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Both Coding Exons of the c-*myc* Gene Contribute to Its Posttranscriptional Regulation in the Quiescent Liver and Regenerating Liver and after Protein Synthesis Inhibition

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In vivo, the steady-state level of c-*myc* mRNA is mainly controlled by posttranscriptional mechanisms. Using a panel of transgenic mice in which various versions of the human c-*myc* proto-oncogene were under the control of major histocompatibility complex *H-2K*^{b} class I regulatory sequences, we have shown that the 5 and the 3 noncoding sequences are dispensable for obtaining a regulated expression of the transgene in adult quiescent tissues, at the start of liver regeneration, and after inhibition of protein synthesis. These results indicated that the coding sequences were sufficient to ensure a regulated c-*myc* expression. In the present study, we have pursued this analysis with transgenes containing one or the other of the two c-*myc* coding exons either alone or in association with the c-*myc* 3 untranslated region. We demonstrate that each of the exons contains determinants which control c-*myc* mRNA expression. Moreover, we show that in the liver, c-*myc* exon 2 sequences are able to down-regulate an otherwise stable *H-2K* mRNA when embedded within it and to induce its transient accumulation after cycloheximide treatment and soon after liver ablation. Finally, the use of transgenes with different coding capacities has allowed us to postulate that the primary mRNA sequence itself and not c-Myc peptides is an important component of c-*myc* posttranscriptional regulation.

In recent years, it has been realized that the rates of RNA decay in the nucleus and in the cytoplasm are important in determining the level of expression of a gene (reviewed in reference 5). In eukaryotic cells, the range of mRNA stability can vary over several orders of magnitude, and numerous studies have indicated that several mRNAs contain within them the information necessary to determine their stability. Transiently expressed genes such as those encoding proto-oncogenes and growth factors have far shorter half-lives than others, such as those encoding β-globin or albumin (15, 32). The mechanisms of differential degradation are an attractive problem which covers several distinct aspects, such as analysis of the structural features of mRNA that determine its susceptibility to decay, identification of the locations of the destabilizing determinants, determination of the *trans*-acting factors with which they interact, and discovery of the physiological signals which alter rates of mRNA decay (reviewed in reference 30).

Several types of destabilizing elements which can confer instability to a normally stable mRNA have been described. In general terms, it appears that removal of one element from its natural context does not necessarily lead to a stabilization of the message, suggesting that destabilizing information may lie within multiple discrete regions of an RNA (33). Resolution of the question of their redundancy is important for a complete understanding of the molecular mechanisms controlling mRNA decay; thus far, a link between translation and mRNA decay has been evidenced in several cases (reference 31 and references cited in references 15, 18, and 30). Unravelling these mechanisms has been hampered by the heterogeneity of the regulatory sequences analyzed and by the nearly complete absence of purified *trans*-acting factors.

The cellular c-*myc* proto-oncogene belongs to the family of early response genes, which encode short-lived mRNAs (half-life of less than 30 min) (4, 12, 24) whose expression is up-regulated following stimulation by growth signals and dramatically increased in cells or animals treated with protein synthesis inhibitors (21, 24–27, 34, 37). Several sequences have been shown to contribute to the modulation of its rate of decay. They are located either in the AU-rich 3′ untranslated region (UTR) (2, 4, 6–8, 19, 29) or in the last part of exon 3 (3, 38). The mechanisms involved in the degradation process are poorly understood, although the poly(A) tail seems to play an important role (7, 21). Removal of the poly(A) tail may be a general mechanism by which the mRNAs containing the AU-rich element are triggered for rapid degradation (reference 10 and references therein). Several proteins which might participate in c-*myc* mRNA protection or degradation have been characterized (3, 6, 17, 40). These results demonstrate the existence of separate stability determinants in c-*myc* mRNA and raise a number of questions regarding how these determinants function under specific circumstances.

Although in vitro or ex vivo experiments may be suitable for delineating the sequences and characterizing the proteins involved in *trans* in c-*myc* mRNA stability, they fail to give information concerning the functions, if any, of these proteins in vivo and their contributions in different physiological situations in the adult and during development. To gain insight into this problem, we decided to construct transgenic lines which express various regions of the human c-*myc* proto-oncogene under the control of the quasi-ubiquitously expressed class I *H-2K* gene regulatory sequences (13) and to compare the expression

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of *H-2/myc* transgenes with that of the endogenous c-*myc* gene in various situations: in quiescent adult organs, during the early steps of liver regeneration, and upon protein synthesis inhibition. We show that the presence of either one or the other of the two coding exons is sufficient for a regulated transgene expression. Moreover, because of the different encoding capacities of the different *H-2/myc* transgenes, our data strongly support the hypothesis that posttranscriptional regulation involving the exon 2 and exon 3 coding regions does not rely on their efficient translation by ribosomes.

MATERIALS AND METHODS

Constructs and transgenic lines. Five *H-2/myc* constructs were prepared; all contain human c-*myc* genomic sequences under the control of 2 kb of 5′ *H-2K*^{b} gene regulatory sequences. The H-2/myc 19 plasmid was derived from the H-2/myc 2 construct, which does not contain 5′ c-*myc* sequences (including exon 1) (28). The H-2/myc 17 and H-2/myc 22 constructs were derived, respectively, from H-2/myc 1 and H-2/myc 5 constructs, which both contain the P2 c-*myc* promoter and the remaining exon 1 sequences but differ in their 3′ UTRs (28). The H-2/myc 21 and H-2/myc 23 constructs were obtained by inserting c-*myc* exon 2 sequences in either orientation inside the *H-2K*^{b} gene.

(i) H-2/myc 19. H-2/myc 2 was cut with *Cla*I. Its ends and those of the *Bcl*I-*Eco*RI 1-kb fragment of the simian virus 40 (SV40) early noncoding region containing the polyadenylation site were blunted with the Klenow fragment of *Escherichia coli* DNA polymerase I and ligated. The 5.5-kb *Xho*I-*Xho*I fragment was recovered from the resulting H-2/myc 19 plasmid and used for microinjection. The donor splice site present in the polylinker between *H-2K* and c-*myc* sequences ensures an efficient splicing of the c-*myc* intron 1 sequences (28).

(ii) H-2/myc 17 and H-2/myc 22. H-2/myc 1 and H-2/myc 5 were cut with *Sac*I and religated, such that the *Sac*I-*Sac*I 2,140-bp fragment of the human c-*myc* gene containing 1,240 bp of intron 1, the entire exon 2, and the first 130 bp of intron 2 was deleted. The resulting plasmids were *Hin*dIII and *Eco*RI restricted to generate, respectively, the 5.5- and the 5.7-kb fragments used for microinjection.

(iii) H-2/myc 21 and H-2/myc 23. H-2/myc 21 and H-2/myc 23 were generated from plasmid H-2 3′$^\Delta$, which contains 6,430 bp of *H-2K*^{b} genomic sequences, including 2 kb of 5′ regulatory region and the polyadenylation site. Plasmid H-2 3′$^\Delta$ was cut with *Pml*I, which cuts once within exon 3. A 770-bp segment that includes all of the human c-*myc* exon 2 sequence was amplified by PCR using the high-fidelity enzyme *Pfu* (Stratagene) and, as 5′ and 3′ primers, 5′-CTCACGT GTCCCGCGACGATACCCC-3′ and 5′-GAGCACGTGCTGGTGGTGGGC GGTG-3′, respectively. The amplified fragment was cut with *Pml*I and cloned in frame into the *Pml*I site of plasmid H-2 3′$^\Delta$. Both *Pml*I junctions were checked by DNA sequencing, and no PCR-induced misincorporation was detected in the 140 and 93 nucleotides (nt) sequenced at the 5′ and 3′ sides, respectively. Plasmid H-2/myc 21 contains the c-*myc* exon 2 sequence in the 5′-3′ orientation. The use of the 5′ primer creates an ATG-ATA substitution such that plasmid H-2/myc 21 contains only the ATG translation initiation codon from the *H-2K*^{b} gene. Plasmid H-2/myc 23 contains the same c-*myc* exon 2 sequence in the 3′-5′ orientation. Both plasmids were cut with *Eco*RI to generate the 9.5-kb fragments used for microinjection.

The 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 × SJL/J)F_1 hybrid mated to identical males as described previously (9). For experiments with cycloheximide (CHX), 3- to 4-month-old transgenic mice were injected with 50 mg of CHX, dissolved in water, per kg of body weight as described previously (25). Partial hepatectomy and sham operations were performed as described previously for rats (14).

DNA analysis. Offspring were analyzed 2 weeks after birth for the presence of the injected fragments (i) by Southern blot analysis of tail DNA *Pst*I restricted, using as a probe either the *Sac*I-*Sac*I 2.1-kb-long fragment encompassing human c-*myc* exon 2 or the *Cla*I-*Eco*RI 1.4-kb fragment containing human c-*myc* exon 3 and (ii) by PCR using either a pair of primers complementary to human c-*myc* exon 2 5′ (5′-TTCGGGGAGACAACGACGGCG-3′) and 3′ (5′-GGCGCTGG AGTCTTGCGAGGCG-3′) sequences or a pair of primers located in the *H-2* promoter (5′-GTCCACGCAGCCCGCAGAACTC-3′) and in the human c-*myc* exon 1 sequence (5′-AAGCAGCTGCAAGGAGAGCC-3′). The sizes of the amplified fragments are 400 and 450 bp, respectively. Transgenic lines were established by mating to C57/BL6 × SJL/J males or females, and transgenic offspring were identified by PCR using the appropriate primers.

RNA isolation and S1 nuclease analysis. Total RNA was extracted from different organs by the LiCl procedure (1), and the amount was determined by measuring the A_{260}. All RNA samples were examined by minigel electrophoresis before being used for hybridization. The murine c-*myc* exon 1 and the human c-*myc* exon 1, exon 2, and exon 2/intron 2 (ex2/in2) probes, as well as the S1 nuclease analysis, have been previously described (26, 28); the two human c-*myc* exon 2 probes are schematized at the bottom of Fig. 2. The c-*myc* in2/ex3 probe used to study the intron 2 splicing efficiency (Fig. 4C) was obtained after subcloning into M13mp19 a 1,578-nt-long fragment (nt 5150 to nt 6728) encompassing the human c-*myc* intron 2/exon 3 boundary. The probe recovered after polymerization and *Pvu*II restriction is 600 nt long. The protected fragment corresponding to fully spliced mRNA is 75 nt long. The L-PK probe used to control RNA loading in experiments represented in Fig. 5A corresponds to a 341-nt-long fragment containing exons 2, 3, and 4 of the murine L-pyruvate kinase cDNA. The nondigested probe obtained after subcloning into M13mp18, polymerization, and restriction by *Bam*HI is 375 nt long. The protected fragment is 341 nt long. The *H-2K* probe used to control H-2/myc 21B and H-2/myc 23B RNAs (Fig. 3 and 5B) includes the *Rsa*I-*Fsp*I 194-nt-long fragment (nt 2832 to 3026) of *H-2K*^{b} exon 3. This fragment has been subcloned into M13mp19. After polymerization and restriction with *Hin*dIII, the nondigested probe is 271 nt long. The protected fragment is 194 nt long. The *H-2/myc* probe used to reveal H-2/myc 21 mRNAs is depicted in Fig. 6C. It corresponds to a *Kpn*I-*Eco*RV 330-nt fragment of plasmid H-2/myc 21 and includes *H-2K* exon 3 and c-*myc* exon 2 sequences. The fragment protected by transgene mRNAs is 307 nt long, while the endogenous *H-2K*^{b} mRNAs protect a 151-nt-long fragment. The probe used to reveal H-2/myc 23 mRNA (αex2) was obtained after subcloning a 570-nt-long fragment (nt 4815 to 5385) including the 3′ part of human c-*myc* exon 2 and the first 98 nt of intron 2 into M13mp18. The probe is recovered after polymerization and *Eco*RI digestion. The protected fragment is 454 nt long.

Reverse transcription-mediated PCR. One microgram of H-2/myc 19B spleen mRNA was reverse transcribed by using 300 ng of oligo(dT) primer (Promega) and 200 U of Moloney murine leukemia virus reverse transcriptase (Gibco-BRL). One-sixtieth of the reverse transcription product was used for PCR performed with the two primers H-2 (5′-AAGTCGTGGTCGACCAGCTG-3′) and SV40 (5′-AGGGGGAGGTGTGGGAGGTT-3′). The expected amplified fragment corresponding to the fully spliced mRNA (without intron 1 and intron 2 sequences) is 881 bp long.

Northern (RNA) blot analysis. Twenty-microgram aliquots of total RNA extracted from livers of transgenic mice after CHX treatment were loaded and migrated on formamide-formaldehyde agarose gels. They were transferred onto a nylon membrane (Hybond N$^+$; Amersham) and hybridized with a human c-*myc* exon 3 riboprobe spanning the *Cla*I-*Eco*RI fragment.

Quantification. Quantification analysis was performed either by scanning the intensity of the bands on X-ray films with a densitometer (Vernon) or, in most cases, by scanning directly the acrylamide gels by using storage phosphor screens and a PhosphorImager apparatus (Molecular Dynamics).

RESULTS

Establishment of *H-2/myc* transgenic strains. To study the sequences involved in the posttranscriptional regulation of the c-*myc* gene in vivo, we have constructed various *H-2/myc* fusion genes in which the human c-*myc* upstream sequences including the P1 (and in some cases P2) promoters were replaced by the mouse *H-2K*^{b} regulatory region. We previously showed that both the 5′ and 3′ UTRs were dispensable for obtaining regulated expression of the transgene in various physiological situations (28). This finding suggested that the coding regions alone were sufficient to confer correct expression of the transgene. In the present study, we dissected the coding regions by constructing new *H-2/myc* transgenes, schematized in Fig. 1, which contained only one of the two coding exons. The H-2/myc 19 construct is derived from H-2/myc 2 (28). It contains exon 2, but exon 3 has been deleted (except for the first 28 nt) and the c-*myc* 3′ UTR has been replaced by the SV40 3′ UTR containing polyadenylation sequences. In H-2/myc 21, the c-*myc* exon 2 sequence was inserted in frame in the 5′-3′ orientation within the coding region of the *H-2K*^{b} gene in order to test the function of this exon independently of any other c-*myc* sequences. H-2/myc 23, in which the same exon 2 sequence was inserted at the same location in the opposite orientation, was used as a control. The H-2/myc 17 and H-2/myc 22 constructs are both derived from H-2/myc 1. They do not contain exon 2 but contain the exons 1 and 3 with the c-*myc* 3′ UTR and SV40 3′ UTR (including polyadenylation signal), respectively. The constructs were microinjected into the pronuclei of one-cell fertilized eggs to derive transgenic lines (9). At least two independent lines were analyzed for each construct except H-2/myc 23, for which only one transgenic line was analyzed. Their main characteristics, established from Southern blot and S1 nuclease analyses, are summarized in Table 1.

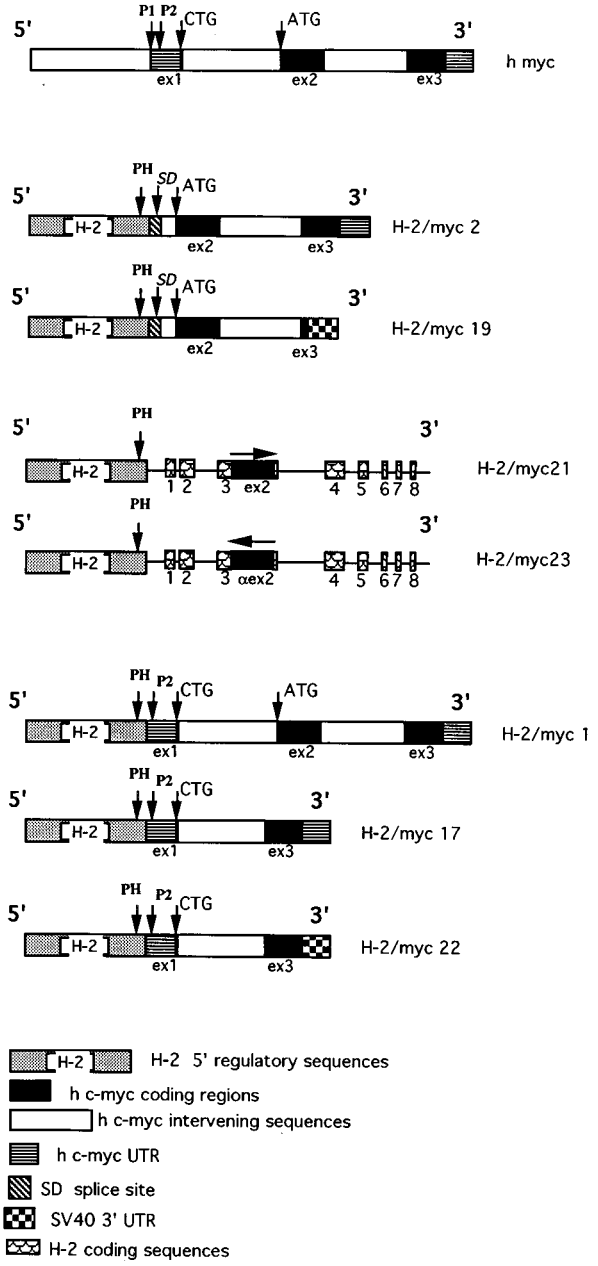

FIG. 1. Schematic representation of the different *H-2/myc* constructs. The structure of the human c-*myc* proto-oncogene (h myc) from which the various constructs have been derived is shown at the top. The H-2/myc 1 and H-2/myc 2 constructs from which H-2/myc 17, H-2/myc 22, and H-2/myc 19, respectively, were derived, are also represented (28). In H-2/myc 19, most of the exon 3 coding region has been deleted. The c-*myc* 3′ noncoding sequences containing polyadenylation sites have been replaced by the 3′ noncoding region of the early region of SV40 containing a polyadenylation signal. The H-2/myc 21 construct corresponds to a full-length H-$2K^b$ gene in exon 3 of which the 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 17 and H-2/myc 22 do not contain exon 2 sequences. In H-2/myc 22, the 3′ UTR of c-*myc* has been replaced by that of SV40 (see Materials and Methods). P1 and P2, P1 and P2 c-*myc* promoters; PH, H-$2K^b$ promoter; CTG and ATG, translation initiation codons of the p67 and p64 c-*myc* proteins (16); SD, donor splice site present in the polylinker between H-$2K^b$ and c-*myc* sequences.

The presence of exon 2 is sufficient to confer a low level of transgene mRNA expression in the liver. Expression of the H-2/myc 19 transgene, which contains exon 2 and the first 28 nt of exon 3, was analyzed in RNA isolated from different adult organs of two strains, using an S1 nuclease protection assay and two different probes, one spanning the exon 2/intron 2 boundary (ex2/in2) (Fig. 2A) and another hybridizing specifically with the central part of human c-*myc* exon 2 (Fig. 2B). The use of the ex2/in2 probe shows that the absence of exon 3 in the H-2/myc 19 construct does not interfere with the splicing efficiency of intron 2 either in the liver or in the spleen. Indeed, the ratios of the intensities of the signals corresponding to unspliced and spliced protected fragments are comparable for a given organ in the two H-2/myc 19 lines and in the other *H-2/myc* lines containing full exon 3 sequences (unspliced/spliced measured ratios are 0.07 and 0.17 for liver and spleen, respectively), as illustrated in Fig. 2A for H-2/myc 2A spleen. The efficiency of intron 1 and intron 2 splicing was also confirmed by performing reverse transcription-mediated PCR on H-2/myc 19B spleen mRNA. With the primers used (see Materials and Methods), the amplified fragment is 881 bp long, which corresponds to the size expected for fully spliced mRNA (data not shown).

The results shown in Fig. 2A also indicate that H-2/myc 19 expression is weaker in the liver than in the spleen. This was confirmed by performing an analysis of transgene expression in various adult organs of two H-2/myc 19 transgenic lines, using the human c-*myc* exon 2 probe. Experiments were run in parallel, using a β_2-microglobulin probe as a control of mRNA quantities (data not shown). Representative results obtained with the H-2/myc 19B line are shown in Fig. 2B. The transgene is expressed at high levels in the lymphoid organs (spleen, thymus, and mesenteric lymph nodes) and lung and is weakly expressed in the other organs analyzed. Similarly, the endogenous c-*myc* transcripts are highly expressed in the lymphoid tissues but barely detectable in the liver, as observed by using a murine c-*myc* exon 1-specific probe which protects P1- and P2-initiated transcripts (Fig. 2B, mmyc; see also Fig. 3, mmyc P2).

To examine whether the c-*myc* exon 2 sequence alone was able to confer a low level of expression of a heterologous mRNA in the liver, we constructed the H-2/myc 21 transgene, in which c-*myc* exon 2 was inserted in frame within exon 3 of the *H-2K*^{b} gene in the 5′-3′ orientation. The ATG initiation codon at the beginning of c-*myc* exon 2 has been mutated to allow translation of this construct only from the ATG located in *H-2K* exon 1. A control was obtained by creating the H-2/myc 23 construct, which is identical to H-2/myc 21 except that the c-*myc* exon 2 sequence is in the opposite direction (Fig. 1 and Materials and Methods). Thus, in both constructs, the insert has the same length and the same sequence at the DNA level. Two H-2/myc 21 transgenic lines and one H-2/myc 23 transgenic line were analyzed. Transgene expression was analyzed in adult organs by using the appropriate antisense or sense human c-*myc* exon 2 probe (see Materials and Methods) and compared with that of the endogenous c-*myc* and *H-2K*^{b} genes. In all of these experiments, the *H-2* exon 3 probe was used concomitantly with the human c-*myc* exon 2 or αex2 probe. As shown in Fig. 3, in both H-2/myc 21A and H-2/myc 21B lines, transgene expression parallels that of the endogenous c-*myc* mRNA and not that of *H-2K*, being high in the lymphoid organs and intestine and low elsewhere, in particular in the liver. For a reason which is still unclear, in H-2/myc 19B, 21B, and 17C lines (Fig. 3 and 4), the transgene is expressed in the lung. By contrast, in the H-2/myc 23B line, transgene

TABLE 1. Main characteristics of *H-2/myc* transgenic lines

Strain	Transgene copy no.[a]	Coding capacity[b]	Initiation codon	Spleen/liver[c]	Liver ± CHX[d]	Transgene[e]	
						Spleen	Liver
19A	30	99 aa (Nter-*myc*)	*myc* ATG	6	5	10	8
19B	30	99 aa (Nter-*myc*)	*myc* ATG	9	5	22	17
21A	1	601 aa (*H-2K/myc*)	*H-2* ATG	3.3	4	NT[f]	NT
21B	3–6	601 aa (*H-2K/myc*)	*H-2* ATG	7	3.2	4	5
23B	3	214 aa (*H-2K*/αmyc)	*H-2* ATG	1.4	1.6	NT	NT
17B	1–2	90 aa (non-*myc*)	*myc* CTG	9	>10	1	1
17C	70	90 aa (non-*myc*)	*myc* CTG	>10	9	6	1
22A	40	90 aa (non-*myc*)	*myc* CTG	4	9	4	2.7
22B	40	90 aa (non-*myc*)	*myc* CTG	4	>10	4	10
Endogenous[g]	2	p67: 454 aa p64: 439 aa	*myc* CTG *myc* ATG	>10	>10		
H-2K[g]	2	349 aa	*H-2K* ATG	1.3	1.2		

[a] Calculated after PhosphorImager scanning of Southern blots loaded with the same amount of DNA.

[b] Theoretical coding capacity of each construct, the initiation starting at either the CTG or ATG codon. The H-2/myc 19A construct has conserved the ATG located at the beginning of exon 2 and is therefore able to encode the first 99 amino acids (aa) of the p64 c-*myc* protein (Nter-*myc*). In the H-2/myc 17 and H-2/myc 22 constructs, the CTG at the end of exon 1 is present, but because of the deletion of exon 2 sequence, there is a frameshift in the reading frame of exon 3 and a stop codon is encountered at amino acid 90. In the H-2/myc 21A construct, the only stop codon is the natural one at the end of *H-2K*[b] sequences, while in the H-2/myc 23B, because of the presence of c-*myc* exon 2 sequences in the 3′-5′ orientation, a stop codon is found after 214 amino acids.

[c] Transgene expression was measured by S1 analysis. Each value represents the ratio of the signal found in the spleen over the signal found in the liver after PhosphorImager scanning of acrylamide gels. In several strains, the level of transgene expression was so weak in the liver that only a minimal value for the spleen/liver (or liver + CHX/liver − CHX) ratio could be given.

[d] Each value represents the ratio of the signal found in the liver after CHX treatment over the signal found in the liver without any treatment and corresponds to the mean of at least two independent experiments for each transgenic line.

[e] Transgene expression among the different transgenic strains. Transgene expression was analyzed by using the appropriate human c-*myc* probe (either exon 1 or exon 2) and a murine c-*myc* probe in spleens and livers of the indicated transgenic mice. The values were obtained after PhosphorImager scanning of the acrylamide gels and correspond to the ratio between exogenous and endogenous c-*myc* signals. These lanes allow direct comparison of transgene expression among the exon 2- or exon 3-containing transgenic mice.

[f] NT, not tested.

[g] Lines representing several characteristics of the murine *H-2K*[b] and c-*myc* genes.

mRNAs are found in all tissues, including the liver, and their expression parallels that of the endogenous *H-2K*[b] gene.

Taken together, these results indicate that c-*myc* exon 2 sequences inserted in the 5′-3′ orientation inside an otherwise stable reporter gene contain sufficient information to confer upon the chimeric mRNAs a posttranscriptional regulation similar to that imposed on the endogenous c-*myc* transcripts. Moreover, as H-2/myc 23 behaves as a negative control, these data support the hypothesis that the regulatory effect observed with c-*myc* exon 2 sequences in their natural orientation relies on specific sequences present in it and involving protein-RNA interaction.

Exon 3 is also involved in maintaining a low level of c-*myc* mRNA in the liver. During the course of our work, it was shown by two independent groups working with either ex vivo or in vitro systems that the c-*myc* carboxy-terminal coding region (CRD) contained determinants involved in either c-*myc* mRNA instability (38) or stability (3). To evaluate the relative importance of this region in vivo independently of exon 2 sequences, we derived four transgenic lines which do not contain exon 2 but contain exon 3 with and without the c-*myc* 3′ UTR, H-2/myc 17B and 17C and H-2/myc 22A and 22B, respectively (Fig. 1 and Table 1).

Transgene expression was analyzed in RNAs extracted from various adult organs of these four transgenic lines by S1 nuclease analysis, using a human c-*myc* probe hybridizing to the exon 1 sequences which are present in these four strains. This probe protects the transcripts initiated at the *H-2* promoter, PH, which is predominantly used, and the transcripts initiated at the c-*myc* P2 promoter. Endogenous c-*myc* mRNA expression was analyzed in parallel, using the mouse exon 1 c-*myc* probe (data not shown). The results (Fig. 4A and Table 1) indicate that the transgenes are highly expressed in lymphoid organs compared with other organs, in particular the liver, independently of the presence or absence of the c-*myc* 3′ UTR.

To analyze whether the intron 1-intron 2 chimeric sequences contained between exon 1 and exon 3 were appropriately spliced in the mature transgene mRNA and to confirm that exon 3 was present in these mRNAs, we performed a Northern blot analysis using RNAs extracted from the liver of the four transgenic lines after treatment with CHX, which inhibits protein synthesis and results in a transient increase in c-*myc* RNA expression (see below). The results shown in Fig. 4B, for which the human c-*myc* exon 3 sequence was used as the probe, indicate that H-2/myc 17B, H-2/myc 17C, and H-2/myc 22A mRNAs contain the exon 3 sequence and correspond to fully spliced mRNAs. Each transgene mRNA population migrates at its appropriate location: H-2/myc 17B and H-2/17C mRNA comigrate; they are larger than the H-2/myc 22 mRNA because the c-*myc* polyadenylation sites are more 3′ than that of SV40; they both differ from H-2/myc 2A transcripts, which, because they contain exon 2 instead of exon 1 sequences, are ≅300 bp longer. As an additional control, an S1 nuclease analysis was performed with a probe which spans the intron 2/exon 3 boundary. The results presented in Fig. 4C show that the protected band has the size expected for spliced mRNAs (75 nt); the same band is observed with H-2/myc 22 mRNA as with H-2/myc 2A mRNA, which contain bona fide exon 2, intron 2, and exon 3 sequences, but not with H-2/myc 19 mRNA, which contain truncated exon 3 sequences.

Transgene expression is induced upon CHX treatment. In rats as in mice, a high level of c-*myc* mRNA expression can be induced transiently in the liver in response to a block of translation provided by CHX injection (25–28). To determine whether the c-*myc* sequences present in the different transgenes were sufficient to confer this property, all of the trans-

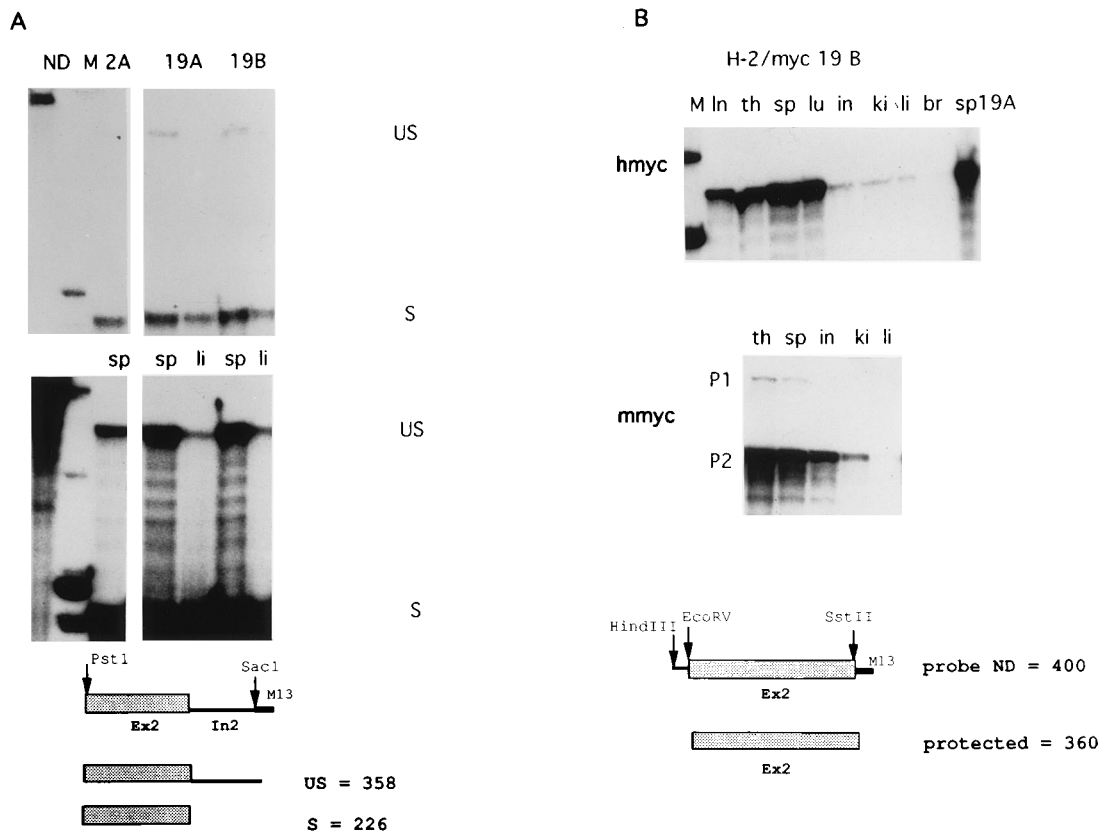

FIG. 2. Transgene mRNA expression in the H-2/myc 19 transgenic lines. (A) Twenty micrograms of RNA extracted from spleens (sp) and 60 μg of RNA extracted from livers (li) of H-2/myc 2A (2A), H-2/myc 19A (19A), and H-2/myc 19B (19B) transgenic mice were hybridized with the ex2/in2 single-stranded probe, and an S1 nuclease analysis was performed as described in Materials and Methods. As shown diagrammatically at the bottom, the ex2/in2 probe protects two species of RNA: the unspliced RNA (US) containing both exon 2 and intron 2 sequences migrating at 358 nt and the spliced RNA (S) containing only exon 2 sequences migrating at 226 nt. Lane ND, nondigested probe; lane, M, *Hpa*II-restricted pBR322. The bottom (27-h exposure) is an overexposure of the upper panel (2-h exposure). (B) Twenty-microgram aliquots of RNA extracted from the indicated organs of H-2/myc 19B transgenic mice were hybridized either with the human c-*myc* exon 2 probe (hmyc), diagrammed at the bottom, or with the murine c-*myc* exon 1 probe (mmyc). ln, mesenteric lymph nodes; th, thymus; sp, spleen; lu, lung; in, intestine; ki, kidney; li, liver; br, brain; sp19A, 20 μg of RNA extracted from the spleens of H-2/myc 19A mice.

genic lines were injected with CHX and sacrificed 2 h later. RNA was extracted from the liver, and an S1 mapping experiment was performed with the suitable human c-*myc* probe (exon 1, exon 2, or αex2). Experiments were carried out in parallel with the mouse-specific c-*myc* exon 1 probe, which was concomitantly used with the L-PK probe (see Materials and Methods) as an internal control. The analysis of H-2/myc 21 and H-2/myc 23 was performed with the *H-2K* probe and either the exon 2 or αex2 probe. The results (Fig. 5) indicate that in all lines examined, transgene expression increases after cycloheximide treatment, although *H-2K* expression does not vary. Induction of murine c-*myc* mRNA is observed in parallel. Quantification of these experiments after PhosphorImager scanning of the corresponding acrylamide gels is shown in Table 1. Taken together, the results confirm that c-*myc* upregulation after the inhibition of translation is controlled at the posttranscriptional level (28) and indicate that either one of the two coding exons is sufficient for transient accumulation of c-*myc* mRNA in the liver after inhibition of protein synthesis. As shown in Fig. 5B, no increase was observed for H-2/myc 23 transgene expression after CHX treatment, indicating that the transient accumulation of c-*myc* mRNA upon inhibition of protein synthesis requires the presence of c-*myc* exon 2 sequences in their natural 5′-3′ orientation.

Induction of transgene expression after partial hepatectomy. We previously showed that c-*myc* mRNA expression was increased 1 to 2 h after partial hepatectomy and that this induction was mainly due to posttranscriptional regulatory mechanisms (27). We also showed that c-*myc* 5′ (including exon 1) and 3′ UTRs were dispensable for this induction and that *H-2K* gene expression was not modified by the ablation (28). To determine whether exon 2 or exon 3 alone could be sufficient for induction of transgenes in the early steps of liver regeneration, we performed partial hepatectomy on the different *H-2/myc* transgenic lines. Total RNA was isolated from livers of mice 1 to 4 h after operation and from sham-operated mice and analyzed by an S1 nuclease protection assay using the appropriate human c-*myc* probe. The results (Fig. 6 and 7) show that the expression of exon 3-containing transgenes increases three- to eightfold 1 h after partial hepatectomy, independently of the presence of the c-*myc* 3′ UTR. The expression of the exon 2 containing constructs increases three- to fivefold, with a peak 2 h after the operation. This holds true not only for the H-2/myc 19A and H-2/myc 19B transgenic mice but also for the H-2/myc 21B mice, in which the c-*myc* exon 2 sequence has been inserted into the *H-2K* gene sequence. By contrast, no induction was observed when partial hepatectomy was performed with the H-2/myc 23B transgenic line, which contains

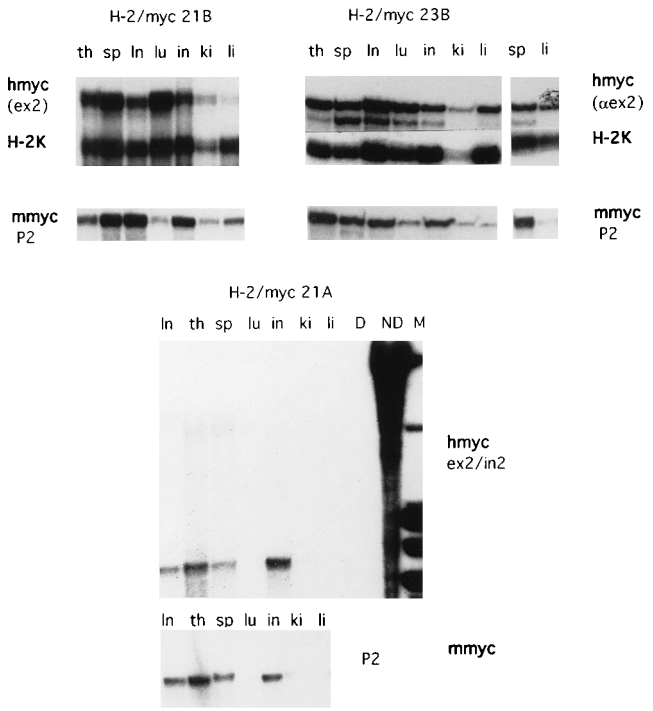

FIG. 3. Transgene mRNA expression in the H-2/myc 21 transgenic lines. Twenty-microgram aliquots of RNA extracted from the indicated organs (see the legend to Fig. 2 for abbreviations) of H-2/myc 21B, H-2/myc 21A, and H-2/myc 23B transgenic mice were hybridized with the human *myc* (hmyc) exon 2 (ex2), ex2/in2, and αex2 probes, respectively (see Materials and Methods and Fig. 2 for descriptions of the exon 2 and ex2/in2 probes). The murine c-*myc* (mmyc) exon 1 probe was hybridized with the same RNA samples in an independent experiment. Only the signals corresponding to the transcripts initiated at the P2 promoter are shown. The *H-2K* probe was coprecipitated with the exon 2 or αex2 probe. In the case of H-2/myc 23, the picture has been cut because of the large difference in the sizes of the protected fragments (454 and 194 nt for αex2 and *H-2K*, respectively). We do not know the significance of the lower band observed under the 454-nt band, which is not found in nontransgenic RNA (data not shown) and the intensity of which is variable from one organ to another and even in the same tissue, as illustrated in the two independent RNA samples extracted from the spleen. Lane D, digested probe; lane ND, undigested probe; lane M, pBR322 cut with *Hpa*II.

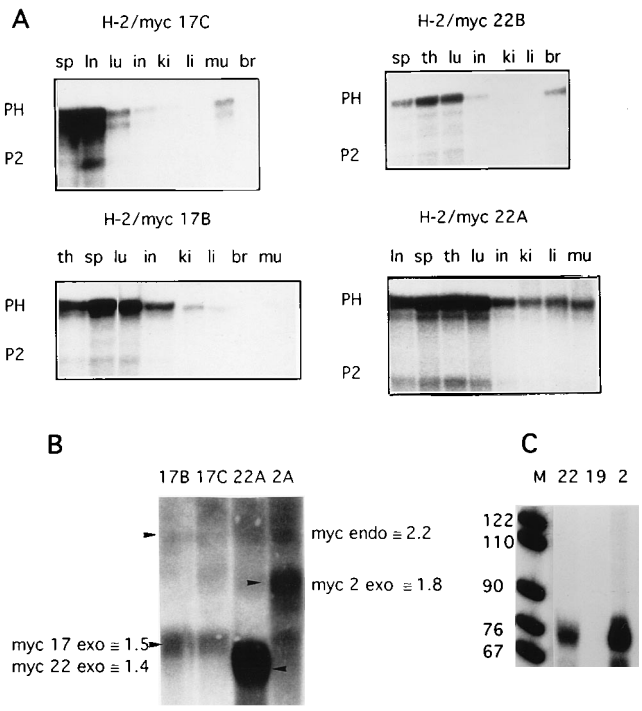

FIG. 4. Transgene mRNA expression in the exon 3-containing H-2/myc 17 and H-2/myc 22 transgenic lines. (A) Twenty-microgram aliquots of RNA extracted from the indicated organs (mu, muscle; see the legend to Fig. 2 for other abbreviations) of H-2/myc 17C, H-2/myc 17B, H-2/myc 22B, and H-2/myc 22A transgenic lines were hybridized with the human c-*myc* exon 1 probe, which protects transcripts initiated at the *H-2K*^{b} (PH) and c-*myc* P2 promoters. (B) Twenty-microgram aliquots of RNA extracted from livers of the indicated transgenic mice after CHX treatment were submitted to Northern blot analysis using a human c-*myc* exon 3 riboprobe. While the endogenous c-*myc* mRNAs (myc endo) migrate at ≅2,200 nt, the exogenous transgene mRNAs (myc 17 exo, myc 22 exo, and myc 2 exo) migrate at ≅1,500, 1,400 and 1,800 nt, respectively. (C) Twenty-microgram aliquots of RNA extracted from livers of myc 22A (lane 22), myc 19A (lane 19), and myc 2A (lane 2) transgenic mice were hybridized with a probe which spans the human c-*myc* intron 2/exon 3 boundary. The expected protected fragment is 75 nt long, corresponding to fully spliced exon 3-containing mRNA. Because of the deletion of most exon 3 sequences in H-2/myc 19 transgenic constructs, H-2/myc 19 mRNAs protect only 24 nt of the probe. Lane M, pBR322 digested with *Hpa*II.

the exon 2 sequence in the reverse orientation (Fig. 7). Thus, exon 2, independently of other c-*myc* sequences, can confer upon a heterologous gene the characteristics of inducibility after hepatectomy observed with the full-length c-*myc* endogenous gene. Since *H-2K* promoter activity does not change after partial hepatectomy, these results confirm that the increase in c-*myc* mRNA expression is a posttranscriptional process and indicate that exon 2 and exon 3 are both targets for this process.

Transgenes differing in coding capacity but similar in posttranscriptional regulation. The different *H-2/myc* transgenes have different coding capacities (summarized in Table 1): the H-2/myc 19 construct encodes the first 250 amino acids of the p64 c-*myc* protein; the H-2/myc 21 construct encodes an *H-2K*/c-*myc* hybrid protein; H-2/myc 17 and 22 transgenes, because of the absence of exon 2 and of the ATG start codon that it contains, may be either not translated at all or translated from the CUG initiation codon located at the end of exon 1. They thus will encode a polypeptide of 90 amino acids, the first 10 of which are present in the p67 c-*myc* protein but not in the p64 protein (16). In this frame, because of the stop codon encountered 90 amino acids after the CUG, the ribosomes cannot reach the end of the exon 3 coding sequences; in particular, they do not translocate across the instability determinant contained in the CRD (3, 17, 38). In one of the two other reading frames, there is no ATG and, in the third, the ATGs are not surrounded by a consensus sequence for mammalian translation initiation (20). Nevertheless, these constructs contain sufficient information to be down-regulated in the quiescent liver (Fig. 2 to 4) and up-regulated after inhibition of protein synthesis (Fig. 5) and partial hepatectomy (Fig. 6).

DISCUSSION

Despite increasing evidence that posttranscriptional mechanisms play important roles in the control of c-*myc* gene expression, relatively few data are yet available concerning the mechanisms, the target sequences, and the components with which they interact. Two discrete sequence elements, one in the 3′ UTR and the other in the carboxy-terminal coding region (the CRD element), have been shown to account for the short half-life of c-*myc* mRNA both by transfection experiments using modified c-*myc* genes and by use of cell-free systems (3, 6, 8, 38, 40). In vitro mRNA decay experiments have suggested that each instability determinant can function on its own by interacting with different regulatory or degradation factors. The 3′ UTR element is recognized by a protein complex, called

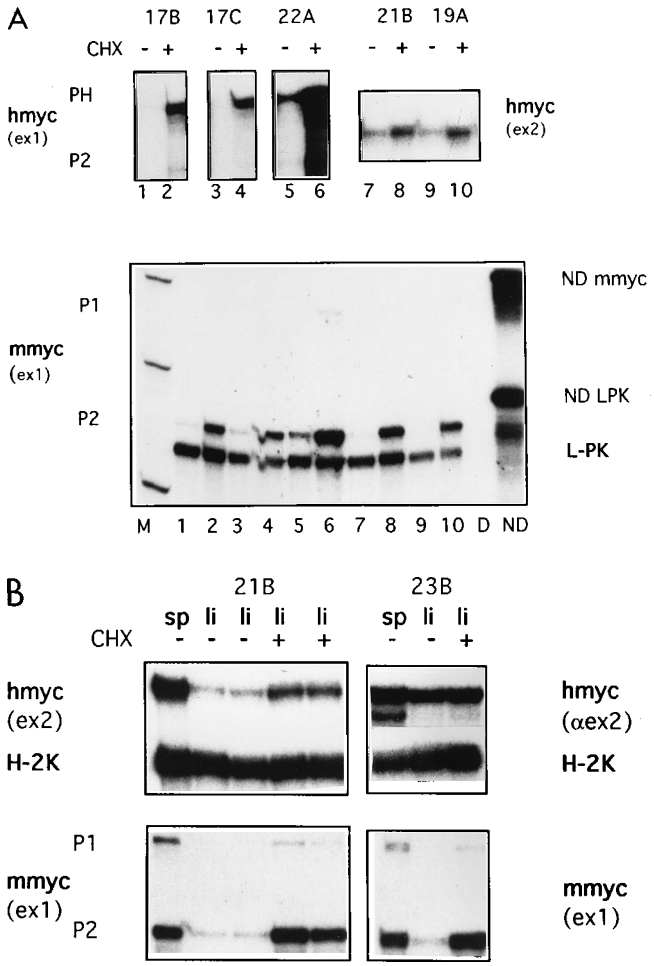

FIG. 5. Induction of c-*myc* mRNA expression in livers of transgenic mice after CHX treatment. (A) Twenty-microgram aliquots of RNA extracted from livers of transgenic mice either not treated (−) or injected 2 h before sacrifice with CHX (+) were hybridized with the human c-*myc* (hmyc) exon 1 (ex1) or exon 2 (ex2) single-stranded probe. The same samples were hybridized in parallel with the murine c-*myc* (mmyc) exon 1 and L-PK probes. ND, nondigested probe. (B) Twenty-microgram aliquots of RNA extracted from spleens (sp) or livers (li) of H-2/myc 21B and H-2/myc 23B transgenic mice either not treated (−) or CHX treated (+) were hybridized simultaneously with the *H-2K*^{b} exon 3 (H-2K) and human c-*myc* exon 2 probes or the αex2 probe. They were hybridized in parallel with the murine c-*myc* exon 1 probe.

AUF1, which seems to selectively accelerate degradation of c-*myc* mRNA. One of its components, the 37-kDa protein, which is found in the cytosol as well as in the nucleus, has recently been cloned and characterized (40). The CRD element, contained in the last 182 nt of the coding part of exon 3, binds an as yet unpurified 75-kDa polysome-associated protein which, in contrast to AUF1, seems to protect the CRD from endonucleolytic cleavage (3). These data raised numerous questions, in particular regarding the way in which these sequences act in various physiological conditions, their relative importance under particular circumstances, and the abundancy of the corresponding *trans*-acting factors. Moreover, these data have been obtained by using in vitro or ex vivo systems, which raises the question of their relevance to the in vivo situation. This is not merely a theoretical question, as several observations have shown that one should be cautious in making such extrapolations (23, 41).

To address this question, we have chosen the transgenic methodology and constructed a series of transgenic mice in which various coding and noncoding regions of the human c-*myc* gene were placed under the transcriptional control of the quasi-ubiquitously expressed *H-2K*^{b} promoter. An extensive analysis of transgene expression compared with endogenous c-*myc* mRNA expression was then undertaken in various situations: in quiescent organs, at the start of liver regeneration, and during inhibition of protein synthesis. From the data described previously concerning *H-2/myc* constructs deleted of the 5′ and the 3′ UTR of the c-*myc* gene (28) and from the data presented in this report concerning constructs containing one of the two c-*myc* coding exons, we can posit three main points. (i) In the liver, all of the *H-2/myc* transgenes are barely detectable, although the *H-2K* gene is actively transcribed in this organ. (ii) Whatever the construct, inhibition of protein synthesis results in a transient increase in both endogenous and exogenous c-*myc* expression, although *H-2K* expression does not vary after such a treatment. (iii) The constructs which contain one or the other of the two coding exons are inducible after partial hepatectomy. Together, these results confirm that in the three situations analyzed, c-*myc* expression is controlled mainly by posttranscriptional mechanisms. They indicate that a regulated expression can be ensured even in the absence of the 3′ and 5′ UTRs (including exon 1) and of c-*myc* intervening sequences; moreover, they show that either of the c-*myc* coding exons can play on its own an active part in this process.

Transgene mRNAs containing only exon 2 sequences are weakly expressed in the liver, and their expression is inducible after inhibition of protein synthesis and in the early steps of liver regeneration, as shown by the analysis of two H-2/myc 19 transgenic mice. The destabilizing effect of c-*myc* exon 2 in the liver was substantiated by analyzing the H-2/myc 21 transgenic lines expressing chimeric transgene mRNAs in which c-*myc* exon 2 is embedded in its natural orientation within an otherwise stable *H-2K* RNA: chimeric mRNA was found to be subjected to the same regulation as the endogenous c-*myc* mRNA. By contrast, when placed in the same context but in the reverse orientation, these sequences have no effect and the chimeric H-2/myc 23 mRNAs behave like *H-2K* endogenous mRNAs. It should be mentioned, however, that in this H-2/myc 23 construct, the polypeptide chain is prematurely interrupted because of a stop codon 50 amino acids after the junction between *H-2K* and c-*myc* inverted sequences; this could theoretically interfere with mRNA abundancy. However, in both prokaryotes and eukaryotes, nonsense mutations are generally reported to mediate mRNA decay and not to induce their stabilization (see, for instance, references 11 and 39 and references therein). Moreover, preliminary results of studies using a construct identical to H-2/myc 21 except for the presence of a stop codon at the beginning of the c-*myc* exon 2 sequence reveal that the premature termination has no effect per se. Indeed, this H-2/myc 24 construct behaves in the liver like H-2/myc 21 but not H-2/myc 23 (unpublished data).

The role of the exon 3 sequence has been highlighted by the study of two H-2/myc 17 transgenic lines which contain this region but lack exon 2 sequences and two H-2/myc 22 transgenic lines which, in addition, lack the c-*myc* 3′ UTR. In these four lines, the steady-state level of transgene expression is similar to that of the endogenous c-*myc* gene in the different situations analyzed. The absence of the c-*myc* 3′ UTR does not alter transgene expression, a result in agreement with those of several ex vivo studies showing that the presence of the AU-rich 3′ UTR was dispensable for a short c-*myc* mRNA half-life (22, 38). However, as we have not constructed a fusion gene containing the c-*myc* 3′ UTR alone, we cannot formally exclude the possibility that this region contains a destabilizing

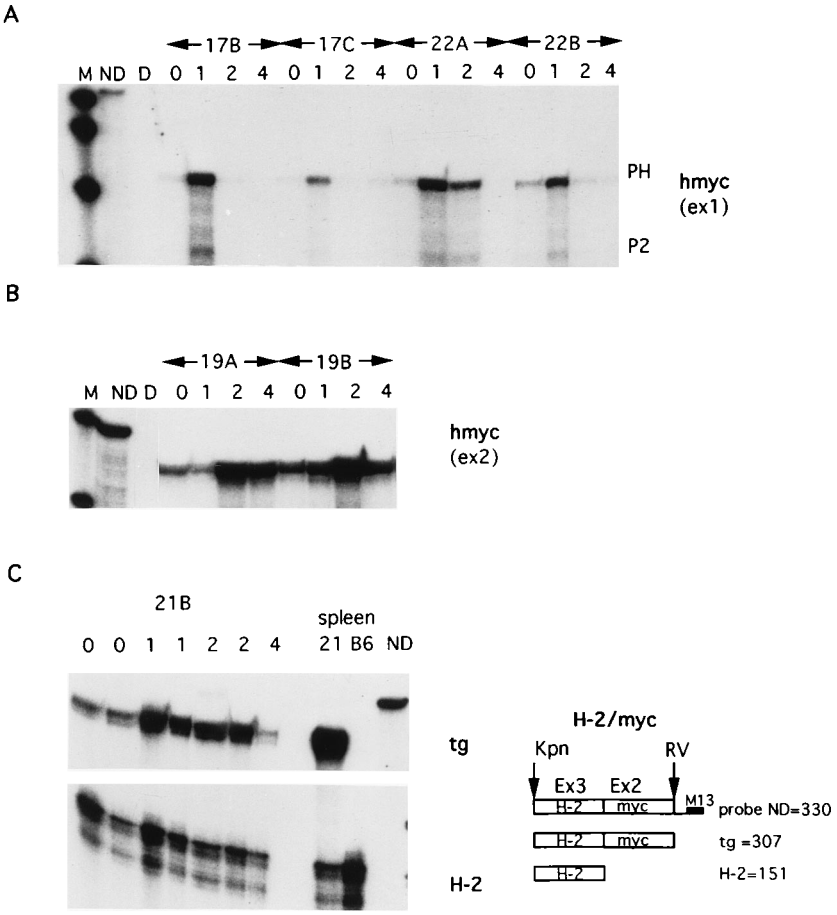

FIG. 6. Expression of transgene mRNA during liver regeneration of exon 2- and exon 3-containing transgenic mice. (A and B) Analysis of exon 3- and exon 2-containing transgenes in the early steps of liver regeneration. For each given time (1, 2 or 4 h), equivalent amounts of RNA extracted from livers of two transgenic mice were mixed and 20 μg of the resulting mixture was analyzed by an S1 nuclease protection assay using the human c-*myc* (hmyc) exon 1 (ex1) or exon 2 (ex2) probe. The time zero corresponds to RNA extracted from sham-operated transgenic mice. Lane M, *Hpa*II-restricted pBR322; lane ND, nondigested probe; lane D, digested probe. (C) Analysis of transgene induction in H-2/myc 21B transgenic mice. Two independent mice were used for each time point. Twenty micrograms of RNA was hybridized with an *H-2/myc* chimeric probe (diagrammed on the right) which within the same RNA preparation is protected by transgene mRNAs (tg; 307 nt) and endogenous *H-2K*^{b} mRNA (H-2; ≅151 nt). Spleen 21 and spleen B6 correspond to 20 μg of RNA extracted from spleens of H-2/myc 21 transgenic and control mice, respectively.

determinant whose activity would be masked by the presence of the CRD contained in exon 3.

Our results demonstrate, therefore, that both c-*myc* coding exons contain an instability determinant(s) that can function independently. As no situation in which they function differently has yet been found, we cannot ascertain whether they constitute redundant systems to ensure rapid c-*myc* mRNA decay or whether one or the other is prevalent in a given physiological context. However, several data suggest that the exon 2 CRD (CRD 2) and the exon 3 CRD (CRD 3) may not behave similarly. In particular, in the liver, the peak of transgene induction in the early steps of liver regeneration is earlier in the exon 3-containing transgenic mice than in the exon 2-containing mice (Fig. 6 and 7). Moreover, the level of induction after CHX treatment is approximately twofold lower in exon 2- than in exon 3-containing lines (Table 1), and compared with the endogenous c-*myc* mRNA, the former show a higher transgene signal in the quiescent liver (Fig. 5A and 6). Experiments are in progress to obtain mice expressing both transgenes simultaneously. The results will allow us to exclude the role of individual fluctuations in response to various stimulations and will be helpful for ascertaining the hypothesis that in the conditions tested, the timing and the levels of expression of CRD 2- and CRD 3-containing transgenes are different.

One of the intriguing points concerning c-*myc* mRNA stability is its relationship with translation. It has been shown in various studies that c-*myc* mRNA is protected from rapid degradation by translation inhibitors (24, 38), suggesting either that ongoing translation of c-*myc* mRNA itself is required for its decay or that c-*myc* mRNA decay requires a highly labile protein with degradation activity that fails to be synthesized upon the inhibition of translation. Wisdom and Lee found that mutation of the translation start codon prevented induction of c-*myc* mRNA by CHX (38) and suggested that a nontranslatable c-*myc* mRNA was more stable than its translatable counterpart. Such a conclusion was also reached by Herrick and Ross: using a chimeric β-globin–CRD 3 construct, they showed that placing a nonsense mutation upstream of the CRD stabilized approximately twofold the chimeric mRNA (17). These results suggest that degradation involving CRD 3 occurs more efficiently when ribosomes are translating this determinant.

The results obtained in vivo with the constructs containing exon 3 sequences are not, however, in agreement with this hypothesis. Indeed, the H-2/myc 17 and myc 22 constructs may

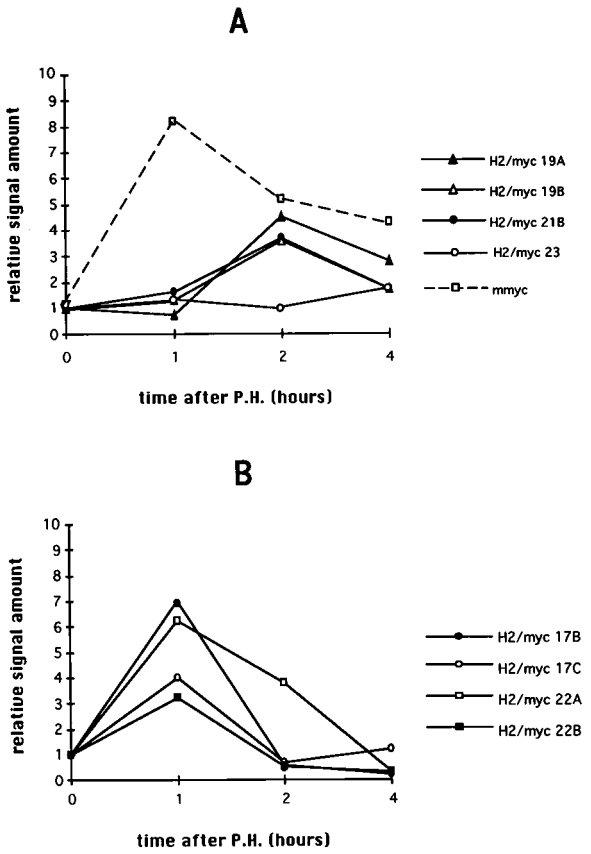

FIG. 7. Induction of transgene expression in the exon 2- and exon 3-containing transgenic lines. Data from acrylamide gels presented in Fig. 6 were quantitated by PhosphorImager scanning. For each sample, the concentration of transgene mRNA was normalized to the β_2-microglobulin or *H-2K* mRNA internal standard. The values obtained were expressed relative to the values found in sham-operated mice, arbitrarily expressed as 1, and plotted against the indicated times. A curve showing the induction of murine c-*myc* (mmyc) transcripts during partial hepatectomy is also shown in panel A. Each point corresponds to the mean of values found in four independent experiments. The results obtained after partial hepatectomy of H-2/myc 23 transgenic mice containing exon 2 sequences in the reverse orientation are also shown. They were obtained after analysis of one or two animals for each time point.

be either not translated at all or prematurely terminated 90 amino acids after the CUG translation initiation codon, such that the ribosomes cannot translocate across CRD 3. Nevertheless these transgene mRNAs are barely detectable in the quiescent liver and up-regulated after inhibition of protein synthesis and partial hepatectomy. Our data imply that in the in vivo situations studied here, the primary RNA sequence and not the c-*myc* exon 3 peptide is important.

Such a conclusion, also recently reached for the destabilization function of the c-*fos* mRNA coding region (36), cannot yet be extrapolated to the CRD 2, since in the two H-2/myc 19 and H-2/myc 21 constructs, exon 2 is potentially read by the polysomes in the same frame as in murine c-*myc* mRNA. However, preliminary results of studies using the H-2/myc 24 construct with a stop codon at the beginning of exon 2 (see above) indicate that the translation of this coding exon is not required to ensure its low level of expression in the liver (unpublished data).

Taken together, our results give support to the hypothesis that what is important with respect to c-*myc* mRNA decay is not its translation per se, the reading of the very first codons which were shown to be important for the degradation of several mRNAs (as illustrated by studies on β-tubulin mRNA [35]), or the reading of common coding sequences, since all *H-2/myc* constructs differ in their coding capacities (see Table 1), but rather its interaction with highly labile components whose expression could be rapidly and transiently abolished after liver ablation and inhibition of protein synthesis. H-2/myc 17 and H-2/myc 22 behave similarly under these conditions, which implies that such labile components do not require the presence of the c-*myc* 3′ UTR to act.

In summary, the detailed in vivo analysis that we have performed with transgenic mice has allowed us to confirm the importance of c-*myc* exon 3 and to uncover a role of exon 2 in regulating c-*myc* mRNA decay. Thus, at least three independent elements, including the 3′ UTR destabilizing determinant, are involved in c-*myc* mRNA half-life. Their dispersed locations on c-*myc* mRNA raise the question of whether they participate in a common pathway for its decay or act independently, being recognition sites for different *trans*-acting factors whose location or synthesis requires directly or indirectly continuous translation. The search for other situations in which the transgenes could be differently stimulated as well as the analysis of the factors with which each determinant reacts in a given physiological context will be the next steps toward reaching an understanding of the underlying molecular mechanisms.

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