoglobulin and T-cell receptor variable-region joining.

MATERIALS AND METHODS

Molecular cloning. A partial *Mbo*I digest of F422 DNA was cloned into λJ1 (39) by using methods described previously (39, 40). Clones were selected which hybridized both to probe exU3, derived from the FeLV U3 region and specific for exogenous, horizontally transmitted FeLV (39), and to probes BBC and HBE, derived from the feline c-*myc* exons 2 and 3, respectively (39). One clone, λFTT, contained two long terminal repeats (LTRs) and was mapped with restriction enzymes by using methods described previously (41).

DNA sequencing. A restriction fragment of the λFTT clone extending from the *Bam*HI site near the 3′ end of the *pol* open reading frame to a *Bgl*II site in cellular DNA downstream from the 3′ LTR was partially digested with BAL 31 double-stranded exonuclease. The resulting overlapping fragments were cloned into M13 and sequenced by using the dideoxynucleotide-chain termination method (47, 57).

Transfection of RECs. We used methods similar to the rat embryo cell (REC) transformation assay of Land, Parada, and Weinberg (31). Fisher rat embryo (14 day) torsos were minced, trypsinized, pooled, and plated at 2.5×10^7 viable cells per 150-cm^2 dish in Dulbecco minimal essential medium, supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 100 μg of gentamycin-sulfate per ml. Cells were grown to confluency (3 to 4 days) and replated at 1.2 ×

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10^6 viable cells per 10-cm dish, and transfections were performed about 20 h later. The transfection mixture consisted of 0.4 mM Na_2HPO_4, 150 mM $CaCl_2$, 140 mM NaCl, 21 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 6.95), 37.5 μg of carrier DNA per dish, 1 μg of pxfMoA*neo* DNA (20) per dish, and 5 μg of each experimental DNA per dish. After $CaCl_2$ was added to this mixture, a precipitate was allowed to form for 15 to 30 min, and then 1.25 ml of this was added dropwise to 5 ml of medium in each plate. After about 10 h, the cells were washed with phosphate-buffered saline (140 mM NaCl, 3.0 mM KCl, 8.0 mM Na_2HPO_4, 1.5 mM KH_2PO_4 [pH 7.2]) and covered with 10 ml of fresh medium. After another 2 h, duplicate plates were trypsinized, pooled, split 1:3, and replated in six 10-cm plates. One day later G-418 (geneticin, GIBCO), a neomycin analog, was added to a concentration of 250 μg/ml to half of the plates. Cells were fed every 3 to 4 days with normal medium or G-418 medium. Transformed foci or G-418-resistant colonies were scored by phase-contrast microscopy 14 to 21 days following transfection.

DNA used in transfections. pFRB, a plasmid containing FRB (Mullins et al., manuscript in preparation), the replication-competent FeLV-Rickard subgroup B provirus, was derived from the same T-cell line, F422, from which the FTT provirus was derived, in plasmid pK125. pEJ6.6, a plasmid containing the tumorigenically active *ras* oncogene, was derived from the human EJ bladder carcinoma cell line (31). pSVc-*myc*-1 is a chimeric *myc* plasmid (31) combining the simian virus 40 early promoter with mouse c-*myc* exons 2 and 3, including the intervening intron. pxfMoA*neo* is a eucaryotic expression plasmid (20) combining the Mo-MuLV promoter with the neomycin resistance gene. Carrier DNA was rat spleen DNA sheared by passage five times through a 21-gauge needle.

In vivo tumorigenicity assay. Transfected or control untransfected RECs were injected subcutaneously into 12-day-old syngeneic rat pups. Injected cells were taken either from eighth-passage subcultures of transformed foci or directly from transformed foci 17 days following transfection. In the former case, 8×10^5 transformed cells were injected per rat, and in the latter case, all (approximately 2×10^6) cells in a 10-cm dish were injected, including a small but variable and unknown number of transformed cells (31). Transformed foci from plates treated with G-418 had few cells and were therefore combined with 2×10^6 control RECs prior to injection.

Soft-agar assay. Cells (1×10^5 per ml) were plated in 0.3% agarose over a base of 0.5% agarose in 6-cm dishes. Colonies were scored by phase-contrast microscopy at 7 and 14 days.

Nucleic acid isolation and analysis. Cells were washed once with phosphate-buffered saline and lysed with a guanidinium isothiocyanate solution (11). RNA was separated from DNA by pelleting through 5.7 M CsCl. The RNA pellet was redissolved and further purified by two ethanol precipitations. The DNA band was further purified by phenol-chloroform extraction and ethanol precipitation. Restriction enzyme digestion, agarose gel electrophoresis, nitrocellulose transfer, hybridization with ^{32}P-labeled nick-translated probes, and autoradiography were as described previously (41) (hybridizations: 42°C in buffer containing 50% formamide–10% dextran sulfate; washes: 50°C in buffer containing 0.2× SSC [1× SSC is 0.15 M NaCl and 0.015 M sodium citrate]). To rehybridize filters, bound probe was first removed by incubation in two changes of 50% formamide–0.2× SSC wash mix at 70°C for 30 min each. RNA was analyzed by methyl mercury gel electrophoresis (3) and detected as described for DNA.

Immunoprecipitation and gel electrophoresis. Cells were incubated for 1 h with 0.5 mCi of L-[^{35}S]methionine per ml, washed with phosphate-buffered saline, trypsinized, and counted in a hemacytometer. Following a second wash, cells were lysed in RIPA buffer (0.05 M Tris hydrochloride [pH 7.2], 0.15 M NaCl, 0.1% sodium dodecyl sulfate, 1% Triton X-100) with 1% sodium deoxycholate and 1 mM phenylmethylsulfonyl fluoride. The lysate was cleared by centrifugation at 100,000 × *g* for 1 h, and the volume was adjusted to 10^6 lysed cells per ml of cleared lysate. Each milliliter of lysate was precleared for 4 h at 4°C with 10 μl of nonimmune rabbit serum preadsorbed to protein A-Sepharose CL-4B beads (Sigma). Anti-human c-*myc* rabbit serum (1 μl) or nonimmune rabbit serum (for controls) was preadsorbed to protein A beads and incubated with 1.0 ml of precleared lysate for 34 h at 4°C. The beads were washed five times with RIPA buffer and once with 50 mM Tris hydrochloride (pH 7.2) and stored overnight at −20°C. Immunoprecipitated protein was released from the beads by incubation in a boiling water bath for 2 to 3 min in loading buffer (0.08 M Tris hydrochloride [pH 6.8], 0.1 M dithiothreitol, 2% sodium dodecyl sulfate, 10% glycerol, 0.2% bromphenol blue) and electrophoresed on a 10% polyacrylamide resolving gel with a 3% stacking gel. The gel was fixed overnight (10% tricarboxylic acid, 10% glacial acetic acid, 30% methanol), incubated 1 h in En^{3}Hance (New England Nuclear), and exposed at −70°C to Kodak SB-5 film (Eastman Kodak).

Antisera. Rabbit antiserum against a synthetic peptide corresponding to the 12 carboxyl-terminal amino acids of human c-*myc* (48) was kindly provided by Gary Ramsay and J. M. Bishop. Rabbit antiserum against complete human c-*myc* protein (25) was kindly provided by R. N. Eisenman.

RESULTS

Structure of the FTT genome and coding potential of v-*myc*. The nucleotide sequence of the 3′ 2.7-kilobase (kb) fragment of FTT is presented in Fig. 1, and its relationship to the feline c-*myc* gene (34, 39, 43, 61) and to its putative FeLV parent, FRB, is schematically shown in Fig. 2. The sequence extends from a *Bam*HI site near the 3′ end of the polymerase open reading frame through v-*myc*, the 3′-most 351 nucleotides of the p15E gene, and the 3′ LTR. One hundred bases upstream of the 5′ v-*myc* junction is a splice acceptor consensus sequence (38) (Fig. 1). It has been found that splice acceptor sites are preceded by one or more 3′ splice signals with the consensus sequence CTGAC (29). The putative splice acceptor site of FTT is preceded by redundant 3′ splice signals, CTGAT and CTCAC, 32 and 47 bases upstream, respectively (Fig. 1). These sequences are conserved in FRB and are the only consensus splice acceptor and 3′ splice signals found in the 739 nucleotides of FRB preceding the start of the predicted gp70 leader sequence (data not shown). Furthermore, RNA blot hybridizations place the actual splice acceptor site within a 250-base-pair sequence including this region (Mullins, unpublished results). Thus, the splice sequences found in FTT upstream of v-*myc* likely correspond to the normal envelope splice acceptor found in the parent virus.

The 5′ FeLV-*myc* junction of FTT occurs 183 bases upstream of the predicted gp70 leader of FRB and 30 bases upstream of the 3′ end of c-*myc* exon 1 (61). The splice acceptor for c-*myc* exon 2 is within the sequence 5′-CCCCCTTCAGCAGGCG-3′ (61), which provides two alter-

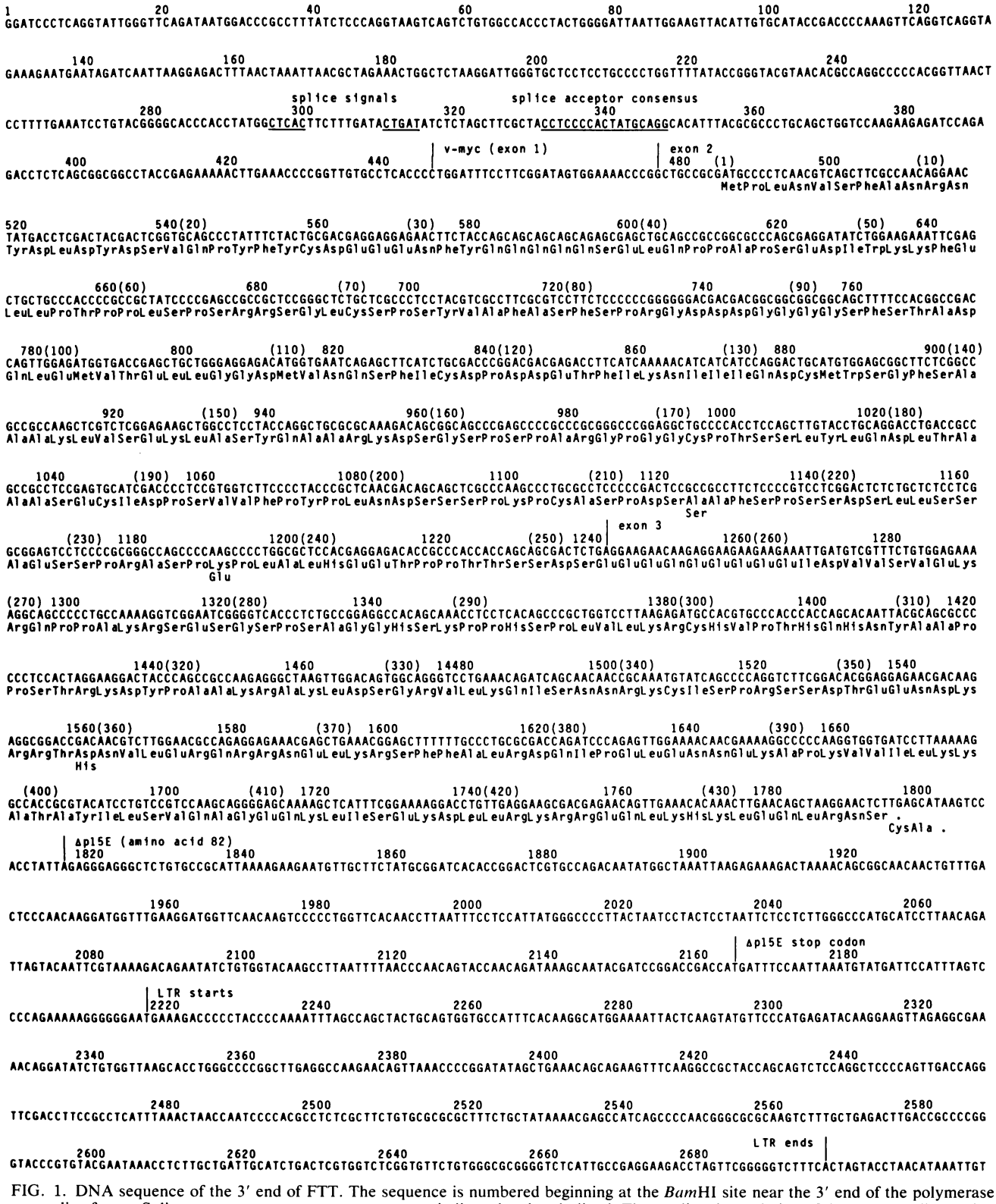

FIG. 1. DNA sequence of the 3′ end of FTT. The sequence is numbered beginning at the *Bam*HI site near the 3′ end of the polymerase open reading frame. Splice acceptor consensus sequences are indicated and underlined. The predicted translation of the v-*myc* coding region is given, and the amino acids are numbered in parentheses. The predicted amino acids in feline c-*myc* (61) which diverge from FTT v-*myc* are given below those of v-*myc*. The viral cellular recombination sites, *myc* exon boundaries, and the beginning and end of the 3′ LTR are indicated by vertical lines above the sequence.

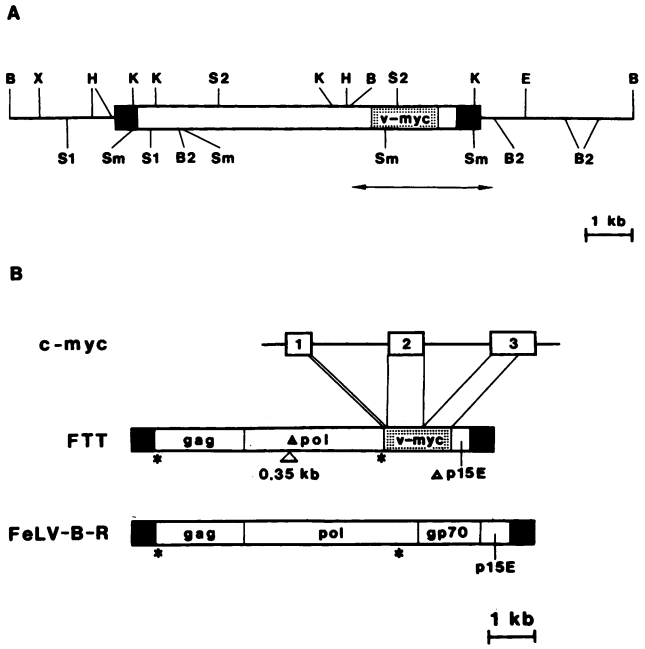

FIG. 2. (A) Restriction enzyme map of FeLV-*myc* recombinant provirus FTT and adjacent cellular DNA. The double-headed arrow under the map indicates the region subcloned for DNA sequencing (Fig. 1). The stippled area in FTT indicates the transduced *myc* sequence. Abbreviations: B, *Bam*HI; B2, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; S1, *Sac*I; S2, *Sac*II; Sm, *Sma*I; X, *Xba*I. (B) Comparison of FTT with the feline c-*myc* region and FeLV-B-R genome. The triangle below FTT *pol* indicates an approximately 0.35-kb deletion in the polymerase open reading frame. The asterisks indicate a splice donor consensus sequence in the leader sequence of the core protein genes (*gag*) of FeLV-61E and FeLV-B-ST (16, 32) and a splice acceptor consensus sequence between the *pol* and *env* genes of FeLV-61E and FeLV-B-R (16; J. Elder, unpublished results). Processing of the FTT genomic RNA at these splice sites would result in a subgenomic transcript, which after polyadenylation would be similar in size to the smaller transcript detected in the Northern (RNA) blots shown in Fig. 4.

native splice sites. Nucleotide sequence comparisons indicate that the FTT splice occurs after the second AG, while in two other FeLV-*myc* viruses the splice occurs after the first AG (10, 61). These alternative splice sites occur in a region believed to be noncoding and so have no effect on the predicted amino acid sequence of v-*myc*. Otherwise, no predicted c-*myc* intron sequences are present in FTT v-*myc*.

The first methionine codon in v-*myc* is found 10 bases within exon 2, beginning an open reading frame of 1,311 bases (437 codons) extending through exons 2 and 3, which are joined in frame at the expected splice junction of c-*myc*. No uninterrupted coding sequences longer than 30 codons are found in any other reading frame. Four codon changes are found in FTT v-*myc* relative to feline c-*myc*, and in three of these cases (positions 214, 236, and 359), FTT v-*myc* is identical to human c-*myc*. The fourth change is a stop codon terminating the FTT v-*myc* open reading frame two amino acids upstream of the c-*myc* termination codon, thus truncating the predicted protein by two amino acids (cysteine and alanine). Sequences identical to c-*myc* extend 18 bases following the v-*myc* stop codon, whereupon FeLV sequences begin in frame at what would be codon 82 of the FRB p15E gene. Over the remaining 882 bases of p15E and the 3′ LTR, there are 13 nucleotide changes between FTT and FRB (98.5% homology).

TABLE 1. Results of transfection of RECs and injection of transfected cells into syngeneic young rats

Transfected DNA	G-418	Transformed foci per 10^6 transfected cells[a]	No. of tumor-bearing rats/ rats injected
FTT + EJ-*ras* + *neo*	−	22	1/3
	+	41	8/9
pSVc-*myc*-1 + EJ-*ras*	−	41	3/3
+ *neo*	+	3	
FeLV-B-R + EJ-*ras*	−	0	
+ *neo*	+	4[b]	
FTT + *neo*	−	0	
	+	0	
pSVc-*myc*-1 + *neo*	−	0	
	+	0	
FeLV-B-R + *neo*	−	0	
	+	0	
EJ-*ras* + *neo*	−	4[b]	0/1
	+	3[b]	0/1
neo	−	0	0/3
	+	0	0/3
Carrier alone	−	0	0/3
	+	0	

[a] Average of three transfected plates, 10^6 cells each.
[b] Could not be maintained in culture.

Transforming activity of FTT. We next investigated the biological function of FTT in transfection experiments. Avian MC29 v-*myc* and simian virus 40 promoter-activated mouse c-*myc* (pSVc-*myc*-1) sequences have been shown to transform primary RECs when cotransfected with human EJ-*ras* DNA (pEJ6.6) (31). Cells which take up and express both *myc* and *ras* sequences become morphologically transformed, grow in soft agar, and cause fatal tumors when injected into irradiated nude mice. To evaluate the potential oncogenic activity of FTT, we carried out similar REC assays using λFTT and pEJ6.6. As a positive control, we cotransfected pSVc-*myc*-1 and pEJ6.6. These results are shown in Table 1.

By 14 days following transfection, dishes of cells transfected with carrier DNA only and grown in medium containing the neomycin analog G-418 were depleted of all cells; however, dishes transfected with a plasmid containing the neomycin resistance gene (pxfMoA*neo*) contained isolated colonies, indicating successful transfection and expression of the *neo* marker. These resistant colonies were not morphologically transformed or immortalized. Similarly, no morphologically transformed or immortalized colonies were observed in dishes individually transfected with pSVc-*myc*-1, λFTT, or pFRB. There were occasional morphologically transformed foci in dishes transfected with *ras* alone or with *ras* in conjunction with FRB; however, these grew slowly and could not be maintained. In contrast, prominent foci of morphologically transformed cells were observed in dishes cotransfected with *ras* and either pSVc-*myc*-1 or λFTT. Subcultures of foci transformed with λFTT plus *ras* grew more rapidly than untransformed RECs and appeared to be immortal (they have subsequently undergone over 200 doublings).

Cells from subcultures of FTT-*ras*-transformed foci also grew in soft agar (data not shown). Growth in soft agar was originally introduced as an in vitro test for malignancy (35) and correlates with tumor formation in nude mice (37). To test directly the in vivo tumorigenicity of FTT-*ras*-transformed cells, they were injected subcutaneously into 12-day-old syngeneic rat pups. Tumors were evident in most

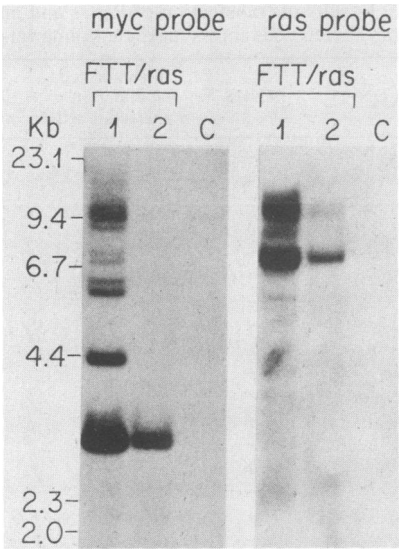

FIG. 3. Detection of transfected DNA in transformed REC DNA. DNA from cells subcultured from two different foci, numbers 1 and 2 (lanes 1 and 2), transformed by cotransfection with λFTT and the *ras* sequence pEJ6.6, was digested with *Kpn*I, and a Southern blot was hybridized with the feline c-*myc* exon 3 probe, HBE (left panel). Lane C was loaded with normal rat spleen control DNA. Lanes 1 and C were loaded with equivalent amounts of DNA (20 μg); lane 2 was loaded with half this amount (10 μg). In the right panel, the same filter as on the left was washed and rehybridized with a probe made from the same *ras* sequence as that used in the transfections.

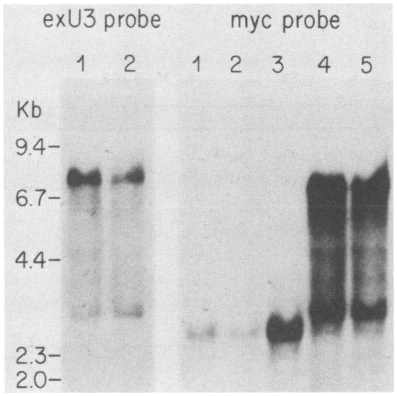

FIG. 4. Detection of RNA transcripts of transfected sequences in transformed-cell RNAs. In the left panel, a Northern blot of RNA from two transformed-cell lines (same as in legend to Fig. 3) was hybridized with exU3, a probe specific for exogenous FeLV (39; Mullins, unpublished results). Additional lanes loaded with normal REC RNA failed to hybridize with this probe and are not shown. In the right panel, RNAs were hybridized with HBE, a probe derived from feline c-*myc* exon 3. Lane 1 was loaded with RNA from normal REC quiescent cells. Lane 2 was loaded with RNA from normal REC asynchronously proliferating cells. Lane 3 was loaded with RNA from normal RECs synchronously stimulated into G1 of the cell cycle. The FTT-*ras*-transformed RECs in lanes 4 and 5 (from the same cells as in lanes 1 and 2 in the left panel) are analogous in growth state to the asynchronously proliferating cells in lane 2. Equivalent amounts of whole-cell RNA were loaded in all lanes (25 μg); however, lanes 1 and 3 (right panel) represent about twice as many cells as all the other lanes.

cases, but their persistence and lethality depended on both the number of cells injected and the particular transformed focus chosen for injection. For example, rats injected with 8×10^5 transformed cells which had been subcultured from a large, fast-growing focus (focus number 1) died within 18 days, while 8×10^5 cells subcultured from a small, slowly growing focus (focus number 2) produced tumors which regressed within 14 days. Rats injected with the entire contents of a plate containing several small, slowly growing foci (approximately 2×10^5 transformed cells) developed large tumors, but some were still alive after 45 days, even though they carried tumors as large as 4 cm in diameter. This type of variability was observed whether the *myc* sequence used in the original transfection was λFTT or pSVc-*myc*-1.

Expression of the FTT provirus in transformed cells. DNA and RNA derived from subcultures of transformed foci were analyzed for evidence of integration and transcription of transfected sequences. In the left panel of Fig. 3, DNA from FTT-*ras*-transformed cells subcultured (six additional passages) from two different foci, numbers 1 and 2 described above, was digested with the restriction endonuclease *Kpn*I, which cleaves within the FTT provirus LTRs, and a resulting nitrocellulose blot was hybridized with the feline c-*myc* exon 3 probe, HBE (39). In lanes loaded with FTT-*ras*-transformed-cell DNA (lanes 1 and 2), the prominent 3.25-kb band corresponds to multiple copies of the internal *Kpn*I fragment found at the 3′ end of the FTT provirus. The additional *myc*-hybridizing bands in focus number 1 (lane 1) apparently represent transfected sequences which had undergone rearrangement. The normal rat c-*myc* *Kpn*I fragment of 9.5 kb is faintly visible in control DNA (lane C).

The right panel of Fig. 3 shows the same filter as above with the previously bound probe removed and rehybridized with a pEJ6.6 *ras* probe. The human *ras* probe does not hybridize to normal rat DNA (lane C) under these conditions. However, both transformed-cell DNAs contain at least one *ras*-hybridizing fragment, indicating the presence of transfected human *ras* DNA.

Expression of FeLV and *myc* RNA in transformed cells was also examined by RNA blot analysis. In the left panel of Fig. 4, RNA from the two transformed-cell lines described in Fig. 3 was hybridized with exU3, a probe specific for exogenous, horizontally transmitted FeLV. The intense 7.2-kb bands correspond to the entire FTT genomic transcript. The faint 3.0-kb bands correspond to a subgenomic RNA, which is expected if processing occurs utilizing the predicted FTT-*env* splice acceptor consensus sequence. In the right panel, the same RNA samples as above were hybridized with the feline c-*myc* exon 3 probe. Lane 1 contains RNA from confluent, normal RECs. Lane 2 contains RNA from continuously, asynchronously proliferating normal RECs. Lane 3 contains RNA from synchronously proliferating normal RECs 3 h after dispersing confluent cultures 1:2; that is, 3 h after stimulating cells from G0 into G1, when c-*myc* transcription is expected to be at a peak level (30). FTT-*ras*-transformed RECs (lanes 4 and 5) are not contact inhibited and so proliferate continuously; thus, they may be considered somewhat analogous in growth state to the asynchronously proliferating normal RECs (lane 2). Equivalent amounts of whole-cell RNA were loaded in all lanes (25 μg); however, cell counts revealed that quiescent RECs (lane 1) and synchronized RECs just entering G1 (lane 3) have only about half as much RNA per cell as do asynchronously proliferating RECs (lane 2) and FTT-*ras*-transformed RECs (lanes 4 and 5) (data not shown). Thus, lanes 1 and 3 represent about twice as many cells as all of the other lanes.

The 2.5-kb rat c-*myc* transcript is detectable at low levels

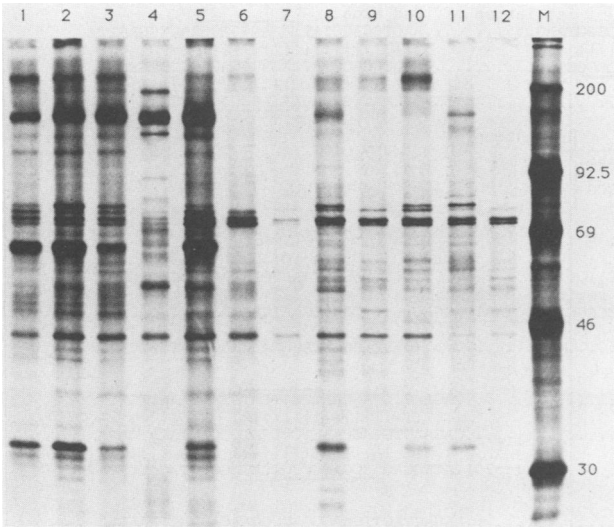

FIG. 5. Cross-reactivity of antiserum against the carboxyl terminus of human c-*myc* with c-*myc* and v-*myc* in RECs and RECs transformed with FTT-*ras*, FEF, and F422 cells. Cell extracts, radioimmunoprecipitations, and gel electrophoreses were prepared as described in Materials and Methods. Since c-*myc* expression is cell cycle dependent, REC and FEF cell extracts were prepared from cells labeled in G0, early G1, and asynchronously proliferating cultures. FTT-*ras*-transformed RECs and F422 were asynchronously proliferating cultures. Cell extracts from 5×10^5 cells were used for each lane except lane 7, in which 2×10^5 cells were used. Lane 1, Untransfected RECs confluent at least 3 days, i.e., in G0 of the cell cycle; lane 2, untransfected RECs subconfluent and asynchronously proliferating; lane 3, untransfected RECs confluent for at least 3 days, then split 1:2 2.5 h before labeling, i.e., synchronized in early G1; lane 4, as in lane 2, but immunoprecipitated with nonimmune rabbit serum; lane 5, FTT-*ras*-transformed RECs asynchronously proliferating; lane 6, as in lane 5, but immunoprecipitated with nonimmune rabbit serum; lane 7, confluent or G0 FEF; lane 8, asynchronously proliferating FEF; lane 9, as in lane 8, but immunoprecipitated with nonimmune rabbit serum; lane 10, FEF synchronized in early G1; lane 11, F422 asynchronously proliferating; lane 12, as in lane 11, but immunoprecipitated with nonimmune rabbit serum; lane 13, molecular weight markers.

in normal quiescent and asynchronously proliferating cell RNA (lanes 1 and 2) and in transformed-cell RNA (lanes 4 and 5) and at a much higher level in normal synchronously proliferating G1 cell RNA (lane 3). In FTT-*ras*-transformed cells (lanes 4 and 5), both feline v-*myc* RNAs (the 7.2-kb full-length transcript and the 3-kb spliced, subgenomic transcript) appear more abundant than the 2.5-kb rat c-*myc* RNA in the same cells and in normal resting or asynchronously proliferating cells. However, imperfect homology between rat c-*myc* and the feline c-*myc* probe results in a consistent underestimation of the relative abundance of rat c-*myc* transcripts.

In an attempt to determine the amount of *myc* protein in the cells under study, we initially carried out radioimmunoprecipitation of cell extracts using a rabbit antiserum against a synthetic peptide corresponding to the 12 carboxyl-terminal amino acids of human c-*myc* (48). Figure 5 shows that the major protein precipitated from REC extracts by this antiserum (lanes 1, 2, and 3) and not by nonimmune rabbit serum (lane 4) has a relative molecular weight (M_r) of 64,000. This corresponds to c-*myc* proteins with M_rs of 62,000 to 67,000 reported in avian, murine, and human cells (25, 48). There is no detectable signal in this autoradiograph exposure or a longer exposure (data not shown) with an M_r in the above range in feline embryo fibroblasts (lanes 7 through 10) or in F422 (lanes 11 and 12), the cat lymphoma T-cell line from which FTT was cloned; thus it appears that this antiserum does not cross-react with cat c-*myc* or FTT v-*myc*. However, a prominent band with an M_r of 64,000 is present in REC cells both untransformed and transformed by FTT-*ras* transfection (lane 5).

In an additional attempt to detect feline *myc* proteins, we carried out analogous immunoprecipitations using a rabbit serum against complete human c-*myc* protein produced in a bacterial expression vector (25). This antiserum failed to precipitate specifically a prominent c-*myc* protein in any of the cells investigated (data not shown).

DISCUSSION

DNA sequence analysis predicts that FTT encodes a v-*myc* protein in place of *env* but utilizing the *env* splice acceptor. In contrast, L115 (10) and the avian tumor viruses MC29, OK10, and CMII (7) encode v-*myc* fusion proteins linked to viral *gag* or *gag-pol* genes. The FTT v-*myc* protein is predicted to be two amino acids shorter at the carboxyl terminus than feline c-*myc*. There are three other coding variations between FTT v-*myc* and feline c-*myc*; however, in each case the FTT amino acid sequence agrees with that of human c-*myc*. Papas and Lautenberger (46) observed that a threonine codon corresponding to position 61 of chicken c-*myc* has been altered in each of three avian v-*myc* sequences, while it is conserved in chicken, human, mouse, and fish c-*myc*. They suggested that this substitution may increase the oncogenic potential of *myc* transducing viruses. Nevertheless, we find that the amino acid at the analogous site (codon 58 in Fig. 1) in both FTT and L115 is identical to feline c-*myc* and to the other known c-*myc* sequences. Our findings are consistent with the prevailing view (53) that altered regulation of *myc* expression can tumorigenically activate *myc* in the absence of protein-coding alterations. While it is not clear whether alterations in FTT v-*myc* enhance its observed tumorigenicity, it does appear that the truncation of the carboxyl terminus does not inactivate the product. This may place constraints on the proposal that the carboxyl-terminal portion of the *myc* product is involved in a DNA-binding function (2).

The level of v-*myc* RNA expression in FTT-*ras*-transformed RECs appears to be much higher than that of c-*myc* in normal quiescent or proliferating RECs; however, it is similar to a peak level of c-*myc* expression found in normal RECs a few hours after quiescent cells have been stimulated to proliferate. Others have observed a temporary peak of c-*myc* expression following the removal of chicken embryo fibroblasts from serum deprivation (13, 15). We found that a similar temporary peak of c-*myc* expression can be observed without serum deprivation, by simply replating cells which have remained confluent for two or more days. However, we do not know whether the peak we observed is of the same magnitude as that observed after serum deprivation.

c-*myc* RNA expression was about the same in normal asynchronously proliferating RECs as in FTT-*ras*-transformed RECs. Since we presume these transformed cells to be somewhat analogous in growth state to the normal asynchronously proliferating cells, this indicates that c-*myc* expression in the transformed cells is not significantly down regulated. This is in contrast to reports that c-*myc* RNA expression is depressed by the presence of extra copies of c-*myc* coupled to the immunoglobulin μ or κ enhancer in cell

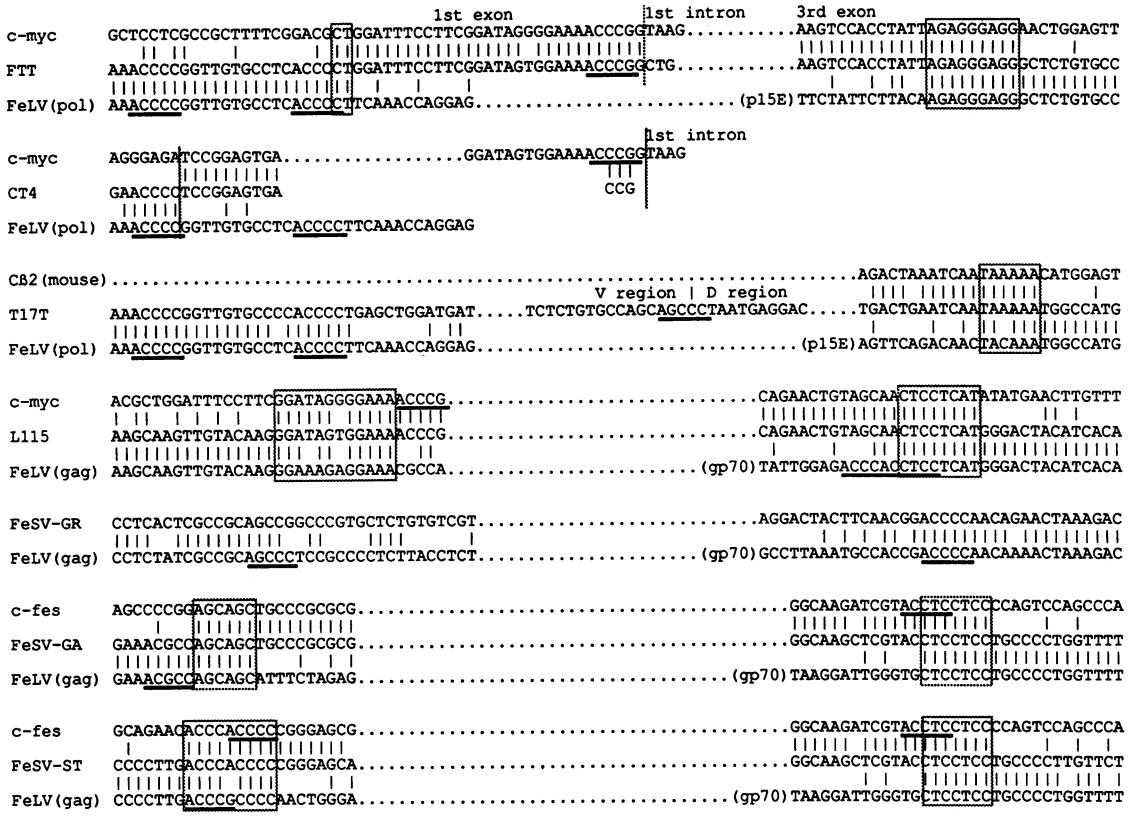

FIG. 6. FeLV-*onc* recombination sites. DNA sequences are given across recombination sites for FTT and FeLV-*myc* isolates L115 (10) and CT4 (61) and FeLV-*tcr* recombinant T17T (22). Also given where appropriate are the corresponding sequences of feline c-*myc* (61), FeLV (16, 18, 19, 32), FeSV-GA, FeSV-ST, and FeSV-GR (42). Sequences resembling the consensus ACCCC are underlined. Regions of homology between viral and cellular sequences at points of recombination are boxed.

lines derived from B-cell tumors of transgenic mice (1) and that feline c-*myc* RNA expression is depressed in feline T-cell tumors containing an FeLV-*myc* virus (21). Our results suggest that recognition sequences involved in down regulation are not present in FTT (perhaps they are located in exon 1) or they may be species specific. Antiserum to the human c-*myc* carboxyl terminus appears to cross-react with rat c-*myc* protein but not with feline c-*myc* or FTT v-*myc*. In agreement with the RNA data, immunoprecipitation with this antiserum indicates that rat c-*myc* protein levels are equivalent in untransfected RECs and FTT-*ras*-transformed RECs; i.e., there appears to be no suppression of rat c-*myc* in the transformed cells. However, these antisera did not permit analysis of feline c-*myc* or FTT v-*myc* protein levels.

The availability of feline c-*myc*, FeLV, and FRB sequence data makes possible a comparison (Fig. 6) of cellular and parental viral sequences at v-*myc* junctions of FTT and two other FeLV-*myc* recombinant viruses, L115 and CT4. We also compared the virus junctions and V-D joining region of the FeLV-*tcr* recombinant virus T17T, which transduced the feline T-cell receptor (*tcr*) β-chain gene and which, like the FeLV-*myc* viruses, was derived from a naturally occurring feline lymphoma (22). Ten bases of a twelve-nucleotide stretch of homology between feline c-*myc* and the parental virus are found at the 5′ recombination point of L115 (boxed in Fig. 6). At 3′ junctions, short regions of homology are found between cellular and parental virus sequences in FTT, T17T, and L115, (9 of 9, 5 of 6, and 8 of 8 bases of homology, respectively). While fortuitous microhomology may facilitate recombination between retroviruses and cellular sequences, it is obviously not required in all cases, since there is no stretch of homology longer than 2 base pairs at the 5′ recombination point of FTT or the CT4 virus. Furthermore, no homology has been found at many other retrovirus-oncogene junctions (data not shown).

When the v-*fes*-containing feline sarcoma viruses FeSV-ST and FeSV-GA were examined, the 3′ recombination site was found to be identical in both the parental viruses and cellular sequences (24, 54), yet the two transducing viruses seem to have arisen separately (10). FeSV-GA (24), FeSV-HZ2 and -HZ4 (containing v-*abl* and v-*kit*, respectively) (4, 5), and L115 have 5′ recombination sites within a 24-base-pair region of *gag* p30. Some cellular recombination sites also occur within close proximity (Fig. 6). In exon 1 of feline c-*myc*, the 5′ L115 junction is approximately 15 bases downstream from that of FTT. In exon 3, the 3′ L115 recombination site is 87 bases downstream from that of FTT. It is possible that the major reasons for the proximity of these recombination sites include (i) positional constraints imposed by the architecture of the viral genome, for example, the location of splice donor and acceptor sites; (ii) protein coding requirements of the oncogene product; or (iii) size constraints imposed on the transduced cellular sequence by the viral packaging process. Nevertheless, certain sequences in these preferred regions may facilitate recombination. The sequence CTCCTC has been noted (10) at the 3′ recombination sites of L115, FeSV-ST, and FeSV-GA, and GAGG or its complement CCTC has been noted (5) near the 5′ recombination site of a number of oncogene-bearing retroviruses. A sequence similar to the consensus ACCCC is

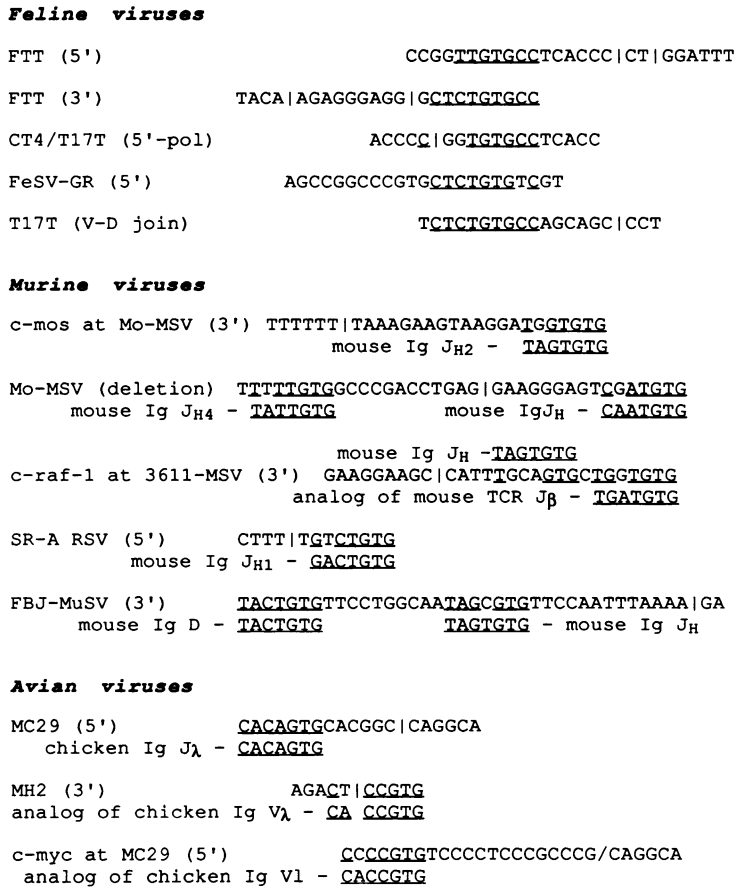

FIG. 7. Potential recombinase recognition sequences found at or near retrovirus-oncogene recombination sites. Junction sites are marked by a vertical bar or by two vertical bars indicating the area over which recombination likely occurred (Fig. 6). The nonamer related to the murine consensus recognition heptamer found near the feline *tcr* V-D junction in T17T is underlined. Bases found in other feline viruses which match this sequence are also underlined. Junction regions of other *onc*-bearing retroviruses are shown along with related *Ig* and *tcr* recombinase recognition heptamer sequences. Sequences are from Mo-MSV (66), mouse immunoglobulin J_{H2} (56), Mo-MSV deletion site (50), mouse immunoglobulin J_{H4} (56), mouse immunoglobulin J_H (17, 56), 3611-MSV (9), TCR J_B (70), SR-A RSV (63), mouse immunoglobulin J_{H1} (56), MC29 (49), chicken immunoglobulin J1λ and V1λ (51), MH2 (62), FBJ-MuSV (67), and mouse immunoglobulin D (55).

found in the FeLV parent at or within a few bases of FeLV-*onc* junctions in 7 of 11 transducing feline retroviruses for which sequences are available and at the deduced V-D joining region found within the T-cell receptor-related sequences in T17T (Fig. 6). Also, the sequence ACCGC is found in both the parental virus and the cellular sequence at the 5′ recombination site of FBJ-MuSV and c-*fos* (67). At all of the virus cell recombination sites above and also in T17T and CT4, we notice regions of purine-pyrimidine strand bias. Similar strand bias can also be found at recombination sites in a number of other published retrovirus sequences (e.g., references 5, 9, 14, 23, 24, 27, 42, 49, 50, 62, 66, 68, 69), and others have noted that purine (or pyrimidine)-repeated sequences (26, 28, 33, 45, 59) and "simple sequence"-repeated sequences with no strand bias (12, 28, 60) occur at recombination hotspots. It has also recently been reported (58) that stretches of guanine-rich DNA can align in a parallel fashion in structures which might facilitate tetrad formation during meiosis. Perhaps the G-rich stretches we note above may be involved in aligning retrovirus and cellular DNAs in a parallel fashion, which facilitates recombination between regions of fortuitous microhomology.

Gene rearrangements within immunoglobulin (*Ig*) and T-cell receptor (*tcr*) loci are known to involve heptamer recombinase recognition sequences (reviewed in reference 65). Interestingly, the nonamer CTCTGTGCC, which includes a seven-base stretch that differs from the murine consensus (CACTGTG) by one base, precedes the V-D joining region in T17T (Fig. 7), and closely related sequences are found at most of the FeLV-*myc* recombination points examined (Fig. 7 [22]). A potential recognition heptamer, CTCTGTG, can also be seen just upstream of the feline *tcr* V-D joining region in the *tcr* sequence captured by T17T (Fig. 6). The identical heptamer is found adjacent to the FTT-v-*myc* 3′ junction and the FeSV-GR-v-*fgr* 5′ junction (Fig. 7). Similar sequences are also found near the FTT 5′ junction and, as earlier noted (61), near the 5′ junction of CT4 and T17T. Most of these heptamers are also followed by CC, suggesting a potential nonameric recognition sequence. Thus, a cryptic recombinase recognition heptamer or similar sequence is located adjacent to a viral oncogene recombination site in 5 of 11 transducing feline retroviruses for which sequences are available. We also notice (Fig. 7) possible recombinase recognition heptamers near recombination sites in 6 of 13 avian and murine transducing retroviruses or their corresponding c-oncogenes. Transcriptional activity of genes such as c-*myc*, immunoglobulin, and *tcr* in pre-B and pre-T cells is believed to enhance their involvement in

recombination (8). This potentially enhanced availability for recombination plus the presence in the same cells of both recombinases and retroviruses containing cryptic recombinase recognition heptamers may partially explain the high rate of oncogene transduction in FeLV-lymphomagenesis.

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