Turnover of primary transcripts is a major step in the regulation of mouse *H19* gene expression

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In the gene expression pathway, RNA biogenesis is a central multi-step process where both message fidelity and steady-state levels of the mature RNA have to be ascertained. An emerging question is whether RNA levels could be regulated at the precursor stage. Until recently, because it was technically very difficult to determine the level of a pre-mRNA, discrimination between changes in transcriptional activity and in pre-mRNA metabolism was extremely difficult. *H19* RNA, the untranslated product of an imprinted gene, undergoes post-transcriptional regulation. Here, using a quantitative real-time RT-PCR approach, we accurately quantify its precursor RNA levels and compare these with the transcriptional activity of the gene, assessed by run-on assays. We find that the levels of *H19* precursor RNA are regulated during physiological processes and this regulation appears to be related to RNA polymerase II transcription termination. Our results provide direct evidence that turnover of polymerase II primary transcripts can regulate gene expression in mammals.

INTRODUCTION

H19 RNA is the product of a mammalian imprinted gene (Bartolomei *et al.*, 1991) with potential riboregulator function (Brannan *et al.*, 1990; Lottin *et al.*, 2002). Despite the fact that this RNA is recruited into polysomes (Li *et al.*, 1998; Milligan *et al.*, 2000), it is not translated. This peculiar molecule has all the characteristics of a nonsense mRNA but, paradoxically, is highly expressed during mouse development (Poirier *et al.*, 1991) and differentiation of cultured C2C12 muscle cells (Davis *et al.*, 1987). Messenger RNAs harbouring premature termination codons (PTC-containing mRNAs) are usually quickly degraded by nonsense-mediated decay (NMD), a post-transcriptional process that is able to detect abnormally interrupted open reading frames

(ORFs) (Maquat, 1995; Li and Wilkinson, 1998; Hentze and Kulozik, 1999; Hilleren and Parker, 1999; Maquat and Carmichael, 2001; Wilusz *et al.*, 2001). Although this surveillance mechanism of message fidelity has not yet been fully elucidated, it appears that, in mammals, splicing events (Le Hir *et al.*, 2001; Neu-Yilik *et al.*, 2001) and reading through ORFs (Li and Wilkinson, 1998; Ishigaki *et al.*, 2001; Muhlemann *et al.*, 2001) are required for NMD activation. *H19* RNA has many stop codons resulting in multiple short ORFs, which are not conserved amongst mammalian species (Brannan *et al.*, 1990). As it undergoes splicing, it should normally be recognised as a PTC-containing RNA and subjected to a regulated NMD process (Ruiz-Echevarria and Peltz, 2000). In previous work, we found that *H19* gene expression is upregulated by a post-transcriptional mechanism during muscle cell differentiation at an early step of RNA biogenesis (Milligan *et al.*, 2000). This raised the question whether repression of a nuclear NMD process is indeed involved or whether a genuine regulation of precursor RNA levels accounts for *H19* gene expression. Here, we use real-time RT-PCR to quantify *H19* precursor RNAs and we demonstrate for the first time that primary transcript levels can be post-transcriptionally regulated during physiological processes, pointing out a novel control step of gene expression in mammals.

RESULTS

H19 precursor RNA levels are upregulated during muscle cell differentiation

Using run-on assays, we showed previously that the transcriptional level of *H19* RNA remains constant during C2C12 muscle cell differentiation and, therefore, that the upregulation, which occurs upon differentiation, is exclusively post-transcriptional

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Fig. 1. Run-on analyses at the 3' end and 3' flanking region of the *H19* gene during C2C12 muscle cell differentiation. Labelled transcripts synthesised by 10^7 nuclei from proliferating (P) or differentiated (D) cells were hybridised to slot blots containing 3' end (left panel) or 3' flanking (right panel) *H19* DNA probes. Gapdh, c-Myc and myogenin were used for normalisation. Myogenin was also used as a muscle differentiation marker. Corrected *H19* signals (see Methods) were plotted in the bar graphs shown below run-on autoradiographs.

(Milligan *et al.*, 2000). However, as we used full-length cDNA probes, we could not formally exclude that this 10-fold upregulation resulted from a release of a block in transcriptional elongation. To rule out this possibility, nascent RNA from run-on assays using nuclei from proliferating or differentiated cells was hybridised to slot blots containing an *H19* probe located in the 3' part of the gene. We found that there is no significant variation in the number of RNA polymerase II complexes engaged at the 3' portion of the *H19* gene during the differentiation process (Figure 1, left panel). Treatment of the nascent RNA with Xrn1 (a 5'→3' exonuclease that degrades 5'-unprotected RNAs) before hybridisation does not change the *H19* patterns in run-on assays (data not shown). Therefore, it follows that *H19* primary transcripts remain protected at the 5' end until RNA polymerase II reaches the 3' end of the gene. We then used a probe flanking the 3' part of the gene and we observed that the transcriptional signal is barely detectable in proliferating cells (Figure 1, right panel). We conclude that polymerase II reaches the 3' end of the gene but does not efficiently transcribe the 3' flanking portion in proliferating cells.

The mouse *H19* gene contains a second poly(A) site that has never been documented because the corresponding transcripts are extremely rare. Real-time RT-PCR quantification confirmed that these transcripts represent no more than 0.1% of the total mature RNA and their expression pattern during C2C12 muscle cell differentiation is like that of the total mature *H19* RNA (Figure 2). Therefore, alternate poly(A) site usage cannot explain *H19* RNA upregulation during C2C12 muscle cell differentiation.

To accurately quantify the relative variations of precursor RNA levels, we then used the real-time RT-PCR technique. Primer pairs covering the junctions between each of the four unspliced introns and the adjacent exon allowed us to quantify *H19* precursor RNA levels. Surprisingly, for each targeted intron, *H19* precursor RNA levels increase dramatically early during the

Fig. 2. Analysis of the post-transcriptional upregulation of mature *H19* RNAs during C2C12 muscle cell differentiation. Strategy to target mature *H19* RNAs is shown at the top of the figure. Combination of primers F (forward) and R2 (reverse 2) amplified mature RNA 2 while F and R1 (reverse 1) amplified both RNA 1 and 2. Total RNA was extracted at serial time from proliferating (P) or differentiated cells (1, 2 or 3 days). Relative levels of mature *H19* RNA were quantified by real-time RT-PCR and are depicted in bar graphs after normalising for RNA loading using Gapdh mRNA levels. In each graph, the highest value was fixed at 100%. It is noteworthy that RNA 2 is very poorly expressed and represents <0.1% of *H19* mature RNA. As a quality control for RNA samples, 10 µg of total RNA were analysed by northern blotting using *H19* and Gapdh cDNA probes (insert).

differentiation process (Figure 3A). Clearly, this increase does not reflect the *H19* transcriptional activity in the body of the gene, which remains constant, but rather correlates with both the transcriptional activity in the 3' flanking portion of the gene and the upregulation of mature *H19* RNA levels. We conclude that the increase of *H19* precursor RNA levels is critical for *H19* gene upregulation while the high stability of the mature RNA (Milligan *et al.*, 2000) leads to its progressive accumulation. This accumulation makes it likely that *H19* precursor RNA remains localised in the nucleus where it is efficiently spliced. In addition, 4 h treatment of proliferating cells with cycloheximide induced a significant decrease in *H19* precursor RNA levels compared with untreated cells (Figure 3B), indicating that degradation of precursors is not prevented by translation inhibition.

H19 precursor RNA levels are downregulated in the heart during mouse development

To assess whether regulation of *H19* precursor RNA takes place in the living organism, we next examined *H19* gene regulation during late mouse development. In most tissues, *H19* gene transcription varies significantly with the developmental stage and consequently it is difficult to appreciate the post-transcriptional contribution to *H19* RNA levels. However, in the heart, run-on experiments using a full-length cDNA probe show that the transcriptional activity of the *H19* gene is almost identical from embryonic day 15 (e.15) to 8 days after birth (Figure 4A), and

Fig. 3. Analysis of H19 precursor RNA levels in C2C12 muscle cells. (A) Using primer pairs covering the junctions between each of the four unspliced introns and the adjacent exon, relative levels of each intron of H19 precursor RNA were quantified by real-time RT-PCR during cell differentiation (see Figure 2). It is noteworthy that H19 precursor RNA levels are at least three orders of magnitude lower than H19 RNA levels. (B) H19 precursor RNA levels were quantified as above in proliferating cells before (grey bars) or after (white bars) 4 h of cycloheximide treatment. In this experiment, levels of intron 4-containing RNA cannot be determined due to low abundance of this RNA in proliferating cells.

phosphoimager quantification on two separate experiments shows no significant difference in transcriptional activity between e.15 and 8 days after birth (83 ± 17 versus 100%, respectively). However, at both stages, transcriptional activity at the 3' flanking portion of the gene is significantly less than at the 3' end (Figure 4B). We conclude that polymerase II does not efficiently transcribe beyond the 3' flanking portion of the *H19* gene, as found for C2C12 proliferating cells (Figure 1). This raised the possibility that H19 precursor RNA levels could be affected. To analyse *H19* gene expression in the heart during the same lapse of time, we quantified by real-time quantitative RT-PCR both precursor and mature H19 RNA levels. As depicted in Figure 4C, the levels of H19 precursor RNA are downregulated and appear to parallel those of the mature RNA (Figure 4D). We conclude that, in the heart during mouse development, the steady-state H19 RNA levels are already downregulated at e.15 and that they result directly from regulation of H19 precursor RNA levels in the absence of any change in transcriptional activity in the body of the gene.

DISCUSSION

In this report, we clearly demonstrate that an efficient post-transcriptional mechanism of regulation able to modulate the amounts of precursor RNA is involved in *H19* gene expression. This process explains H19 upregulation during C2C12 muscular cell differentiation as well as H19 downregulation in the heart during development. It plays a critical role in determining the turnover of H19 primary transcripts and, consequently, the amount of H19 precursor RNA available to produce the steady-state levels of mature RNA. To our knowledge, this study provides the first direct evidence that, beyond transcriptional activity, the regulation of precursor RNA levels can be an important step of gene regulation in mammals. This novel event in gene expression, like all other known from transcription initiation to translation, appears regulated during physiological processes. It has been known for some time that only 2% of nuclear pre-RNA (hnRNA) produce mature RNA in mouse cells

(Brandhorst and McConkey, 1974); it seems inconceivable that most of this nuclear degradation acts on aberrant transcripts, therefore regulation of precursor RNA levels could be a general regulatory mechanism for determining mature RNA levels in mammals. In addition to quality control systems as NMD that are involved in complete degradation of aberrant mRNAs, other RNA surveillance systems, fundamentally different in that they govern normal RNA levels, are probably required as 'fine tuning systems' in eukaryotic cell nuclei (Hilleren and Parker, 1999). The possibility that gene expression could be controlled by pre-mRNA decay is suspected for artificially mutated α -globin RNA (Provost and Tremblay, 2000), for *N-myc* expression in N-type neuroblastoma cells (Lazarova *et al.*, 1999) and for the α -fetoprotein (Vacher *et al.*, 1992), alkaline phosphatase (Kiledjian and Kadesch, 1991) and peptidylglycine monooxygenase (El Meskini *et al.*, 1997) gene expression. Taken together, with our own data, showing that, during physiological processes, a regulated balance between stabilisation/destabilisation of H19 precursor RNAs accounts for mature RNA levels, this suggests that turnover of RNA polymerase II primary transcripts could be a general step in the regulation of gene expression in eukaryotic cells.

Two features argue against the involvement of the NMD in this regulatory pathway. First, H19 precursor RNA levels are directly targeted and, more importantly, upon translation inhibition with cycloheximide neither the H19 precursor (Figure 3B) nor the mature H19 RNA (Milligan *et al.*, 2000) accumulate in proliferating C2C12 cells, as it should be expected if NMD was involved (Zhang *et al.*, 1997). Our data show that the destabilisation of H19 primary transcripts occurs when polymerase II does not efficiently transcribe to the 3' flanking portion of the gene, and therefore we propose that the regulation step take places at the coupled transcription termination/3' maturation processes. A plausible explanation would be that proper 3' end maturation of the primary transcript failed when the polymerase prematurely disengages from the DNA template and does not reach its functional downstream transcription termination site. Unfortunately, these incorrectly processed transcripts are very short lived

Fig. 4. Analysis of the post-transcriptional downregulation of *H19* gene expression in the heart during late mouse development. Nuclei (2×10^6) isolated from heart tissue at the indicated developmental stages were used for *in vitro* transcription and slot-blot hybridisation. (A) Autoradiograph of run-on experiment using a full-length cDNA probe and (B) phosphorimager quantification of run-on using probes to the 3' end or the 3' flanking region of the *H19* gene. (C) Relative *H19* precursor RNA levels (intron 3-containing *H19* precursor RNA) and (D) mature *H19* RNA levels were analysed, at the indicated developmental stages, as described in Figures 3A and 2, respectively. The ratios between the relative levels of *H19* precursors and mature RNAs are very similar to those in C2C12 cells (see Figure 3A). As a quality control, 10 μ g of total RNA from each samples were analysed by northern blotting using *H19* cDNA and 18S rRNA DNA probes (insert in D).

products that are detected as nascent RNA by run-on assays but are extremely difficult to further visualise by standard RNA mapping techniques (Proudfoot *et al.*, 2002). Consequently, we have no indication as to whether this premature release of the polymerase depends on the gene sequence or on other regulatory elements at the locus. We are also unable to evaluate how far the poly(A) signal is involved but we suspect that the RNA polymerase II complex could be itself decisive since it is important for the integration of nuclear events, hence providing an avenue of regulation (Hirose and Manley, 2000). According to a recent model that predicts that polymerase II transcription termination requires a cotranscriptional precleavage event of the nascent transcript (CoTC) (Dye and Proudfoot, 2001; Proudfoot *et al.*, 2002), it could be possible that *H19* downregulation results in simultaneous CoTC and polymerase II release leading to a 3' unprotected RNA, which is quickly degraded. This hypothesis reinforces the proposal that CoTC would be a general mechanism for the targeting and degradation of some nascent transcripts by stimulating polymerase II transcription termination (Dye and Proudfoot, 2001).

A regulated pathway for nuclear pre-mRNA turnover has been identified in yeast involving a multi-enzymatic complex of 3'→5' exonucleases called the exosome (Bousquet-Antonelli *et al.*, 2000). The exosome also contributes to a quality control system that monitors correct 3' end formation of mRNAs (Hillgren *et al.*, 2001; Butler, 2002). Its mammalian counterpart (Mitchell *et al.*, 1997; Allmang *et al.*, 1999; Chen *et al.*, 2001) is a reasonable candidate to play a role in the regulation of *H19* precursor RNA levels; however, further work will be required to specify the exact mechanism and to investigate how precisely it is regulated.

Speculation

The observation that constant and high rates of transcription are maintained while the major part of the transcript produced is quickly degraded remains probably the more intriguing biological aspect of our work. Why does the cell, in some physiological conditions, favour the post-transcriptional downregulation of *H19* precursor RNA instead of a transcriptional repression? This

could be due to transcriptional constraints at the *H19* locus. *H19* is an imprinted gene, expressed only from the maternal allele (Bartolomei *et al.*, 1991), and it shares common regulatory elements with other imprinted genes in the same chromosomal region. Among these elements, *H19* transcriptional enhancers, located downstream of the gene, are also necessary for *Igf2* gene expression on the paternal allele (Leighton *et al.*, 1995). Therefore, any change in the transcriptional activity relating to the *H19* enhancers would also affect *Igf2* expression, for which dosage is very important for normal mouse development (Sun *et al.*, 1997). Finally, since genes often share regulatory sequences and most transcriptional factors form a common pool, such constraints on transcription may be a regular feature of gene expression in mammals. Therefore, regulation of precursor RNA levels could be one way to compensate for such possible transcriptional constraints and to allow appropriate gene expression.

METHODS

Cell culture, RNA extraction, northern blots, probes and cycloheximide treatment. The experimental procedures were described previously (Milligan *et al.*, 2000). Briefly, the mouse skeletal muscle cell line C2C12 was grown in 50% DMEM, 50% HAM'S F12 containing 10% fetal calf serum and myoblastic differentiation was induced by serum starvation. Total RNA was isolated by the guanidinium monothiocyanate procedure, electrophoresed into 1.1% agarose/formaldehyde gels, blotted on positively charged nylon membranes and hybridised with random priming labelled probes.

Run-on experiments. The procedures for nucleus preparation and run-on assays in Figure 4A are as described previously (Milligan *et al.*, 2000). For nuclear run-on in Figures 1 and 4B, a pBluescript construct containing the last 728 bp essentially from the last *H19* exon (3' end probe) and a PCR product containing 752 bp downstream from the cleavage site (3' flanking probe) were used as probes. The smaller size of these *H19* probes compared to the size of the other probes we used previously (Milligan *et al.*, 2000), leads to a dramatic decrease in the *H19* hybridisation signals. To partially compensate for this decrease,

we used 500 μCi [α - ^{32}P]UTP at 6000Ci/mmol versus 100 μCi [α - ^{32}P]UTP at 800 Ci/mmol in standard assays (Figure 4A). Nuclei from heart tissue were isolated from 23 newborns, 15 3-day-old, 12 8-day-old and eight 19-day-old individuals. For the e.15 stage, 39 embryos were dissected, a primary culture of cardiac cells was performed and used in the experiments. Run-on values for specific *H19* hybridisation were obtained from phosphoimager quantifications after subtracting unspecific hybridisation (line 'plasmid') and correcting for incorporation of [α - ^{32}P]UTP. The relative levels of the nascent *H19* transcripts are the average between two experiments after normalisation to the levels of the other control transcripts (the highest value was fixed at 100%). Primers used to prepare the 3' flanking probe were: forward, cagtcattggtctctgtggac; reverse, ctctgctgtggcccttcag.

Xrn1 treatment. *In vitro* UTP-labelled transcripts were treated with Xrn1 before hybridisation to slot blots according to previously described conditions (Boeck et al., 1998).

Real-time quantitative RT-PCR. Ten micrograms of RNA samples were treated by DNase I (1 U for 30 min at 34°C) and incubated with hexanucleotide random primers (1 μg) in the presence (+RT) or in the absence (–RT) of M-MLV reverse transcriptase (200 U for 1 h at 37°C). Dilutions (10%) of these reactions were then subjected to real-time quantitative PCR (SYBR Green, Light-Cycler; Roche) using specific primers (0.5 μM each). Primers used for quantitative RT-PCRs were as follows. *H19* intron1: forward, ggcttgcatgacagacagaac; reverse aactgtcgtggaggagactg. *H19* intron2: forward, gagcactactccctgccacagg; reverse, cagacggctctctacgacaagg. *H19* intron3: forward, cctgtcgtgaagccgtctg; reverse, gggcagaagagaactcacctt. *H19* intron4: forward, ggaccctttggaagcttgcc; reverse, ggcttggtccaggatgatgtg. *H19* mature RNAs: forward, ggagactaggccaggtctc; reverse, ctttcagcttcaccttgagcag. *H19* mature RNA 2: forward, ggagactaggccaggtctc; reverse, caacctcccccatgagtcg. *Gapdh* mRNA: forward, acagtcattgcatcactgcc; reverse, gcctgcttcacaccttcttg.

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