Elongation and maturation of c-*myc* RNA is inhibited by differentiation inducing agents in HL60 cells

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ABSTRACT

Maturation of c-*myc* mRNA proceeds in a given order in HL60 cells. It starts with splicing of intron 2, continues with splicing of intron 1 and ends with 3' cleavage and polyadenylation of the primary transcript. This process is inhibited, when HL60 cells were induced to terminal differentiation by dimethylsulfoxide (DMSO). DMSO interferes specifically with maturation of c-*myc* but not c-*abl* RNA in HL60 cells. Simultaneously, DMSO induces a block to RNA elongation at the boundary of c-*myc* exon 1 and intron 1 in HL60 cells. Participation of the same factor(s) in the regulation of c-*myc* RNA elongation and splicing is supposed.

INTRODUCTION

Activation of the proto-oncogene c-*myc* by several different mechanisms is a characteristic of a variety of tumors (1–7). In order to understand the nature of the abnormal activation of c-*myc* in tumor cells it is essential to first understand normal regulation of the gene, since c-*myc* expression is also a characteristic of proliferation in normal cells.

Regulation of the dual c-*myc* promoter P_1/P_2 has been studied in great detail. Transcription from the P_1/P_2 promoter is regulated by two distinct mechanisms, transcription initiation and RNA-elongation. Early down-regulation of c-*myc* expression in differentiating HL60 cells is predominantly regulated by blocking RNA elongation of c-*myc* RNA at the boundary of c-*myc* exon 1 and intron 1 (8,9), whereas c-*myc* expression is shut off by inhibition of transcription initiation during the late phase of differentiation (10). The same transcriptional mechanisms are indicated in c-*myc* activation (11–15). The *cis*-elements involved in regulation of c-*myc* transcription are being intensively studied in many laboratories (16–21) and positively (22,23) and negatively (24) acting factors have been identified.

In serum-deprived hamster fibroblasts (25), differentiating mouse teratocarcinoma cells (26) and mouse erythroleukemia (MEL) cells (27,28), the c-*myc* gene is assumed to be regulated post-transcriptionally. A substantial decrease in the already short half life of c-*myc* mRNA (29) has been reported for Burkitt's lymphoma cells treated with interferon (30) and for MEL cells induced to differentiation by DMSO (27). The cellular localisation and precise mechanisms leading to the absence of cytoplasmic c-*myc* mRNA in cells with a high transcription rate of the gene has, however, been poorly studied. It is unknown, whether reduced stability of cytoplasmic c-*myc* RNA contributes solely to post-transcriptional regulation of c-*myc*.

In the following report maturation of c-*myc* RNA has been studied. Evidence is presented showing that splicing and 3' cleavage occur in a coordinated fashion and are subject to regulation in differentiating HL60 cells.

MATERIALS AND METHODS

Cell lines and cell culture

HL60 is a human promyelotic cell line (31), BL67 a Burkitt's lymphoma cell line carrying a t(8;14) translocation with the breakpoint in the first exon of the c-*myc* gene (32). Cells were grown to $3-6\times10^5$ cells/ml in 10% fetal calf serum, RPMI-1640 medium, supplemented with penicillin, streptomycin and L-glutamine.

Extraction and Northern analysis of total cellular RNA

Total cellular RNA was extracted by the lithium chloride-urea method as described by Auffray and Rougeon (33). RNA blot analysis was performed by fractionation of RNA samples on a 1% agarose-formaldehyde gel (34). Standard procedures were followed for hybridization of blots (35) with multiprime ^{32}P-labelled probes (36), for washing and autoradiography (34).

S1 mapping

Single-stranded uniformly labelled probes were prepared by primer extension of M13 clones. 5'-labelled probes were made by dephosphorylation followed by kinase reaction and 3'-labelled probes with T4- polymerase (34). Hybridization of labelled DNA fragments to total RNA was carried out in a modified method of Berk and Sharp (37). Hybridization mixtures of 20 µl containing ~100 000 c.p.m. of the probe (sp. act. $\sim10^7-10^8$ c.p.m./µg); 40 µg RNA in 90% formamide, 400 mM NaCl, 40 mM Pipes pH 6.5, 1 mM EDTA were denatured at 90°C for 5 min and immediately transferred to 58°C. After 15h the hybridization was terminated by addition of 180 µl ice-cold buffer

containing 250 mM NaCl, 30mM Na-acetate pH 4.5, 2 mM Zn-acetate, 5% glycerol, and 400 U nuclease S1 (Boehringer, Mannheim). The samples were incubated at 25°C for 1h, extracted with TE-saturated phenol, and precipitated with ethanol. Protected DNA fragments were separated on 5% polyacrylamide gels with 7M urea.

RESULTS

Steady state levels of cytoplasmic and nuclear c-*myc* RNA in HL60 cells

DMSO (dimethylsulfoxide) is a potent inducer of granulocytic differentiation in HL60 cells and causes a rapid decrease of cytoplasmic steady state c-*myc* RNA (38). This decrease is mainly regulated at the level of transcript elongation at the end of the first exon (8,9). In order to analyse whether post-transcriptional regulation in the nucleus also contributes to down-regulation of c-*myc* the levels of spliced and unspliced c-*myc* RNA were studied in differentiating HL60 cells. Figure 1a shows a Northern blot of total cellular RNA harvested at different times after addition of 1.25% DMSO which induces granulocytic differentiation within four days in almost 100% of the cells. Apparently, the steady state c-*myc* RNA starts to decrease immediately after the addition of the inducer and is down to less than 5% of the initial amount of uninduced cells after 2h.

The level of c-*myc* precursor RNA was studied with an intron 1-specific probe. Unspliced but 3′ cleaved c-*myc* precursor RNA should give rise to molecules of ~ 3.8 kb (containing intron 1 or intron 2) and ~ 5.2 kb (containing intron 1 + 2). Surprisingly, neither a 3.8 kb nor a 5.2 kb c-*myc* RNA was detected in total RNA of HL60 cells. Instead, intron 1 sequences hybridized to a smear of high molecular weight RNA (5 to 15 kb) which did not significantly decrease in HL60 cells after treatment with DMSO (Figure 1b). This suggests that the majority of c-*myc* precursor RNA molecules exists in a 3′ uncleaved configuration preventing the detection of distinct RNA size classes. These precursor molecules may be stabilized and not further processed, as *de novo* synthesis of c-*myc* RNA is blocked in HL60 cells after addition of DMSO.

A comparison of the hybridization signals obtained with exon 3 (3h exposure, Figure 1a) and intron 1 (7d exposure, Figure 1b) probes indicates that approximately 5% of c-*myc* RNA in untreated HL60 cells is unspliced. To validate this estimation, the ratio of spliced and unspliced c-*myc* RNA was studied in S1 protection experiments. An S1 probe spanning the entire first exon (including the boundary to intron 1) allowed the discrimination of spliced and unspliced P_1- and P_2-transcripts in total cellular RNA (Figure 2a). In HL60 cells, the c-*myc* gene is preferentially transcribed from the P_2-promoter. Densitometric scanning of the autoradiogram revealed that about 5% of P_2-specific transcripts contained an unspliced first intron. The amount of this unspliced RNA did not significantly decrease after addition of DMSO. A similar result was obtained with an S1 probe spanning the boundary of exon 2 to intron 2 (Figure 2b). Thus, S1 analysis confirmed the results of Northern analysis that about 5% of c-*myc* RNA in HL60 cells is in an unspliced configuration. The structure of this RNA was studied in further S1 experiments.

Structural characterization of nuclear c-*myc* RNA: splicing precedes 3′ cleavage of c-*myc* RNA in HL60 cells

Two S1 probes were constructed in order to elucidate the sequential steps of c-*myc* RNA maturation in HL60 cells. Probe

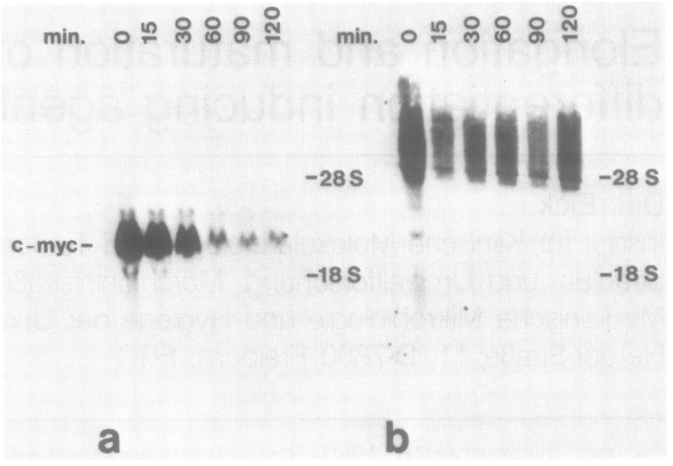

Figure 1. Northern blot analysis of total RNA of HL60 cells after DMSO treatment. HL60 cells were incubated in the presence of 1.25% DMSO. RNA was extracted from aliquots at various times after addition of DMSO and analysed on Northern blots hybridized with a third exon c-*myc* probe (*Cla*I-*Eco*RI, 1.4 kb) (a), or with a first intron probe (*Sac*I-*Sma*I, 0.35 kb) (b). The time of exposure of the autoradiograms was 3h (a) and 7 days (b).

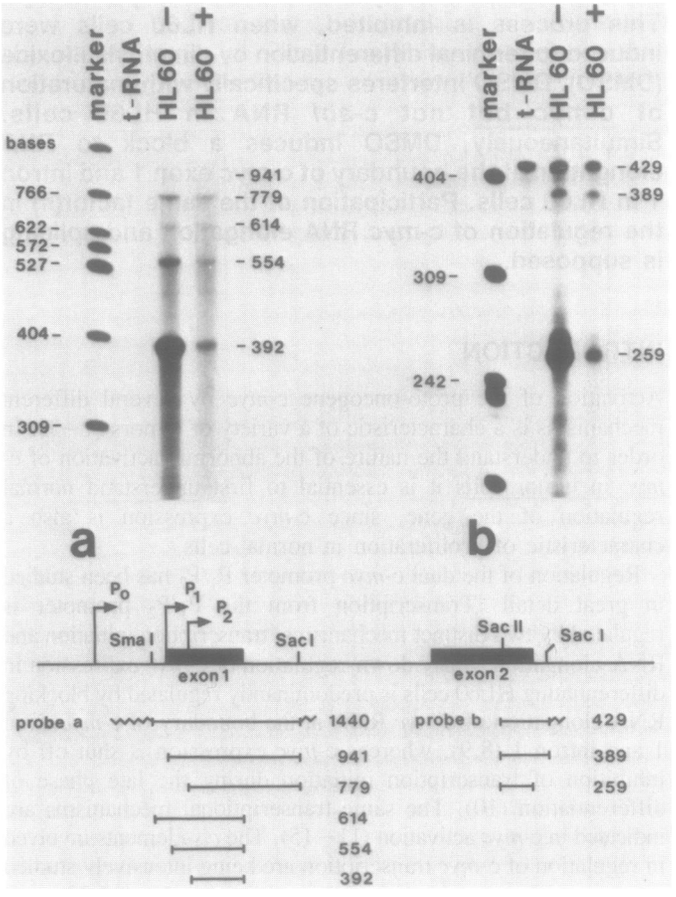

Figure 2. S1 analysis of total c-*myc* RNA. HL60 cells were incubated in the absence (−) or presence (+) of DMSO for 1h before RNA extraction. Labelled DNA probes were hybridized to total cellular RNA or yeast tRNA (Boehringer, Mannheim). M13 clones containing a *Sma*I-*Sac*I fragment (protecting the first exon derived RNA completely) (probe a), and a *Sac*II-*Sac*I fragment (probe b) were labelled with [^{32}P] by primer extension. The probes and the size of the protected fragments are described in the map shown below the autoradiograms. Molecular weight standards are end-labelled *Hpa*II fragments of the lymphotropic papova virus (LPV) cloned in pBR322.

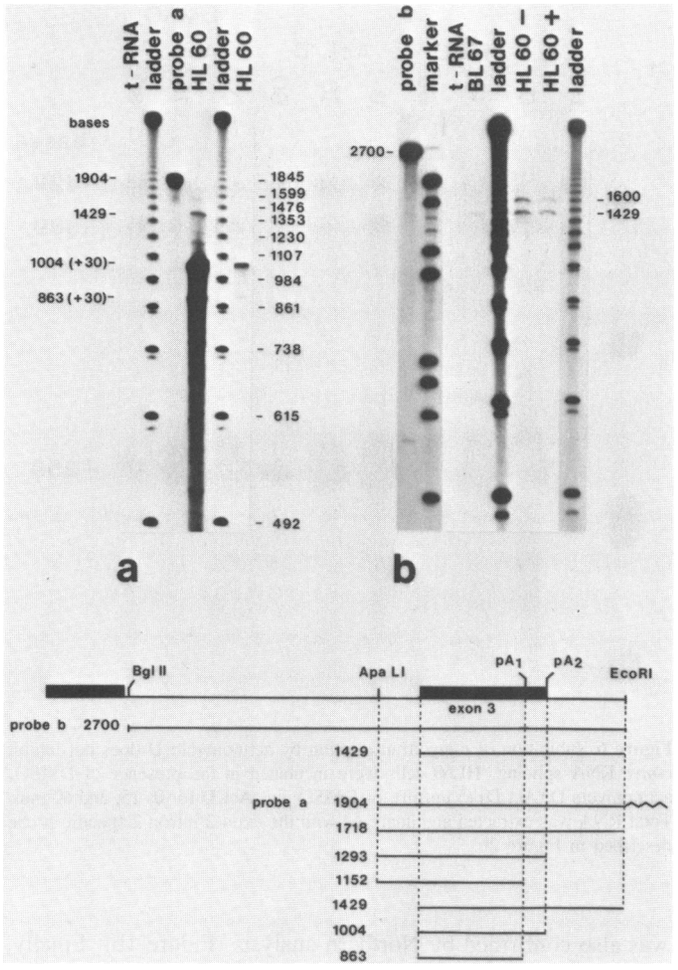

Figure 3. Order of exon 2 splicing and 3′ cleavage of c-*myc* RNA in HL60 cells. Total RNA was prepared from HL60 cells treated for 1h with (+) or without (−) DMSO or from BL67 cells and studied by S1 analysis. Double stranded DNA probes were labelled with [^{32}P] by T4-polymerase starting at the *Apa*LI site (probe a), or by T4-polynucleotide kinase at the *Eco*RI site (probe b). The probes and the size of expected fragments are described in the map shown below the autoradiograms. The minor and major polyadenylation sites are designated pA1 and pA2, respectively (Cleavage of the primary transcript occurs approximately 30 nucleotides downstream of the polyA site). Two lanes with a long and short exposure are shown for HL60 RNA hybridized with probe a. The 123 bp ladder (Gibco/BRL) served as molecular weight standard.

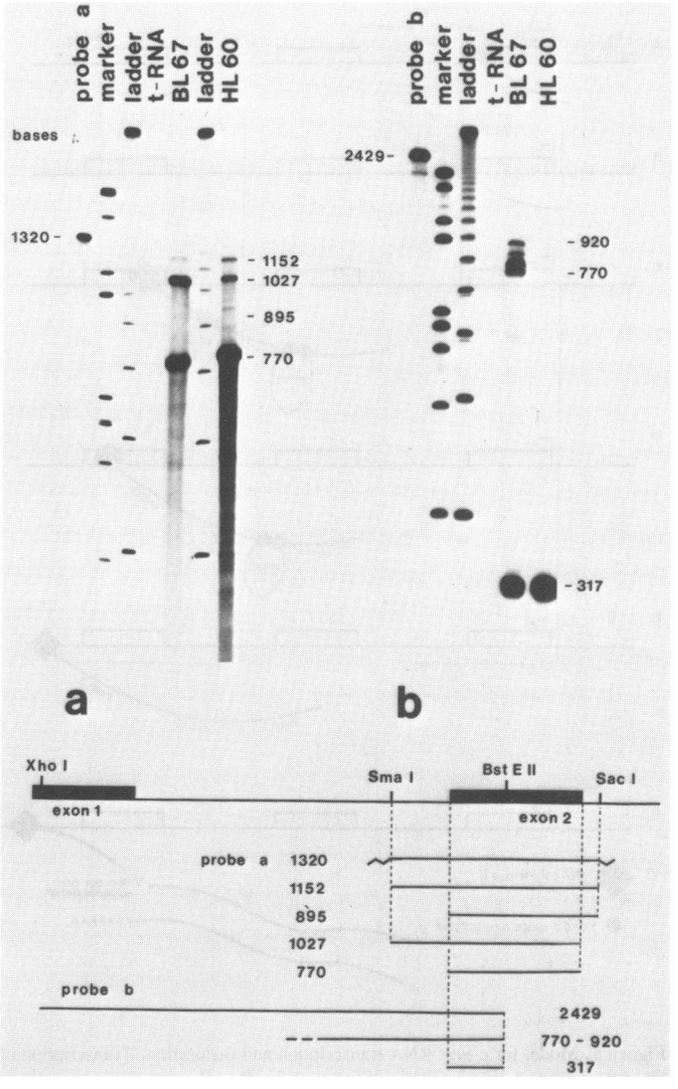

Figure 4. Temporal order of intron 1 and intron 2 splicing. Total RNA was prepared from HL60 and BL67 cells and studied by S1 analysis. The 1152 bp *Sma*I-*Sac*I fragment was cloned in M13 and uniformly labelled with [^{32}P] by primer extension (probe a). The probe detected spliced and unspliced c-*myc* RNA as well as splicing intermediates. Probe b (the 2429 bp *Xho*I-*Bst*EII fragment labelled at the *Bst*EII restriction site by T4 polynucleotide kinase) detects c-*myc* RNA initiated at the cryptic promoters within intron 1.

a could discriminate spliced and unspliced as well as 3′ cleaved and uncleaved c-*myc* RNA. The probe contained part of intron 2, the entire exon 3 and 400 bp downstream of exon 3 up to the *Eco*RI site (Figure 3a). The label was incorporated into the 3′ part of the probe by T4-polymerase. Digestion of probe a with frequently cutting enzymes revealed that about 80% of the label was incorporated in intron 2 and about 20% in exon 3. The label in the opposite strand was removed by an asymmetrical cut within vector sequences and subsequent gel purification of the probe. Probe a generated two fragments of ~890 and ~1030 bases with RNA of HL60 cells corresponding to the minor (pA1) and major (pA2) polyadenylation sites of the c-*myc* gene, respectively (39). Unspliced and 3′ cleaved molecules of c-*myc* RNA are expected to have a size of approximately 1180 and 1320 bases. Such fragments were visible only as faint bands. Additionally, the probe detected one major precursor molecule of c-*myc* RNA. This fragment migrated at the position of a spliced and 3′ uncleaved precursor of c-*myc* RNA (1429 bases). Thus, the majority of nuclear c-*myc* precursor RNA molecules has lost intron 2 but still contains an uncleaved 3′ end.

The second S1 probe (probe b) was constructed to confirm this assumption. Probe b was 5′ labelled at the *Eco*RI site and could only detect 3′ uncleaved c-*myc* RNA (Figure 3b). Two fragments of about 1429 and 1600 bases were protected by HL60 RNA. The smaller one corresponds to the band of 1429 bases in Figure 3a and confirmed the presence of spliced and 3′ uncleaved c-*myc* RNA in HL60 cells. The nature of the larger fragment is not yet clear.

Splicing of intron 2 precedes splicing of intron 1 in HL60 cells

To study the pathway of c-*myc* RNA maturation further, the time course of splicing intron 1 and intron 2 was determined. An S1 experiment was performed with an uniformly labelled probe carrying the complete exon 2 and adjacent parts of intron 1 and intron 2, respectively. A fragment of 770 bases was generated

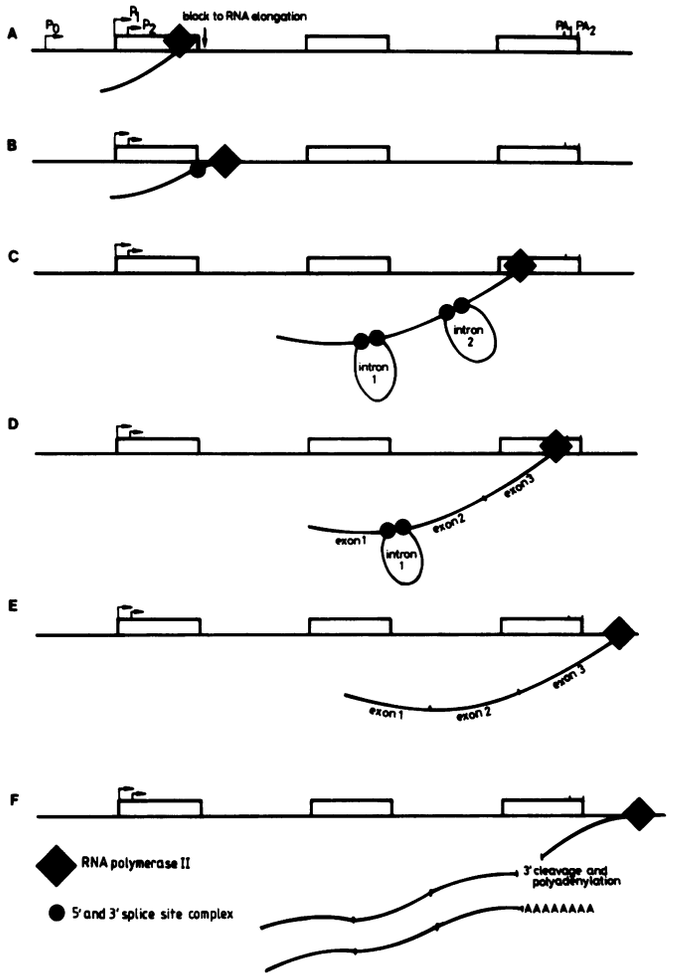

Figure 5. Model for c-*myc* RNA transcription and maturation. Transcription of the c-*myc* gene in HL60 cells is initiated at the P_0-, P_1- and predominantly at the P_2-promoter. Elongation of c-*myc* RNA is blocked at the boundary of exon 1/intron 1 by an yet unknown mechanism. The arrow marks the major termination site for RNA polymerase II (52) (A). The model suggests that binding of a 5′ splice site component at the nascent c-*myc* transcript may be involved in the release of the elongational block of c-*myc* RNA transcription (B). The polymerase then moves on and passes intron 1 and 2 (C). From the primary transcript intron 2 and intron 1 are then removed by splicing (D and E). Finally, the c-*myc* RNA is 3′ cleaved and polyadenylated (F). The model proposes that splicing and 3′ cleavage of c-*myc* RNA takes place co-transcriptionally. This notion seems likely but is not proven (see discussion).

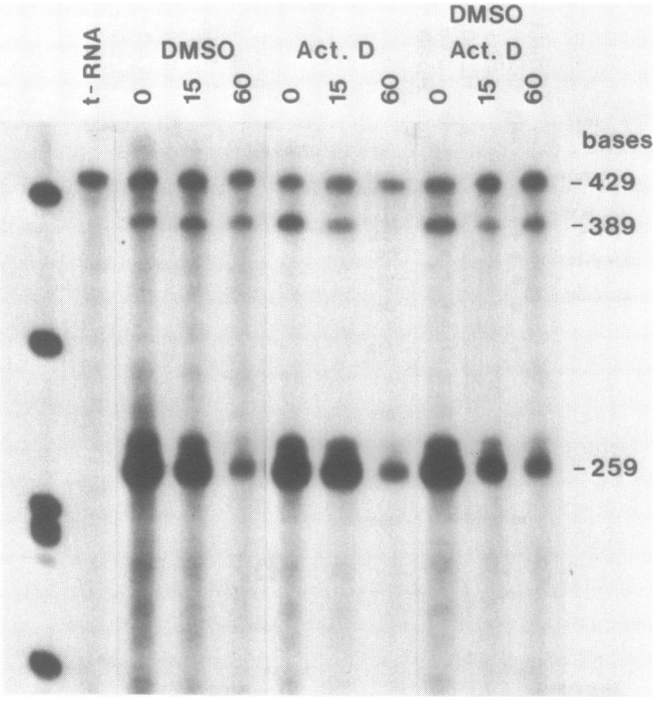

Figure 6. Inhibition of c-*myc* transcription by actinomycin D does not inhibit c-*myc* RNA splicing. HL60 cells were incubated in the presence of DMSO, actinomycin D (Act.D) (5 μg/ml), or DMSO plus Act.D for 0, 15, and 60 min. Total RNA was extracted and analysed with the exon 2/intron 2 specific probe described in Figure 2b.

which corresponds to entirely spliced c-*myc* RNA in HL60 cells (Figure 4a).

Additionally, two types of c-*myc* precursor RNA molecules giving rise to bands of 1027 and 1152 bases were detected. The larger fragment (1152 bases) corresponds to c-*myc* RNA still containing both introns, the smaller one (1027 bases) to c-*myc* RNA carrying an unspliced intron 1 and spliced intron 2. A precursor molecule of 895 bases which is expected only when intron 1 is spliced could not be detected. To rule out the possibility that the fragment of 1027 bases was derived from c-*myc* RNA initiated at a cryptic promoter in intron 1, the activity of intron 1 promoters was tested in HL60 cells. An end-labelled S1 probe clearly detected cryptic c-*myc* RNA in the Burkitt's lymphoma cell line BL67 (32) but not in HL60 cells (Figure 4b). The absence of considerable amounts of cryptic c-*myc* RNA in HL60 cells was also confirmed by Northern analysis (Figure 1b). Finally, the ratio of spliced to unspliced c-*myc* RNA of exon 1 to intron 1 (Figure 2a) coincides with the ratio of exon 2 to intron 1 which is estimated to be 20:1. Thus, about 5% of total c-*myc* RNA carries a first intron.

The results are summarized in a model for the pathway of c-*myc* RNA maturation (Figure 5). The model proposes that c-*myc* RNA of HL60 cells is first spliced and then cleaved at its 3′ end and polyadenylated. It is, however, not yet clear whether splicing and 3′ cleavage occur co-transcriptionally at the nascent transcript. Alternatively, the primary transcript could be spliced and 3′ cleaved after release from the transcriptional complex.

Maturation of c-*myc* RNA is specifically inhibited in DMSO-induced HL60 cells

DMSO induces a block to RNA elongation in the c-*myc* gene of HL60 cells leading to a dramatic decrease of cytoplasmic c-*myc* RNA. At the same time, the level of nuclear c-*myc* RNA remains almost unaffected. This implies that the c-*myc* precursor RNA becomes stabilized and is no longer targeted for splicing. To test whether the pool of nuclear c-*myc* RNA studied above is the one which is normally processed and transported to the cytoplasm, HL60 cells were treated for various times with DMSO, actinomycin D, or with a combination of DMSO plus actinomycin D. Total cellular RNA was prepared and analysed with an exon 2/intron 2 specific probe. One hour after addition of DMSO the spliced form of c-*myc* RNA was reduced about 10 fold in HL60 cells, whereas the amount of unspliced precursor RNA was reduced only by a factor of 1.5 (Figure 6). When transcription was blocked by actinomycin D, the levels of spliced

and unspliced c-*myc* RNA decreased with similar kinetics and unspliced c-*myc* RNA was no longer detectable after 1h. This ruled out the possibility that blocking of c-*myc* transcription abolishes splicing of c-*myc* RNA and demonstrated that the pool of nuclear c-*myc* precursor RNA has actually disappeared. After treatment of HL60 cells with a combination of DMSO and actinomycin D, cytoplasmic c-*myc* RNA disappeared with similar kinetics as with DMSO alone. This indicates that (i) the stability of cytoplasmic c-*myc* RNA is not significantly reduced in DMSO-induced HL60 cells and (ii) that DMSO has a specific inhibiting effect on c-*myc* RNA splicing.

To test if DMSO has a general inhibiting effect on RNA splicing, expression of the c-*abl* gene was studied in DMSO-treated HL60 cells. The c-*abl* gene was selected, because its expression does not show significant variations in differentiating HL60 cells (38). As shown in Figure 7, the levels of spliced and unspliced c-*abl* RNA remained unchanged in HL60 cells in the presence of DMSO. When transcription was blocked by actinomycin D, the level of unspliced c-*abl* RNA was at least 10 fold reduced. The level of spliced c-*abl* RNA decreased only about 1.5 fold within 1h implying a half life of more than 2 hours for c-*abl* mRNA. Splicing of c-*abl* RNA was not affected when HL60 cells were treated with DMSO plus actinomycin D. Thus, DMSO did interfere with transcription and splicing of c-*myc* but not c-*abl* RNA in HL60 cells.

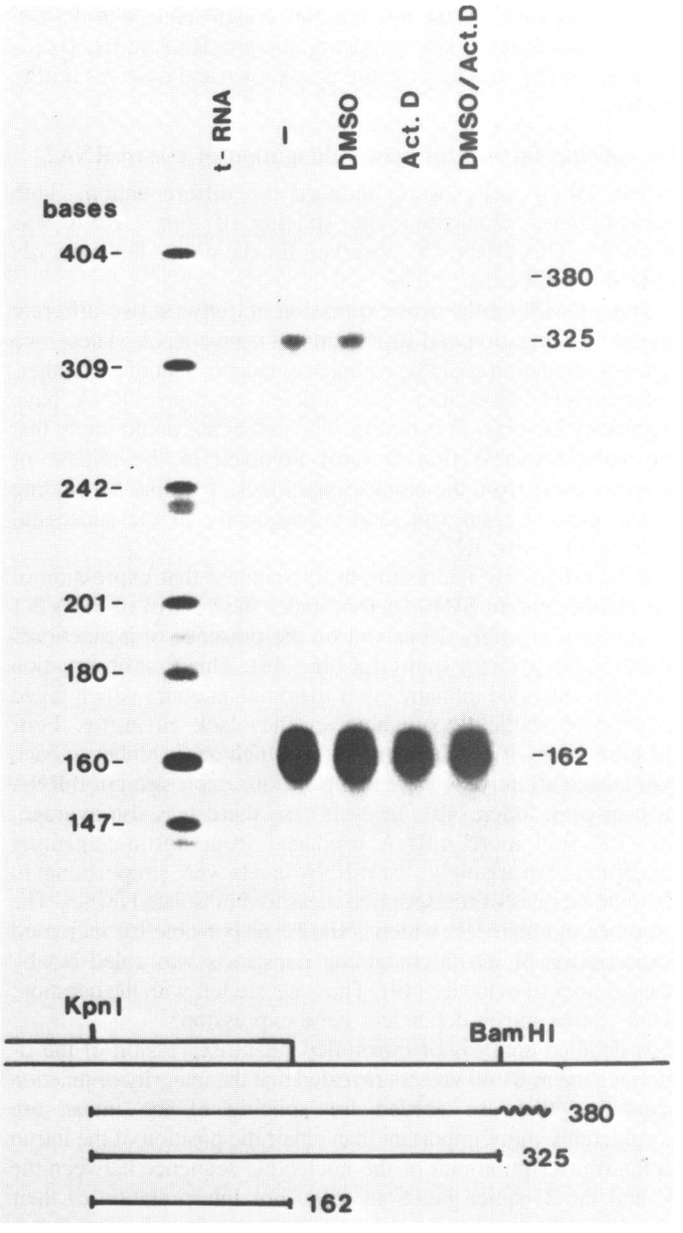

Figure 7. Transcription and splicing of c-*abl* RNA is not affected by DMSO in HL60 cells. HL60 cells were incubated for 1h in the absence (–) or presence of DMSO, actinomycin D (Act.D), or DMSO plus Act.D. S1-analysis was performed with total RNA using a M13 cloned uniformly labelled 325 bp *Kpn*I-*Bam*HI (55) fragment containing exon a_2/intron a_2 sequences of the c-*abl* gene.

DISCUSSION

Maturation pathway of c-*myc* RNA in HL60 cells

The high molecular weight of the primary c-*myc* transcript indicates that several kilobases downstream of the third exon are transcribed by RNA polymerase II. In this regard the c-*myc* gene in HL60 cells does not appear to differ from the mouse c-*myc* gene (27) and other cellular genes (40,41) with primary transcripts elongated up to several kilobases downstream of the polyadenylation site, before cleavage and subsequent polyadenylation occurs. The results obtained for HL60 cells indicate further that splicing of c-*myc* RNA is an early post-transcriptional event and precedes 3′ cleavage and poly-A addition. Whether splicing of the c-*myc* transcript occurs already co-transcriptionally or after release from the transcriptional complex remains, however, unclear.

Splicing of RNA before 3′ cleavage and polyadenylation has been observed for several other genes. The secreted and the membrane-bound immunoglobulin heavy chains are expressed in B cells in a differentiation specific manner. Creation of the μ_{mem}-specific RNA requires the removal of the polyadenylation signal for μ_s-specific RNA. This is accomplished when splicing of the $C_{\mu 4}$-$C_{\mu mem}$ intron precedes cleavage and polyadenylation at the μ_s-polyA site (42).

Direct proof for co-transcriptional splicing has recently been presented for the Drosophila chorion genes s36-1 and s38-1 (43). Using electron microscopical methods, the authors demonstrated that spliceosomes were loaded on the nascent transcript, followed by removal of introns upstream of the transcriptional complex.

Another example is the gene for dihydrofolate reductase (dhfr). Nonsense mutations in this gene affect not only translation but also processing and nuclear transport of dhfr-RNA (44). According to the model of the authors, translation of the spliced 5′ end of the dhfr-mRNA already starts outside the nucleus, pulling the message out of a nuclear pore, while the 3′ end of the dhfr-RNA is still being synthesized in the nucleus.

The c-*myc* gene in HL60 cells is thus an additional member in the family of genes where transcripts are spliced before 3′ cleavage and polyadenylation.

The temporal order of intron splicing of c-*myc* RNA does not follow the sequence of intron arrangement in the gene. In HL60 cells the second exon is preferentially spliced first. Similar observations have been reported for *in vitro* splicing of a δ-crystallin mRNA precursor containing three exons and two introns. Whenever uncapped precursor RNA was used as substrate the downstream intron was removed first. After capping of the precursor, the upstream intron was spliced more efficiently. Preincubation of the extract with cap analogues inhibited splicing

of the upstream intron but not the downstream intron (45). Whether the order of intron splicing of c-*myc* RNA in HL60 cells is influenced by the cap structure is unknown and deserves further studies.

Do splicing factors influence elongation of c-*myc* RNA?

When HL60 cells were induced to differentiation, both transcriptional elongation and splicing of c-*myc* RNA was inhibited. This effect was observed for the c-*myc* but not c-*abl* gene of HL60 cells.

Thus, DMSO influences expression of c-*myc* at two different levels: i) elongation and ii) splicing of c-*myc* RNA. These two types of regulation could be completely independent of each other. Alternatively, elongation and splicing of c-*myc* RNA have regulatory factor(s) in common. The latter case could imply that the same factor(s) first is (are) involved in the release of polymerase II from the elongational block, possibly by binding to the nascent transcript, and subsequently in the successful splicing of c-*myc* RNA.

It is particularly interesting in this context that expression of the globin gene in SV40 vectors under the control of the VP-1 promoter is strongly dependent on the presence of a functional intron in the primary transcript (46–48). This first observation has been extended to many other mammalian genes which failed to produce stable RNA whenever they lack an intron. Four different pairs of gene constructs, in which one member of each pair lacked all introns, were compared for expression of mRNA in transgenic mice (49). In each case there was, on average, 10–100 fold more mRNA produced from intron-containing constructs. Surprisingly, the mRNA levels were proportional to the relative rates of transcription measured in isolated nuclei. The presence of enhancers which could be responsible for increased transcription of intron-containing constructs was ruled out by several lines of evidence (49). Thus, we are left with the question: what causes intron-dependent gene expression?

A detailed analysis of intron-dependent expression of the β-globin gene in SV40 vectors revealed that the integrity of junction sequences that are needed for splicing of the intron are considerably more important than either the position of the intron in the transcription unit or the nucleotide sequence between the 5′ and the 3′ splice junctions (50). For interpretation of their data the authors propose that spliceosomes in eukaryotes may have a similar role in gene transcription as ribosomes in prokaryotes. In attenuated bacterial genes the stalled ribosome allows read-through transcription and prevents premature termination (51). Similarly, binding of splicing components (or other factors) to the nascent transcript near the 5′ splice site may allow RNA polymerase II to traverse the region of an elongational block in eukaryotic genes.

Recently, the sequence requirements for premature termination of transcription in the human c-*myc* gene have been determined. The major termination site for RNA polymerase II has been mapped 23 bp downstream of exon 1 (52). A region of 95 bp located −130 to −35 bp relative to the exon1/intron1 boundary is also necessary for termination (53). Interestingly, RNAs initiated at the dual c-*myc* promoter P_1/P_2 or at the heterologous HSV-TK promoter are terminated downstream of the 95 bp sequence, whereas transcripts containing sequences originating in the vector upstream of P_1/P_2 or derived from the major late promoter of adenovirus 2 (Ad2) did not terminate (53). This implies, that the structure of the RNA or the promoter utilized is an additional important factor for premature termination.

If regulatory factors of splicing are somehow involved in RNA elongation, then it is surprising that the promoter of the HSV-TK gene, which naturally is devoid of introns, can cooperate with the 95 bp termination sequence of the c-*myc* gene. A detailed analysis, however, has revealed that sequences within the transcribed region of the HSV-TK gene can substitute for the intron requirement in other genes and reconstitute RNA production (50). This raises the issue of whether introns and TK sequences accomplish their effects by similar or different mechanisms. In both instances, binding of factors acting positively on transcription is likely. For the release of the elongational block in the c-*myc* gene, binding of positively acting factor(s) must be assumed since premature termination is an intrinsic property of the first exon when transcribed *in vitro* by purified RNA polymerase II (54).

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