Exon 2-Mediated c-myc mRNA Decay In Vivo Is Independent of Its Translation

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Received 15 April 1996/Returned for modification 22 May 1996/Accepted 25 June 1996

We have previously shown that the steady-state level of c-myc mRNA in vivo is primarily controlled by posttranscriptional regulatory mechanisms. To identify the sequences involved in this process, we constructed a series of H-2/myc transgenic lines in which various regions of the human c-MYC gene were placed under the control of the quasi-ubiquitous H-2K class I regulatory sequences. We demonstrated that the presence of one of the two coding exons, exon 2 or exon 3, is sufficient to confer a level of expression of transgene mRNA similar to that of endogenous c-myc in various adult tissues as well as after partial hepatectomy or after protein synthesis inhibition. We now focus on the molecular mechanisms involved in modulation of expression of mRNAs containing c-myc exon 2 sequences, with special emphasis on the coupling between translation and c-myc mRNA turnover. We have undertaken an analysis of expression, both at the mRNA level and at the protein level, of new transgenic constructs in which the translation is impaired either by disruption of the initiation codon or by addition of stop codons upstream of exon 2. Our results show that the translation of c-myc exon 2 is not required for regulated expression of the transgene in the different situations analyzed, and therefore they indicate that the mRNA destabilizing function of exon 2 is independent of translation by ribosomes. Our investigations also reveal that, in the thymus, some H-2/myc transgenes express high levels of mRNA but low levels of protein. Besides the fact that these results suggest the existence of tissue-specific mechanisms that control c-myc translatability in vivo, they also bring another indication of the uncoupling of c-myc mRNA translation and degradation.

The cellular c-myc proto-oncogene is a member of the family of early-response genes, which encode short-lived mRNAs whose expression is upregulated after stimulation by growth signals and superinduced after inhibition of protein synthesis (for review, see reference 19). The mechanisms contributing to c-myc mRNA expression have been extensively studied in vitro and, more recently, in vivo. These studies have allowed the identification of numerous cis-acting sequences (reviewed in references 32 and 38) and a few trans-acting factors involved in transcriptional regulation of c-myc expression (5, 22, 44, 49, 58). On the other hand, regulation at the posttranscriptional level has shown to be an essential component of c-myc expression in various situations (12, 30, 34, 41, 54) (reviewed in references 29 and 51), the stability of c-myc transcripts being the most studied regulatory parameter. The rates of c-myc mRNA turnover are controlled by AU-rich elements (ARE), including two AUUUA motifs, contained in its 3' untranslated region (UTR), of which a variable number of copies are found in the AREs of many labile mRNAs (7, 28). However, deletion of this element does not lead to a fully stable transcript (30). This result can be explained by more recent in vitro and ex vivo experiments which showed that the sequences encoding the carboxy-terminal part of c-MYC protein contain an element that specifies rapid mRNA turnover (3, 18, 31, 57).

In adult mice, although the rates of c-myc transcription are roughly similar among various tissues (40), c-myc mRNA expression is variable from one organ to another. Although the level of expression is high in lymphoid organs and intermediate in intestine, it is very low elsewhere, including in muscle, liver, brain, and kidney tissue. However, in the liver, c-myc mRNA expression can be dramatically and rapidly induced in response to partial hepatectomy or treatment with inhibitors of translation with no noticeable change in the rate of transcription (42). Taken together, these findings indicate the existence of an important posttranscriptional component in the regulation of c-myc expression in vivo. In order to evaluate the role of various portions of c-myc mRNA in the control of its expression in vivo, we derived a series of transgenic lines containing different regions of the human c-MYC gene under the transcriptional control of the quasi-ubiquitous H-2K class I 5' regulatory sequences. The analysis of H-2/myc transgenic mice confirmed that the tissue-specific level of c-myc mRNA is mainly controlled by posttranscriptional events. It also showed that transgenes containing only one of the two coding exons, exon 2 or exon 3, independently of the presence of the 3' UTR, are expressed with the same tissue specificity as endogenous c-myc mRNA and that they are also inducible in the regenerating liver and after inhibition of protein synthesis (43). These results indicate that c-myc coding sequences contain at least two independent cis-acting elements involved in its posttranscriptional regulation, a situation also described for another early response gene, c-fos (52).

The results obtained with H-2/myc transgenic mice prompted us to analyze the mechanisms by which these coding elements act to ensure proper regulation of c-myc mRNA abundance. The observation that c-myc transcripts are dramatically stabilized in cultured cells or in the livers of mice after treatment with protein synthesis inhibitors raises the question
of the possible mechanisms linking c-myc mRNA turnover to the process of translation.

In the present study, we have tested whether ribosome translocation across the coding region of exon 2 is required for the correct posttranscriptional regulation in vivo of c-myc mRNA. For this purpose, we have constructed new H-2/myc transgenes which contain either a mutation disrupting the AUG initiation codon, from which the c-MYC 2 (p62) protein is normally synthesized, or stop codons at the beginning of exon 2. The comparison of expression of these transgenes with that of their nonmutated counterparts indicates that translation of exon 2 is not required for correct c-myc mRNA turnover in the quiescent liver, after cycloheximide (CHX) treatment, or after partial hepatectomy.

MATERIALS AND METHODS

Constructs and transgenic lines. All of the constructs were derived from the myc 12 construct (Fig. 1) which corresponds to the HindIII-EcoRI 8,080-bp-long fragment of the human c-myc gene (14).

(i) H-2/myc 21 and H-2/myc 24. The H-2/myc 21 construct was generated as previously described (31). Briefly, a PCR-amplified fragment including all human c-myc exon 2 sequences was inserted into the unique PmlI site present in the exon 3 of the H-2 3′3 plasmid (11). For H-2/myc 24, a PCR fragment including all human c-myc exon 2 sequences was obtained by using as a 5′ primer, 5′-GTCA CGTGATGACCCGCCGAGAATCCG-3′, which includes a PmlI site (CAGCGT) and two successive stop codons (TGATGA), and by using as a 3′ primer, 5′-GAGCACGTGCTGGTGCGGTG-3′, which also includes a PmlI site. The amplified fragment was digested with PmlI and inserted into frame into the PmlI site of the H-2 3′3 plasmid. Junctions and mutation sites were checked by sequencing. Both plasmids were cut with EcoRI for microinjection.

Transgenic mice were obtained by microinjection of the purified H-2/myc fragments without vector sequences into the pronuclei of fertilized eggs derived from a C57BL/6 × SJLJ F1 hybrid mated to identical males as described in reference 4. Several transgenic lines were derived from each construct. Offspring were analyzed 2 weeks after birth for the presence of the injected fragments (i) by Southern blot analysis of tail DNA and (ii) by PCR, with probes and primers specific to human c-MYC exon 2 as previously described (31). For experiments with CHX, 3- to 4-month-old transgenic mice were injected with 50 mg of CHX by intraperitoneal injection.

(ii) H-2/myc 15 and H-2/myc 25. To construct H-2/myc 15, the ATG initiation codon at the beginning of exon 2 was replaced by an ATC codon (Fig. 1) by the method described in references 26 and 27 with the H-2/myc 2 plasmid (43) used as a template and the 5′-TAGGCCGAGAATCCG-3′ primer. To construct H-2/myc 25, the H-2/myc 15 plasmid was digested with ClaI and the excised 1,600-bp fragment was replaced with a ClaI-ClaI fragment from plasmid H-2/myc 6 (43), which includes a BclI-EcoRI 1-kb-long fragment containing the simian virus 40 polyadenylation site. Both plasmids were linearized with NotI for microinjection.

RNA isolation and S1 nuclease analysis. Total RNA was extracted from different organs by the LiCl procedure (2), and the amount was determined by measuring the A260. All RNA samples were analyzed by minigel electrophoresis before hybridization. The S1 nuclease analysis and the probes used in this study have been previously described (31). The results of S1 experiments were analyzed quantitatively by direct scanning of the acrylamide gels with storage phosphor screens and the PhosphorImager apparatus (Molecular Dynamics).

Protein synthesis in vitro. (i) Preparation of synthetic RNAs. Reverse transcription was performed with total RNA samples extracted from the spleens of H-2/myc 21 and H-2/myc 24 mice with Moloney murine leukemia virus reverse transcriptase and an oligo(dT) primer. The cDNAs obtained were used as templates for PCR amplification with a 5′ primer corresponding to the 5′ end of H-2/2K transcript (5′-CAGAAGTGCGAATCCGCGG-3′) and a 3′ primer complementary to c-myc exon 2 sequences from nucleotides 5,144 to 5,165 (5′-GG CGCTGGAATCCGCGG-3′). The resulting 1,200-bp-long amplified DNA fragments include sequences from the transcription start site of H-2/myc 21 or H-2/myc 24 mRNA to the 5′66th nucleotide of c-myc exon 2. The amplified fragments were subcloned into the pCR vector with the TA Cloning Kit (Invitrogen). The resulting plasmids were linearized with BamHI and used as templates for in vitro transcription with T7 RNA polymerase and the cap analog 7-mGpppG (Pharmacia) to obtain capped synthetic RNAs.

(ii) Translation. Two micrograms of H-2/myc 21 or H-2/myc 24 synthetic RNA was used for in vitro translation with the rabbit reticulocyte lysate system (nu- clear extract; Promega) with [35S]methionine according to the manufacturer’s instructions. One-tenth of the translation reaction mixture was run on a denaturating sodium dodecyl sulfate (SDS)-12% polyacrylamide gel. Gels were fixed, dried, and autoradiographed to detect translation products.

Preparation of proteins, lysis and immunoblotting. Thymi, mesenteric lymph nodes, and spleens of control and H-2/myc mice were dissected; lymphocytes were isolated, counted, aliquoted, lysed in electrophoresis sample buffer containing 100 mM dithiothreitol and boiled for 8 min. The samples were then subjected to SDS-polyacrylamide gel electrophoresis (PAGE). The proteins were transferred on to a nitrocellulose filter which was subsequently stained with Ponceau S for protein loading estimation. The filters were blocked with 5% lyophilized nonfat milk in Tris-buffered saline (TBS) and incubated overnight at 4°C with the anti-c-myc monoclonal antibody 9E10 (raised against human c-MYC amino acids 408 to 439 [13]) or rabbit polyclonal antibody N-262 (raised against human c-MYC amino acids 1 to 262, Santa Cruz Biotechnology) in TBS. After washing, the anti-MYC antibodies were revealed by incubation with goat

FIG. 1. Schematic representation of the different H-2/myc constructs. The structure of the myc 12 construct, which corresponds to the 8,080-bp-long HindIII-EcoRI fragment of the human c-MYC gene, is shown at the top. The localization of the two probes used in S1 nuclease analysis, which either protect 360 nucleotides of exon 2 sequences (probe exon 2) or span the exon 2-intron 2 boundary (probe exon2in, detailed in Fig. 5D), is shown below the myc 12 construct. The H-2/myc constructs contain various portions of the human c-MYC gene. The H-2/myc 15 and H-2/myc 25 constructs were derived from H-2/myc 2 and H-2/myc 6 constructs, respectively (43). They both contain an ATC codon instead of the ATG initiation codon at the beginning of c-MYC exon 2. In H-2/myc 25, c-myc 3′ noncoding sequences containing polyadenylation sites have been replaced by the 3′ noncoding region of the early region of simian virus 40 (SV40), including a polyadenylation signal. The H-2/myc 21 construct corresponds to a full-length H-2K gene, in exon 3 of which c-MYC exon 2 has been inserted in-frame. In the H-2/myc 23 construct, the same c-MYC exon 2 sequences are in the reverse orientation. H-2/myc 24 is identical to H-2/myc 21, except that two TGA stop codons have been inserted at the beginning of c-MYC exon 2 sequences. P1 and P2, P1 and P2 c-myc promoters; PH, H-2K promoter; CTG and ATG, translation initiation codons of the c-MYC 1 and 2 proteins.
anti-mouse or donkey anti-rabbit peroxidase-labeled antibodies (Amersham), respectively. Peroxidase activity was revealed by chemiluminescence with the enhanced chemiluminescence Western blotting (immunoblotting) detection system (Amersham). In several experiments, the tissue sample was divided and used both for RNA and protein analysis. For the immunoblot shown in Fig. 7C, bound antibody 9E10 was revealed by incubation with rabbit anti-mouse immunoglobulins followed by 125I-labeled protein A and autoradiography.

RESULTS

Experimental approach. In the present study, we have focused our analysis on the possible role of translation in the control of mRNA stability by the c-myc exon 2 coding region determinant (referred to as CRD2). To that end, we constructed the untranslatable counterparts of two previously described transgenes which both contain CRD2, H-2/myc 21 and H-2/myc 21 (43) and H-2/myc 21 (31) (see Fig. 1). We analyzed transgene mRNA expression in the resulting transgenic lines by S1 nuclease assay in various situations and compared it with that of the endogenous c-myc. In theory, a reduction or an increase in cytoplasmic mRNA abundance may be attributable to several factors, including altered efficiency of transcription, premRNA processing, nuclear mRNA export, and nuclear and/or cytoplasmic mRNA decay or stabilization. However, because of the relatively constant transcriptional activity of the H-2K promoter in all of the situations analyzed (43), any variation in transgenic mRNA steady-state levels should be attributable to posttranscriptional events. Moreover, we performed S1 nuclease analysis of total RNA with c-myc exon 2 probes; this allowed us to measure a single signal corresponding to both nuclear and cytoplasmic transcripts. Thus, any variation of this signal will reflect changes in the stability of transgene mRNAs, regardless of their intracellular localization and/or export to the cytoplasm.

Translation of c-myc exon 2 is not required for controlled transgene expression. We have previously described two transgenic lines, H-2/mic 21A and 21B, which encode chimeric mRNAs containing all H-2Kb coding sequences plus c-MYC exon 2 sequences inserted in-frame within H-2Kb exon 3. As previously reported (31), this insertion was able to confer on transgene mRNA a pattern of expression similar to that of endogenous c-myc (i) in adult tissues (as illustrated in Fig. 2A), (ii) in the liver after CHX treatment (Fig. 2C), and (iii) during the early steps of regeneration (Fig. 3B). These results pointed to a role for exon 2 sequences in the posttranscriptional regulation of c-myc and stressed the importance of translation in this process. To determine whether preventing translation of c-myc exon 2 in this context would have any effect on transgene expression, we derived H-2/myc 24 transgenic mice, in which two stop codons were placed right at the beginning of c-MYC exon 2 (Fig. 1). Thus, the mRNAs transcribed from the H-2/myc 21 transgene and its mutated H-2/myc 24 counterpart should be translated from the AUG initiation codon present in the first exon of the H-2Kb gene, but in the case of H-2/myc 24,
translation should be prematurely terminated at amino acid 188 when the stop codons are encountered.

We analyzed H-2/myc 24 transgene expression at the mRNA level by S1 nuclease protection assay in different tissues of four independent transgenic lines by using a probe hybridizing specifically to human c-myc exon 2 sequences and the H-2Kb exon 3 probe as an internal standard. As a control for correct splicing, we used an H-2Kb probe encompassing intron 2-exon 3 boundaries which allowed us to verify that the insertion of c-MYC exon 2 sequences does not affect splicing of chimeric mRNAs (data not shown). As shown for the two H-2/myc 24A and 24C lines, the patterns of expression of H-2/myc 24 and of H-2/myc 21 mRNAs are nearly identical (Fig. 2A and B). In particular, their level of expression is high in lymphoid organs and weak in the liver, while H-2K is expressed at a similar level in both organs. We also examined two other aspects of post-transcriptional regulation: the transient accumulation of c-myc mRNA after inhibition of protein synthesis and that during the early steps of liver regeneration. As shown in Fig. 2C, H-2/myc 24 mRNA expression increases in the liver 2 h after CHX treatment to levels comparable to those of H-2/myc 21 and murine c-myc mRNAs. Similarly, its expression increases 1 h after partial hepatectomy and parallels that of endogenous c-myc transcripts (Fig. 3). Taken together, these results indicate that the nonsense mutation does not interfere with post-transcriptional tissue-specific regulation of transgene expression. However, it was important to test the effectiveness of this mutation in blocking ribosome progression. As shown in Fig. 4A, the only phase of H-2/myc 24 mRNA which contains no multiple stop codons is phase 1, in which we inserted the two UGAs at the beginning of exon 2 in the correct H-2/c-myc reading frame. To test the ability of the nonsense mutation to stop ribosome progression, we performed reverse transcription with total RNA samples extracted from the spleens of H-2/myc 21 and H-2/myc 24 mice. The cDNAs obtained were used as templates for PCR amplification to obtain 1,200-bp-long amplified DNA fragments which included sequences from the transcription start site of H-2/myc 21 or H-2/myc 24 mRNA to the 665th nucleotide of c-myc exon 2 (see Materials and Methods). The amplified fragments were subcloned and used as templates for in vitro transcription to obtain capped synthetic RNAs. We then compared their translation efficiencies in rabbit reticulocyte lysates with [35S]methionine (see Materials and Methods). As shown in Fig. 4B, translation of H-2/myc 21 synthetic RNA, which does not contain stop codons, resulted in a peptide of the expected size of ~40 to 45 kDa, while only a small product, of the size expected after translation of H-2K sequences up to the stop codons (~20 kDa), was observed with H-2/myc 24 synthetic RNAs.

A more direct proof of the effectiveness of the mutation was obtained in a second series of experiments with immunoblotting of protein lysates obtained from the spleens of H-2/myc 21B and H-2/myc 24C mice (Fig. 4C [see Materials and Methods]). The qualitative and quantitative consistency of the loadings was checked by Ponceau S staining of the blotted proteins (data not shown). Because no antibody directed against the amino-terminal portion of H-2K protein common to both H-2/myc 21 and H-2/myc 24 gene products was available, we used a polyclonal antibody (N-262) raised against a peptide corresponding to the entire human c-myc exon 2. A molecule with an apparent size of ~88 kDa was recognized by these antibodies in extracts of H-2/myc 21 cells but not H-2/myc 24 cells, even when eight times more cells were used for preparing lysates. The comparatively lower level of transgene mRNA expression in H-2/myc 24C than in H-2/myc 21B spleen (approximately fourfold, as measured by PhosphorImager scanning) (Fig. 4D) cannot account for such quantitative differences at the protein level. Identical results were obtained with extracts from mesenteric lymph nodes (data not shown). These data indicate that the nonsense mutation introduced in the H-2/myc 24 construct is able to prevent ribosome progression through c-myc exon 2 in vitro and in vivo; together with the analysis of transgene mRNA expression (the results of which are summarized in Table 1 and Fig. 3B for partial hepatectomy), the data demonstrate that the correct posttranscriptional regulation of the transgene in the three situations analyzed does not rely on translation of c-myc exon 2.
A mutated AUG initiation codon does not alter transgene expression. In order to examine the behavior of a transgene which cannot be translated at all, we derived new constructs which contain human c-MYC exon 2 and exon 3, in association with c-myc or the simian virus 40 3' UTR, respectively. H-2/myc 15 and H-2/myc 25. In these transgenes, the unique AUG initiation codon, located at the beginning of exon 2, has been mutated for an AUC. This choice was dictated by the results obtained from transfection experiments performed with myoblasts in which a mouse c-myc construct containing this mutation was reported to be untranslatable (57).

Transgene mRNA expression in different adult tissues was analyzed by S1 nuclease assay with either a probe encompassing the c-MYC exon 2/intron 2 boundary, which indicates that H-2/myc 15 transgenic RNAs are efficiently spliced (Fig. 5D), or a probe hybridizing specifically with human c-myc exon 2 sequences. The results obtained with the H-2/myc 15D and 25A lines are shown in Fig. 5A. The pattern of expression of both transgenes is similar to that of their nonmutagenized counterparts, H-2/myc 24 and H-2/myc 6, respectively (43). Similarly to endogenous c-myc transcripts, H-2/myc 15 and 25 transgenic mRNA levels are increased after CHX treatment (Fig. 5B) and in the early steps of liver regeneration after partial hepatectomy, as shown in Fig. 5C for the only line tested, H-2/myc 15D.

The dramatic decrease observed 2 h after hepatectomy has been reproducibly found in several independent experiments; we do not yet know its significance.

In order to verify that H-2/myc 15 and H-2/myc 25 mRNAs are indeed not translated in transgenic mice, we prepared protein lysates from the lymphoid tissues, which express the highest level of transgenic mRNAs, and performed an immunoblot with the 9E10 monoclonal antibody (13), which recognizes specifically an epitope located in the carboxy-terminal part of human c-MYC proteins. As seen in Fig. 6A, the normal c-MYC reading frame (frame 1) is the only one which does not contain multiple stop codons. As a positive control, we analyzed extracts from the human fibrosarcoma 2TGH cell line (48), which expresses the two human proteins c-MYC 1 and 2 (Fig. 6B, lane H). These proteins are not present in the spleen lysates of control mice (Fig. 6B, lanes C), while only a very weak signal corresponding to c-MYC 2 protein is observed in H-2/myc 15 and 25 spleen extracts (Fig. 6B, lanes 15 and 25). However, in these transgenic animals, besides some unspecific bands, a product with an apparent size of ~44 kDa (del-MYC) is reproducibly detected in the spleen (Fig. 6B) as well as in the lymph nodes (Fig. 7C, lanes 15D). Taken together, these results show that introduction of the mutation has not altered the splicing efficiency of the constructs and demonstrate that in

![Figure 4](http://mcb.asm.org/may9/5111/fig4.png)

**Figure 4.** Translational efficiency of H-2/myc 21 and H-2/myc 24 transgenic mRNAs. (A) Schematic representation of the encoding capacities of H-2/myc 24 mRNAs on the basis of the presence of open reading frames. Nucleotide numbers are shown. Phase 1, c-myc open reading frame. Short rods, ATG codons; long rods, nonsense codons. The asterisk and the open circle indicate the normal H-2Kb initiation and stop codon, respectively. The two primers used in reverse transcription-PCR experiments to obtain synthetic RNAs for the in vitro translation experiment shown in panel B are indicated by arrows. (B) In vitro translation products from H-2/myc 21 (lane 21) and H-2/myc 24 (lane 24) synthetic RNAs. Kd, molecular mass in kdaltons. The peptides derived from translation of synthetic RNAs are indicated by the arrows. (C) Immunoblot analysis of protein lysates from H-2/myc 21B and H-2/myc 24C splenocytes with polyclonal N-262 antibodies against the first 262 amino acids of the human c-MYC protein. The number of cells from which the lysates were prepared is indicated at the top. After dissection, the tissue was divided into two parts: one was used for mRNA detection, and the other was used for protein analysis. Lane C, lysates from the spleen of a nontransgenic mouse; lane H, lysates from the human 2TGH cell line, expressing both c-MYC 1 and 2 proteins, used as positive control. (D) Analysis of the level of transgenic mRNA expression in the same samples as those used in panel C. Twenty micrograms of total RNA was hybridized simultaneously with the human c-myc exon 2 (hmyc) and H-2Kb (H2) probes.
lymphoid organs expressing high levels of transgene mRNAs, a smaller c-MYC protein is synthesized, probably arising from translation initiation at downstream start sites in the exon 2. In the thymus, some transgene mRNAs are strongly expressed. As shown with all H-2/myc constructs, transgene mRNAs are expressed at a similar level in the spleen, thymus, and lymph nodes (Fig. 2A and B, 5A and B, and 7B). However, when H-2/myc 2B transgenic mice were examined, c-MYC 2 protein was barely detectable in their thymus (Fig. 7A, lanes th1, th2, and th3), in contrast to their lymph nodes and spleens, where it could be readily revealed (Fig. 7A, lanes sp1, ln2, and ln3). This unexpected result was consistently found in many independent experiments performed with thymic proteins, even when extracted from H-2/myc 2A mice, which express higher levels of transgene mRNAs (Fig. 7C and data not shown). Interestingly, similar results could be extended to H-2/myc 15D mice, which express the del-MYC protein in their spleens (Fig. 6B) and lymph nodes (Fig. 7C) but not in their thymus (Fig. 7C). This is not due to technical difficulties in detecting c-MYC proteins in thymic lysates, since we could easily observe c-MYC 1 and 2 proteins in the thymi of myc 12A transgenic mice (Fig. 1), which contain the complete human c-MYC sequences, including exon 1 (Fig. 7C, lane 12A, and reference 33).

**DISCUSSION**

One critical issue pertaining to the function of RNA elements which contribute to mRNA instability is whether their action is coupled to protein translation. Indeed, early observations emerged from ex vivo studies showing that protein synthesis inhibitors, like puromycin or CHX, superinduced the accumulation of granulocyte-macrophage colony-stimulating factor, c-fos, and c-myc transcripts (35, 39, 53). With regard to this, at least two non-mutually exclusive models can be considered. In the *cis* model, translation of the determinants of instability present in the mRNA is necessary for its rapid turnover. This could be mediated by factors associated with ribosomes, as in the case of histone (45), or by specific sequences present in the nascent peptide, similarly to what occurs in the case of β-tubulin (9). In the *trans* model, the translation of specific sequences is not required, and mRNA stability is necessary for its rapid turnover. This schematic view can be refined by taking into account, for instance, the cellular location of these proteins, i.e., nuclear and/or cytoplasmic and their association or lack of association with polysomes.

Novel approaches have recently been designed to try to distinguish between a cotranslational decay mechanism (*cis* effect) and one requiring a labile nuclease(s) (*trans* effect). These include inhibition of translation by hairpin insertion (8, 10), target mutation (30, 57), insertion of an internal ribosomal entry site to restore translation (1) and the use of the ferritin iron-responsive element to shuttle chimeric mRNAs from ribonucleoproteins to polyribosomes (56). Several controversial
Results have been obtained concerning the ARE-mediated decay: in the case of granulocyte-macrophage colony-stimulating factor and c-fos, ARE has been shown either to function as a decay element only when the mRNA bearing it is translated by ribosomes (1, 10, 50, 56) or, on the contrary, to be uncoupled from translation by ribosomes (8, 24). Because different experimental systems have been used in these studies, the discrepancy between these conclusions might reflect the requirement of cell- or cell-cycle-specific factors for ARE to exert its translation-(in)dependent destabilization effect. On the other hand, ongoing translation is necessary for the c-fos protein coding region to direct rapid mRNA degradation through deadenylation (8). Whether translation of c-myc coding region determinants is required for decay is still controversial. Although mutation of the AUG codon led to stabilization of mRNA containing the c-myc coding region (57), the half-life of c-myc transcripts containing a frameshift which causes premature termination was identical to that of their translatable counterparts (30). As for c-fos ARE, the differences in the experimental systems used make it difficult to draw definitive conclusions.

Here, we have employed targeted inhibition of translation to examine in vivo the possible coupling between translation and...
the destabilizing activity of c-myc exon 2. In our experimental system, the use of the H-2K promoter, together with the analysis as a whole of both nuclear and cytoplasmic transgene mRNAs, allowed us to conclude that the variations observed in the H-2myc mRNA steady-state levels are due to changes in their stability. We have previously shown that the lability of hybrid H-2/c-myc transgene mRNAs in the liver (H-2/myc 21) was due to the insertion of the c-MYC exon 2 sequences in the 5’→3’ orientation into the coding region of the H-2Kb gene (31). In the present study, we have introduced a stop codon mutation into the same reporter mRNA, giving rise to the H-2/myc 24 transgene, and the consequences of the mutation on exon 2 destabilizing function were analyzed under the same experimental conditions. Moreover, we have verified that the mutation effectively prevented translation of the target sequences in vivo. For this purpose, we directly tried to detect the possible peptides derived from translation of transgene mRNAs by performing immunoblot experiments with lysates from lymphoid tissue. Two reasons prompted us to use this approach instead of undertaking a gradient analysis of polysomal distribution of H-2/myc mRNA: first, the lymphoid organs in which transgene mRNAs are highly expressed are rich in nonspecific nucleases and, second, because of their relatively large size, H-2/myc hybrid transcripts would tend to sediment as large particles even in the absence of translation (55). Comparative analyses of H-2/myc 21 and H-2/myc 24 protein lysates and mRNA expression confirm that the nonsense mutation is effective and show that inhibition of ribosome translocation through c-MYC exon 2 does not interfere with its ability to properly regulate hybrid transgene transcript expression in the different situations analyzed.

Theoretically, the premature termination of translation could expose H-2/myc 24 mRNA to degradation by triggering nonsense-mediated mRNA decay. This mechanism of degradation of aberrant mRNAs in yeast (reviewed in reference 23) and mammalian (see reference 37 and references therein) cells has been well studied. However, several arguments indicate that such a mechanism is not responsible for the observed pattern of H-2/myc 24 expression. The first argument relies on the fact that the expression patterns of H-2/myc 21 and H-2/myc 24 in different adult tissues are very similar; therefore, if a nonsense-mediated degradation mechanism operated for H-2/myc 24, its tissue specificity should follow that of normal CRD2-mediated instability, an occurrence which is highly improbable. The second relies on the analysis of another transgenic line, H-2/myc 23, in which exon 2 is inserted at the same location within the H-2Kb coding region but in the opposite orientation (Fig. 1 and reference 31). Despite the fact that multiple stop codons prevent ribosome translocation through these antisense c-myc sequences, transgene mRNAs are expressed in the liver at levels comparable to that found in lymphoid organs (31).

The finding that translation of exon 2 sequences is not required for its regulatory role in the half-life of c-myc mRNA in different organs and physiological situations is important in several respects. First, it implies that the regulatory determinant or determinants present in c-myc exon 2 are recognized as RNA sequences, ruling out any involvement of the nascent c-MYC peptide in exon 2-mediated mRNA decay, such as that found, for instance, for β-tubulin mRNA (9). Second, it excludes any direct involvement of the reading ribosome in this process, giving strength to the hypothesis that the degradation of c-myc mRNAs observed in the liver relies on a trans-acting mechanism mediated by a labile factor or factors which might be located in the cytoplasm and/or in the nucleus. An important goal in future work will be to identify these trans-acting factors. Our results suggest that these factors are more abundant in hepatocytes and in other low-c-myc mRNA-expressing tissues than in lymphoid tissues and that they disappear or are rapidly inactivated after protein synthesis inhibition or in the very early steps after partial hepatectomy. Alternatively, degradative factors may be ubiquitous, and protective factors could counteract their effect specifically in lymphoid organs.

In H-2/myc 24 transcripts, the presence of stop codons should not interfere with their ability to be translated from the H-2K initiation codon located 564 nucleotides upstream and thus to be loaded on polysomes in the same way as H-2/myc 21 with no change in the subcellular compartment in which the translation occurs. Therefore, even if ribosomal translocation is not involved, it is possible that loading on the polysomes favors localization of these mRNAs into a compartment where they could be easily degraded via the CRD2. In order to study the behavior of the whole c-myc coding region in a transcript which would not be loaded on polysomes, we derived constructs H-2/myc 15 and 25, both of which contain a mutated AUG initiation codon but which differ in their 3’ UTRs. In these constructs, the CUG initiation codon in exon 1 that gives rise to the c-MYC 1 protein (17) is not present. The point mutation that changes the translation initiation codon from AUG to AUC was expected, from a previous report (57), to impede polyribosome loading. To our surprise, analysis of protein lysates from transgenic spleen or lymph nodes revealed that H-2/myc 15 or 25 mRNA could be translated, giving rise to a smaller c-MYC protein (del-MYC). This observation is, however, in good agreement with a recent report showing the
existence of a functional initiation codon downstream from the normal c-MYC AUG, which, by a “leaky scanning” mechanism, can produce a minor N-terminal-truncated c-MYC protein (called Δ-c-MYC) (16). The synthesis of this protein, which does not normally occur when the upstream CUG or AUG initiation codons are first encountered by the translation machinery, is dramatically favored when the transcripts do not contain these codons, as in the case of H-2/myc 15 or 25. It should be noted that the synthesis of the Δ-c-MYC proteins, which have been suggested to function as dominant-negative inhibitors of the full-length c-MYC 1 and 2 proteins, since they lack a significant portion of the transactivation domain of c-MYC (16), is not accompanied by any disturbance in lymphocyte maturation, differentiation, or proliferation in the different transgenic lines analyzed (our unpublished results). It is also interesting that the synthesis of the H-2/myc 15 and H-2/myc 25 show similar expression patterns and translational efficiencies, despite the fact that they carry different 3′ UTRs. This indicates that the c-myc 3′ UTR, which seems to play an important role in the localization of c-myc transcripts (21), does not have any major effect either on controlling the differentabilities of transgene mRNAs in the different tissues and situations studied or in modulating their translational efficiency.

Another indication of the uncoupling between c-myc mRNA translation and degradation was provided by the analysis of transgene mRNA translation products in the thymus. Although it is normally assumed that there is a good correlation between c-myc mRNA abundance and the amount of c-MYC proteins synthesized (13), we found a low level of human MYC 2 protein in the thymus of H-2/myc 24 and 28 mice, despite high levels of these nonmutated transgene mRNAs. Similar results were also obtained for H-2/myc 15D mice, in which the truncated del-MYC protein was barely expressed in the thymus. These observations indicate that efficient translation of transgene mRNAs in this organ is not required to protect them from degradation. However, it should be noted that the translational machinery is able to translate c-myc mRNA in the thymus. We could not directly test the synthesis of endogenous c-MYC proteins in this organ because of the low efficiency of various anti-c-MYC antibodies we have tested for detecting these proteins. However, we could easily reveal the presence of human c-MYC 1 and 2 in thymus extracts from the myc 124 line (Fig. 7C and reference 33), in which transgene mRNAs are expressed at a level similar to that found in H-2/myc 24 thymus (data not shown). One explanation for the different translatabilities of these two constructs could rely on the fact that the 5′ portion of myc 12 mRNA, like the endogenous c-myc mRNAs but in contrast to H-2/myc 2 and 15 transgene transcripts, contains the entire c-myc exon 1 and thus exhibits a long 5′ UTR. Indeed, the 5′ UTR of many mRNAs has been shown to exert important controls on their translational efficiency (for examples, see references 15, 20, 46, and 47 and the references cited therein). While this hypothesis awaits further experimental proof, the data we have obtained suggest the existence of tissue-specific mechanisms affecting c-myc mRNA translatability and reveal another degree of complexity in the regulation of c-myc expression.

ACKNOWLEDGMENTS

We thank A. Lavenu for excellent technical assistance and S. Pourrin for the production of transgenic mice. We are also grateful to C. Kean and A. Borman for advice concerning in vitro translation experiments and S. Pellegrini for the gift of the 2TGH cell line. We thank J. Smith for critical reading of the manuscript.

This work was supported by ARC grant 6780. S. Pisto received financial support from CNP and ARC.

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