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Alternate structures and stabilities of c-*myc* RNA in a bursal lymphoma cell line

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ABSTRACT

A bursal lymphoma cell line, BK25, had been shown to be haploid at the c-myc locus and to have undergone an alteration of chromatin structure upstream from the c-myc coding region. In BK25 DNA at least the 3' half of an ALV provirus is integrated 160bp upstream from exon 2. As a result of this integration event, the first and second exons are separated by at least 17kb. Approximately 90% of c-myc transcription begins in the promoter of the ALV proviral long terminal repeat (LTR) and this mRNA has a half-life of ~25 minutes in actinomycin D chase experiments. Approximately 10% of c-myc transcription initiates at the normal promoter of c-myc. The latter message has an unusually long half-life of >100 minutes. By contrast, in MSB-1 cells, which lack any c-myc rearrangments, transcription begins at the normal promoter in exon 1 and c-myc RNA has a half-life of ~15 minutes. These results suggest that factors in addition to the structure of the 5' end of chicken c-myc RNA determine its stability in vivo.

INTRODUCTION

Local rearrangements of the c-myc proto-oncogene have been implicated as causative or potentiating events in the development of B cell neoplasms of chickens[1], rodents[2-8], and man[2,8-11]. In avian bursal lymphomas the c-myc gene is altered by the nearby integration of an avian leukosis virus (ALV) provirus. In most cases sequences in the ALV long terminal repeat (LTR) act as efficient promoters of transcription into c-myc[1] while in other cases sequences in the LTR act as transcriptional enhancers of c-myc expression[12,13].

We have studied the structure and expression of the c-myc region of several avian bursal lymphoma cell lines in order to understand the contributions of promoter insertion and transcriptional enhancement in determining constitutively high steady state levels of c-myc expression. One of these cell lines, BK25, had been established from an RAV-2 induced bursal lymphoma[14]. We had previously shown that BK25 is haploid at the

c-myc locus in that only the provirus-associated allele of c-myc is present, while the normal, unrearranged allele apparently has been spontaneously lost during propagation in culture. This finding facilitated an analysis of the chromatin structure of c-myc in this and several other bursal lymphoma cell lines in comparison with that found in normal uninfected bursa. This study showed that an alteration in the chromatin structure of c-myc accompanies proviral integration in ALV-induced bursal lymphoma cell lines[15]. This alteration results in the loss of a family of DNase I hypersensitive sites upstream from the c-myc coding exons that is present in uninfected bursa, and the appearance of a novel distribution of DNase I hypersensitive sites with new sites mapping within proviral sequences, both within and outside the LTR. This finding suggested that one possible mechanism of transcriptional enhancement by the ALV provirus could be the reorganization of chromatin by proviral integration.

In an effort to define the structural determinants of c-myc expression in this cell line we have studied its DNA sequence and the relative utilization of potential c-myc promoters in BK25 cells. We have compared these findings with a similar analysis in MSB-1 cells, a Merek's disease virus transformed T-cell line in which there is no c-myc rearrangement. The half life of c-myc RNA originating from unrearranged c-myc alleles has generally been found to be short[16]. The truncation of the c-myc gene that results in the loss of exon 1 from the predominant c-myc transcript in several tumor cell types results in increased mRNA stability[17-19], suggesting that alteration of the structure of the 5' end of c-myc RNA influences its stability in vivo. By contrast, we have found that an mRNA species initiated from the normal c-myc promoter of BK25 cells has a greatly prolonged half-life compared with that observed for an LTR-initiated c-myc RNA in the same cells. Our results suggest that factors other than the structure of the 5' end of chicken c-myc RNA influence its stability in vivo.

MATERIALS AND METHODS

Cells

BK25[14] and MSB-1 cells[20] were grown in RPMI 1640 supplemented with 10% fetal calf serum.

Molecular Cloning and DNA Sequence Analysis

A fragment encompassing LTR and c-myc sequence was cloned from high molecular weight BK25 DNA. DNA was prepared as previously described[15] and

10kb BamHI fragments were isolated on a neutral 10-40% sucrose gradients, ligated to alkaline phosphatase treated lambda phage L47.1 arms, packaged in vitro, and plaque lift filters were probed with a 3.2 kb SstI fragment of c-myc[15]. All cloning procedures were as described[21]. Fragments of this clone were subcloned into M13mp18 and M13mp19[22] and sequenced by the dideoxy chain termination method[23].

Quantitative S1 Analysis

Total cellular RNA was isolated by the guanidine thiocyanate method[24]. Uniformly labeled, single-stranded "prime cut" DNA probes were prepared in M13 phage as described[25]. Each probe (50,000 cpm) was incubated with 50 µg of total cellular RNA in a 15 µl reaction mixture containing 400 mM NaCl, 80% formamide, 40 mM PIPES [piperazine-N,N'-bis(2-ethanesulfonic acid)] (pH 6.4), 1 mM EDTA. These were hybridized at 58 degrees for 14 hours. Samples were diluted into a volume of 200 µl in 30 mM sodium acetate (pH 4.6), 280 mM NaCl, 4.5 mM $ZnSO_4$, and 300 units per ml S1 nuclease and incubated for 45 minutes at 37°. Samples were phenol extracted, chloroform extracted, ethanol precipitated, electrophoresed on 5% acrylamide-urea gels, dried, and exposed as autoradiographs.

RNase Protection Analysis

Approximately 100,000 dpm of ^{32}P-labeled RNA was mixed with either 1 µg of polyadenylated RNA or 50 µg of total cellular RNA in 80% formamide, 40 mM PIPES, (pH 6.8), 0.4 M NaCl, 1 mM EDTA in a total of 20 µl. Samples were heated at 80° for 5 minutes and hybridized overnight at 42°. Nuclease treatment with RNase A and T1, RNA extraction, and electrophoresis were as previously described by Nottenberg and Varmus[26].

Nucleic Acid Probes and Primers

The probes used in these analyses were generated utilizing the sequence and numbering system of Shih et. al.[27]. The primer extension probes used were the following: A 24 nucleotide (nt) synthetic fragment that begins at position 1229 was used to identify the 5' end of exon 1. A 21 nucleotide synthetic fragment that begins in intron 1 at position 2151 was used to identify the start site for LTR-initiated transcription. These were end labeled with ^{32}P-γ-ATP using T4 polynucleotide kinase. A 117nt HinfI-SstI fragment beginning at position 2317 was prepared as a "prime-cut" probe[25] and isolated from a 5% acrylamide-urea gel prior to use.

Hybridization probes were the following: For exon 1 a 305bp AvaII fragment from 1233 to 1538; for exon 2+3 a 3.2kb SstI fragment previously

described[15]; and for intron 2 a 1kb SalI-ClaI fragment. For chicken glyceraldehyde-phosphate dehydrogenase (GAPDH) a 1.3kb PstI fragment of the cDNA clone was used[28]. These were labeled by nick translation[29].

For RNase protection analysis a 433 bp ApaI-SmaI fragment in plasmid pT7-1 provided by C. Nottenberg[26] was used. The plasmid was linearized with DraI and labeled in vitro with T7 RNA polymerase as described by Nottenberg and Varmus. For S1 protection analysis a 590bp SphI-SstI fragment of cloned BK25 DNA was used. This was cloned into M13mp19 and prepared as a continuously labeled prime-cut probe.

Primer Extension Analysis

Approximately 50,000 cpm (20 pg) of probe were hybridized with either

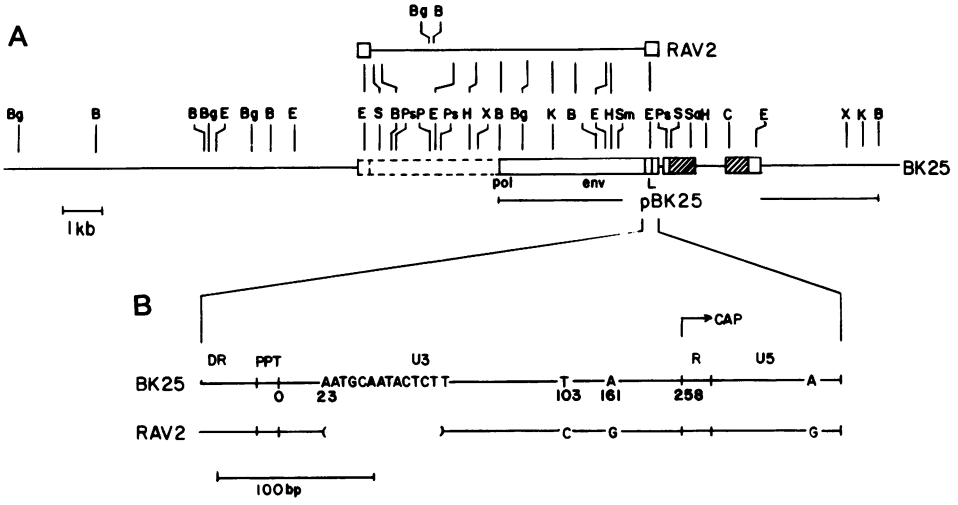

Figure 1: Structure of the c-myc coding region in BK25 cells.
(A) Composite of the results of restriction mapping and sequence analysis of BK25 DNA. The top line shows a partial restriction map of RAV2 DNA (41). The next line shows a restriction map of BK25 DNA. Sequence and restriction analysis of the cloned region (pBK25) have confirmed the arrangement of pol, env, and LTR sequences depicted. The segment drawn with the dashed lines is presumed to represent contiguous proviral sequences because of the correspondence of restriction sites between RAV2 and BK25.
(B) Sequence of the LTR of BK25 DNA compared with RAV2 LTR (32). In the LTR sequence the following abbreviations are used: DR = direct repeat; PPT = polypurine tract; U3 = unique 3' region; R = 21 nucleotide terminal direct repeat; U5 = unique 5' region. The following abbreviations are used for restriction sites: H, HindIII; C, ClaI; P, PstI; E, EcoRI; K, KpnI; X, XbaI; Sa, SalI; Sm, SmaI; Bg, BglII; and B, BamHI.

1 µg of polyadenylated or 50 µg of total cellular RNA in 10 µl of 280 mM KCl, 20 mM TRIS (pH 8.3), 1 mM EDTA for 4 hours at 65°. This was adjusted to a final concentration of 10 mM $MgCl_2$, 10 mM TRIS (pH 8.3), 5mM DTT, 0.5 mM deoxynucleotide triphosphates, 100 µg actinomycin D per ml, in a volume of 50 µl containing 2.5 units RNasin, and 10 units of AMV reverse transcriptase and incubated at 42° for 30 minutes. Samples were then phenol extracted, chloroform extracted, ethanol precipitated, and electrophoresed on 5 or 10% acrylamide-urea gels.

Blot Hybridization

DNA blot hybridizations were performed using standard techniques[21]. RNA blot hybridizations were done on 1% agarose-formaldehyde gels as describe[21,30].

RESULTS

DNA Structure and Sequence of the c-myc Region of BK25 Cells

Our previous studies on the chromatin structure of the c-myc region in BK25 cells showed that a prominant DNaseI hypersensitive site was found within or very near the proviral LTR just upstream from exon 2. We also found a family of DNaseI hypersensitive sites upstream from the LTR. These upstream hypersensitive sites were distinct from those found in uninfected bursa or in several other bursal lymphoma cell lines[15]. In order to identify the DNA sequences on which these new hypersensitive sites form, we both molecularly cloned a segment of the region and analyzed adjacent sequences by restriction mapping. A 10kb BamHI fragment of the c-myc region was cloned into lambda L47.1 by standards techniques[21] and subcloned into pBR322. The insert from this clone is designated pBK25 in Figure 1A. Partial sequence analysis and restriction mapping of this clone are shown in Figure 1 and these results demonstrate that the 3' half of the provirus has integrated 160 base pairs upstream from exon 2. Proviral pol, env, and LTR sequences lie just upstream from exon 2 as depicted in Figure 1A. No exon 1 sequences were found within this clone. Using a variety of enzymes and probes we were able to generate the restriction map of the c-myc coding region depicted in Figure 1A. In this analysis we were unable to map exon 1 on the contiguous 17kb upstream from exon 2 in BK25 cells. We were also unable to locate exon 1 on overlapping 10kb BamHI or BglII fragments by chromosomal walking. This suggests that either exon 1 lies >17 kb upstream from exon 2 or that exon 1 has been translocated in BK25 cells; however, the analysis of c-myc transcription (see below) and translation[31] in these

cells shows that exon 1 lies upstream from exon 2 and that there is no chromosomal translocation involving c-myc.

Sequence and restriction analysis of the cloned region of BK25 (pBK25 in fig.1A) confirm that proviral pol, env and LTR sequences lie upstream from the c-myc coding exons as indicated in figure 1A. Restriction mapping of the region upstream from this clone suggests that a complete ALV provirus may be integrated in the region. The relative position of restriction sites in BK25 and RAV2 are shown for comparison (Fig.1A). The region of BK25 drawn in dashed lines is likely to represent contiguous LTR and gag proviral sequences, although this has not been demonstrated unequivocally.

Mapping of internal restriction sites in exon 1 and sites lying 1kb upstream from exon 1 showed no internal rearrangements of the restriction sites in this region (data not shown), and the results of RNase protection experiments confirm that there are no sequence alterations within exon 1 in BK25 cells (see below). Furthermore, blot hybridization shows that exons 1 and 2 are equally represented in BK25 cells (data not shown).

Sequence Analysis of the LTR-c-myc Region in BK25

Segments of the c-myc clone from BK25 cells were subcloned into M13 vectors and sequenced by the dideoxy chain termination method[23]. The results of this analysis are summarized in Figure 1B. The c-myc-associated LTR of BK25 differs from the published sequence of RAV2[32] by the presence of a 14bp insert that occurs 23bp from the beginning of U3. There are three other transitions, as noted in Figure 1B. The LTR ends abruptly in normal c-myc sequences of intron 1 in an AT-rich region where the sequence $(AT)_{11}$ occurs[27]. The DNA sequence of c-myc in BK25 cells is identical to the normal c-myc proto-oncogene from the end of the LTR through the first 60 codons of exon 2[33].

Analysis of c-myc Transcription in BK25 and MSB-1 Cells: Mapping 5' Ends

The analysis of DNA in Figure 1 shows that while BK25 cells are haploid at the c-myc locus, they contain at least two potential transcriptional start regions - - the normal promoter(s) in exon 1 and the promoter of the proviral LTR adjacent to exon 2. In undertaking an analysis of c-myc trancription in BK25 cells we used MSB-1 cells as a control. MSB-1 is a Marek's disease virus-transformed T-cell line with an unrearranged c-myc gene (our unpublished results).

Figure 2 shows the results of primer extension and S1 nuclease protection analysis of c-myc transcription in BK25 and MSB-1 cells. Both

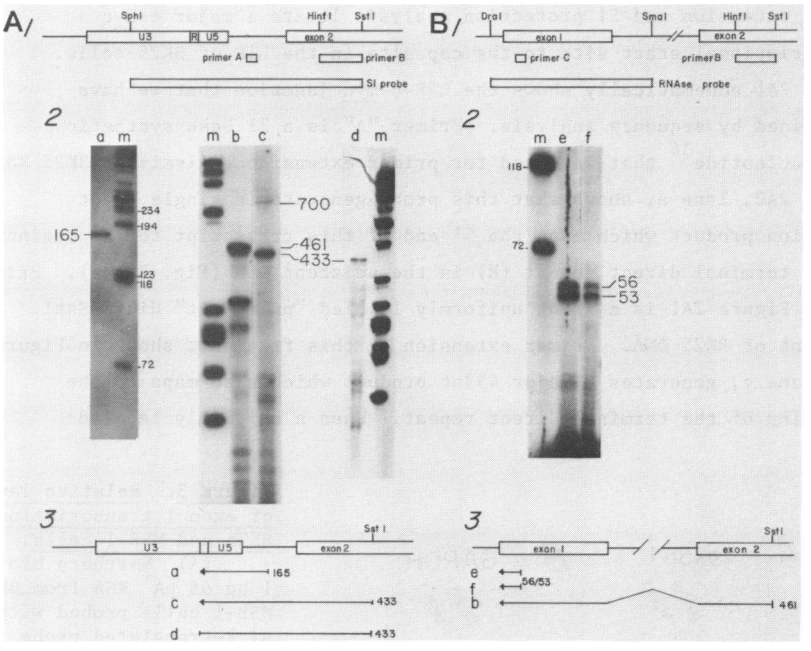

Figure 2: Primer extension and S1 nuclease protection analysis of c-myc transcription in BK25 cells.

(A) Transcription of the LTR-c-myc junction in BK25 cells. (1) The drawing shows a schematic of the LTR-c-myc junction of BK25. In this analysis two primers were used. Primer "A" is a kinase-labeled synthetic 21nt fragment. Primer "B" is a 117nt continuously labeled, single stranded HinfI-SstI fragment. The S1 probe is a 590nt SphI-SstI fragment prepared as a continuously labeled single stranded probe. (2) Autoradiographs of reaction products. (a) Primer extension reaction of 2 µg of poly-A^+ BK25 RNA using primer A and electrophoresed on a 6% acrylamide-urea gel. (b) Primer extension reaction of 50 µg of MSB-1 total cellular RNA using primer "B". (c) Primer extension reaction of 50 µg of BK25 total cellular RNA using primer "B". (d) S1 protection of 50 µg of BK25 total cellular RNA using the S1 probe run on the same gel. (3) Interpretation of the results. Primer extension products from reactions (a) and (c) and S1 protection product from reaction (d) are shown.

(B) Primer extension and RNase protection analysis of exon 1 transcription in BK25 and MSB-1 cells. (1) The drawing depicts the arrangement of exon 1 and the 5' end of exon 2 in both cell types. Primer "B" is a 117nt HinfI-SstI continuously labeled fragment. Primer "C" is a synthetic 24nt kinase-labeled fragment. The RNase protection probe is a 430 nucleotide DraI-SmaI RNA probe continuously labeled with ^{32}P by T7 RNA polymerase in the vector pT7-1 (26). (2) Autoradiographs of reaction products. Primer extension of primer "C" using 50 µg total cellular BK25 RNA as template (e) or 50 µg of total cellular MSB-1 RNA (f) run on a 10% acrylamide urea gel. (3) Interpretation of results. Primer extension products of BK25 RNA are shown in (e) and those for MSB-1 RNA in (b) and (f). Lane (c) shows a minor primer extension product of 700 nucleotides in BK25 RNA using primer "B". Lanes marked "m" contain kinase-labeled HaeIII cleaved ØX174 or a mixture of these fragments and those from a 123 bp ladder.

primer extension and S1 protection analysis locate a major c-myc transcriptional start site to the cap site in the LTR of BK25 cells. Figure 2A1 schematically shows the LTR-c-myc junction that we have determined by sequence analysis. Primer "A" is a 21 base synthetic oligonucleotide[34] that was used for primer extension analysis of BK25 RNA. Figure 2A2, lane a, shows that this probe generates a single 165nt extension product which maps the 5' end of this transcript to the beginning of the terminal direct repeat (R) in the adjacent LTR (Fig. 2A3,a). Primer "B" in Figure 2A1 is a 117nt uniformly labeled "prime-cut" HinfI-SstI fragment of BK25 DNA. Primer extension of this fragment, shown in Figure 2A2, lane c, generates a major 433nt product which also maps to the beginning of the terminal direct repeat. When a uniformly labeled

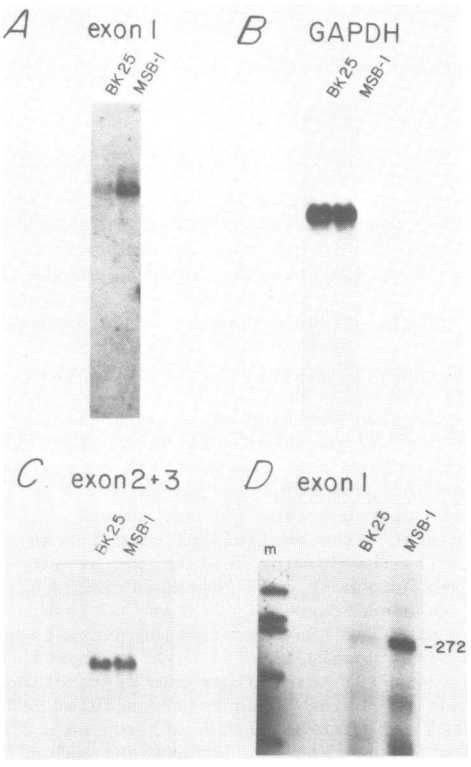

Figure 3: Relative levels of exon 1 transcription in BK25 and MSB-1 cells.

(A) Northern blot of 1 µg of pA^+ RNA from BK25 or MSB-1 cells probed with a nick-translated probe for exon 1 of c-myc. This sample was blotted onto DBM paper and exposed autoradiographically for 3 days.

(B) The same blot depicted in (A) was washed and rehybridized with a probe for GAPDH and exposed for 3 hours.

(C) Northern blot of 1 µg of pA^+ RNA from BK25 or MSB-1 cells probed with a nick-translated probe for exons 2+3 of c-myc and exposed for 24 hours.

(D) Quantitative RNase protection analysis of relative levels of exon 1 transcription in BK25 and MSB-1 cells. In both lanes 1 µg of pA^+ RNA was hybridized to the DraI-SmaI ^{32}P-labeled RNA fragment, treated with RNase, extracted, and electrophoresed on a 6% acrylamide-urea gel, dried and exposed autoradiographically for 6 hours with enhancement screens. The marker lane (m) contains kinase-labeled HaeIII-cleaved fragments of ØX174 DNA.

"prime-cut" probe prepared from a SphI-SstI fragment of BK25 DNA (the "S1 probe" in Fig.2A1) is used in an S1 protection assay, a fragment of 433nt is protected (Figure 2A2, lane d). This is consistent with the primer extension results and maps the 5' end of the LTR initiated mRNA to the cap site in R. These results are summarized in Figure 2A3 and show that c-myc transcription in BK25 cells begins at the cap site in the upstream LTR. The S1 probe migrates with an apparent mobility of 700nt (data not shown).

We next wished to determine whether c-myc transcription was also initiated in exon 1 of BK25 cells. Figure 3A shows that a probe for exon 1 detects a band of ~2.4 kb in BK25 cells. This message is slightly larger than that detected by the same probe in MSB-1 cells and is less abundant in BK25 cells than it is in MSB-1 cells. Overall levels of c-myc coding exon transcription in BK25 and MSB-1 are comparable (Fig. 3C). The Northern blot in Figure 3A was performed using DBM paper in an attempt to detect any discrete low molecular weight exon 1 transcripts in BK25 cells; however, none was found.

Primer extension using a terminally labeled 24nt synthetic oligonucleotide ("primer C" in Fig. 2B1) was done using BK25 RNA (Fig. 2B2, lane e) or MSB-1 RNA (Fig. 2B2, lane f). In both cases, fragments of 56 and 53nt were generated. This maps the start site of exon 1 transcription to the same sites in both BK25 and MSB-1 cells, as depicted in Figure 2B3, e and f. These start sites are in general agreement with previous results[34]. Nottenberg and Varmus had mapped exon 1 to include 272 to 275bp beginning very near the same start site that we have observed in BK25 and MSB-1 cells. To define the limits of exon 1 in BK25 and MSB-1 cells, we performed RNase protection experiments using a 430nt SmaI-DraI fragment cloned into pT7-1[26] shown in Figure 2B1, "RNase probe." RNA polymerase was used to synthesize continuously labeled anti-sense RNA. This labeled RNA was hybridized with BK25 or MSB-1 RNA, unhybridized sequences digested with RNase A and T1, and the protected, labeled RNA electrophoresed on acrylamide-urea gels and autoradiographed. The results of this RNase protection experiment are shown in Figure 3B. In both BK25 and MSB-1 cells the probe protects a fragment of 272nt. The probe migrates with an apparent mobility of 420nt and is not shown on the autoradiograph.

These primer extension analyses and RNase protection experiments show that exon 1 is transcribed in BK25 cells, but they do not show whether exon 1 transcription continues into exon 2. In an attempt to demonstrate this we performed primer extension experiments using a primer in exon 2. In

MSB-1 cells when an exon 2 primer (primer B in Fig. 2A1) is used, a 461nt fragment is generated (Fig. 2A2, lane b). Such a fragment is the size predicted from the splice junction of exons 1 and 2 reported by Nottenberg and Varmus[26] and is depicted in Figure 2B3,b. In addition, an extension product of 330nt seen with MSB-1 RNA. Because no corresponding RNase protected fragment is found, we feel that the 330nt extension product represents a premature stop site in the primer extension reaction. Also, when primer extension experiments are done with MSB-1 RNA using primer A (Fig. 2A1), we do not observe any extension products corresponding to the projected promoter in intron 1 that would correspond to the 330nt primer extension product. When the exon 2 primer (primer B) is used for extension of BK25 RNA a major extension product of 433nt is detected (Fig. 2A2,c), as shown previously, which identifies the major transcriptional start site at the LTR promoter shown in Figure 2A3,c and correlates with the S1 protection results. At this level of autoradiographic exposure we see no signal corresponding to a physiologically spliced mRNA encompassing the normal splice junctions of exons 1 and 2. On the same gel (Fig. 2A2, lane c), a minor extension product of 700nt is seen. This band is not seen in long exposures of the MSB-1 primer extension products, and is apparently unique to BK25 cells.

Relative Levels of Exon 1 Transcription in BK25 and MSB-1 Cells

To determine the relative steady-state levels of c-myc expression in BK25 and MSB-1 cells, Northern blot hybridization was performed using a probe that includes the c-myc coding region. Figure 3C shows that comparable levels of c-myc RNA are detected by a probe for the coding region (a 3.2 kb SstI fragment of c-myc that includes exons 2 and 3). In addition, comparable levels of mRNA for the universally expressed gene for glyceraldehyde-phosphate dehydrogenase are found in the two cell types (Fig. 3B).

Expression of c-myc RNA in BK25 cells results from transcription initiated in both the LTR and from the normal start site in exon 1, while all of the c-myc RNA in MSB-1 cells originates from exon 1 transcription. In order to compare the relative proportion in BK25 cells of exon 1 initiated mRNA with that originating from the LTR we performed quantitative RNase protection using an exon 1-specific probe. In this analysis, a 430bp SmaI-DraI fragment spanning exon 1 and cloned into pT7-1 was used to prepare a uniformly labeled anti-sense RNase probe. This probe is depicted in Figure 2B1 and labeled "RNase probe". This probe was hybridized to 1 µg

of polyadenylated RNA from BK25 or MSB-1 cells. Hybridized RNA was digested with RNase A and T1, phenol extracted, electrophoresed, and exposed autoradiographically. Figure 3D shows that in both BK25 and MSB-1 cells a fragment of 272nt is protected with the exon 1 probe. This agrees with the size of exon 1 reported by Nottenberg and Varmus[26] and corroborates the result shown in Figure 3A. Furthermore, taken in conjunction with the primer extension data in Figure 2B2, lanes e and f, this result further supports the conclusion that exon 1 is transcribed in a normal fashion, albeit at a low level in BK25 cells. Densitometric tracing of the autoradiograms in Figure 3A and D show that the steady state levels of exon 1 mRNA is 10-fold lower in BK25 cells than in MSB-1 cells. Since both cell types express equal levels of c-myc RNA, this suggests that about 10% of c-myc RNA initiates in exon 1 while about 90% initiates in the LTR in BK25 cells.

Half-Life of c-myc RNA in BK25 Cells

The half-life of c-myc RNA has generally been observed to be short, approximately 15-30 minutes[16,34], suggesting that one component of elevated steady state levels of c-myc RNA in bursal lymphomas may result from increased half-life, hence from post-transcriptional regulation. For two bursal lymphoma cell lines, S13 and 243L, the half-life of c-myc RNA was found to be short (around 30 minutes) which was comparable to that found in MSB-1 cells and chick embryo fibroblasts[34]. In mouse plasmacytomas and Burkitt's lymphomas, truncated c-myc transcripts lacking exon 1 have been found to have a significantly prolonged half-life[17,18]. Because BK25 cells produce two c-myc mRNAs by alternative promoter utilization, one initiating in exon 1 and the other in LTR sequences, we wished to determine the half-lives of the two alternative mRNA species in order to determine

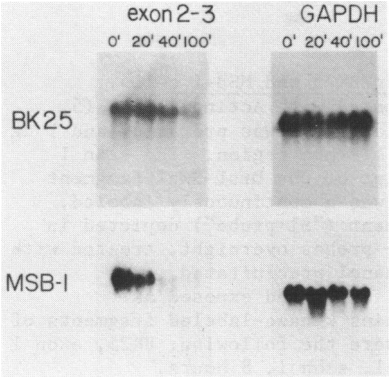

Figure 4: Stability of c-myc RNA in BK25 and MSB-1 cells.
BK25 or MSB-1 cells were treated with 5 µg/ml Actinomycin D for from 0 to 100 minutes, washed, RNA isolated, and 5 µg of total cellular RNA were electrophoresed on duplicate formaldelyde-agarose gels. These gels were blotted onto nitrocellulose and probed with the nick-translated ^{32}P-labeled probes for c-myc exons 2 and 3 or GAPDH, washed and exposed as autoradiographs for 18 hours with enhancement screens.

whether the presence or absence of exon 1 or LTR sequences significantly altered c-myc mRNA half-life.

BK25 cells were treated with 5 µg/ml actinomycin D, rapidly washed, total cellular RNA isolated at intervals, electrophoresed on formaldehyde gels, blotted onto nitrocellulose and probed with probes for exons 2 and 3 of c-myc or a control gene, glyceraldehyde-phosphate-dehydrogenase (GAPDH). The results of this experiment, shown in Figure 4, show that the half life of c-myc RNA in both BK25 and MSB-1 cells is short, approximately 25 minutes in BK25 cells and 15 minutes in MSB-1 cells.

In Figure 5 we have used quantitative nuclease protection of the 5' ends of the c-myc transcripts to determine the half lives of the alternate c-myc mRNAs from BK25 cells compared with that found in MSB-1 cells. An RNA probe spanning exon 1 (the "RNase probe" depicted in Fig. 2B1) was hybridized with 1 µg of polyadenylated RNA from cells treated with

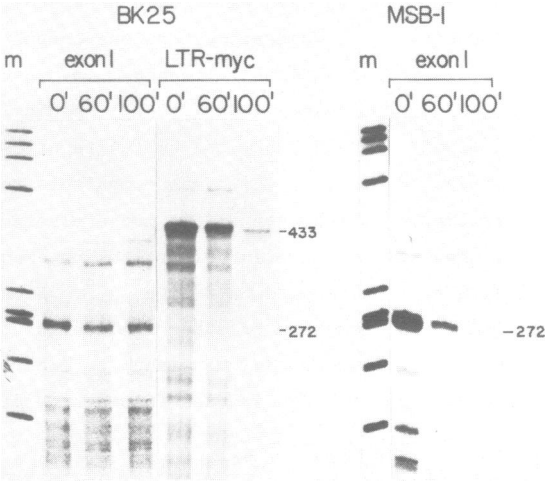

Figure 5: Half life of c-myc transcripts in BK25 and MSB-1 cells.
Logarithmically growing cells were treated with Actinomycin D (5 µg/ml) for the times indicated. Polyadenylated RNA was prepared, and 1 µg was hybridized to probes for exon 1 or the LTR-myc region. The exon 1 probe was the in vitro labeled RNA transcript of the DraI-SmaI fragment depicted in Figure 2B1. The LTR-myc probe was a continuously labeled, single stranded "prime cut" SphI-SstI fragment ("S1 probe") depicted in Figure 2A1. Samples were hybridized to the probes overnight, treated with RNase or S1 nuclease, phenol extracted, ethanol precipitated, and electrophoresed on 6% acrylamide-urea gels, dried and exposed as autoradiographs. The marker lane (m) contains kinase-labeled fragments of HaeIII-cleaved ØX174 DNA. Exposure times were the following: BK25, exon 1 probe, 3 days; BK25, LTR-myc 10 hours; MSB-1, exon 1, 8 hours.

Actinomycin D for various times, treated with RNaseA and RNase T1, electrophoresed on 6% acryamide-urea gels, autoradiographed, and scanned with a densitometer. In MSB-1 cells a fragment of 272nt is protected by this probe and disappears with a half life of 25 minutes. This probe protects a fragment of identical size in BK25 RNA; however, in the latter case this species has a half life of greater than 100 minutes. Additional background bands that appear in this autoradiograph are seen as a result of the long exposure times used.

To determine the stability of the c-myc RNA in BK25 cells that initiates in the LTR, we performed quantitative S1 protection analysis using the same RNA from Actinomycin D treated BK25 cells. In this case the probe used was a continuously labeled "prime cut" probe spanning the LTR-c-myc junction (the "S1 probe" in Fig. 2A1). An S1 protected fragment of 433nt is seen in the autoradiograph of this gel (Fig. 5). Densitometric tracing of this autoradiograph shows that the LTR-initiated c-myc has a half life of 25 minutes. These same results have been obtained in three separate experiments using both total cellular and polyadenylated RNA.

Thus in BK25 cells the major c-myc RNA species is transcribed starting at the LTR and decays with a half life of about 25 minutes, similar to the kinetics of disappearance of c-myc RNA in MSB-1 cells which lack any c-myc rearrangement. A minor RNA species in BK25 cells, initiated in exon 1, has a long half life and is initiated at the same transcriptional start site in exon 1 as that utilized in MSB-1 cells.

DISCUSSION

We have studied the DNA structure and pattern of transcription of the c-myc oncogene in the bursal lymphoma cell line BK25 and compared it with that found in MSB-1, a T-cell line having an unrearranged c-myc gene. In BK25 exon 1 is displaced >17kb upstream from exon 2 with a complete or nearly complete provirus integrated between exons 1 and 2. The DNA analysis alone has not definitely shown that exons 1 and 2 lie on the same DNA segment; however, the transcriptional analysis is most consistent with this interpretation. In addition, BK25 cells have been shown to express the larger (p62/63) form of the myc protein which initiates in a CTG codon in exon 1[31,35]. Exon 1 DNA in BK25 cells is identical to that found in CEF and MSB-1 in the region that encompasses exon 1 and including the 5' flanking 1 kb.

The ALV provirus has integrated in a segment of c-myc DNA where the

sequence $(AT)_{11}$ occurs. An extensive survey of bursal lymphomas has shown a clustering of proviral integrations in or near this region[36-37]. The poly-AT sequence may be a hot spot for proviral recombination in other systems as well. For instance, the integrated Epstein-Barr virus provirus of the Burkitt lymphoma cell line Namalwa occurs at a similar AT-rich segment[38].

BK25 cells are haploid at the c-myc locus and produce two c-myc mRNA species. One is an unspliced message that initiates in the cap site of the adjacent proviral LTR. The other initiates at the normal c-myc start site(s) in exon 1. The LTR initiated mRNA is the major c-myc transcript accounting for about 90% of the steady state levels of c-myc RNA and this message has a short half life of approximately 25 minutes measured in Actinomycin D chase experiments. The exon 1 initiated mRNA comprises about 10% of the c-myc RNA and has a prolonged half life of >>100 minutes. By contrast, in MSB-1 cells, c-myc mRNA initiated in exon 1 has a short half life of about 15 minutes, similar to that found in chick embryo fibroblasts[34]. These observations demonstrate that the presence of the first exon is not sufficient to confer a short half life on the mature message and contrasts with what has been suggested from observations in mouse myelomas[18], Burkitt lymphoma[17], and COLO320 cells[19], in which truncation of c-myc RNA my loss of exon 1 results in increased mRNA stability. Our observations are consistent with results obtained with human c-myc constructs transfected into murine fibroblasts demonstrating that sequences from the 3' untranslated segment of c-myc are required for mRNA instability[39]. It will therefore be important to determine the sequence of the stable c-myc RNA.

The exon 1 transcript in BK25 cells most likely encompasses the whole coding region. As mentioned above, the cells express the larger p62/63 myc protein initiated in exon 1. Also, in a standard Northern blot, probes for both exon 1 and exons 2+3 detect comparable size ~2.4 kb RNA species in BK25 cells while an exon 1 probe detects ~2.4 kb RNAs in both MSB-1 and BK25 cells (Fig. 3A). The primer extension experiments using primer B derived from exon 2 (Fig 2A1) shows the LTR-c-myc that is transcribed through the normal 5' splice site of exon 2 and does not detect a product consistent with a processed transcript identical to that found in MSB-1 cells. Instead a minor band at 700nt is seen. The latter may represent an unprocessed intermediate, an aberrantly spliced mRNA, or an experimental artifact. It is formally possible that exon 1 is a polyadenylated "orphon"

in BK25 cells in which transcription does not extend into exon 2 either because of translocation or because of failure to splice to the 5' splice acceptor site of exon 2; however, we have not detected other exon 1 transcripts in standard Northern blots (c.f. Fig. 3A).

The relative excess of LTR-initiated c-myc RNA in BK25 cells could result from preferential use of the strong promoter in the proviral LTR acting to produce high levels of c-myc which represses expression from the normal promoter in exon 1. Such a negative feedback mechanism for the control of c-myc expression has been proposed previously[11,40]. Alternatively, the transcriptional enhancer in the LTR[12] may be displaced too far downstream from exon 1 to exert an effect on transcription from the normal promoter.

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