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Intragenic pausing and anti-sense transcription within the murine c-*myc* locus

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We present a detailed analysis of strand-specific transcription in different regions of the murine c-*myc* locus. In normal and transformed cell lines, RNA polymerase II directed transcription occurs in the sense and anti-sense direction. Three noncontiguous regions show a high level of transcription in the anti-sense orientation: upstream of the first exon, within the first intron and in the 3′ part of the gene (intron 2 and exon 3). In a cell line carrying a c-*myc* amplification (54cl2), anti-sense transcription is not uniformly increased throughout the locus and is differentially affected by inhibition of protein synthesis. These results suggest that anti-sense transcription in various parts of the locus is independently regulated. In the sense orientation, transcriptional activity is higher in the first exon than in the rest of the gene indicating that transcription pauses near the 3′ end of the first exon. The extent of this intragenic pausing varies among different cell lines and is most severe in cells with a c-*myc* amplification. Transcription initiation and pausing are both negatively regulated by labile proteins.

Key words: Murine c-*myc* locus/nuclear run-on assays/pausing/anti-sense transcription

Introduction

The c-*myc* proto-oncogene is the cellular homologue of the avian myelocytomatosis virus transforming gene (Hayward *et al.*, 1981). DNA sequence analysis indicate that the c-*myc* gene is made up of three exons and two introns (Stanton *et al.*, 1983, 1984; Battey *et al.*, 1983; Bernard *et al.*, 1983; Watt *et al.*, 1983). The last two exons encode a protein of 439 amino acids. No definite role has been assigned to the first exon, which is clearly non-coding in mouse (Stanton *et al.*, 1984), although the possibility of a long open reading frame in human c-*myc* has been raised by Gazin *et al.* (1984).

It appears that alteration of this gene's pattern of expression either by gene amplification (Alitalo *et al.*, 1983; Little *et al.*, 1983; Schwab *et al.*, 1985), retroviral insertion (Corcoran *et al.*, 1984; Li *et al.*, 1984) or chromosome translocation may be an important step in the development of a variety of neoplasms. C-*myc* can co-operate with other proto-oncogenes to transform primary fibroblasts (Land *et al.*, 1983). Transgenic mice harboring c-*myc* genes driven by tissue-specific regulatory sequences developed malignancies at high frequency in the corresponding tissue (Stewart *et al.*, 1984b; Adams *et al.*, 1985).

The c-*myc* proto-oncogene is expressed from two promoters (P_1 and P_2) in a wide variety of cell types (Stewart *et al.*, 1984a; Yang *et al.*, 1985). Normal c-*myc* RNAs (Dani *et al.*, 1984) and the 62–64-kd c-*myc* polypeptide (Hann and Eisenman, 1984) are highly unstable *in vivo*. The c-*myc* gene product has been reported to be a DNA-binding protein and has been localized within the nucleus (Donner *et al.*, 1982; Abrams *et al.*, 1982). C-*myc* is expressed at the RNA and protein levels throughout the cell cycle, and c-*myc* expression seems to correlate with the competency of cells to enter and progress through the cycle (Hann *et al.*, 1985; Thompson *et al.*, 1985). Transcriptional and post-transcriptional mechanisms are responsible for alterations in c-*myc* expression. The addition of serum or defined growth factors to growth-arrested fibroblasts results in transient increases in c-*myc* RNA levels as a consequence of both increased transcription and enhanced messenger stability (Greenberg and Ziff, 1984; Blanchard *et al.*, 1985). However, the relative contributions of these two factors to c-*myc* induction remain controversial. The repression of normal c-*myc* expression in transformed cells harboring c-*myc* chromosome translocations or *myc* retroviruses has been proposed to be an autoregulatory phenomenon which may be mediated by a repressor at the level of the gene's first exon (Leder *et al.*, 1983; Dunnick *et al.*, 1983; Rabbitts *et al.*, 1984; Rapp *et al.*, 1985). A *cis*-acting negative control element with the opposite properties of a transcriptional enhancer has recently been localized upstream of the murine c-*myc* gene (Remmers *et al.*, 1986).

In this report, we have performed run-on transcription assays with nuclei isolated from normal and transformed murine cells. We show that anti-sense transcription occurs in several regions of the locus and sense transcription pauses near the end of the first exon. The results from cells that contain a c-*myc* amplification, strongly suggest that pausing can be used as a control device to modulate the level of sense transcription in the coding region of the c-*myc* gene.

Results

Small, single-stranded subclones originating from different regions of the c-*myc* locus were prepared in M13 vectors and used as probes in run-on transcription assays to assess the level and polarity of transcription in various parts of the locus. The location of these probes in the c-*myc* locus is shown in Figure 5. Nuclei were isolated from murine fibroblast (NIH3T3), lymphoid (70Z3, ABPC4) and myeloid (FDC-P1) cell lines with normal c-*myc* copy numbers and A-MuLV transformed fibroblasts (54cl2 and N25) with amplified c-*myc* loci (Nepveu *et al.*, 1985). Run-on transcriptions were carried out for 10 min in the presence of [α-^{32}P]UTP. We estimated that the nascent nuclear transcripts were extended by 100–200 nucleotides with this protocol (data not shown). Labelled RNAs were purified and hybridized to an excess of single-stranded M13 c-*myc* DNAs immobilized on nitrocellulose filters. The intensities of these hybridization signals provide a measure of the steady-state distribution of RNA polymerases in c-*myc* chromatin (Schibler *et al.*, 1983). All transcription which we have detected in the c-*myc* locus is directed by RNA polymerase II since it was 98% inhibited by 2 μg/ml of α-amanitin (Figure 1). This concentration of α-amanitin is two orders of magnitude below that required to inhibit RNA poly-

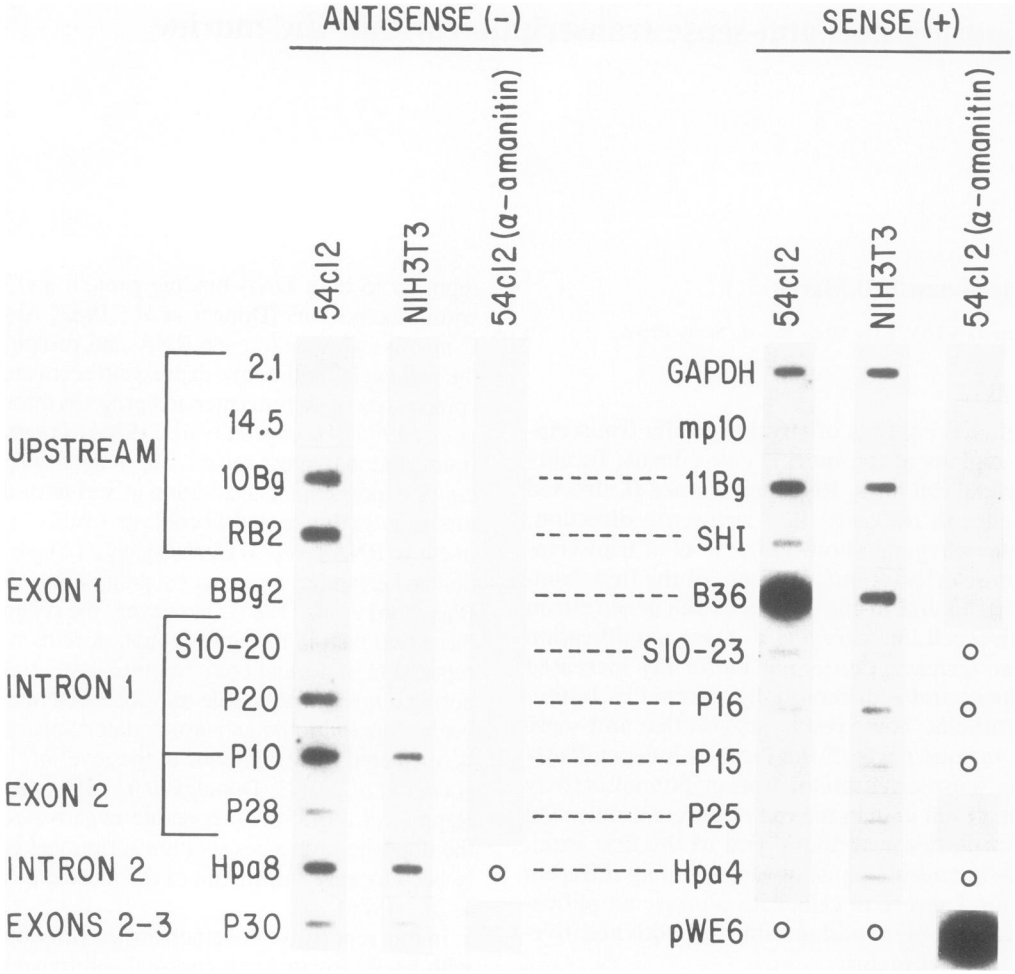

Fig. 1. Run-on transcription analysis of the c-*myc* locus in NIH3T3 and 54cl2 cells. The nascent transcripts were labelled in isolated nuclei (Schibler *et al.*, 1983) and hybridized to an excess of c-*myc* M13 ssDNAs immobilized on nitrocellulose filters. The c-*myc* M13 probes are described in Figure 5. The GAPDH probe is the *PstI* fragment of the rat glyceraldehyde-3-phosphate-dehydrogenase cDNA clone, pRGAPDH13 (Piechaczyk *et al.*, 1984). pWE6 is a hamster ribosomal probe containing most of the 28S sequences plus the spacer between the 18S and 28S genes (Hassouna *et al.*, 1984). Where indicated, α-amanitin was present in the run-on reaction mixture, at a concentration of 2 μg/ml. The level of ribosomal gene transcription was comparable in the presence and absence of α-amanitin (data not shown). The notations 'sense(+)' and 'anti-sense(−)' indicate the orientation of transcription. 54cl2 cells are derived from NIH3T3 and contain a 19-fold amplification of the c-*myc* locus (Nepveu *et al.*, 1985).

merase III whereas RNA polymerase I is virtually unaffected by this peptide (Roeder, 1976).

Sense transcription within the c-myc locus

Transcription is discernible upstream of the c-*myc* promoters in the sense orientation. As shown in Figure 1 for NIH3T3 and 54cl2 cells, it is very intense in the region delimited by the 11 Bg probe (located 424−1140 nt 5′ of P1) and then decreases just before exon 1 as shown with the SHI probe (see Figure 5 for probe locations). It is conceivable that a fraction of these transcription complexes that initiated far upstream also proceed through the gene. However, Northern and S1 nuclease analyses have failed to reveal significant amounts of sense transcripts initiating upstream of the normal c-*myc* initiation sites, P1 and P2 (Yang *et al.*, 1985; Nepveu *et al.*, 1985; data not shown). Clearly, if these transcripts accumulated, they would represent a very small fraction of the c-*myc* mRNAs in the steady-state pool.

The signal obtained for sense transcription in exon 1 (B36 probe) is several fold more intense than that seen in the remaining 3′ portions of the gene (Figure 1 and Table I). It is striking that the lowest level of transcription is found within the coding region of the gene and that it does not notably increase when the gene is amplified. The fact that the first exon probe (B36) gives a stronger signal indicates that the concentration of elongating RNA polymerase II complexes is much higher in the first exon than in the rest of the gene. These results suggest that strong intragenic pausing (or premature termination) occurs *in vivo*. An alternative explanation is that *in vivo* (but not in isolated nuclei), the rate of elongation is slower in exon 1 than in the rest of the gene, leaving the first exon loaded with transcriptional complexes that would resume a fast rate of elongation upon incubation *in vitro*. If this were the case, we would expect the *in vitro* transcriptional activity in intron 1 to increase to the level of that in exon 1, when the elongation is allowed to proceed for a longer period of time. The latter result was not obtained (see Figure 2).

We considered that a high rate of transcription initiation *in vitro* could be responsible for the difference in signals between exon 1 and the remaining portions of the gene. The contribution of re-initiation to run-on transcription assays is generally considered negligible (Weber *et al.*, 1977; Groudine *et al.*, 1981), especially when nuclei are incubated for a short period of time (10 min) as in these cases. To investigate this point, we incubated nuclei from N25 cells, which possess an 8-fold c-*myc* gene amplification (Nepveu *et al.*, 1985), for 35 min with various concentrations of Sarkosyl (see Figure 2). Studies on *in vitro* transcription with nuclear extracts and reconstituted systems, have shown that

Table I. Quantitation of transcription within the c-*myc* locus

DNA probes		Location in gene	NIH3T3	54c12	54c12/NIH3T3
2.1	(−)	Upstream	0	0.8	–
14.5	(−)	Upstream	0.2	0.1	0.5
10Bg	(−)	Upstream	0.1	2.0	20.0
RB2	(−)	Upstream	0.8	7.8	9.8
BBg2	(−)	Exon 1	0.1	0.2	2.0
S10-20	(−)	Intron 1	0.3	0.5	1.7
P20	(−)	Intron 1	0.4	4.1	10.3
P10	(−)	Intron 1	2.7	8.8	3.3
P28	(−)	Exon 2	0.2	1.4	7.0
Hpa8	(−)	Intron 2	2.5	6.3	2.5
P30	(−)	Exon 2–3	0.5	2.0	4.0
11Bg	(+)	Upstream	1.8	3.0	1.7
SHI	(+)	Upstream	0.2	2.5	12.5
B36	(+)	Exon 1	6.3	50.0	7.9
S10-23	(+)	Intron 1	0.6	0.8	1.3
P16	(+)	Intron 1	1.1	2.5	2.3
P15	(+)	Intron 1	1.0	1.8	1.8
P25	(+)	Exon 2	1.0	1.8	1.8
Hpa4	(+)	Intron 2	0.8	1.7	2.1

Run-on transcription assays have been repeated five times for each cell line. The intensity of the signals have been measured by densitometric scanning and the data from different experiments have been normalized using the GAPDH control included in each case. The average value of the signals has then been adjusted by taking into account the number of uridine residues present in the corresponding portions of the gene. Finally, to facilitate the comparison of the data, all transcriptional activities have been expressed relative to the P25 signal in NIH3T3 (exon 2 sense orientation) which has been given an arbitrary value of 1.0.

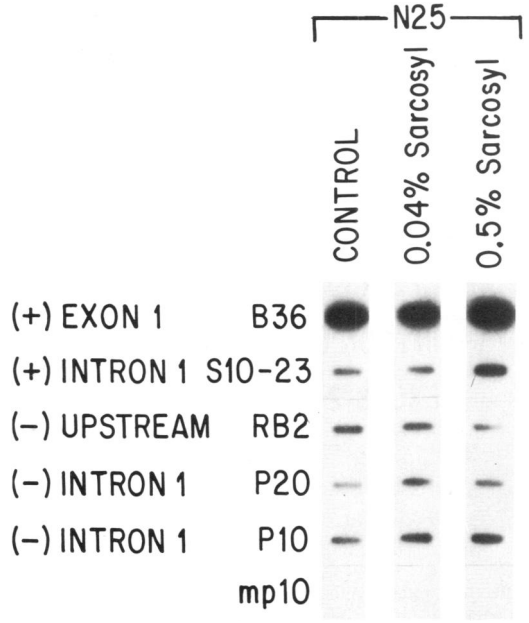

Fig. 2. Run-on transcription analysis in the presence of Sarkosyl. Nuclei were isolated from N25 cells. These cells are derived from NIH3T3 and contain an 8-fold amplification of the c-*myc* gene (Nepveu *et al.*, 1985). Nascent transcripts were elongated *in vitro* for 35 min. Where indicated, Sarkosyl was included in the run-on reaction mixture. The c-*myc* M13 ssDNA probes are described in Figure 5. The signs (+) and (−) signify sense and anti-sense transcription respectively.

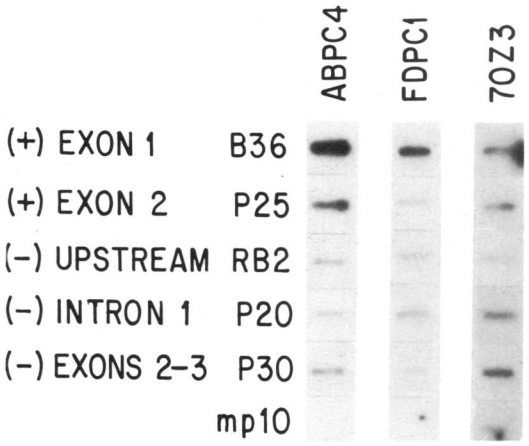

Fig. 3. Run-on transcription analysis of the c-*myc* locus in lymphoid and myeloid cell lines. ABPC4 is a plasma cell tumor with a 6;15 chromosome translocation which contains two intact c-*myc* genes (Ohno *et al.*, 1984). FDPC1 is a myeloid cell line (Dexter *et al.*, 1980). 70Z3 is a pre-B lymphoma (Paige *et al.*, 1978). C-*myc* M13 ssDNA probes are described in Figure 5.

initiation is completely blocked by 0.015% Sarkosyl, whereas elongation is not hampered (Hawley and Roeder, 1985). At higher Sarkosyl concentrations (0.5%), elongation is occasionally stimulated, presumably because most of the chromatin-bound proteins are released (Gariglio *et al.*, 1981; Green *et al.*, 1975). In the absence of Sarkosyl, the transcriptional activity in exon 1 is 12-fold higher than in intron 1. In the presence of 0.04% Sarkosyl, this difference is reduced to 9-fold and, at 0.5% Sarkosyl, it is further reduced to 4.2-fold. However, the absolute level of exon 1 transcription is generally unaffected at both low and high levels of Sarkosyl in this and other similar experiments. Therefore, these results exclude the possibility that re-initiation of transcription *in vitro* is responsible for the unequal transcriptional activities observed for exon 1 and the more 3′ portions of the gene. Rather, these observations confirm that the progression of RNA polymerase II complexes is hindered in the vicinity of the exon 1/intron 1 boundary. It remains to be determined whether this block is associated with the release of transcripts and polymerase II complexes from the DNA template, in which case this would represent premature termination. Until this can be clarified, we will use the term 'pausing' to refer to this intragenic reduction in c-*myc* sense transcription.

Nuclear run-on assays performed with other cell types demonstrated that pausing is a common feature of c-*myc* transcription. Results of experiments with nuclei from a plasma cell tumor with a 6;15 variant translocation (ABPC4), a pre-B lymphoma (70Z3) and an IL-3 dependent myeloid line (FDC-P1) are presented in Figure 3. Pausing is observed in all three cell lines. The transcriptional activity in exon 1 varies substantially but the level of activity in exon 2 is comparable in different lines. This phenomenon was even more apparent for NIH3T3 and 54cl2 (or N25) cells. These results strongly suggest that intragenic pausing is a mechanism of c-*myc* gene control that differentially modulates transcription in various cellular contexts.

Anti-sense transcription within the c-myc locus

In the anti-sense direction, three regions of the c-*myc* locus show high levels of transcription: upstream sequences (probes 10 Bg and RB2), intron 1 (probes P20 and P10) and the 3′ half of the gene (probes Hpa8 and P30) (see Figure 5 for probe locations and Figure 1 and Table I for run-on data in 54cl2 and NIH3T3). The presence of a major DNase I hypersensitive site at the end of the first intron (Fahrlander *et al.*, 1985) could be related to the high level of anti-sense transcription in this region. Reproduc-

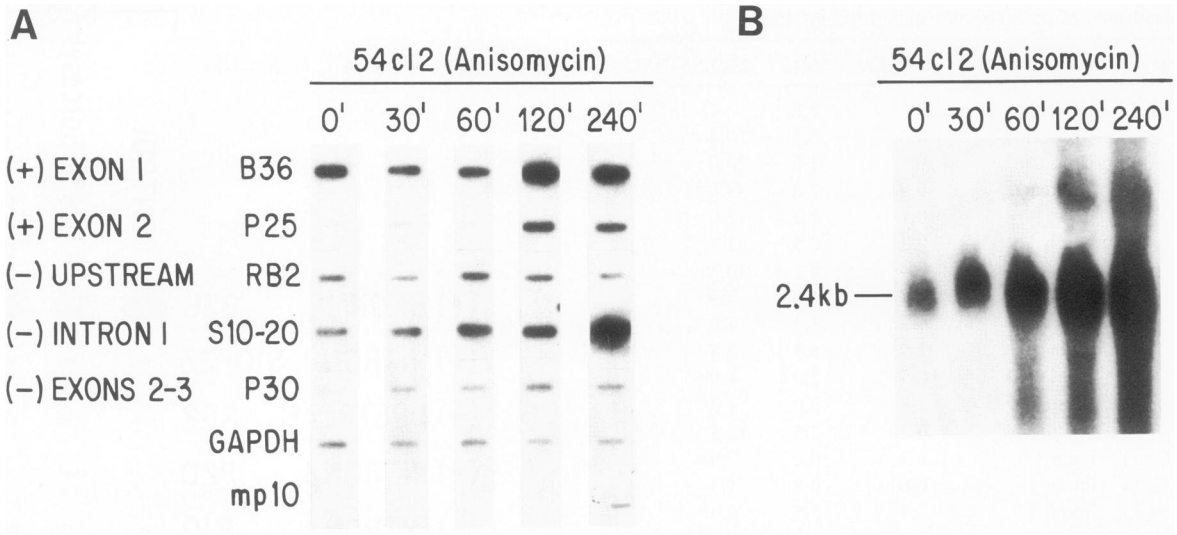

Fig. 4. Effect of protein synthesis inhibition on the expression of the c-*myc* gene in 54cl2 cells. 54cl2 cells were treated with 100 μM anisomycin for various periods of time. Nuclei and cytoplasmic RNAs were purified and analyzed for c-*myc* expression. **(A)** Run-on transcription analysis. DNA probes are described in Figures 5. **(B)** Northern analysis. 30 μg samples of total cytoplasmic RNA were electrophoresed through a 1% agarose–formaldehyde denaturing gel, blotted (Thomas, 1980), and hybridized to a uniformly labeled sense-specific DNA probe complementary to the P28 exon 2 clone (Stanton *et al.*, 1983) (Figure 5).

ible variations in the level of transcription detected by these small, non-overlapping DNA probes suggests that independent anti-sense transcription units exist in different portions of the locus. These observations cannot simply be explained by different degrees of competition between sense and anti-sense RNAs in the hybridization reaction since these results were obtained with RNase pretreated nuclei which do not retain an endogenous pool of unlabelled c-*myc* RNAs.

Similar experiments with lymphoid (ABPC4 and 70Z3) and myeloid (FDC-P1) cell lines in Figure 3 reveal that anti-sense transcription is a common feature of c-*myc* loci. We note that the relative intensities of signals for different anti-sense transcripts fluctuate somewhat in different cell types. However, variations in the level of anti-sense transcription are not correlated with any obvious changes in sense transcription suggesting that they are independently regulated.

Involvement of labile proteins in the negative regulation of c-myc transcription

The increase in c-*myc* mRNA levels after serum stimulation of growth arrested fibroblasts was further augmented upon incubation with a protein synthesis inhibitor (Kelly *et al.*, 1983). Subsequent work has indicated that this effect may largely be due to enhancement of c-*myc* RNA stability (Dani *et al.*, 1984; Greenberg *et al.*, 1986; Thompson *et al.*, 1986). A 2- to 3-fold increase in the induction of c-*myc* transcription in response to inhibition of protein synthesis suggested that a labile repressor may regulate c-*myc* transcription in some cells (Greenberg *et al.*, 1986).

We investigated the effect of inhibition of protein synthesis on c-*myc* expression in 54cl2 cells. The cells were treated with 100 μM anisomycin for various periods of time after which nuclei and cytoplasmic RNAs were prepared for analysis. Anti-sense transcription displays a variety of responses to anisomycin (Figure 4a): (i) it does not significantly change upstream of the gene; (ii) it is enhanced in the 3′ part of the gene up to 5-fold after 120 min; and (iii) it increases up to 9.5-fold in intron 1. These results suggest that anti-sense transcription in various regions of c-*myc* is differentially regulated by negative factors. For sense

Table II. Effect of protein synthesis inhibition on c-*myc* transcription

DNA probe		Location in c-*myc*	0′	30′	60′	120′	240′
B36	(+)	Exon 1	1	0.4	0.6	2.6	1.7
P25	(+)	Exon 2	1	1.2	1.3	14.5	10.0
RB2	(−)	Upstream	1	0.6	1.3	1.2	0.6
S10-20	(−)	Intron 1	1	1.3	2.5	2.9	9.5
P30	(−)	Exons 2–3	1	2.0	3.6	4.9	4.4
B36/P25			28.3	9.5	11.6	5.7	4.9

The run-on transcription assays have been repeated five times for each time point and the relative intensities of different signals were determined by densitometric scanning. Signals with different probes were independently normalized to the values obtained with the same probe at 0 time which were all given an arbitrary value of 1.0. The relative intensities of the signals observed for each probe in the absence of anisomycin are the same as shown in Table I.

transcription, we reproducibly observe a slight diminution in the exon 1 signal (1.8- to 2.6-fold) after a 30–60 min pre-incubation with anisomycin, but this is compensated by a roughly equivalent reduction in pausing (2.4- to 3-fold) such that the transcriptional activity downstream of exon 1 is unaffected (Figure 4a and Table II). However, Northern analysis reveals that enhancement of cytoplasmic c-*myc* RNA levels begins after 30 min of protein synthesis inhibition (see Figure 4b). Therefore, we conclude that this early increase in the c-*myc* mRNA pool is due to post-transcriptional phenomena. After 120 min of anisomycin treatment, sense transcription is elevated 2.5-fold in exon 1 and 14.5-fold in exon 2, and the ratio of the signals from the first and second exons (B36/P25) drops from 28.3 at time 0′ to 5.7 at 120 min. After 4 h treatment with anisomycin, the signals of exon 1 and 2 are augmented 1.7- and 10-fold respectively relative to the levels seen at time 0 and the ratio of the two signals (B36/p25) is 4.9. Therefore, changes occurring at two levels of control, initiation and pausing, contribute to augment c-*myc* sense transcription in response to prolonged treatment with anisomycin. Differential effects in the magnitudes of transcription initiation and pausing during the anisomycin time course suggest that additional fac-

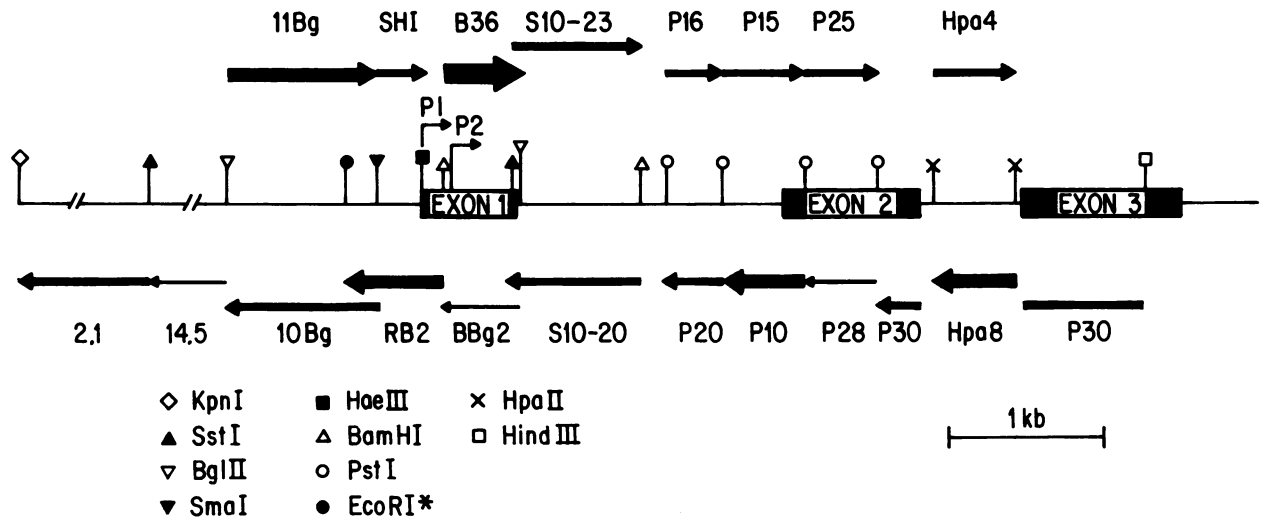

Fig. 5. Transcriptional activity of the c-*myc* locus in 54cl2 cells and locations of DNA probes. Arrow thicknesses approximate the relative levels of transcription in various regions of the c-*myc* locus. The locations and boundaries of c-*myc* DNA segments cloned in M13 vectors are indicated. Single-stranded M13 clones complementary to sense transcripts are shown above the c-*myc* gene map while those complementary to anti-sense transcripts are below.

tors may be required for initiation and these could be depleted by protein synthesis inhibition. We infer from these observations that, in cells containing a c-*myc* amplification, both transcription initiation and pausing are negatively regulated by a labile protein or proteins. Future studies will assess whether the same or different factors are involved in these two levels of transcriptional control.

Discussion

Modulation of c-myc expression by intragenic pausing

The progression of sense-transcription beyond the c-*myc* first exon was hindered to some extent in all cells analyzed. This stresses the importance of using single-stranded probes covering only the coding region of the gene to assess the regulation of c-*myc* gene expression transcriptionally. Probes containing exon 1 sequences would mask the weaker signal from the coding region, which is the most pertinent for the synthesis of the c-*myc* gene product. It is remarkable that in most instances only a minor fraction of RNA polymerase II transcriptional complexes (that initiate at the normal c-*myc* start sites, P1 and P2), reach the coding region of the gene. Therefore, intragenic pausing (or premature termination) has a profound effect on c-*myc* transcription. The degree of pausing was shown to vary in different cellular contexts and was proportional to the level of sense-transcription within the first exon. Consequently, only minor variations in sense transcription downstream of exon 1 were detected. These observations strongly suggest that pausing is a control device used by the cell to modulate the level of sense transcription (in the coding region) of the c-*myc* gene. Since the extent of pausing varies in different cellular contexts, its modulation is very likely to involve cellular factors.

We suggest two alternative models for c-*myc* pausing which involve either positive or negative regulatory factors. The first model would stipulate that a DNA sequence in the vicinity of the exon 1−intron 1 boundary imposes a secondary structure that is not conducive to the progression of transcription. RNA polymerase II complexes may stall when they encounter such a configuration and progression beyond this point would presumably require the action of some transcriptional activators, possibly analogous to anti-termination factors found in *Escherichia coli* (Greenblatt, 1981). Premature termination also takes place in the major late transcription unit of adenovirus type 2. This has been shown to occur *in vivo*, in isolated nuclei and in reconstituted systems (Maderous and Chen-Kiang, 1984; Hawley and Roeder, 1985). The addition of the IIs transcription factor to a reconstituted system increased the efficiency of RNA polymerase II complexes to progress through this site in the adenovirus genome, while the extent of pausing was augmented by levels of Sarkosyl exceeding 0.015% (Reinberg *et al.*, in preparation; Hawley and Roeder, 1985). In our system, we did not observe augmentation of pausing when we added 0.04% or 0.5% Sarkosyl to the run-on reaction mixture. In addition, pausing was reduced after the cells had been treated with a protein-synthesis inhibitor, which is not easily reconciled with the concept of an activator like the anti-termination factors (Greenblatt, 1981). These results are likely to reflect fundamental differences in the mechanisms of pausing and read-through transcription in adenovirus and the c-*myc* gene.

The second model may or may not involve a particular secondary structure, but in this case, the association of negative regulatory factors with the c-*myc* chromatin would be required to halt the progression of transcription. The relief in pausing that we observe after treatment of cells with anisomycin tends to favor such a mechanism. This result further suggests that at least one of the negative factors is a labile protein. The fact that the transcriptional activity in the first exon was also increased, after prolonged treatments with anisomycin, indicates that transcription initiation is also negatively affected by a labile protein. It is possible that the same labile protein takes part in the down-regulation of initiation and pausing. However, the kinetics and magnitude of the changes in initiation and pausing were not the same suggesting that positive as well as negative regulatory factors are involved.

Existence of anti-sense transcription within the c-myc locus

The discovery that transcription also takes place in the opposite or anti-sense orientation raises provocative questions about its role, if any, in gene expression. It is striking that the regions of highest anti-sense transcription (upstream of the first exon, intron 1 and intron 2) consist of sequences which are not present in mature c-*myc* mRNAs. Therefore, it is tempting to consider

that such anti-sense transcripts could play some role in the maturation of c-*myc* sense RNAs. Promoter interference has been evoked to explain why a promoter located downstream of another one often shows weaker activity (Cullen *et al.*, 1984). It is not known if the same kind of interference could occur when transcription units are opposing each other. Overlapping transcription units of opposite polarity are frequently found in DNA tumor viruses, but in these cases one unit is preferentially or exclusively expressed early in infection whereas transcription in the other orientation becomes prevalent late in infection (Acheson, 1980; Flint and Broker, 1980).

We have not detected distinct species of anti-sense transcripts by Northern or S1 analysis. S1 nuclease mapping of nuclear transcripts was performed with various uniformly labelled single-stranded M13 probes. With some anti-sense specific probes, we reproducibly observed a protected band corresponding to the full size of the insert, but we never detected a smaller band (data not shown). These results confirm the existence of anti-sense transcripts, already revealed by the run-on transcription assay. However, this also strongly suggests that the ends of the anti-sense transcripts are heterogeneous. This would be the case if anti-sense transcription initiated at many sites and if no precise cleavage took place at their 3′ ends. In this regard, a computer assisted search for sequences often found near the 5′ and 3′ ends of RNA polymerase II transcripts, did not reveal any sequence with a perfect match.

It has been proposed that the presence of a polyadenylation signal determines not only where but whether a transcript is going to be cleaved and processed (Birnstiel *et al.*, 1985). It follows that in the absence of such a signal within a transcription unit, the RNA product could be rapidly degraded. C-*myc* anti-sense transcripts have been found to accumulate in a number of murine plasma cell tumors with translocated, broken *myc* genes. These anti-sense RNAs initiated in the c-*myc* first intron and terminated at polyadenylation sites 3′ of immunoglobulin (Ig) heavy chain constant region (C_H) genes (Dean *et al.*, 1983; Keath *et al.*, 1984; Calabi and Neuberger, 1985). Our results clearly show that anti-sense transcription within the *myc* gene's first intron was already present prior to translocation. We conclude that the products of c-*myc* intron 1 anti-sense transcription accumulated in these tumors solely as a consequence of acquiring IgC_H gene polyadenylation signals.

Finally, the existence of anti-sense transcription within the c-*myc* gene's introns and coding exons indicates that caution should be exercised in interpreting c-*myc* transcription data obtained with duplex DNA probes. This is especially true in situations where c-*myc* regulation was interpreted to be entirely post-transcriptional (Blanchard *et al.*, 1985; Dony *et al.*, 1985; Dean *et al.*, 1986).

Transcriptional down-regulation of amplified c-myc loci

A comparison of nuclear run-on transcription analysis in fibroblastic cells with (54cl2) or without (NIH3T3) a c-*myc* amplification reveals that different regions of this locus are subject to distinct regulatory controls. Anti-sense transcription upstream of the gene and in the first intron is enhanced 10-fold in 54cl2, whereas it is increased only 2- to 4-fold in the 3′ part of the gene. Sense transcription is increased 2-fold upstream of the gene, 8-fold in the first exon but only 1- to 2-fold in the remaining 3′ part of the gene, indicating that pausing is stringently controlled in cells carrying a c-*myc* amplification. These results may explain our previous observation that the steady-state levels of c-*myc* RNAs were elevated only 2-fold in 54cl2 over the level seen in NIH3T3 even though the c-*myc* gene copy number is amplified 19-fold in the former cell line (Nepveu *et al.*, 1985). Similar results were obtained with N25 cells which carry an 8-fold c-*myc* amplification (Nepveu *et al.*, 1985). We conclude that the down-regulation of c-*myc* expression in these cell lines occurs mainly at two stages of the transcriptional process: initiation and pausing. It would follow from these findings that c-*myc* DNA amplifications need not directly result in commensurate increases in c-*myc* RNA levels and that other factors responsible for normal c-*myc* transcriptional control must also be disrupted to cause elevated c-*myc* expression.

After the submission of this manuscript, an independent study presented evidence that c-*myc* down-regulation in differentiating HL60 human promyelocytic leukemia cells largely results from a block in transcriptional elongation (Bentley and Groudine, 1986). The site of this block was localized to the c-*myc* exon 1 −intron 1 boundary (Bentley and Groudine, 1986). These results together with those presented here suggest that pausing of c-*myc* transcription is a general control mechanism for modulating c-*myc* mRNA levels in differentiating and proliferating cells as well as in transformed cells with amplified *myc* loci.

Materials and methods

Cell lines

The cell lines used in these experiments have been described elsewhere: NIH3T3 (Jainchell *et al.*, 1969); 54cl2 and N25 (Rosenberg and Witte, 1980; Nepveu *et al.*, 1985); ABPC4 (Ohno *et al.*, 1984); FDPC1 (Dexter *et al.*, 1980); 70Z3 (Paige *et al.*, 1978).

RNA preparation and Northern analysis

Total RNA was prepared by the guanidinium thiocyanate/hot phenol method essentially as described by Feramisco *et al.* (1982). RNAs were separated by electrophoresis in 1% agarose gels containing 1 M formaldehyde and were transferred to nitrocellulose membranes according to Thomas (1980). Hybridization and washing conditions have been described elsewhere (Marcu *et al.*, 1983).

Nuclei isolation and elongation of transcripts in vitro

Nuclei were purified and nascent transcripts were elongated essentially as described (Schibler *et al.*, 1983). The nascent labelled transcripts were purified according to Groudine *et al.* (1981), except that the unincorporated nucleotides were removed by centrifugation through a G-50 spin column and then the eluate was TCA precipitated in Eppendorf tubes as described (McKnight and Palmiter, 1979).

Slot-blot hybridizations of in vitro labelled nuclear RNAs

DNA samples were fixed onto nitrocellulose filters as described (Piechaczyk *et al.*, 1984) using a BRL Hybri-Slot apparatus. The filters were pre-hybridized for 2 days at 42°C in hybridization buffer which consisted of 50% formamide, 50 mM Hepes at pH 7.0, 0.75 M NaCl, 0.5% SDS, 2 mM EDTA, 0.2% ficoll, 0.2% polyvinylpyrrolidone, 0.2% BSA and 0.5 mg/ml of salmon sperm DNA. Hybridization with run-on transcription products was peformed at 42°C for a minimum of 48 h. The volume of the reaction was 1.5 ml and typically contained 1×10^7 c.p.m./ml. Before addition to the filter the whole hybridization mixture was heated at 70°C for 7 min and cooled on ice for 1 min. After hybridization, the filters were washed in 0.1 × SSC, 0.1% SDS at 65°C. The washing solution was changed every 20 min, at least five times. Filters were covered with Saran wrap and exposed for various times to Kodak XAR film at −70°C with an intensifier screen.

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References

Abrams,H.D., Rohrschneider,L.R. and Eisenman,R.N. (1982) *Cell*, **29**, 427–439.

Acheson,N.H. (1980) In Tooze,J. (ed.), *DNA Viruses: Molecular Biology of Tumor Viruses*. Cold Spring Harbor Laboratory Press, New York, pp. 182–185.

Adams,J.M., Harris,A.W., Pinkert,C.A., Corcoran,L.M., Alexander,W.S., Cory,S., Palmiter,R.D. and Brinster,R.L. (1985) *Nature*, **318**, 533–538.

Alitalo,K., Schwab,M., Lin,C.C., Varmus,H.E. and Bishop,J.M. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 1707–1711.

Battey,J., Moulding,C., Taub,R., Murphy,W., Potter,H., Lenoir,G. and Leder,P. (1983) *Cell*, **34**, 779–787.

Bernard,O., Cory,S., Gerondakis,S., Webb,E. and Adams,J. (1983) *EMBO J.*, **2**, 2375–2383.

Bentley,D.L. and Groudine,M. (1986) *Nature*, **321**, 702–706.

Birnstiel,M.L., Busslinger,M. and Strub,K. (1985) *Cell*, **41**, 349–359.

Blanchard,J.-M., Piechaczyk,M., Dani,Ch., Chambard,J.-C., Franchi,A., Pouyssegur,J. and Jeanteur,Ph. (1985) *Nature*, **317**, 443–445.

Calabi,F. and Neuberger,M.S. (1985) *EMBO J.*, **4**, 667–674.

Corcoran,L.M., Adams,J.M., Dunn,A.R. and Cory,S. (1984) *Cell*, **37**, 113–122.

Cullen,B.R., Lomedico,P.T. and Ju,G. (1984) *Nature*, **307**, 241–245.

Dani,Ch., Blanchard,J.M., Piechaczyk,M., El Sabouty,S., Marty,L. and Jeanteur,Ph. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 7046–7050.

Dean,M., Kent,R.B. and Sonenshein,G.E. (1983) *Nature*, **305**, 443–446.

Dean,M., Levine,R.A. and Campisi,J. (1986) *Mol. Cell. Biol.*, **6**, 518–524.

Donner,P., Greiser-Wilke,I. and Moelling,K. (1982) *Nature*, **296**, 262–265.

Dony,C., Kessel,M. and Gruss,P. (1985) *Nature*, **317**, 636–639.

Dunnick,W., Shell,B.E. and Dery,C. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 7269–7273.

Fahrlander,P.D., Piechaczyk,M. and Marcu,K.B. (1985) *EMBO J.*, **4**, 3195–3202.

Feramisco,J.R., Smart,J.E., Burridge,K., Helfman,D.M. and Thomas,G.P. (1982) *J. Biol. Chem.*, **257**, 11024–11031.

Flint,S.J. and Broker,T.R. (1980) In Tooze,J. (ed.), *DNA Tumor Viruses: Molecular Biology of Tumor Viruses*. Cold Spring Harbor Laboratory Press, New York, pp. 485–487.

Gariglio,P., Bellard,M. and Chambon,P. (1981) *Nucleic Acids Res.*, **9**, 2589–2598.

Gazin,C., Dupont de Dichenin,S., Hampe,A., Masson,J.-M., Martin,P., Stehelin,D. and Galibert,F. (1984) *EMBO J.*, **3**, 383–388.

Green,M.H., Buss,J. and Gariglio,P. (1975) *Eur. J. Biochem.*, **53**, 217–225.

Greenberg,M.E. and Ziff,E.B. (1984) *Nature*, **311**, 438–442.

Greenberg,M.E, Hermanowski,A.L. and Ziff,E.B. (1986) *Mol. Cell. Biol.*, **6**, 1050–1057.

Greenblatt,J. (1981) *Cell*, **24**, 8–9.

Groudine,M., Peretz,M. and Weintraub,H. (1981) *Mol. Cell. Biol.*, **1**, 281–288.

Hann,S.R. and Eisenman,R.N. (1984) *Mol. Cell. Biol.*, **4**, 2486–2497.

Hann,S.R., Thompson,C.B. and Eisenman,R.N. (1985) *Nature*, **314**, 366–369.

Hassouna,N., Michot,B. and Bachellerie,J.P. (1984) *Nucleic Acids Res.*, **12**, 3563–3583.

Hawley,D.K. and Roeder,R.G. (1985) *J. Biol. Chem.*, **260**, 8163–8172.

Hayward,W.S., Neel,B. and Astrin,S. (1981) *Nature*, **290**, 475–480.

Jainchill,J.L., Aaronson,S.A. and Todaro,G.J. (1969) *J. Virol.*, **4**, 549–553.

Keath,E.J., Kalekar,A. and Cole,M.D. (1984) *Cell*, **37**, 521–528.

Kelly,K., Cochran,B.H., Stiles,C. and Leder,P. (1983) *Cell*, **35**, 603–610.

Land,H., Parada,L.F. and Weinberg,R.A. (1983) *Nature*, **304**, 596–602.

Leder,P., Battey,J., Lenoir,G., Moulding,G., Murphy,W., Potter,H., Stewart,T. and Taub,R. (1983) *Science*, **222**, 765–771.

Li,Y., Holland,C., Hartley,J. and Hopkins,N. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 6808–6811.

Little,C.D., Nau,M.M., Carney,D.N., Gazdar,A.F. and Minna,J.D. (1983) *Nature*, **306**, 194–196.

Maderious,A. and Chen-Kiang,S. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 5931–5935.

Marcu,K.B., Harris,L.J., Stanton,L.W., Erikson,J., Watt,R. and Croce,C. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 519–523.

McKnight,G.S. and Palmiter,R.D. (1979) *J. Biol. Chem.*, **254**, 9050–9058.

Nepveu,A., Fahrlander,P.D., Yang,J.-Q. and Marcu,K.B. (1985) *Nature*, **317**, 440–443.

Paige,C.J., Kincade,P.W. and Ralph,P. (1978) *J. Immunol.*, **121**, 641–647.

Piechaczyk,M., Blanchard,J.M., Marty,L., Dani,Ch., Panabieres,F., ElSabouty, S., Fort,Ph. and Jeanteur,Ph. (1984) *Nucleic Acids Res.*, **12**, 6951–6963.

Rabbitts,T.H., Forster,A., Hamlyn,P.H. and Baer,R. (1984) *Nature*, **309**, 592–597.

Rapp,U.R., Cleveland,J.L., Brightman,K., Scout,A. and Ihle,J.N. (1985) *Nature*, **317**, 434–438.

Remmers,E.F., Yang,J.-Q. and Marcu,K.B. (1986) *EMBO J.*, **5**, 899–904.

Roeder,R.G. (1976) In Losick,R. and Chamberlain,M. (eds), *RNA Polymerase*. Cold Spring Harbor Laboratory Press, New York, p. 285.

Rosenberg,N. and Witte,O.N. (1980) *J. Virol.*, **33**, 340–348.

Schibler,N., Hagenbuchle,O., Wellauer,P.K. and Pittet,A.C. (1983) *Cell*, **33**, 501–508.

Schwab,M., Ramsey,G., Alitalo,K., Varmus,H.E., Bishop,J.M., Martinson,T., Levan,G. and Levan,A. (1985) *Nature*, **315**, 345–347.

Stanton,L.W., Watt,R. and Marcu,K.B. (1983) *Nature*, **303**, 401–406.

Stanton,L.W., Fahrlander,P.D., Tesser,P. and Marcu,K.B. (1984) *Nature*, **310**, 423–425.

Stewart,T.A., Bellve,A.R. and Leder,P. (1984a) *Science*, **226**, 707–710.

Stewart,T.A., Pattengale,P.K. and Leder,P. (1984b) *Cell*, **38**, 627–637.

Thomas,P.S. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 5201–5205.

Thompson,C.B., Challoner,P.B., Neiman,P.E. and Groudine,M. (1985) *Nature*, **314**, 363–366.

Thompson,C.B., Challoner,P.B., Neiman,P.E. and Groudine,M. (1986) *Nature*, **319**, 374–380.

Watt,R., Stanton,L.W., Marcu,K.B., Gallo,R.C., Croce,C.M. and Rovera,G. (1983) *Nature*, **303**, 725–728.

Weber,T., Jelinek,W. and Darnell,J.E. (1977) *Cell*, **10**, 611–616.

Yang,J.-Q., Bauer,S., Mushinski,J.F. and Marcu,K.B. (1985) *EMBO J.*, **4**, 1441–1447.

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