Enhanced Transcription of c-*myc* in Bursal Lymphoma Cells Requires Continuous Protein Synthesis

Maxine Linial, Nancy Gunderson, Mark Groudine

Abstract. *In several bursal lymphoma cell lines in which* c-myc *transcription is regulated by avian leukosis virus (ALV) long terminal repeat (LTR) sequences, protein synthesis inhibition decreases the transcriptional activity of* c-myc *as well as other LTR driven viral genes. This decrease in transcription is associated with a change in the chromatin structure of* c-myc, *as measured by deoxyribonuclease I (DNase I) hypersensitivity, and a shift of transcription from the LTR to the normal* c-myc *promoter. In contrast, cycloheximide had little or no effect on the transcription of LTR driven genes in infected chicken embryo fibroblasts treated with the drug. These results suggest that a labile, cell type–specific protein may interact with the retroviral LTR and regulate transcription of genes under LTR control. Further, the results demonstrate that the increase in intracellular concentration of* c-myc *RNA induced by cycloheximide treatment of normal cells is the result of stabilization of this message.*

The cellular-*myc* (c-*myc*) gene of mouse, man, and birds appears to be transcribed in all actively dividing cells. Much interest has been focused on this gene since altered gene arrangement or expression has been demonstrated in several tumor types, including Burkitt's lymphomas in humans, plasmacytomas in mice, and avian leukosis virus (ALV)–induced lymphomas in the bursa of Fabricius in chickens (*1–4*). The c-*myc* gene is composed of three exons, two of which encode c-*myc* protein (*5–8*). The first exon, which is lacking in some mammalian and avian tumor cells (*3–9*), has been implicated in control of c-*myc* gene expression (*10*). The c-*myc* is remarkable for the short half-life of both its RNA and protein products (*11, 12*).

Experiments with inhibitors of protein synthesis such as cycloheximide (CH) (*13, 14*) have suggested that c-*myc* transcription might be controlled by a labile repressor protein. However, since CH treatment in some cells results in an increase in stability in c-*myc* messenger RNA (mRNA) (*12*), the CH effect could be at the posttranscriptional level. It has also been proposed that the promoter utilization or secondary structure of the 5′ end of c-*myc* RNA might be involved in the CH effect (*12*).

Using a number of avian cell types (*8, 15*), we find that the amount of c-*myc* RNA is controlled posttranscriptionally at the level of message stability, and find no association between RNA stability and the structure of the 5′ end of c-*myc* RNA. In addition, we have observed that, in cell lines in which transcription of the c-*myc* gene is controlled by sequences in the enhancer or promoter of the ALV long terminal repeat (LTR) (*16*), protein synthesis is required for the increased transcription of c-*myc*. If protein synthesis is inhibited in these lines, the level of LTR-enhanced c-*myc* and viral gene transcription, as measured by nuclear runoff assays, decreases, and a deoxyribonuclease I (DNase I)–hypersensitive site in the LTR is lost. In one cell line, inhibition of protein synthesis leads to a shift in c-*myc* transcription from the viral LTR promoter to the normal c-*myc* promoter.

Cycloheximide increases c-*myc* RNA in normal cells, but not in most bursal lymphoma cells. We have been studying c-*myc* transcription in cell lines derived from tumors induced by ALV in which c-*myc* transcription is augmented by the insertion of the viral LTR in the region of the c-*myc* gene. Promoter utilization (LTR or cellular), as well as the structure of the c-*myc* transcripts (*8*) (Fig. 1), have been determined in some of these lines. The chicken system is particularly useful for study of transcription of the c-*myc* gene, because gross translocations have not occurred in the vicinity of c-*myc* in avian cells exhibiting altered c-*myc* transcriptional patterns. Furthermore, promoter utilization differs among the cell lines (Fig. 1). For example, c-*myc* transcription in cell line S13 is initiated at the normal cell promoters P1 and P2, whereas in cell line H1, the viral LTR promoter is utilized. In line 293S, initiation of c-*myc* RNA appears to occur both in intronic sequences and at P1, possibly from the two different alleles, while in 243L initiation does not occur either at the LTR or at P1. It was thus of interest to use these cell lines to attempt to correlate promoter utilization with stabilization of c-*myc* RNA in the absence of protein synthesis. If, for instance, only cell lines utilizing the normal c-*myc* promoter respond to inhibition of protein synthesis by increasing the amount of c-*myc* RNA, then increases in steady state RNA after CH treatment would be observed in S13 cells, but not HI or 243L cells.

Steady-state RNA was measured by Northern blot analysis in normal cells and transformed cell lines (Fig. 2). In these experiments, we used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (*17*) as a control for the amount of RNA placed on each lane, since all chicken cells examined contained reasonably high steady-state levels of GAPDH RNA, which did not vary with CH treatment. We found that chicken embryo fibroblasts (CEF) contain little c-*myc* RNA, but this RNA increased dramatically after the cells were treated for 3 hours with CH (Fig. 2A). We also examined lymphocytes derived from bursa, spleen, or thymus (Fig. 2C). All contained low steady-state levels of c-*myc* RNA, but these were increased by CH treatment. The amount of c-*myc* RNA in the four normal cell types examined increased between 12 and 30 times relative to GAPDH mRNA (*18*). In CEF, both polyadenylated and nonpolyadenylated c-*myc* RNA's increased with CH treatment (*19*). We also examined the effect of CH on the c-*myc* RNA in MSB cells (Fig. 2A), a chicken T-cell line transformed by Marek's disease virus (*20*), where the structure of the c-*myc*

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gene and transcript is normal (*8*). In MSB cells, we found about a 12-fold increase in c-*myc* RNA after the cells were treated with CH. The level of chicken c-*myc* RNA also increased after CH treatment of Rat-2 cells stably transfected with a plasmid containing the genomic chicken c-*myc* gene. These transfected cells synthesize c-*myc* RNA to an amount comparable to that of normal chicken cells (*19*). Thus, there is an increase in steady-state level c-*myc* gene transcripts in the absence of protein synthesis in all cells examined in which c-*myc* expression is dependent on normal control signals.

We next examined the amount of c-*myc* RNA in the six bursal lymphoma cell lines whose c-*myc* genes are shown in Fig. 1. In five of the cell lines—S13, BK25, HI, 243L, and 293S (Fig. 2, A and B)—little or no quantitative change in c-*myc* RNA was seen after treatment; we found a 1- to 2.7-fold increase in the amount of c-*myc* RNA relative to that of GAPDH RNA. The apparent lack of induction of c-*myc* RNA by CH was independent of the promoter used (Fig. 1). In S13 cells, the level of poly(A)$^+$ and poly(A)$^-$ c-*myc* RNA's both remained constant after CH treatment (*19*). In contrast, treatment with CH for 3 hours increased the c-*myc* RNA by a factor of more than 10 relative to GAPDH RNA in cell line BK3A (Fig. 2A). We also looked at the effect of CH on the steady-state level of chicken c-*myc* RNA on a Rat-2 cell line stably transfected with a plasmid containing the LTR and c-*myc* exons 2 and 3 isolated from a genomic library of DNA from BK25 cells (*21*). In these cells (Fig. 2D) there was an eightfold increase in c-*myc* RNA after treatment.

Short half-life of c-*myc* RNA in normal and transformed avian cells. One interpretation of the above results is that the insertion of viral sequences in the c-*myc* domain somehow affects the turnover of c-*myc* RNA. To examine this, the half-life of the c-*myc* RNA in MSB and S13 cells was compared, with actinomycin D being used to prevent RNA synthesis (*12*) (Fig. 3A). The c-*myc* RNA in both cell types was equally unstable with a half-life of about 30 minutes. Furthermore, an actinomycin D decay experiment, performed in the presence of the

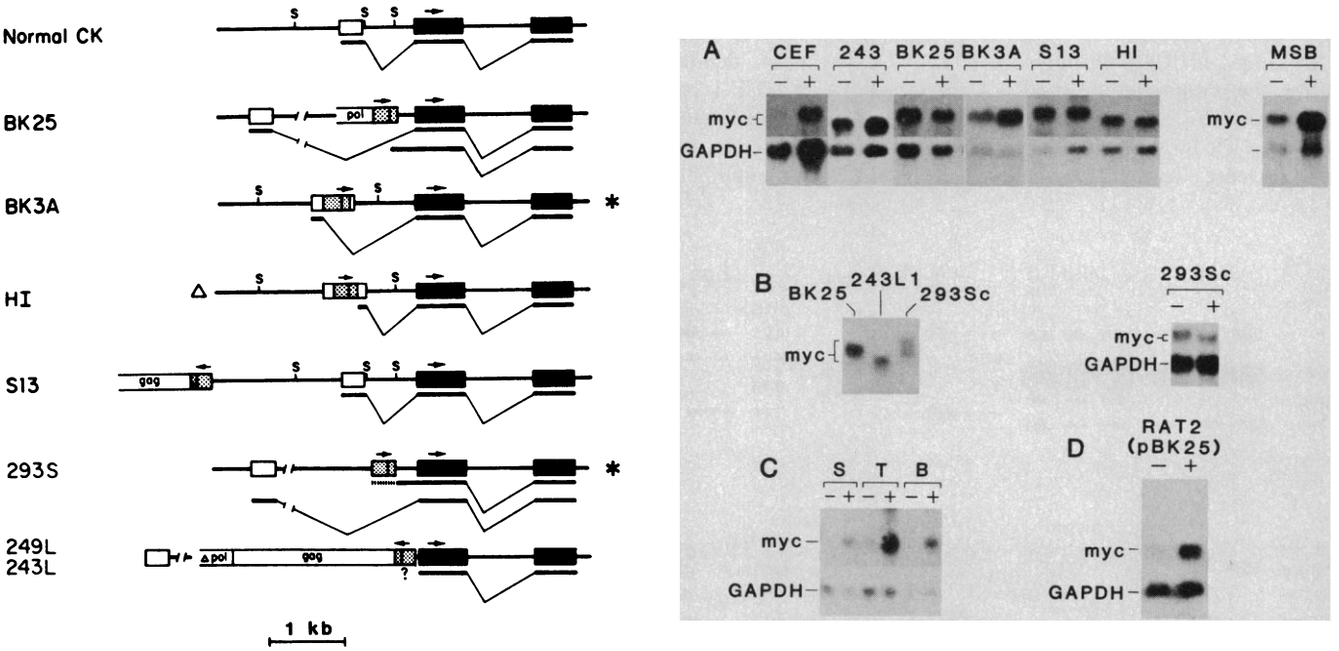

Fig. 1 (left). Structure of the c-*myc* alleles in normal chicken cells and the bursal lymphoma cell lines used in this study. Mapping data for lines S13, BK25, BK3A, and HI are from restriction enzyme analyses, as well as transcriptional analyses (*8, 15*) (Fig. 5). The origin of HI, BK25, S13, and BK3A has been described (*15, 39*). Lines 243L and 293S were established in our laboratory from tumors induced in chickens (line 15×7) by the avian leukosis viruses RAV-1 (line 293S) or MCAV-A (line 243L). The 293S cells were derived from a metastatic spleen tumor, and the 243L cells were derived from a metastatic liver tumor from chickens bearing large bursal tumors. In the case of 293S, the viral integration in the vicinity of c-*myc* is identical in the bursal and splenic derived cell lines and the original tumors. Solid boxes indicate the c-*myc* coding exons 2 and 3; the open box indicates the noncoding first exon. Stippled boxes indicate the viral LTR; other known viral sequences are indicated by gene name (*gag*, viral structural protein gene; *pol*, RNA dependent DNA polymerase). (Δ) indicates a known deletion in the gene. In the case of line HI, sequences upstream of c-*myc* exon I are missing from the genome. The broken line indicates that portions of the locus are located further upstream in these transformed cells. The S indicates restriction endonuclease Sma I sites. The arrows denote the direction of transcription from both the LTR and c-*myc*. The major c-*myc* RNA transcripts are drawn beneath the structure of the c-*myc* gene. A dotted line denotes uncertainty in the start site for the transcript. Asterisks indicate cell lines that harbor a normal c-*myc* allele in addition to the rearranged allele depicted. Although originally we reported only one c-*myc* transcript in BK25 cells (*8*), recent experiments with the intronic primer shown in Fig. 5 show the presence of a second transcript initiating at or near the viral LTR. Fig. 2 (right). Steady-state RNA's in normal and transformed cell lines treated with CH or untreated controls. The MSB cells used are derived from a Marek's disease–induced thymic tumor and have been described (*20*). Chicken embryo fibroblasts (CEF) were obtained from *ev*-1 embryos, and were used at passage 3 and beyond. Rat-2 (pBK25) are Rat-2 cells stably transfected by the plasmid pBK25, and lymphocytes were obtained from the spleen, thymus, and bursa of 4- to 6-week-old chickens (*40, 41*). Total RNA's, approximately equalized for ribosomal RNA content, were denatured with formamide and subjected to electrophoresis on denaturing agarose gels (*42*). Gels were blotted to nitrocellulose and probed. All cells were exponentially growing when RNA was extracted with guanidium isothiocyanate (3.7*M*) (*42*). The CH treatment period was 3 hours; CH at 10 μg/ml was used, except for 293S (20 μg/ml) or 243L cells (30 μg/ml). In all cases the inhibition of protein synthesis was more than 90 percent. The probe for the chicken glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) was a 1.3-kb from the cDNA clone pGAPDH-1 (*17*), and the probe for c-*myc* was a Cla I–Eco RI 0.8-kb subclone of chicken c-*myc* exon 3. Excised fragments were labeled with ^{32}P by nick translation. In (A), the 243L RNA samples were run on a separate gel from the other bursal lines. 243L does encode a smaller c-*myc* RNA than CEF or BK25 (B) and the relative location of the c-*myc* bands has been determined from normal CEF markers. The relative ratio of c-*myc* to GAPDH was determined from densitometer tracings of this and other Northern blots.

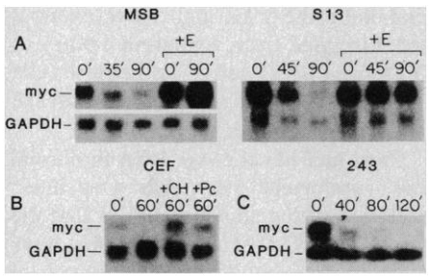

Fig. 3. Determination of the half-life of c-*myc* RNA in the presence or absence of protein synthesis inhibitors. (A) Exponentially growing MSB or S13 cells were treated with actinomycin D (0.5 μg/ml) for the indicated times and then rapidly lysed; the RNA was then extracted. Identical portions of MSB or S13 cells were first treated with 0.1 m*M* emetine (E) for 20 minutes before the addition of actinomycin D (+E). (B) Identical numbers of CEF were treated for 60 minutes with actinomycin D (0.5 mg/ml), either with no protein synthesis inhibitor, or with cycloheximide (CH) (20 μg/ml) or pactamycin (Pc) (10 m*M*) added 20 minutes before the actinomycin D was added. Control cells received no inhibitor. (C) Bursal lymphoma cell line 243L cells were treated with actinomycin D (0.5 μg/ml) for the indicated times and processed as in (A). RNA extraction and Northern blot analysis was as for Fig. 2.

protein synthesis inhibitor emetine, shows that the turnover rate of both MSB and S13 c-*myc* RNA is reduced in the absence of protein synthesis. Both CH and emetine inhibit protein synthesis by similar mechanisms and lead to "freezing" of RNA on polysomes (*22*). Thus, the stability of c-*myc* mRNA could be caused by the sequestering of the RNA in a compartment inaccessible to ribonuclease. To test this, we examined the effect of treatment with pactamycin, an inhibitor of initiation of translation at the concentration used (*23*). In Fig. 3B, CEF's were treated with actinomycin D for 60 minutes with or without prior treatment with pactamycin or CH. This experiment demonstrates that c-*myc* RNA in CEF is stabilized to a similar extent in the presence of pactamycin as in the presence of CH. Prior treatment with either inhibitor followed by treatment with actinomycin D for 60 minutes led to about a 100-fold increase in c-*myc* RNA. Thus, nontranslated c-*myc* mRNA which is not frozen on polysomes is also protected from degradation.

To test whether exon 1 is an essential component of the instability of c-*myc* RNA, we measured the half-life of c-*myc* mRNA in 243L cells, which contain only a rearranged copy of c-*myc* (Fig. 1). Using RNA from these cells, we detected no primer extension products with an exon 1 probe or an intronic probe (see below, as well as Fig. 5 below), although the same RNA could protect a DNA fragment containing exon 2 sequences as measured by nuclease S1 analysis (*19*). We found that the c-*myc* RNA in 243L cells completely lacking exon 1 has a half-life of about 10 minutes (Fig. 3C), identical to that measured for CEF in the same experiment (Fig. 3B).

Transcriptional activity of c-*myc* is inhibited by cycloheximide in ALV transformed cells. In order to examine whether differences in the observed steady-state levels of c-*myc* RNA in normal and bursal lymphoma cells, in the presence or absence of protein synthesis, are a result of differences in transcriptional rates or posttranscriptional events, we performed nuclear runoff experiments. As was previously described, while no new initiation occurs under these conditions, relative rates of elongation and polymerase density along specific genes can be determined (*24*). In all experiments, the rate of c-*myc* transcription was normalized (*18*) to that of either *fos* (*25*) or GAPDH (*26*). Transcription from all three genes is sensitive to concentrations of α-amanitin that specifically inhibit polymerase II transcription (Fig. 4A). In initial analyses (Fig. 4A), the rate of transcription was measured in MSB and S13 cells, either untreated, or treated with cycloheximide or emetine for 3 hours. In the untreated cells, we found that the transcription of c-*myc* in S13 cells was about ninefold greater than that of MSB cells, consistent with the observed differences in steady-state levels of c-*myc* RNA in the two cell types (Fig. 2). The use of inhibitors had little or no effect on the c-*myc* transcription observed in MSB cells. Thus, the increase in steady-state c-*myc* RNA in these cells in the absence of protein synthesis, can be accounted for by stabilization of RNA. In contrast, protein synthesis inhibition decreased the relative level of c-*myc* transcription about sevenfold in S13 cells (Fig. 4A). The rate of transcription in S13 cells in the absence of protein synthesis was found to be approximately

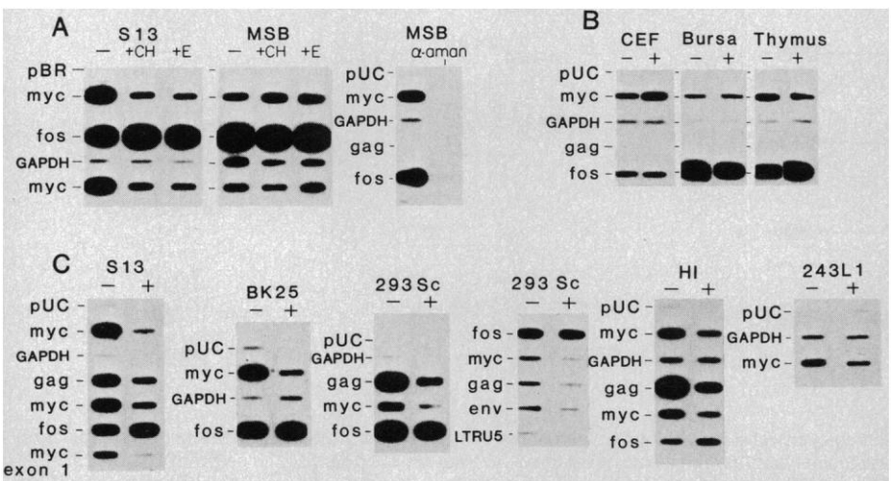

Fig. 4. Runoff transcription analysis of normal and transformed cells. Cells were treated with cycloheximide (CH) or emetine (E) as in Figs. 2 and 3 for 3 hours. α-Amanitin (α-aman) at 2 μg/ml was added to nuclei as described (*24*). Nuclei were isolated (*28*) and resuspended at a concentration of 2×10^8 nuclei per milliliter in nuclear freezing buffer (40 percent glycerol, 50 m*M* tris-HCl, *p*H 8.3, 5 m*M* $MgCl_2$, and 0.1 m*M* EDTA). The nuclei were either used directly or frozen at −70°C. The nuclear runoff reactions, isolation of ^{32}P-labeled RNA, hybridization conditions, and analyses of hybrid were performed by combining several features of two previously described techniques (*24, 43*) and are described in (*44*). Nitrocellulose filters containing 5 μg of the indicated plasmids were hybridized to the runoff products for 36 hours. Equal amounts, measured by the number of counts per minute (1×10^7 to 2×10^7), of runoff products were added to each filter for nuclei that were being directly compared. Plasmids used for these experiments were *myc* (a 2.4-kb Sst I–Eco RI fragment of chicken c-*myc* containing exons 1 and 2 and intron 2 cloned in SP65); U5 LTR (a 0.6-kb Eco RI–Bam HI fragment from the U3 region to *gag* of RSV cloned in Sp63); *gag* (a 1.3-kb Bam HI fragment cloned in pBR); GAPDH, a 1.3 kb cDNA insert in PBR (*17*); *fos*, a 1.1-kb Pst I–Bgl II fragment of v-*fos* (*25*); *env*, a 2.1-kb Bam HI–Xba I fragment in PBR (obtained from E. Hunter); control plasmid pUC19 (*45*).

the same as that in treated or untreated MSB cells. Thus, we conclude that the differences in steady-state levels of c-*myc* RNA observed in MSB and S13 cells is a result of different rates of transcription of a rapidly degraded message.

To determine whether the results with S13 and MSB cells were generally true for bursal lymphoma cell lines and non-established normal cells, runoff transcription assays were performed with nuclei from the five other cell lines (Fig. 1), as well as CEF, and lymphocytes from normal chicken bursa (B) and thymus (T). We found no decrease in c-*myc* transcriptional activity after protein synthesis inhibition in any of the normal cells examined. In fact, a small increase in c-*myc* transcription was noted in the CEF (less than twofold after normalization to the *fos* control) (Fig. 4B). Small increases were also noted in the MSB cells and the thymic lymphocytes, but not bursal cells. In contrast, five of the cell lines showed decreases (ranging from five- to tenfold) in the level of c-*myc* transcript measured in the runoff assay (S13, BK25, 293S, 243L, and HI). The BK3A cell lines, which behaved like normal cells after CH treatment with respect to the c-*myc* steady-state levels, also behaved like normal cells in runoff transcription levels (Fig. 4D). All of the bursal lymphoma cell lines contain numerous copies of integrated ALV and produce infectious virus. Thus, we could also measure the transcriptional activity of the LTR promoted viral structural gene (*gag*), and found that changes in transcription of viral *gag* sequences after CH treatment paralleled that of c-*myc* sequences in the cell lines (Fig. 4B). For one cell line, 293S, we examined transcription of the viral LTR sequences and the envelope genes as well (Fig. 4C). All of the viral sequences and c-*myc* exhibited a concomitant decrease in transcription after CH treatment.

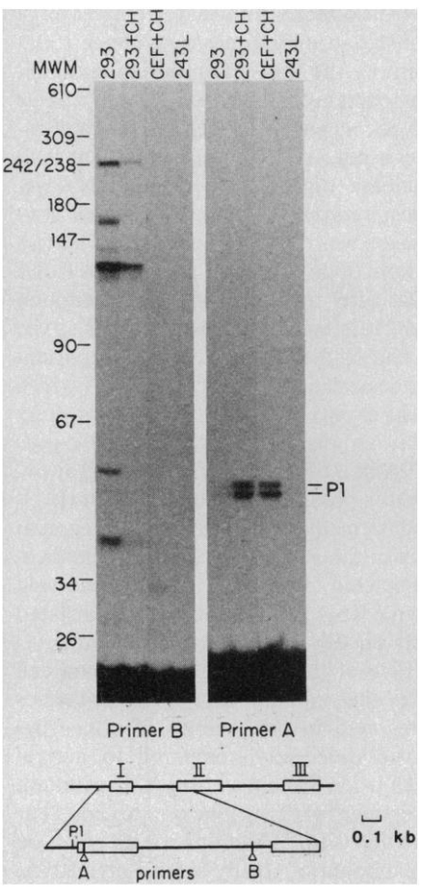

Fig. 5. Primer extension analysis of c-*myc* transcripts encoded by 293S cells. Synthetic oligonucleotides based on the chicken c-*myc* sequence were synthesized commercially. The location of the primers relative to the c-*myc* locus is shown at the bottom. The oligonucleotides were end-labeled with [γ-^{32}P] ATP and T4 polynucleotide kinase. RNA's used were from 293S cells (+) or (−) CH treatment for 3 hours, chick embryo fibroblasts (CEF) treated with CH, or untreated 243L cells. Hybridization to total cell RNA was at 65°C for 3 hours and elongation of primers utilized avian myeloblastosis virus reverse transcriptase as described (*46*). Products were analyzed on an 8 percent polyacrylamide denaturing gel. Molecular weight markers (MWM) used were Msp I–digested lambda DNA end-labeled with ^{32}P-labeled TTP and CTP.

The cycloheximide-sensitive factor, presumably a protein, which interacts with the viral LTR to regulate transcription could be either of viral or cellular origin. Since we found that the level of viral genes in most bursal lines was CH-sensitive, we analyzed the transcription of the viral *gag* gene in CEF infected with the avian leukosis virus RAV-1, or the ALV released from S13 cells [(S13 ALV); Fig 4E)]. As in the bursal lymphoma experiments, more than 90 percent of protein synthesis in the infected CEF was inhibited by CH (*19*). However, no difference in the transcription of *gag* was noted between control and CH-treated infected CEF (*27*). Further evidence for the cell-specific nature of the CH-sensitive protein comes from the experiment with BK25 c-*myc* transfected Rat-2 cells (Fig. 2D). In these cells, unlike bursal lymphoma BK25 cells, there was increase in the chicken c-*myc* transcript steady-state level on CH treatment, suggesting that transcription mediated by the identical LTR is under different control in the two cell types.

Qualitative changes in c-*myc* transcripts after cycloheximide treatment. The 293S cells contain two c-*myc* alleles, the rearranged allele with viral LTR sequences inserted within intron 1 (Fig. 1), and a normal allele. We detected at least two distinct c-*myc* RNA's in 293S cells and have found that, after CH treatment of 293S cells, the larger species of c-*myc* RNA is preferentially lost from the steady-state RNA pool (Fig. 2B) (*19*). To ascertain which sequences comprise the different 293S c-*myc* RNA's, primer extension experiments were performed with a synthetic oligonucleotide primer (primer A) specific for the normal c-*myc* promoter at the end of exon 1, P1 (*8*), and another oligonucleotide primer (primer B) specific for intron 1 sequences (Fig. 5).

In untreated 293S cells we found a

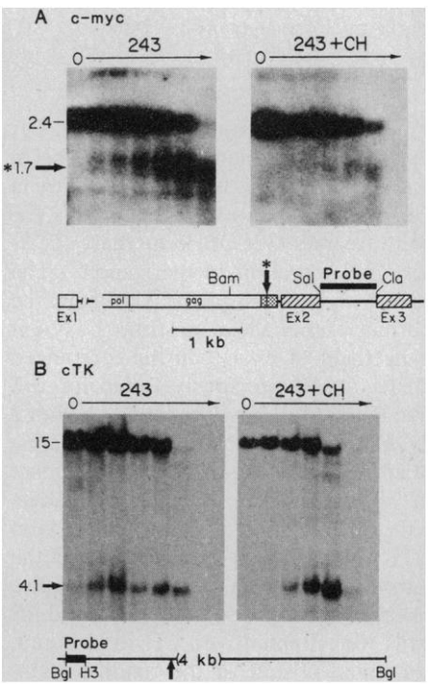

Fig. 6. DNase I digestion of 243L nuclei. Nuclei from cell line 243L either untreated or treated with CH (30 μg/ml) for 3 hours were digested with increasing concentrations of DNase I (as designated by the direction of the arrow: 0, 0.3, 0.6, 1.2, 2.4, 4.8, 7.2 μg/ml); DNA was isolated, digested with the enzymes denoted, and subjected to blot hybridization after electrophoresis in 1 percent agarose gels. The blots were probed with the nick-translated probes designated below the autoradiograms. Nuclear isolation, DNase I digestion of nuclei, DNA purification, restriction enzyme digestion, and blot hybridization were performed as described (*28*). (A) DNA samples were digested with Cla I and Bam HI and blots were hybridized to a 1-kb Cla I–Sal I fragment that essentially spans the second c-*myc* intron as indicated in the line drawing. A 2.4-kb parent band and 1.7-kb subband mapping to the LTR sequences are evident in the control samples. (B) Duplicates of samples used in (A) were digested with Bgl II and blots were hybridized to a 675-bp fragment from the 3′ region of the chicken TK (cTK) gene (*47*).

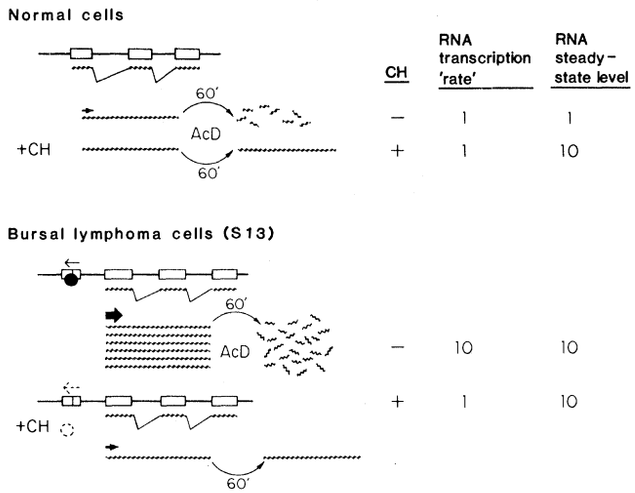

Fig. 7. Model for the effect of cycloheximide on c-*myc* transcription in normal and bursal lymphoma cells. Open boxes and solid lines represent the c-*myc* gene and, in the case of S13 cells, the placement of the viral LTR upstream of exon 1 in the opposite transcriptional orientation of c-*myc* (<--). The wavy lines represent the c-*myc* transcript in the presence or absence of cycloheximide (CH) before or after treatment of cells with actinomycin D for 60 minutes to inhibit synthesis of new transcripts. The size of the arrows above the wavy lines indicates the transcription rate. The solid circle represents the putative labile LTR binding protein which ceases to be synthesized in the presence of CH (dotted circle). The RNA transcription "rate" is derived from runoff transcription analyses (Fig. 4) and the steady-state RNA level from Northern blot analyses (Figs. 2 and 3).

small amount of transcription from P1, yielding an extension product identical in size to that of the bona fide P1 start in CEF (*8*). (In this experiment, the CEF were treated with CH to increase the c-*myc* RNA present in the sample.) The 293S c-*myc* transcripts could be from the normal c-*myc* allele, or from LTR enhancement of exon 1 in the rearranged allele, as we previously found for cell line BK25 (*8*). Using primer B, we found 293S cell-specific transcription initiating in intron 1, yielding an extension product of about 250 bases, which represents either transcripts originating in the viral LTR (*28*), or at a cryptic promoter in the intron, as has been seen in some mouse plasmacytomas (*29*). When the 293S cells were treated with CH for 3 hours, we found a shift in the relative abundance of transcripts detected by primers A and B. There was about a tenfold increase in the (approximately) 50-base P1 extension products and an approximately fivefold decrease in the 250-base product from the intronic region. These results, in conjunction with the nuclear transcription analyses (Fig. 4) demonstrate that in the absence of protein synthesis there is a shift in c-*myc* promoter utilization in 293S cells.

Changes in chromatin structure after cycloheximide treatment. Since protein synthesis inhibition led to both quantitative and qualitative changes in c-*myc* transcription in bursal lymphoma cells, we examined the structure of chromatin surrounding the c-*myc* alleles. We previously reported that after integration of the ALV LTR into the c-*myc* region of bursal lymphoma cells, the major hypersensitive site within this chromosomal domain is within the proviral LTR (*30*). If a labile protein were involved in the enhanced transcription directed by the ALV LTR, this protein might be involved in the formation of the hypersensitive site within the LTR, and the diminution of this protein by CH treatment might result in the loss of this altered chromatin structure. To test this, we chose 243L cells, which contain only one c-*myc* allele, and in which the LTR integration has occurred in the first intron (Fig. 1), sufficiently distant from the 5′ end of the first c-*myc* exon to permit us to distinguish the major c-*myc* hypersensitive sites from that of the LTR. When DNA from 243L nuclei digested with DNase I is redigested with Cla I and Bam HI and assayed for (Southern) subbands, a prominent subband (indicated by the arrow) is found within the LTR region (Fig. 6A). When the same assay is performed on 243L cells that have been treated with CH for 3 hours, this major site is barely detectable. As a control, when the same DNA samples are digested with Bgl II and blot hybridized to a thymidine kinase (TK) probe (Fig. 6B), the major TK hypersensitive site in the 3′ region of the TK locus (*31*) is detectable in both treated and untreated nuclei, indicating that the loss of the LTR hypersensitive site is not due to the inability to detect such sites in DNA from the CH-treated 243L cells.

Implications for regulation of c-*myc* expression. Regulation of the gene products of the c-*myc* locus occurs at several levels. Since both the c-*myc* mRNA (*12*) and (Fig. 3) and the c-*myc* proteins (*11*) are unstable, it appears that cells have dual mechanisms to prevent accumulation of high levels of c-*myc* proteins. We have now found that treatment of all normal avian cells by inhibitors of protein synthesis dramatically increases the steady-state level of c-*myc* RNA, as had been previously found for some mammalian cells and regenerating rat liver (*12, 13, 32*). This is caused by stabilization of c-*myc* RNA that continues to be synthesized at the normal level. It is possible that c-*myc* RNA is degraded by a specific ribonuclease with a high turnover rate; however, this does not appear likely since treatment of MSB cells with emetine for only 20 minutes resulted in a 20-fold increase in the steady-state level of c-*myc* RNA (Fig. 3A). A more attractive hypothesis is that degradation of c-*myc* RNA is directly coupled to completion of translation of the message. Since treatment by the translation initiation inhibitor pactamycin also increased c-*myc* RNA concentrations (Fig. 3B), stabilization is not caused merely by resistance to nuclease digestion of c-*myc* RNA sequestered on polysomes. A similar conclusion was reached in the case of stabilization of histone H4 mRNA in HeLa cells after protein synthesis inhibition (*33*). It is unlikely that sequences at the 5′ end of the c-*myc* RNA are important for degradation, since c-*myc* RNA which lacks exon 1 sequences (243L cells) (Fig. 5) is as unstable as the normal c-*myc* RNA in CEF (Fig. 3, B and C). However, it is possible that sequences at the 3′ end of the message could signal degradation of the RNA only after translation is completed. This would predict that each c-*myc* RNA molecule may be translated only once.

Five of the six bursal lymphoma cell lines that contain viral LTR sequences integrated in the vicinity of c-*myc* behaved differently compared to normal cells when protein synthesis is inhibited. Treatment with cycloheximide (Fig. 2) or emetine (Fig. 3) had little or no effect on the amount of steady-state c-*myc* RNA, but a five- to tenfold decrease was observed in c-*myc* transcription as measured in nuclear runoff experiments (Fig. 4). In these bursal lymphoma cell lines, c-*myc* RNA is unstable, and the RNA turnover can be reduced by CH treatment as in normal cells. However, in these cell lines, transcription of c-*myc* is further controlled by proximal viral LTR sequences acting as enhancers (*16*) on the normal c-*myc* promoter, or as promoters and enhancers. We suggest that in the bursal lymphoma cell lines a rapidly turning over protein interacts with the viral LTR to increase the transcription from the c-*myc* gene. In the absence of protein synthesis, the puta-

tive regulatory protein disappears, and the transcription level reverts to that seen in normal cells (Fig. 5). Thus, the lack of change in the steady-state level of c-*myc* RNA in CH-treated bursal lymphoma cell lines can be explained by a decrease in the rate of c-*myc* transcription and a concomitant increase in the half-life of c-*myc* RNA (Fig. 7).

This putative regulatory protein could interact either with LTR enhancer sequences alone (S13) or with promoter and enhancer sequences (293S, BK25) to increase transcription of genes under LTR control, including c-*myc* and *gag* protein. Such a protein could be either of cellular or viral origin. Recently, an alternately spliced mRNA from the Rous sarcoma virus *gag* gene region which might encode a protein that interacts with LTR sequences to increase transcription was described (*34*). An alternative possibility is that the control protein is a cellular protein found in the bursal target cells for ALV-induced transformation. This normal cellular protein might then fortuitously interact with regions of the ALV LTR containing sequences similar to cellular DNA sequences that are normal binding sites for the protein.

The loss of the LTR-associated hypersensitive site in cell line 243L (Fig. 6) is reminiscent of similar changes in the murine mammary tumor virus (MMTV) LTR upon withdrawal of hormone (*35*), and is compatible with the idea that a labile protein involved in the enhanced transcription directed by the LTR might also be involved in the generation of the hypersensitive site. That protein may be involved in the formation of hypersensitive sites has been suggested by reconstitution experiments with the chicken β^A globin gene and nuclear extracts from avian red blood cells (*36*). In addition, a HeLa cell factor which interacts with the sequences in the SV40 enhancer has been described (*37*), as has a cellular protein which binds to the MMLV LTR and the BK virus enhancer, as well as to sequences 5′ to the chicken lysozyme gene (*38*).

One bursal lymphoma cell line, BK3A, was found to behave like normal cells in response to protein synthesis inhibition, in that steady-state c-*myc* RNA level increased, and the level of c-*myc* nuclear runoff transcription was not affected. It is possible that BK3A cells represent a different stage in B-cell development from the other lines, or that the putative LTR binding protein is not labile in these cells. Alternatively, these cells could originally have been infected with an ALV with an altered LTR.

Our results measuring runoff transcription in ALV-infected CEF (Fig. 6D) show that the *gag* gene is actively transcribed in these cells, but that transcription of *gag* and c-*myc* genes is not cycloheximide-sensitive. The level of *gag* transcription in infected CEF is at least as high as that in the bursal lymphoma cell lines. We have also obtained similar results with ALV–infected thymic and peripheral blood lymphocytes obtained from mature chickens infected in vivo (*19*). This would tend to suggest that the CH-sensitive protein is tissue- or cell type–specific, rather than viral-specific. However, this leaves us with a paradox, since the viral LTR functions in CEF's and other normal cells to drive *gag* gene transcription. There are at least three different hypotheses to explain this: (i) Transcription from the LTR does not normally require any specific regulatory proteins but in bursal cells a tissue-specific unstable protein fortuitously interacts with the LTR. This model seems unlikely since it would predict that in the absence of the protein (as after CH treatment) the level of transcription from the LTR would remain high. (ii) The same regulatory protein functions in all cell types, but the protein is modified in the target cells for ALV-induced bursal lymphomas so that it becomes unstable, or regulation of the gene coding for this protein differs in bursal and other cells. (iii) Different cellular regulatory proteins which recognize LTR sequences are present in different cells or tissues. Although at present we have no data to support this last idea, it appears to be the most attractive of the three.

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27. The CEF contained primarily intact proviruses, and the bursal cell lines contained a mixture of truncated and full-length proviruses. Since the total LTR driven transcription in the cell lines decreases five- to tenfold with CH treatment, we suspect that the LTR's of both types of proviruses behave similarly. In fact, sequence analysis of the BK25 c-*myc* associated LTR shows it to be very similar to functional ALV LTR's (*21*).
28. Although we could not detect any LTR U5 sequences on 293S mRNA by Northern blot analysis, the size of the 250-bp primer extension product and the approximate location of the LTR as mapped by restriction enzyme digestion of 293S cellular DNA is consistent with promotion from the normal ALV LTR R region promoter.
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40. Rat-2 (pBK25) cells were obtained by co-transfection with pRSV-neo plasmid DNA. This DNA contains the neomycin resistance gene (*41*) under RSV LTR control (obtained from B. Howard) and the pBK25 plasmid, which contains 9 kb of sequences from the Bam HI site in the ALV *pol* gene to the Bam HI site 3′ of the c-*myc* gene (obtained from W. S. Schubach). Clones resistant to G418 were screened for intact BK25 sequences by Southern blotting and chicken c-*myc* RNA by Northern blotting. One positive clone, N132C, was used for the CH experiment. Splenic, thymic, and bursal lymphocytes were obtained by gentle teasing of the organs from a 4- to 6-week-old chicken and filtering the cells through nylon mesh to remove clumps. Contaminating nonlymphoid cells were removed by centrifuging the cell suspension over Ficoll-Hypaque. More than 95 percent of the cells prepared from the bursa stained positively with monoclonal antibodies specific for HLA class II and surface immunoglobulin antigens, and more than 95 percent of the cells from the thymus stained positively for thymocyte antigens (*19*).
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44. Each reaction consisted of 210 μl of nuclei, 60 μl of 5× runoff buffer (5× consists of 25 m*M* tris HCl, *p*H 8.0, 12.5 m*M* $MgCl_2$, 750 m*M* KCl, and 1.25 m*M* triphosphates of A, G, and C). α-^{32}P-labeled uridine triphosphate (30 μl; ~450 μCi;

3000 Ci/m*M*) (UTP) was then added, and the nuclear suspension was incubated at 30°C for 30 minutes, after which time 15 μl of DNase I (5 μg/ml) in 10 m*M* $CaCl_2$ (5 μg/ml) was added. After 5 minutes at 30°C, the reaction was made 1× SET (1 percent sodium dodecyl sulfate (SDS), 5 m*M* EDTA, 10 m*M* tris-HCl, *p*H 7.4), and proteinase K was added to a concentration of 200 μg/ml. After incubation at 37°C for 45 minutes, the solution was extracted with an equal volume of a mixture of phenol and chloroform, and the interphase was again extracted with 100 μl of 1× SET. Ammonium acetate (10*M*) was added to the combined aqueous phases (original plus reextraction) to a final concentration of 2.3*M*, an equal volume of isopropyl alcohol was added, and nucleic acid was precipitated (−70°C for 15 minutes). The precipitate was centrifuged in a microcentrifuge for 10 minutes, and the pellet was resuspended in 100 μl of TE (10 m*M* tris-HCl, 1 m*M* EDTA) and centrifuged through a G-50 (medium) spin column. The eluate was made 0.2*M* in NaOH and after 10 minutes on ice, HEPES was added to a concentration of 0.24*M*. Two and one-half volumes of ethanol were then added, and the solution containing the precipitate held overnight at −20°C. After centrifugation in a microcentrifuge for 5 minutes, the pellet was resuspended in hybridization buffer, which consisted of [10 m*M* TES, *p*H 7.4, 0.2 percent SDS, 10 m*M* EDTA, 0.3*M* NaCl, 1× Denhardt's, and *Escherichia coli* RNA (250 μg/ml)]. Nitrocellulose filters containing plasmid DNA's were prepared with a Schleicher & Schuell Slot Blot Apparatus under conditions suggested by S and S, except that wells were washed with 10× SSC (saline sodium citrate). These filters were first hybridized in the hybridization solution described above for a minimum of 2 hours at 65°C. After this preliminary hybridization, the filters were hybridized to the runoff products in hybridization solution for 36 hours. A typical reaction contained 2 ml of hybridization solution with 1 × 10^7 cpm/ml. After hybridization, filters were washed for 1 hour in 2× SSC at 65°C. The filters were then incubated at 37°C in 2× SSC with RNase A (10 mg/ml) for 30 minutes and were subsequently washed in 2× SSC at 37°C for 1 hour. Alternatively, after hybridization the filters were washed twice for 15 minutes in 0.1 percent SDS, 2× SSC at room temperature, and then washed at 60°C (0.1 percent SDS, 0.1× SSC) for 30 minutes. Either protocol for processing of the filters after hybridization yielded the same specificity in signal. Filters were then exposed to Kodak XAR film in cassettes containing Lightening-Plus screens at −70°C for various times.

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RESEARCH ARTICLE

Tyrosine Kinase Receptor with Extensive Homology to EGF Receptor Shares Chromosomal Location with *neu* Oncogene

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Growth factors and their receptors are involved in the regulation of cell proliferation, and several recent findings suggest that they also play a key role in oncogenesis (*1–4*). Of approximately 20 identified oncogenes, the three that have been correlated with known cellular proteins are each related to either a growth factor or a growth factor receptor. The B chain of platelet-derived growth factor (PDGF) is encoded by the proto-oncogene c-*sis* (*2*), the erb-B oncogene product gp68 is a truncated form of the epidermal growth factor (EGF) receptor (*3*), and the proto-oncogene c-*fms* may be related or identical to the receptor for macrophage colony-stimulating factor ($CSF\text{-}1^R$) (*4*).

The receptor-related oncogenes are members of a gene family in that each has tyrosine-specific protein kinase activity, and is associated with the plasma membrane (*5*). Such features are also shared by several other polypeptide hormone receptors, including those for insulin (*6*), PDGF (*7*), and insulin-like growth factor 1 (IGF-1) (*8*); hence more connections may be found between tyrosine kinase growth factor receptors and tyrosine kinase oncogene products.

Comparison of the complete primary structure of the human EGF receptor (*9*) with the sequence of the avian erythroblastosis virus (AEV) transforming gene, v-erbB (*10*), revealed close sequence similarity; in addition, there were amino and carboxyl terminal deletions that may reflect key structural changes in the generation of an oncogene from the gene for a normal growth factor receptor (*3, 9*). Another oncogene, termed *neu*, is also related to v-erbB and was originally identified by its activation in ethylnitrosourea-induced rat neuroblastomas (*11*). In contrast to v-erbB, which encodes a 68,000-dalton truncated EGF receptor, the *neu* oncogene product is a 185,000-dalton cell surface antigen that can be detected by cross-reaction with polyclonal antibodies against EGF receptor (*11*); *neu* may itself be a structurally altered cell surface receptor with homology to the EGF receptor and binding specificity for an unidentified ligand.

Using v-erbB as a screening probe, we isolated genomic and cDNA clones coding for an EGF receptor-related, but distinct, 138,000-dalton polypeptide having all the structural features of a cell surface receptor molecule. On the basis of its structural homology, this putative receptor is a new member of the tyrosine-specific protein kinase family. It is encoded by a 4.8-kb messenger RNA (mRNA) that is widely expressed in normal and malignant tissues. We have localized the gene for this protein to q21 of chromosome 17, which is distinct from the EGF receptor locus, but coincident with the *neu* oncogene mapping position (*12*). We therefore consider the possibility that we have isolated and characterized the normal human counterpart of the rat *neu* oncogene.

Tyrosine kinase–type receptor gene and complementary DNA. As part of our attempts to isolate and characterize the chromosomal gene coding for the human cellular homologue of the viral erbB gp68 polypeptide, AEV-ES4 erbB sequences (2.5-kb Pvu II fragment of pAEV) (*13*) were used as a ^{32}P-labeled hybridization probe for the screening of a human genomic DNA library at reduced stringency

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