Rapid c-*myc* mRNA degradation does not require (A+U)-rich sequences or complete translation of the mRNA

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ABSTRACT

The c-*myc* proto-oncogene encodes a highly unstable mRNA. Stabilized, truncated *myc* transcripts have been found in several human and murine tumors of hematopoietic origin. Recently, two tumors expressing 3′ truncated c-*myc* mRNAs that were five times more stable than normal *myc* transcripts, were described. We have tried to determine the cause of the increased stability of the 3′ truncated *myc* transcripts by studying the half-life of mutated c-*myc* mRNAs. The c-*myc* 3′ untranslated region has been shown to contain sequences that confer mRNA instability. Possible candidates for such sequences are two (A+U)-rich regions in the 3′ end of the c-*myc* RNA that resemble RNA destabilizing elements present in the c-*fos* and GMCSF mRNAs. We show that deletions in the (A+U)-rich regions do not stabilize c-*myc* messengers, and that hybrid mRNAs containing SV40 sequences at their 3′ ends and terminating at an SV40 polyadenylation signal decay as quickly as normal c-*myc* transcripts. Our results indicate that neither the loss of (A+U)-rich sequences nor the mere addition of non-*myc* sequences to the 3′ end of the mRNA lead to stabilization. We also show that rapid degradation of c-*myc* mRNA does not require complete translation of the coding sequences.

INTRODUCTION

The product of the c-*myc* proto-oncogene, which is thought to have a regulatory role in cell growth and differentiation, can contribute to oncogenic transformation when it is overexpressed (reviewed in 1 and 2). Classic examples are Burkitt's lymphoma, a human tumor of B-cell origin, and murine plasmacytoma. In these hematopoietic tumors the *myc* gene is activated by translocation to chromosomes carrying immunoglobulin genes, genes that are highly active in B-lymphocytes. The level of *myc* mRNA in these tumors is increased by the presence of strong immunoglobulin enhancers but can be further elevated by the stabilization of the normally labile transcript. c-*myc* mRNA normally decays with a half-life of approximately 15 minutes (3, 4). The rapid degradation of the transcript is thought to be an important factor in c-*myc* gene regulation. In lymphomas in which the c-*myc* gene has lost part or all of the non-coding first exon, truncated mRNAs that are abnormally stable are formed (5−10). These mRNAs start either in non-*myc* sequences or are transcribed from cryptic promoters in intron 1. Studies with hybrid constructs consisting of sequences from the c-*myc* gene and the neomycin resistance gene (neo), have shown that *myc* exon 1 does not contain destabilizing sequences (11). However, the presence of a small region from intron 1 was shown to prolong the half-life of truncated *myc* mRNAs (5, 6, 11). It therefore appears that the addition of certain sequences that are normally not present in the mature c-*myc* transcript can retard the decay of *myc* mRNA.

Recently, two human cell lines derived from a myeloma and a T-cell leukemia, containing translocations leading to loss of part of the c-*myc* 3′ untranslated region (UTR), were described (12, 13). c-*myc* mRNA from these tumor cell lines decayed substantially more slowly than normal *myc* mRNA. The increased stability of these 3′ truncated transcripts has several possible causes. First, destabilizing sequences located in the 3′ UTR may have been lost. The neo/*myc* hybrid experiments mentioned above had also shown that, in contrast to exon 1, exon 3 did contain destabilizing sequences. Fusion of most of the non-coding sequences of the third *myc* exon to the neo gene led to the production of hybrid mRNAs with a half-life that was at least five-fold shorter than that of the original neo transcripts (11). The c-*myc* 3′ UTR contains two polyadenylation (pA) signals, each preceded by an (A+T)-rich region (4, 14). The (A+T)-rich regions resemble (A+T)-rich elements found in the 3′ UTR of the granulocyte-macrophage colony-stimulating factor (GMCSF) gene and a number of other genes encoding unstable mRNAs, such as c-*fos* and c-*myb* (15). The elements are 40−60 basepairs (bp) long and contain one or more ATTTA pentamers. Introduction of the (A+U)-rich element of the GMCSF or the c-*fos* mRNAs into the stable β-globin mRNA was shown to be sufficient to reduce the half-life from several hours to 30 minutes or less (15−17). In the two translocations leading to loss of 3′ c-*myc* sequences mentioned above, both (A+T)-rich regions and pA sites were lost in the myeloma (13) and all sequences

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downstream of the first (A+T)-rich region were lost in the T-cell lymphoma (12). In both cases, the resulting *myc* mRNAs were at least five-fold more stable. This suggests that the loss of one or both (A+U)-rich regions could be the cause of the stabilization of 3′ truncated transcripts. However, the role of the (A+U)-rich regions in *myc* mRNA instability has not yet been proven. Next to the loss of 3′ c-*myc* sequences, the stabilized 3′ truncated c-*myc* transcripts also contained additional non-*myc* sequences at their ends, and terminated at pA sites outside the c-*myc* gene. Thus, it cannot be excluded that the presence of non-*myc* sequences in the mRNA rather than the absence of the (A+U)-rich regions is the cause of the altered stability.

Sequences that are normally not present in c-*myc* mRNA could affect the stability of the hybrid mRNAs in two ways. They could contain specific sequences that function as stabilizing elements, somehow interfering with the normal mechanism of degradation. This appears to be the case with 5′ truncated *myc* transcripts beginning in intron 1. Alternatively, the mere alteration of the position of important sequence elements, such as the pA site, with respect to other elements such as the stopcodon and the (A+U)-rich regions, may disrupt rapid degradation. That the position of sequence elements may be important for mRNA degradation is illustrated by cell cycle-dependent histone mRNA decay. For proper decay to occur, ribosomes must reach the normal stopcodon, which is located at a specific distance upstream of a hairpin loop at the end of the mRNA (18). Lengthening the distance between the stopcodon and the hairpin abolishes regulated histone mRNA degradation. The rapid degradation of c-*myc* mRNA has also been found to be dependent on translation (19). It is not known whether the requirement of translation results from the need (like histone mRNAs) for the ribosomes to reach the normal stop codon. The inhibition of translation stabilizes c-*myc* mRNA by slowing down poly(A) tail shortening, a process that normally preceeds *myc* mRNA breakdown (20). How translation and poly(A) tail shortening are coupled remains unclear. Complete translation of the coding sequences of the mRNA may be of importance for an interaction between ribosome-bound factors and elements in the 3′ UTR, such as the (A+U)-rich region or the poly(A) tail. In that case, alterations in the length of the 3′ UTR of truncted *myc* mRNAs may account for the longer half-life of these messengers.

To determine whether the presence of the (A+U)-rich regions, the nature of the 3′ untranslated sequences, the position of the pA sites and the position of the stopcodon, are relevant to c-*myc* mRNA decay, we constructed a number of human *myc* genes with deletions of either (A+T)-rich region, substitution of the normal pA signal by non-*myc* sequences, or frameshifts leading to premature termination of translation. The mutated genes were introduced into rat fibroblasts and the half-life of the resulting mRNAs was measured. Our results show that (A+U)-rich regions are dispensable for rapid *myc* mRNA decay. We also show that the replacement of 3′ c-*myc* sequences, including the pA signals, by non-*myc* sequences does not per se stabilize the *myc* transcript. This suggests that the stabilization of 3′ truncated *myc* mRNAs is not a result of loss of the (A+U)-rich regions, polyadenylation outside the *myc* gene or an altered distance between the stop codon and other elements in the 3′ UTR. Our results suggest that c-*myc* mRNA contains destabilizing sequences outside the (A+U)-rich regions. We also show that complete translation of *myc* mRNA is not required for instability of the mRNA, although the rapid decay of both normal *myc* mRNAs and *myc* mRNAs lacking (A+U)-rich sequences is dependent on translation.

MATERIALS AND METHODS

Plasmid constructs

To make the different deletion mutants, the normal human *myc* gene (*Kpn*I-*Eco*RI fragment, 21) was inserted into pIC 20H (a pUC derivative (22)) in such a way that the SV40 enhancer (*Hind*III-*Pvu*II fragment) was present at its 5′ end (to ensure detectable mRNA levels) and the SV40 pA signal (*Sau*IIIA fragment) was at its 3′ end (to provide a pA signal when no *myc* pA was present). The plasmid also contained the hygromycin resistance gene as a selectable marker, just downstream of c-*myc*. This basic construct (A592, Fig. 1A) was used as a cassette into which the mutations were introduced. The actual mutations were made in a plasmid containing the 3′ *Cla*I-*Eco*RI fragment of human c-*myc* (pIE-Cla), followed by replacement of the normal fragment with the mutated one in plasmid A592. A summary of the constructs is given in Fig 1. In dAU1 the sequences between the *Nsi*I and *Ssp*I sites were deleted. dAU2 contained a deletion from the most 5′ *Rsa*I fragment in pIE-Cla to the downstream *Dra*I site. AU1-2 was made by deleting the region between the two *Dra*I sites. In dSsp the region between the *Ssp*I site and a *Hpa*I site located 30 bp upstream of the SV40 pA signal, was deleted. dDra was made by deleting all *myc* sequences downstream of the 5′ *Dra*I site. Frameshifts leading to premature termination of translation were insterted into c-*myc* as follows: plasmid A592 was linearized with either *Bst*EII (G′GTGACC) or *Cla*I (AT′CGAT). The protruding ends were filled in and the plasmid was religated. The mutations were confirmed by the loss of the *Bst*EII (GGTGAC/GTGACC) and *Cla*I (ATCG/CG-AT)sites and by the formation of a new *Mae*II and *Nru*I site respectively (bold lettering, above). The religation of the blunted sites led to a frameshift so that a stopcodon was encountered 4 nucleotides after the point of ligation with *Bst*EII (underlined, above) and 215 nucleotides downstream of the ligation site with *Cla*I.

Transfection of cells and RNA isolation

All constructs were transfected to Rat-2 fibroblasts (23) by the calcium phosphate method (24). Selective medium containing 200 μg/ml hygromycin B was added to the cells on the day following transfection. After about 2 weeks, the colonies that had formed were pooled. These polyclonal cell lines were seeded on six 15-cm dishes. The next day the translation-blocker actinomycin D (ActD) was added to the subconfluent cultures (at 5 μg/ml) and RNA was isolated at different times, using the Nonidet P-40 method (25). To study the effect of a translation block, the cells were preincubated for 2 hours with medium containing 10 μg cycloheximide per ml, before the addition of ActD.

RNase protection analysis

Samples of cytoplasmic RNA (20 μg) were coprecipitated with a ^{32}P-labeled RNA probe, hybridized overnight and digested with RNase T1 as described (20). All probes were made by cloning the desired fragments into pEP 30, a plasmid containing the SP6 and T7 promoters. The exon 2 probes were internal exon 2 fragments. For rat *myc* we used an *Eco*RV-*Pst*I fragment, giving rise to a probe of 482 nucleotides and a protected band of 394 nucleotides. For human *myc* we used a *Pst*I-*Pvu*II fragment, giving rise to a probe of 270 nucleotides and a protected band of 240 nucleotides. The control probes were a 110-bp fragment of the human elongation factor gene cDNA (hEF) and a 140-bp fragment of the glyceraldehyde dehydrogenase gene cDNA (GAPDH) (4). Under the digestion conditions used, the

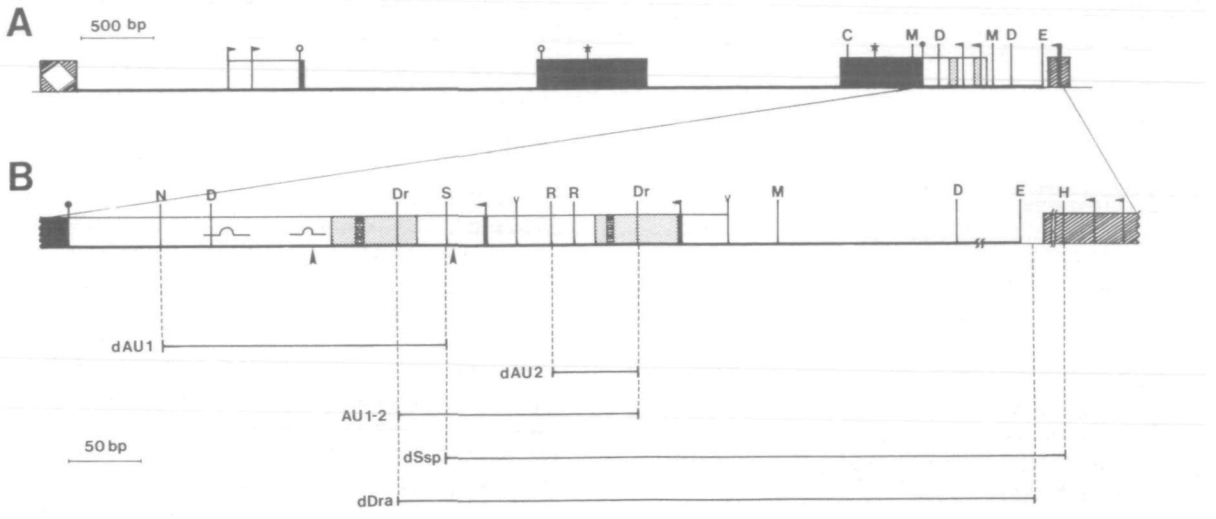

Figure 1. Schematic representation of normal and mutated human c-*myc* genes. A. Restriction map representing a fragment from plasmid A592, containing the human c-*myc* gene flanked by the SV40 enhancer (diamond shape, left) and SV40 pA signal (right). The thick black line represents genomic c-*myc* sequences, thin lines are pIC 20H polylinker sequences. Hatched boxes: SV40 sequences, all other boxes are *myc* exons. Black boxes: translated region. Stippled areas: (A+U)-rich regions, the striped segments within these regions are ATTTA pentamers, conserved between rat an human. White pin-heads: translation start codons, black pin-head: translation stop codon, stars: first stopcodon encountered after *Bst*EII (left) or *Cla*I (right) frameshifts. Flags, pointing right: cap sites, pointing left: pA signals. C= *Cla*I, M= *Mbo*I, D= *Dde*I, E= *Eco*RI. B. Schematic representation of 3′ UTR. The areas deleted in the mutated genes are marked by the bars under the map. The signs within the leftmost white box denote the position of the predicted stem-loop structures. The forks just 3′ to each pA signal mark the poly(A) addition sites. The two arrowheads show, from left to right, the site of translocation in the myeloma (13) and the T-cell lymphoma (12). Restriction sites: as above, N: *Nde*I, Dr: *Dra*I, S: *Ssp*I, R: *Rsa*I, H: *Hpa*I.

GAPDH probe always gave rise to 2 bands of about 140 and 145 nucleotides. The 3′ probes that were used to analyse the termini of the mRNAs were different for each mutated plasmid. To analyze *myc* RNA from cells containing the dAU1 construct, an *Mbo*II fragment containing the deletion was cloned into pEP 30. For the analysis of dAU2 and dAU1/2 RNA, *Dde*I fragments containing the deletions were cloned into pEP 30. To determine whether the pA sites of the mutant mRNAs were identical to the normal *myc* pA sites, HeLa RNA was used as a reference. Under the conditions used (RNase T1 only), most of the hybrids between wildtype *myc* mRNA (from HeLa cells) and the deletion-containing probes are not cleaved at the site where the non-hybridizing RNA loops out, giving rise to protected bands with a length corresponding to *myc* mRNA carrying the various deletions but ending at the normal pA sites. A small fraction of the hybrids are cut, giving rise to shorter fragments.

Calculation of mRNA half-life

Autoradiograms in which the signal from the bands was in the linear range of the film were scanned densitometrically on an LKB 2222-020 UltroScan XL laser densitometer, coupled to a line printer. The peak areas, corresponding to the bands, were measured by intergration by the computer and were verified by weighing the cut out peaks. *myc* signals were corrected for RNA content in each sample by comparison with hEF or GAPDH bands. hEF and GAPDH mRNAs do not decay detectably during the time period studied (4, 20). The percentage remaining *myc* signal was plotted logarithmically against the time. The bestfit lines were calculated using linear regression. The variation in the determination of the half-life within one experiment was estimated to be about 5 min. Some variability between experiments was also observed, leading to half-life measurements of 14 to 33 min for the endogenous rat *myc* mRNA. This is in accordance with the half-lives observed by others, ranging from 15 min (3) to 30 min (11). In half-life measurements using the exon 2 probes, several exposures of the gels were always made so that the rat and human bands could be compared optimally.

RESULTS

Rapid degradation of c-*myc* mRNA can proceed in the absence of (A+U)-rich regions

To analyze whether the (A+U)-rich regions are required for rapid *myc* mRNA degradation, we made several deletions in a plasmid containing the complete human *myc* gene (Fig 1). Due to the location of each (A+U)-rich region just upstream of a pA signal, the presence of these regions in an mRNA is dependent on the pA site used. The majority of the mRNAs terminate at the second poly(A) site (pA2) and thus contain two (A+U)-rich regions (AU1 and AU2) (4). mRNAs ending at the first pA site (pA1) are as unstable as mRNAs ending at pA2 (4) yet contain only the upstream (A+U)-rich region (AU1). Therefore, if the (A+U)-rich regions are required for rapid degradation of c-*myc* mRNA, this function must be fulfilled by AU1 for mRNAs terminating at the first pA site. Therefore, dAU1 should be a very instructive mutant. mRNAs transcribed from this construct lack the complete AU1 region and sequences directly upstream of it (Fig 1B). The AU1 region is highly conserved among species and contains the 11-bp sequence UUUGUAUUUAA (containing an AUUUA pentamer), which is identical in all mammalian c-*myc* genes sequenced (14, 21, 26, 27), and in the chicken (28) and *Xenopus* (29) c-*myc* genes. This sequence is preceded by a U-rich region in all these species. A computer RNA-folding program (30) predicted two stable stem-loop structures upstream of AU1. The hairpin closest to AU1 is completely conserved between human, mouse and rat c-*myc* RNAs, the upstream hairpin shows some variation in the loop sequences. These features suggest that AU1 and sequences in the vicinity could

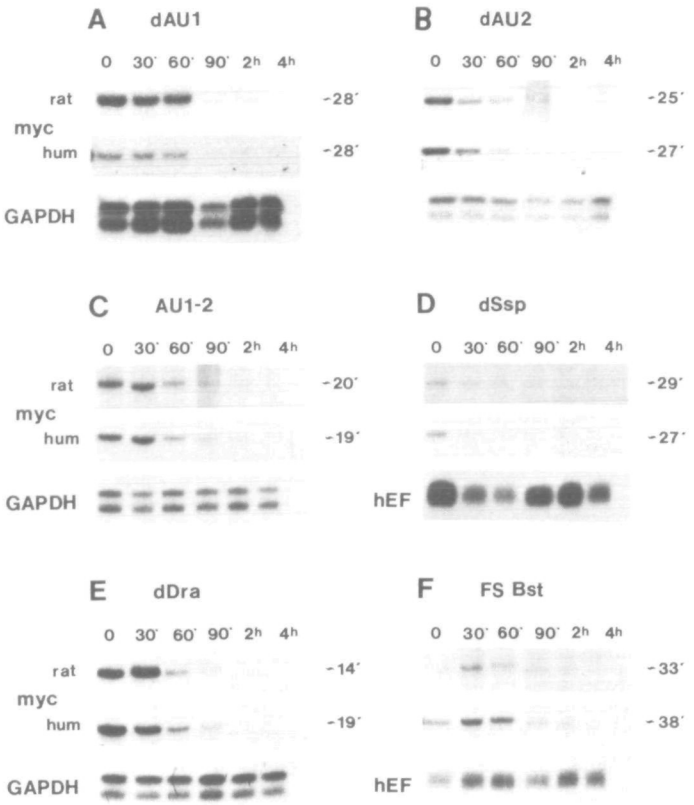

Figure 2. RNase protection analysis of RNAs from cell lines carrying the mutated *myc* genes. After addition of ActD, the cells were incubated for the times shown, after which RNA was extracted, purified, and hybridized simultaneously to rat and human *myc* probes for exon 2 and a control probe (GAPDH or hEF). Following RNase treatment, the samples were loaded on a denaturing polyacrylamide gel and subjected to electrophoresis. The gel was exposed to film for various times. The parts of the film containing the rat (top) and human (hum, middle) exon 2 and control bands (bottom) are shown. Exposures were chosen in which the signal of the human and rat bands was comparable. The exposure times were: A, rat: 7 days, hum: 2 days, GAPDH: 16 hrs; B, rat: 5 days, hum: 16 hrs, GAPDH: 6 hrs; C, rat: 2.5 days, hum: 1.5 days, GAPDH: 6 hrs; D, rat: 5 days, hum: 6 hrs, hEF: 6 hrs; E, rat: 2 days, hum: 2 days, GAPDH: 6 hrs; F, rat: 7 days, hum: 3 days, hEF: 6 hrs. Exposures suitable for scanning were used to calculate the half-life of the mRNAs (given to the right of the *myc* bands). The apostrophe stands for minutes, the h for hours.

have an important function. The dAU1 construct should give rise to two types of *myc* mRNAs: those terminating at pA1, lacking an (A+U)-rich region, and those terminating at pA2, lacking AU1 but still containing AU2. RNA half-lives were determined by blocking transcription in the cells by the addition of actinomycin D (ActD), and extracting RNA at different times. No difference was found in the rate of disappearance of *myc* mRNA from the endogenous (rat) and transfected (human) genes, as measured by RNase protection analysis with exon 2 probes (Fig. 2A). This could be explained by the fact that no human *myc* transcripts terminated at pA1 and that all of the longer mRNAs were unstable because they contained AU2. Therefore, we analyzed the same mRNAs with a 3′ probe that enabled us to distinguish between the pA sites used. We found a low level of transcripts that terminated at pA1 (Fig. 3A), comparable to the normal situation in which the 3′ pA site is used six-fold more frequently than the upstream one (4). The sites of polyadenylation were identical to those of normal *myc* RNA in HeLa cells, with one major and two minor bands at pA2 and one band at pA1. Both the long and the short transcripts decayed at the same rate (Fig. 3B), with a half-life of about 25 min. Thus, AU1 is not required for the rapid decay of c-*myc* mRNA.

To study whether the presence of AU2 determines the instability of mRNAs ending at pA2, we deleted a large part of this region, including the AUUUA motif (dAU2, Fig. 1B). Sequence comparison of c-*myc* genes that have been sequenced beyond pA1 shows that the region between the pA sites is highly conserved. For example, the rat and human sequences show a conservation of 92% in the AU2 region (4). Again, we found no difference in the overall half-life of the mutant mRNAs compared to endogenous rat *myc* mRNAs (Fig. 2B). Analysis with a 3′ probe revealed that mRNAs terminating at pA1 and pA2 did not have a different half-life (Fig. 3). Apparently, mRNAs lacking more than half of AU2 are still unstable. Of course, these mRNAs still contain the complete AU1 region. Surprisingly, use of the poly(A) addition site at pA1 appeared to be affected by the downstream deletion, because we consistently observed two bands of equal strength, derived from mRNAs ending in this region.

Fusion of both (A+U)-rich regions (AU1-2, Fig. 1B) did not affect the decay rate of the human mRNA (Fig. 2C), which terminated at the normal pA2 site (not shown). Therefore, we conclude that internal deletions in the part of the 3′ UTR that contains the (A+U)-rich sequences does not in any way affect the half-life of c-*myc* mRNA in our system.

Replacement of the c-*myc* pA signal by the SV40 pA signal does not affect the half-life of c-*myc* mRNA

Whether exchange of the c-*myc* pA signals for a non-*myc* pA signal would affect the rate of mRNA degradation was studied by deleting all *myc* sequences downstream of the *Ssp*I site (dSsp, Fig. 1B). This mutated gene should produce mRNAs whose c-*myc* portion is comparable to the stabilized truncated transcripts observed in the T-cell lymphoma (Fig. 1). A pA signal was provided by the SV40 fragment, containing two AATAAA motifs 30 bp apart, located directly downstream of the deletion. The distance between the c-*myc* stopcodon, AU1 and the pA signals was slightly longer than the distance between these elements in the normal *myc* mRNA (the first SV40 pA signal was located just 6 nucleotides further downstream, compared with *myc* pA1). No difference in the rate of decay of the human and rat c-*myc* mRNAs was found (Fig. 2D). A similar result was obtained when a construct with an even larger deletion, starting at the upstream *Dra*I site (dDra, Fig. 1B), was used (Fig. 2E). The SV40 pA signals were located 70 bp further downstream, compared to the position of *myc* pA1, and mRNAs produced by this construct contained more SV40 sequences (188 nucleotides) than those of dSsp. Apparently, addition of non-*myc* sequences and alterations in the pA site and signal, do not stabilize *myc* mRNA per se.

Termination at the normal stopcodon is not required for proper *myc* mRNA decay

Truncated c-*myc* mRNAs from tumors could have an increased stability due to structural changes in the 3′ end, such as alterations in the distance between the stop codon and the pA signal. What the effect would be of altering the position of the stop codon was studied as follows. Two constructs were made which produced mRNAs in which a frameshift caused premature termination of translation. The stopcodons were located in exon 2 (FS *Bst*EII) and exon 3 (FS *Cla*I) and were 986 and 324 nucleotides upstream of the normal stopcodon, respectively (Fig 1A). Neither the

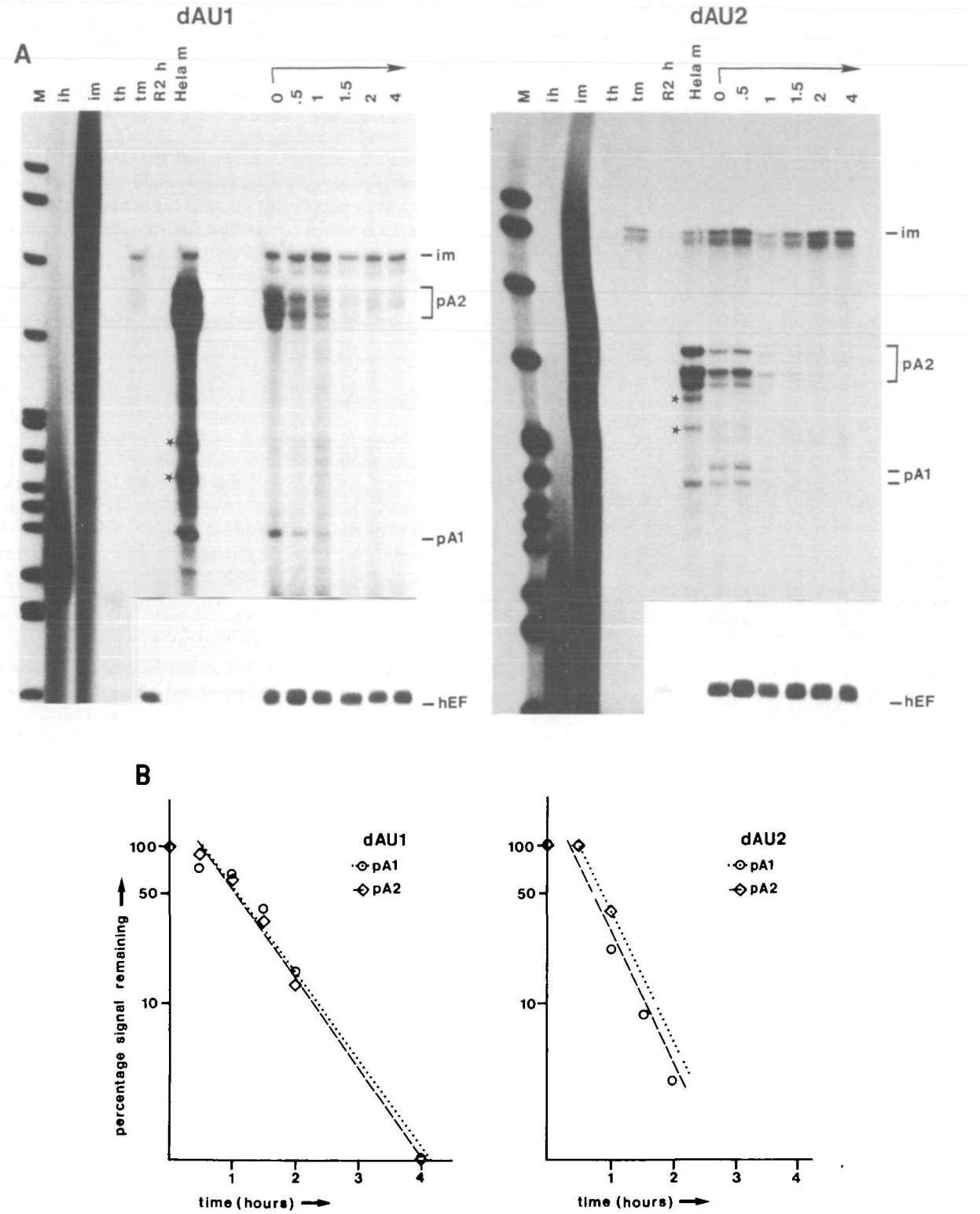

Figure 3. Determination of the half-lives of mRNAs from dAU1 and dAU2, terminating at pA1 and pA2. A. RNase protection assay. M: marker (^{32}P-labeled pBR322 digested with MspI, fragment sizes from top: 623, 527, 404, 309, 242/238, 217, 201, 190, 180, 160, 140, 122 bp); ih: input hEF probe; im: input *myc* 3′ probe; th: tRNA plus hEF probe; tm: tRNA plus *myc* probe; R2h: Rat-2 RNA plus hEF probe; Helam: HeLa RNA plus *myc* probe; pA2: bands arising from *myc* RNA terminating at pA2; pA1: bands arising from *myc* RNA terminating at pA1. Stars indicate bands normally not found in HeLa RNA probed with a 3′ *myc* probe and arising from partial digestion of the deletion-containing probe where non-hybridizing RNA loops out. Samples from the ActD chase were loaded on the right half of each gel. The numbers indicate the incubation time with ActD, in hours. The dAU1 autoradiogram was exposed for 14 days, the dAU2 autoradiogram for 10 days. The insets show a 6 hour exposure of the hEF control bands. B. Plots used for the calculation of the half-life of pA1 and pA2 RNAs. For dAU1 RNAs the half-lives were approximately 30 min for both pA1 and pA2. For dAU2 RNAs the half-lives were approximately 20 min for both pA1 and pA2.

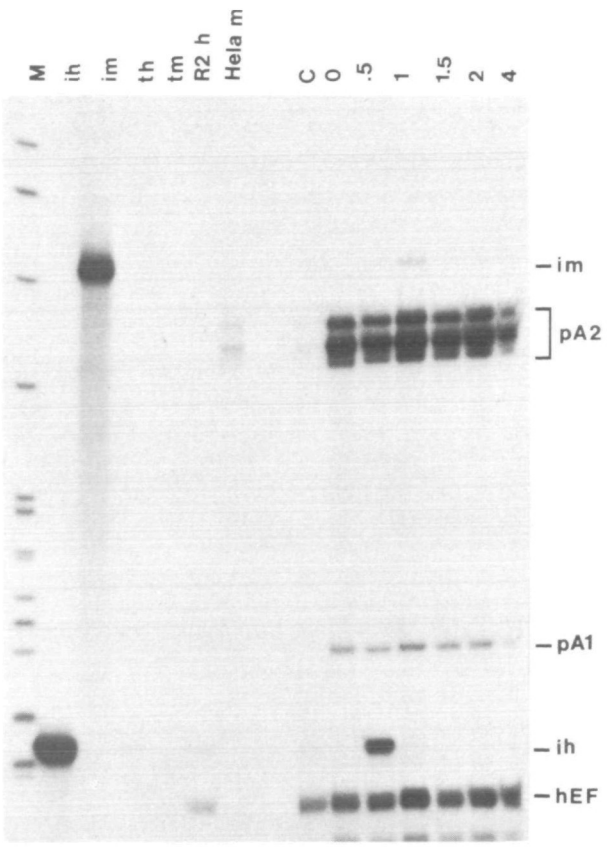

Figure 4. RNase protection of RNA from dAU1 cells treated with cycloheximide for 2 hours (lane C) before the addition of ActD and incubation for the times specified (in hours). M: marker (as in Fig. 3A), ih and im: input hEF and *myc* probes respectively, th and tm: tRNA plus hEF and *myc* probes respectively, R2h: Rat-2 RNA plus hEF probe, Helam: HeLa RNA plus *myc* probe. pA2 and pA1: bands from RNAs terminating at pA2 and pA1 respectively, hEF: band from RNA protected by hEF probe. The autoradiogram was exposed to the gel for 16 hrs.

*Bst*EII construct (Fig. 2F) nor the *Cla*I construct (not shown) gave rise to mRNAs with an altered half-life. In the most upstream frameshift, a stopcodon was encountered after only 100 amino acids. This stopcodon was followed by others in the same reading frame, making readthrough translation highly unlikely. We therefore conclude that the degradation of c-*myc* mRNA does not require the ribosomes to reach the correct stop codon and that the observed stabilization of 3′ truncated *myc* mRNAs does not stem from the disruption of putative interaction between ribosome-coupled factors at the stopcodon and the 3′ terminus of the mRNA. In addition, the demonstrated dependence of c-*myc* mRNA decay on translation (19, 20) does not appear to stem from the necessity of the ribosomes to completely translate the coding portion of the *myc* messenger.

Degradation of c-*myc* mRNAs lacking an (A+U)-rich region is still translation-dependent

We have previously reported that *myc* mRNAs ending at either of the two pA sites have identical half-lives and are both stabilized to the same extent by translation-blocking drugs, suggesting that they are degraded by a similar mechanism (4). Although they have the same half-life as mRNAs ending at pA2, it is theoretically possible that pA1 mRNAs, lacking an (A+U)-rich region, are being degraded by a different mechanism. The (A+U)-rich regions have been shown to play a role in poly(A) tail shortening of the unstable c-*fos* mRNA (31). The shortening of c-*fos* and c-*myc* mRNA poly(A) tails preceeds the degradation of the mRNA, and is dependent on translation (4, 31). Thus, at least for c-*fos* mRNA, there seems to be a link between translational requirement and (A+U)-rich mediated degradation. We were curious to see whether the decay of a *myc* mRNA lacking (A+U)-rich sequences was still translation-dependent. Therefore, we incubated the cells containing the dAU1 construct with cycloheximide, an inhibitor of translation, for 2 hours before adding ActD. mRNAs terminating at pA1 showed the same pattern of stabilization as mRNAs terminating at pA2 (Fig. 4). Apparently, *myc* messengers lacking an (A+U)-rich region are still degraded in a translation-dependent manner.

DISCUSSION

The observed increased stability of 3′ truncated *myc* transcripts found in tumors could have a number of causes. Firstly, the truncation could have resulted in the loss of destabilizing sequences, possibly the (A+U)-rich regions. Secondly, the addition of non-*myc* sequences, which might contain stabilizing elements or which might disrupt primary or secondary structures that are required for rapid degradation, could interfere with the normal pattern of decay.

We have tried to asses the influence of the various alterations in the 3′ end of truncated mRNAs by studying the half-life of altered *myc* transcripts. The effect of deletions in the (A+U)-rich regions was studied using the dAU1, dAU2 and AU1−2 constructs. From our results we can draw several conclusions. Firstly, that deletions and rearrangements in the (A+U)-rich regions do not affect mRNA decay. Secondly, that *myc* mRNAs lacking any (A+U)-rich sequences (dAU1 mRNAs ending at pA1) are stll rapidly degraded and thus, that (A+U)-rich sequences are dispensable for *myc* mRNA instability. This idea is supported by the recently published work of Bonnieu et al. (32), who show that mutation of both AUUUA pentamers to A-GGGA does not affect the turnover of murine c-*myc* mRNA and who do not observe any stabilization when internal deletions in the (A+U)-rich area are made. These results are in contradiction with the previous suggestion that deletions in the c-*myc* (A+U)-rich regions lead to an increase in mRNA half-life. However, these latter studies were performed with constructs in which the deletions started in the c-*myc* coding region and extended into the 3′ UTR (11). Recently, evidence for the presence of a destabilizing element in the C-terminal coding region of c-*myc* RNA was presented (33). The presence of such an element would explain why mRNAs carrying large deletions, including part of the coding region, are stabilized, while mRNAs with deletions in the (A+U)-rich regions remain unstable. The question then arises whether the (A+U)-rich regions encode destabilizing elements at all or whether the sequences that determine the short half-life of c-*myc* mRNA are located entirely outside these regions. Recent experiments with hybrid mRNAs performed in our laboratory show that a c-*myc* RNA fragment containing the (A+U)-rich regions can have a destabilizing effect, although its activity may differ under various conditions (Laird-Offringa *et al.*, submitted). This suggests that, like c-*fos* mRNA, which has been shown to contain destabilizing elements in the coding region and the 3′ UTR (17), c-*myc* mRNA instability is determined by

more than one region in the mRNA. Deletion of the *c-fos* (A+U)-rich element does not affect the turnover of *fos* mRNA under the normal conditions for *fos* expression, i.e. growth factor induction (17). However, when constitutively expressed c-*fos* constructs under control of a foreign promoter are studied, deletion of the (A+U)-rich regions does slow down *fos* mRNA decay (34, 35). Thus, the two instability determinants may have different activities under different conditions. By deleting the (A+U)-rich sequences in c-*myc* mRNA, we have shown that other sequences in the RNA can be sufficient to confer rapid c-*myc* mRNA decay. However, as with c-*fos* mRNA, it is possible that these other sequences are inactive under certain conditions. Under those conditions, *myc* mRNA may be dependent on the (A+U)-rich sequences for its short half-life. This could be the case in the two human tumors expressing 3′ truncated *myc* transcripts. Removal of the (A+U)-rich sequences would then be the cause of the increased half-life of these transcripts.

Alternatively, the fusion of non-*myc* sequences to the 3′ ends of these truncated messengers may have disrupted the function of destabilizing elements located outside the (A+U)-rich region. The effect of loss of c-*myc* 3′ sequences in combination with the addition of non-*myc* sequences on the rate of mRNA decay was studied using the dSsp and dDra constructs. In the dSsp construct, the truncation of the *myc* gene was similar to the one found in the T-cell lymphoma (Fig. 1). We found no adverse effect of the loss of the AU2 region, the pA signals, and (in dDra) part of AU1. Thus, we conclude that the deletions in this part of the gene, and polyadenylation in non-*myc* sequences, does not necessarily lead to an increase in mRNA half-life. The possiblity remains that the potential to stabilize the c-*myc* mRNA is limited to certain sequences. Indeed, it is conceivable that there is a selection for the addition of stabilizing sequences to translocated *myc* genes. Cells in which a translocation leads to stabilization of the *myc* transcript as well as enhanced transcription, may express more *myc* protein than cells in which the mRNA remains unstable, and thus may have a selective advantage. The best way to clarify this issue would be to clone the 3′ truncated *myc* genes, or the cDNAs corresponding to their truncated mRNAs, from the two human tumor cell lines, and to transfect these cDNAs or genomic fragments into cells to determine the half-life of the resulting mRNAs. In addition, more extensive experiments with *myc* genes carrying single or combined deletions in the coding region and the 3′ UTR will be required to determine the contribution of each region to *myc* mRNA instability.

To analyze whether the position of the stop codon with respect to the pA signal or site is relevant to mRNA stability and to asses the role of translation in c-*myc* mRNA degradation, we studied the half-life of mRNAs whose translation stopped prematurely. We found no effect on mRNA half-life, even when no more than 25% of the coding region was translated. This shows that complete translation of the coding region is not necessary for rapid mRNA degradation. Thus, if translation of c-*myc* mRNA itself is a requirement for rapid degradation and demonstrates that a coupling between ribosome termination at the correct site and decay signals in the 3′ end of the mRNA (as is the case for histone) is absent. Thus, if translation of *myc* RNA itself is a requirement for rapid degradation, as is suggested by the extremely rapid effect of protein synthesis inhibitors on *myc* mRNA breakdown (20), this requirement is restricted to translation of only a small part of the coding sequence. Experiments with various protein synthesis inhibitors have suggested that ribosomal movement along the mRNA may be of importance for proper decay (19, 20). In that case, the length of the translated region may not be relevant. The similar stabilization of normal *myc* mRNAs and *myc* mRNAs lacking any (A+U)-rich region (dAU1 mRNAs ending at pA1) by the inhibition of translation indicates that *myc* mRNA degradation requires translation, irrespective of the sequences that mediate its instability.

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