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Epigenetic regulation of an IAP retrotransposon in the aging mouse: progressive demethylation and de-silencing of the element by its repetitive induction

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ABSTRACT

The recent insertion of a murine intracisternal A-particle (IAP) retrotransposon within one of the introns of a housekeeping gene, the circadian *m.nocturnin* gene, revealed a singular expression profile, both throughout the daytime and the mouse life span. Measurement of the levels of transcripts from this element by quantitative real-time RT–PCR, in organs of 1–24-month-old mice, disclosed that the inserted element—which is part of a large family of otherwise severely repressed mobile elements—becomes active upon aging, specifically in the liver where the *m.nocturnin* housekeeping gene is expressed in a circadian manner and induces a circadian expression of the IAP sequence. This age-dependent induction is cell-autonomous, as it persists in hepatocytes in primary culture. We further show, using methylation-sensitive enzymes, a correlation between the life-time kinetics of this process and a liver-specific demethylation of the IAP promoter. These results strongly support a model whereby the progressive demethylation and turning on of the IAP sequence is the sole result of the transient, daily activation—throughout the mouse life span—of its promoter. This phenomenon, which develops on a timescale of months to years in the aging mouse, might reveal a general epigenetic—and stochastic—process, which could account for a large series of events associated with cell and animal aging.

INTRODUCTION

Intracisternal A-particle (IAP) sequences are moderately reiterated transposable elements (~1000 copies in the mouse genome) which are closely related to retroviruses (1) and transpose through the reverse transcription of an RNA intermediate (2). They are flanked by two long terminal repeats (LTR), with a U3-R-U5 organization, that contain the signals for the initiation and regulation of transcription (5′-LTR) and for the polyadenylation of the transcripts (3′-LTR). Their insertion may provoke mutations that are various in nature and include activation of the tagged gene by an enhancer effect (reviewed in 1,3). These elements are severely repressed in the living animal possibly as a biological requisite for genomic stability. Actually, transposable elements are severely methylated in most species, and their expression is hardly detectable (4,5). It has been shown that repression is removed under definite conditions, including conditions of normal development, with evidence for very specific induction of IAP in the stem cells of the male germline, at undifferentiated stages (in gonocytes and undifferentiated spermatogonia) (6), where they are demethylated. Induction of expression also takes place under conditions of artificial manipulation of the cells, including treatment with demethylating agents such as 5-azacytidine (7,8) and knocking out of methylase genes in transgenic mice (9). These conditions result in the massive induction of IAP transcription, with an increase in RNA levels of >100-fold, and a parallel demethylation of the IAP promoter. Despite the severe control for IAP expression *in vivo*, we have previously demonstrated that IAP transcripts can still be detected in some, non-germline, tissues of normal mice (10). It is likely that these transcripts are associated with the clonal expression of a limited number of elements by a 'position effect', induced by a nearby gene. This has been previously illustrated by the demonstration that a major IAP transcript observed in the liver of aged mice originates from a single element (IAP-AR, for age-related IAP) inserted in one of the introns of the *m.nocturnin* gene (11). This gene, which we have cloned and characterized (12), is a housekeeping gene expressed in most organs, with a circadian expression observed essentially in the liver (and to some extent in the photoreceptors) (13,14). The inserted IAP element has transposed recently, as it is not observed in all laboratory mouse strains, being present in BALB/c and absent in CBA/J mice (12). The insertion is not deleterious for the tagged gene, since *m.nocturnin* transcripts are still observed, although the presence of the IAP in the first intron of the gene should have prevented full-length transcript as a result of the presence of a polyadenylation sequence within the IAP LTR. Furthermore, circadian expression of *m.nocturnin* is still observed in the former strains, with properties similar to those in the latter (14). In fact, for some still unresolved reasons not associated with the LTR sequence *per se* but most probably relevant to strong selection pressure for *m.nocturnin* gene function, the

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IAP polyadenylation signal is not recognized efficiently, thus resulting in read-through transcripts for both the *m.nocturnin* and the IAP transcripts. IAP transcription can therefore be easily assayed in the mouse tissues, as the corresponding transcripts can be unambiguously probed with the 3′ flanking DNA, and this insertion provides a rather unique opportunity to analyze the control of IAP repression in a living animal.

Accordingly, a rather unexpected feature of the expression of this IAP was the observation of its specific induction in the aged mouse (10). Here, we have therefore analyzed the expression profile of this IAP element in the course of mouse aging (from 1 to 24 months) together with that of the tagged *m.nocturnin* gene. The methylation status of the IAP promoter, followed in parallel, disclosed a specific and progressive demethylation with aging, only observed in the liver. The effect is not linked to general factors (e.g. circulating hormones, nutrients, etc.) that would be modified in the aged mouse, as IAP induction is still observed in hepatocytes in primary culture that we isolated from aged mice. Rather, we propose that IAP activation—and demethylation—is the consequence of the activity of the element itself, resulting from its presence within the *m.nocturnin* gene which induces throughout the mouse life a circadian activation of the IAP element and its progressive demethylation with age. The remarkable feature of this regulation is its unusual timescale, i.e. months to years, suggesting simple molecular processes possibly accounting for a series of phenotypic alterations associated with aging. A model is presented whereby the regulation of the level of gene activation would be mainly dependent on the level of 'gene use', thus providing a simple self-adaptative response.

MATERIALS AND METHODS

Animal care and handling

BALB/c male mice were obtained from Janvier, and were 4 weeks or 8 months old at the time of arrival in our animal facilities. They were housed in a strict 12:12 h light/dark regimen (lights on at 7 a.m and lights off at 7 p.m., corresponding to Zeitgeber time ZT 0 and ZT 12, respectively) for at least 2 weeks before they were sacrificed. All mice were fed *ad libitum*. They were killed by cervical dislocation and had no evident pathology at the time of death.

RNA extraction and northern blot analysis

Tissue specimens were frozen in liquid nitrogen and stored at –80°C before use. Total RNA was isolated using the RNeasy Mini kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). The quality of RNA samples was assessed by electrophoresis through denaturing gels and staining with ethidium bromide. The extraction yield was quantified spectrophotometrically. For northern blot analysis, 10–15 µg of total RNA/lane was fractionated on 1.2% (w/v) agarose/0.66 M formaldehyde gels in 1× MOPS/0.66 M formaldehyde buffer. RNAs were transferred to a charged nylon filter (Hybond N^+, Amersham Pharmacia Biotech) in 0.15 M NH_4Ac buffer. Filters were prehybridized for 30 min, hybridized for 20 h at 65°C in Church solution (7% SDS, 1 mM EDTA pH 8, 0.5 M Na_2HPO_4 pH 7) without bovine serum albumin, and washed twice for 15 min at 65°C in 0.5× SSC, 0.1% SDS. The probes used were a 1.9 kb *Pvu*II–*Pvu*II fragment (nucleotides 897–2779; GenBank accession no. AF183960) encompassing exon 2 and part of exon 3 of the *m.nocturnin* cDNA, and a 4.5 kb *Xmn*I–*Mlu*I fragment encompassing the almost complete sequence of a canonical IAP element (nucleotides 602–5081; GenBank accession no. X04120). They were ^{32}P-labeled using a random-priming kit (Amersham). Filters were exposed to X-ray films (Kodak, AR) and transcripts were quantitated by phosphorimager analysis.

Determination of mRNA levels using real-time 'TaqMan' RT–PCR

A fluorescence-based real-time quantitative RT–PCR method developed by Perkin Elmer ABI (TaqMan) was used to measure mRNA levels in mouse tissues. One microgram of total RNA for each sample was reverse transcribed in a 20 µl volume reaction using 50 U Moloney murine leukemia virus reverse transcriptase (Perkin Elmer Corp. Applied Biosystems), 20 U ribonuclease inhibitor (Perkin Elmer Corp. Applied Biosystems), 1 mM dA/T/C/G (Amersham Pharmacia Biotech, Uppsala, Sweden), 5 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.3), 10 mM KCl and 50 pM random hexamers (Perkin Elmer Corp. Applied Biosystems). After an initial step at room temperature for 10 min, reverse transcription was performed at 42°C for 45 min, ended by a step at 99°C for 7 min. The complementary DNA strand (cDNA) was then diluted 1:20 in nuclease-free H_2O (Promega Corp., Madison, WI).

Quantitative PCR was carried out with a cDNA equivalent of 15 ng total RNA per reaction, using the TaqMan core reagent kit according to the manufacturer's instructions: 1× buffer A, 5 mM $MgCl_2$, 200 µM dA/C/G, 400 µM dU, 1.25 U AmpliTaq Gold polymerase, 2.5 U uracil *N*-glycosylase, 1 µM TaqMan probe and 2 µM of each primer in a 25 µl final reaction volume. PCRs were carried out in a Perkin Elmer ABI 5700 machine, with a first denaturation step at 95°C for 10 min, followed by 40 cycles comprising denaturation at 95°C for 15 s and annealing–elongation at 60°C for 1 min. Negative controls demonstrated an absence of carryover. Additionally, for each type of amplicon, amplification products were electrophoresed on agarose gels and disclosed unique bands at the expected size.

To normalize for differences in the amount of total RNA in each RT–PCR, amplification of 18S ribosomal RNA was performed as an internal control. Primers and probes for 18S were purchased from Perkin Elmer Corp. Applied Biosystems. The following primers were synthesized (MWG-biotech): *m.nocturnin* ex1, 5′-CGGAACTGCAGGCGG; *m.nocturnin* ex2–3, 5′-GCCTTCTCCGAGAGCTTGG; IAP-AR 10 kb, 5′-GACTGACAGCTGGCTATGGGA. The following TaqMan probe (labeled with 6-FAM at the 5′ end and with TAMRA at the 3′ end; MWG-biotech) was synthesized: *m.nocturnin* ex2, 5′-CCCGAACATCTGGAGCCCATCG.

Isolation of genomic DNA and Southern blot analyses

Animals were sacrificed at the indicated ages and Zeitgeber times. Tissues were treated overnight in 50 mM Tris–HCl (pH 8.0), 100 mM EDTA, 0.5% SDS, and 300 µg/ml proteinase K at 50°C, and DNA was extracted twice with phenol/chloroform/isoamylalcohol (25:24:1). DNA concentrations were quantified spectrophotometrically. Genomic DNA (20 µg) was digested overnight with a 3-fold excess of *Bgl*II (New England

Biolabs) and a methylation-sensitive restriction enzyme in the appropriate buffer, and then precipitated. For Southern blot analysis, 12 µg of total DNA/lane was fractionated on 1% (w/v) agarose gels in 1× TBE buffer. DNAs were transferred to a charged nylon filter (Hybond N^+; Amersham Pharmacia Biotech) in 10× SSC buffer. Filters were prehybridized for 30 min, hybridized for 20 h at 65°C in Church solution (7% SDS, 1 mM EDTA pH 8, 0.5 M Na_2HPO_4 pH 7) without bovine serum albumin, and washed twice for 15 min at 65°C in 0.5× SSC, 0.1% SDS. The probe used was a 1.2 kb *Bgl*II–*Nae*I fragment encompassing part of the *m.nocturnin* first intron. It was ^{32}P-labeled using a random-priming kit (Amersham). Filters were exposed to X-ray films (Kodak AR), and transcripts were quantitated by phosphorimager analysis.

Isolation and primary culture of hepatocytes

Hepatocytes were isolated from young (2 month) or old (24 month) C57BL/6 × DBA/2 mice by perfusing the liver with a collagenase solution (1 mg/ml). Cells were collected in 199 medium (Life Technologies) supplemented with 10% fetal calf serum, centrifuged at low speed, and deposited in a culture dish for 5 min to eliminate the rapidly attaching fibroblastic cells. The hepatocyte-enriched culture medium was then recovered and cells counted for viability by trypan blue exclusion. Cell viability was close to 70% for old mice and 80% for young mice. An aliquot (3×10^6 cells) of the hepatocytes was then frozen at –80°C for RNA extraction and the remaining cells were plated at a density of 1.5×10^6 cells/6 cm^2 dish in a final volume of 3 ml of 199 medium with 5 mM glucose and 3% fetal calf serum. After 5 h for attachment, the medium containing unattached cells was removed and replaced every 24 h. Hepatocytes were harvested 96 h after plating.

RESULTS

Structure and transcriptional regulation of the IAP–*nocturnin* locus in the aging mouse

Figure 1A illustrates the structure of the *m.nocturnin* gene with the IAP sequence inserted within the first intron of the gene, and the corresponding transcripts. These include the two *nocturnin* mRNAs, of 2.7 and 3.1 kb, and the IAP-promoted mRNAs of 6 and 10 kb (IAP-AR transcripts), resulting from a readthrough across the IAP 3′-LTR into exons 2 and 3 of *m.nocturnin* (11,12). As illustrated in the northern blot analysis in Figure 1B, all four transcripts can be detected in the liver of aged mice, using a *nocturnin* probe (Fig. 1B, left lane). Figure 1B also shows that the 6 and 10 kb bands are associated with the IAP sequence inserted within the *nocturnin* locus, as they are no more observed in mice lacking this IAP insertion (12), whereas the two *nocturnin*-associated bands are unaltered (Fig. 1B, right lane). Northern blot analysis, using an IAP probe and RNAs from the liver of young and old mice (Fig. 1C), confirms that the 6 and 10 kb transcripts are associated with the IAP-AR sequence and further shows that they are strongly induced upon mouse aging, both transcripts being undetectable in the liver of young mice. Moreover, the blot shows that IAP transcription originating from the other IAP elements present in the mouse genome (~1000 copies, either full-length or internally deleted, and resulting in the observed 7.2 and 5.4 kb bands, respectively) is (i) weak (as expected for these highly repressed elements) relative to their high copy number, and (ii) constant with age (at variance with that for the *m.nocturnin*-tagging IAP).

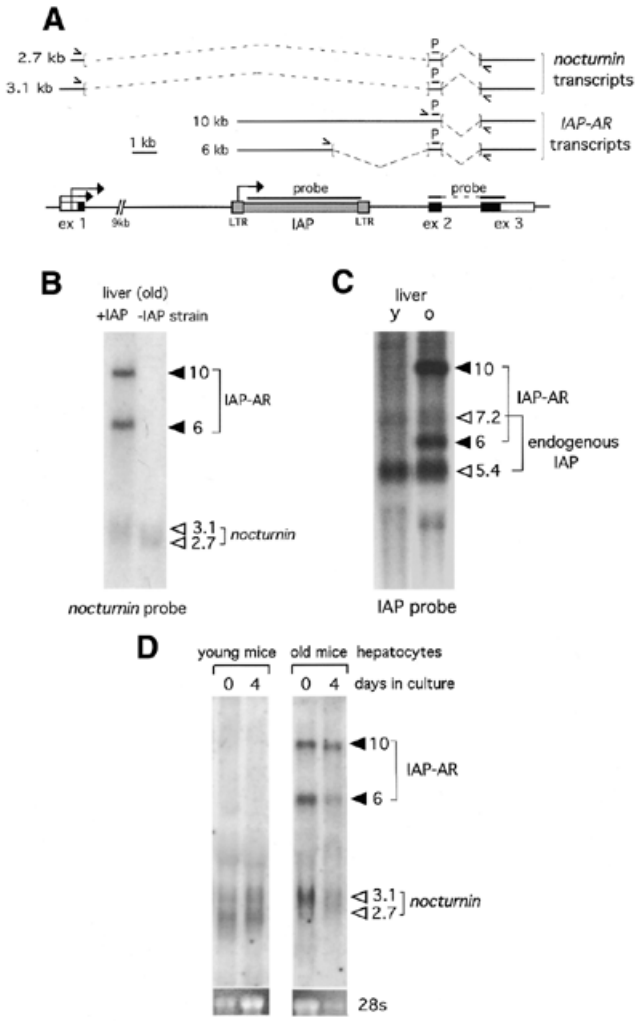

Figure 1. Structure of the *m.nocturnin* locus and transcriptional status *in vivo* and *ex vivo* in hepatocytes from the aging mouse. (**A**) Genomic organization of the IAP-tagged *m.nocturnin* gene with the *m.nocturnin* and the IAP-AR transcripts indicated. The exon–intron sequences are drawn to scale, with the *m.nocturnin* coding and untranslated regions represented by black and white boxes, respectively, and the IAP sequence in the first intron with a gray box. Arrows indicate transcription start sites for the *m.nocturnin* gene and the IAP sequence. The structure of the previously characterized *m.nocturnin* and IAP-AR transcripts is schematized, with the intronic sequences as dotted lines. The positions of the probes used for the northern blot analyses are indicated, as well as the position of the primers (small arrows) and the fluorescent probe (P) used for real-time TaqMan RT–PCR. (**B**) Northern blot analysis of total RNAs (12 µg per lane) extracted from the liver of 2-year-old BALB/c (+IAP) and CBA/J (–IAP) mice (the latter not containing the IAP insertion at the *nocturnin* locus). The *m.nocturnin* probe used is shown in (A), and the *m.nocturnin* and IAP-AR transcripts are indicated by empty and filled arrowheads, respectively. (**C**) Northern blot analysis of total RNAs (12 µg per lane) from the liver of young (6-week-old) and old (24-month-old) mice, hybridized with an IAP probe [see (A)]. Positions of the IAP-AR transcripts and of transcripts from the other IAP elements (i.e. 5.4 and 7.2 kb elements) are indicated by filled and empty arrowheads, respectively. (**D**) Northern blot analysis of total RNAs (12 µg per lane) from hepatocytes isolated from the liver of young (2-month-old) and old (24-month-old) mice and maintained in culture for the indicated number of days (0 and 4 days, see Materials and Methods). Positions of the IAP-AR and *m.nocturnin* transcripts are indicated as in (B). The intensity of the BET-labeled 28S rRNA band is given below each lane.

An important issue was to determine whether the age-related induction of IAP expression is a cell-autonomous property of the IAP–*nocturnin* locus or whether it corresponds to a systemic induction due to, for instance, circulating hormones or factors the amounts of which would increase or decrease in the aging mouse. To answer this question, we isolated hepatocytes—the cells expected to be responsible for the observed IAP induction—from the liver of aged mice, together with cells from the liver of young mice as a control, and established them in primary cultures. IAP-AR transcripts levels were then assayed on freshly isolated hepatocytes and on cells that were maintained in primary culture for at least 4 days. As illustrated in the northern blot analysis in Figure 1D, the IAP-AR transcripts are observed in the hepatocytes isolated from old mice, with levels close to that observed in the liver in toto (Fig. 1B), thus suggesting that the IAP-AR transcripts actually originate from this cell type. Moreover, the hepatocytes after 4 days of primary culture disclose the same profile of IAP expression as the freshly prepared hepatocytes, with high level expression in cells from 'old' livers, and undetectable levels in cells from 'young' livers. These features are maintained along the cell culture and are therefore most probably cell-autonomous.

Quantitative analysis of IAP induction with age

A quantitative characterization of the differences in the levels of the IAP-AR transcripts between young and old mice has to take into account that the IAP sequence is inserted within a gene whose expression fluctuates in a circadian manner, as previously reported (13,14), and that this circadian variation has an influence on the level of the IAP-AR transcripts. Actually, as illustrated in Figure 2A where transcripts levels are quantitated using a real-time TaqMan RT–PCR assay as a function of Zeitgeber time (ZT, a light/dark reference cycle where ZT 0 and ZT 12 are when lights are turned on and off, respectively), the level of the IAP-AR transcripts displays a circadian variation (5–10-fold amplitude, with a peak at dusk) which strictly follows the circadian expression of the embedding *m.nocturnin* gene (~30-fold variation and identical acme position). This influence is maintained thoughout the mouse life-time, as illustrated in Figure 2B where we measured the circadian extent of both IAP-AR and *m.nocturnin* variations: in agreement with the northern blot data (Fig. 1C), induction of IAP-AR with age is clearly visible when comparing 3-month and 14-month-old mice, with conservation of the influence of the circadian *nocturnin* gene on the IAP promoter. The induction of IAP-AR transcripts levels with age manifests itself essentially for the 'basal' level of IAP-AR expression, with >15-fold induction in the aged mouse. Maximal transcripts levels are still 5–8-fold higher in old mice, where they most probably correspond to a plateau value for IAP-AR expression. A parallel quantitative analysis of *m.nocturnin* transcripts levels (Fig. 2B, right) shows that the amplitude of their circadian fluctuations remains unaltered with age, indicating that the increased level of IAP expression with age is not due to an increase in the level of expression of the *m.nocturnin* gene.

A quantitative analysis of the levels of IAP transcripts was therefore performed in the course of mouse aging (1–24 months), with animals that were kept under controlled conditions of day/light regimen several weeks before they were sacrificed. For each age point, three animals were killed at identical hours

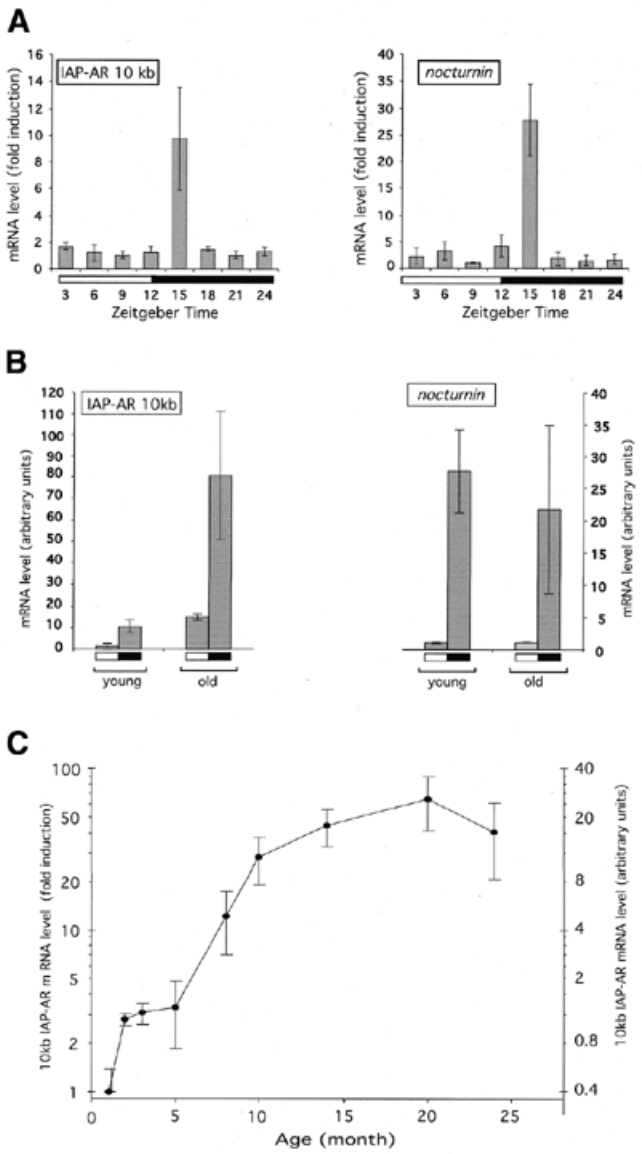

Figure 2. Daily, transient induction of the *m.nocturnin* locus and quantitation with age of the IAP-AR transcripts level. (**A**) Real-time TaqMan quantitative RT–PCR analysis of the 10 kb IAP-AR (left) and *m.nocturnin* (right) transcripts levels in the liver of mice sacrificed at different Zeitgeber times (open and filled box below the *x*-axis indicates light and dark periods, respectively). Mice were kept under standard light/dark conditions for at least 2 weeks before sacrifice. Each value is the mean for three animals (3-month-old BALB/c mice), with error bars indicating standard deviations (see Fig. 1A for primers and probe). (**B**) Age-dependent daily variations of the 10 kb IAP-AR (left) and *m.nocturnin* (right) transcripts levels as measured by real-time TaqMan quantitative RT–PCR analysis using RNA extracted from the liver of young (3-month-old) or old (14-month-old) mice. Same experimental conditions as in (A). The horizontal bars below the *x*-axis for each panel represent light (open bars) and dark (black bars) periods, each plotted value being the mean for three animals sacrificed at ZT 4 and ZT 15 (for the peak value), respectively, with error bars indicating standard deviations. The mRNA levels in the ordinates are in arbitrary units, taken as unity the values of the light period for young mice. (**C**) Quantitation of the increase of the 10 kb IAP-AR transcript level with age as measured by real-time TaqMan quantitative RT–PCR. Same experimental conditions as in (A) and (B). Mice were kept under standard light/dark conditions for at least 2 weeks before sacrifice (at ZT 4). Each plotted value is the mean for three animals, with error bars indicating standard deviations. The *y*-axes correspond to a logarithmic scale [left, fold induction; right, mRNA level, with the same arbitrary units as in (B)]. The circadian (A and B) and age-dependent (B and C) inductions reported for the IAP-AR 10 kb transcripts are similarly observed for the IAP-AR 6 kb transcripts, with a slightly reduced extent (data not shown).

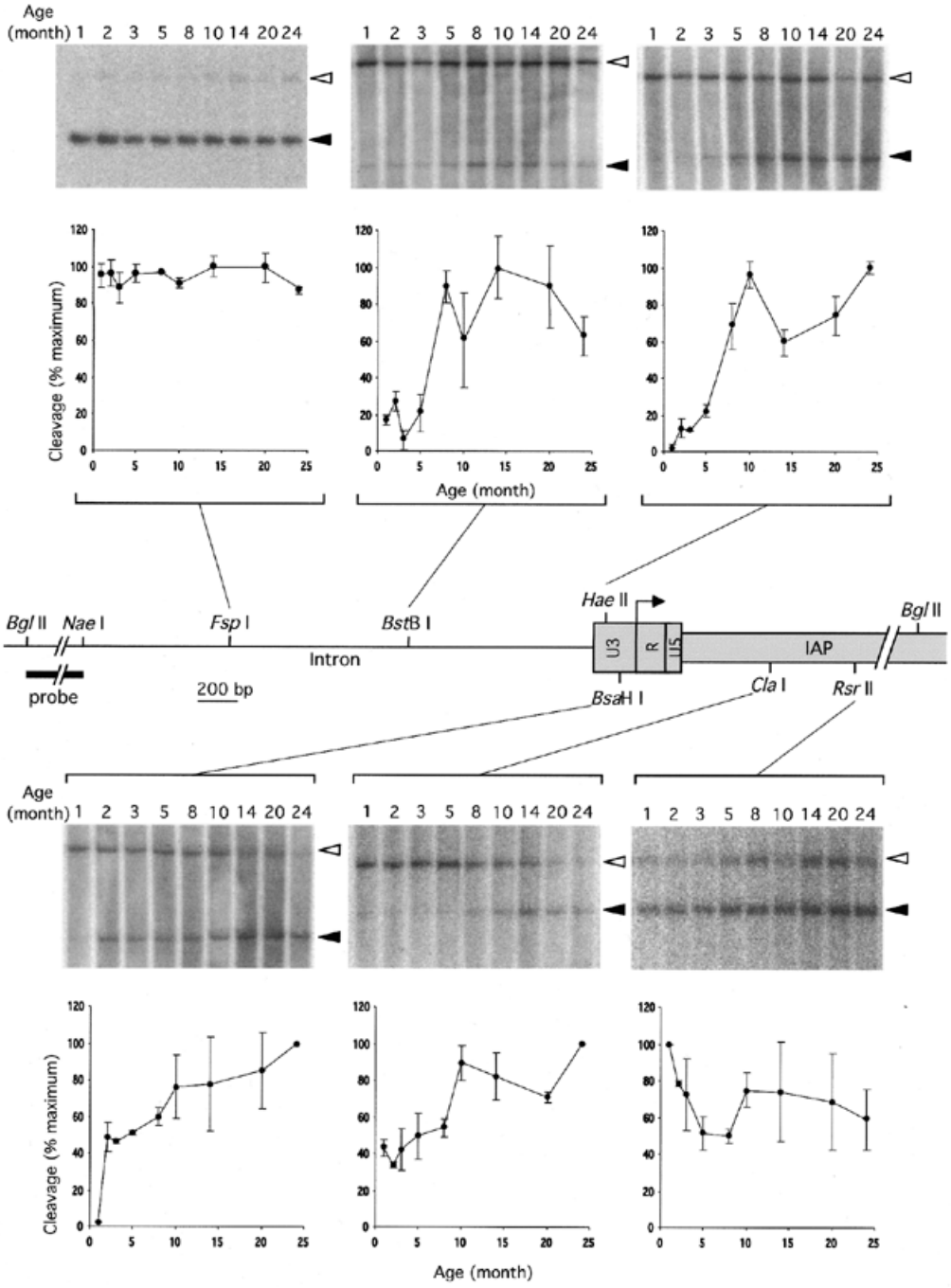

Figure 3. Methylation status of the IAP in the *m.nocturnin* locus as a function of age. Methylation-sensitive digestions were performed on DNAs extracted from the liver of mice kept under standard light/dark conditions and sacrificed at different ages (same tissues as in Fig. 2C). The enzymes used and the positions of their restriction sites are indicated on the schematic representation of the IAP LTR and surrounding sequences, in the central part of the figure. DNAs were first restricted with the methylation-insensitive *Bgl*II enzyme, which delineates a 6470 bp fragment, then with the indicated methylation-sensitive enzymes; digested DNAs were run on an agarose gel, blotted onto a membrane, and hybridized with the 1240 bp *Bgl*II–*Nae*I probe indicated in the figure. For each enzyme, the corresponding Southern blot is shown, together with a quantitation corresponding to the mean for two independent experiments (performed on two series of aging mice). The open arrowheads correspond to the *Bgl*II–*Bgl*II fragment, the filled arrowheads to the *Bgl*II–X fragment, where X indicates the methylation-sensitive enzyme used. For the quantitations, each value corresponds to the ratio of the intensity of the lower band over the sum of the intensities of the two bands, as measured using a Phosphoimager apparatus (see Materials and Methods). Results are expressed as percentages of maximal effect.

of the day (ZT 4) and both their DNA (for measurement of the levels of methylation, see below) and their RNA (for real-time quantitative RT–PCR analysis of IAP levels) were extracted. Figure 2C illustrates the kinetics of IAP induction with age. A marked increase of the levels of the 10 kb IAP-AR transcript is observed from 1-month to 24-month-old mice (~50-fold induction, with possibly a slight 2–3-fold decrease after 20 months). Interestingly, the kinetics of IAP induction is not linear and displays, in a reproducible manner, a sharp 2–3-fold increase between 1 and 2 months of age, followed by a lag period of at

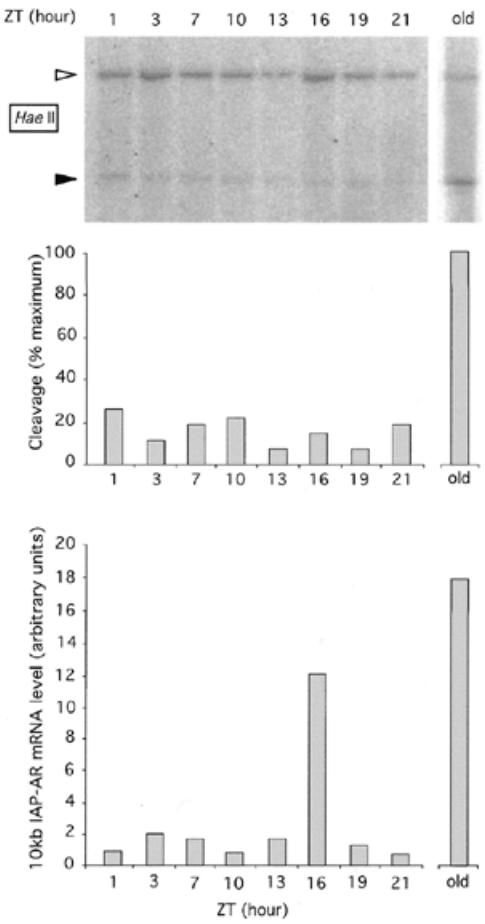

Figure 4. Circadian induction of the IAP LTR is not associated with circadian changes in its methylation status. (Upper panels) Methylation status of the IAP LTR as a function of Zeitgeber time. Same experimental conditions as in Figure 3, using the *Hae*II methylation-sensitive restriction enzyme. Mice were kept under standard light/dark conditions for at least 2 weeks before they were sacrificed (when 3 months old, with 24-month-old mice sacrificed at ZT 4 as a positive control, see right lanes). The livers of three mice were pooled for each measurement. (Lower panel) Real-time TaqMan quantitative RT–PCR analysis of the 10 kb IAP-AR transcript levels in the same organs as those used in the upper panels. Same experimental conditions as in Figure 2.

least 3 months, after which a marked increase is again observed from 5 to 20 months of age.

Demethylation of the IAP promoter with age and methylation status on the daytime scale

The methylation status of the IAP promoter and flanking DNA in the liver was followed using a series of methylation-sensitive restriction enzymes and an appropriate probe (Fig. 3), in the course of mouse aging. Two independent experiments were performed, giving identical results. As illustrated in Figure 3, we selected a series of methylation-sensitive enzymes, whose restriction sites are located within the IAP 5′-LTR itself (*Hae*II, *Bsa*HI), as well as in the internal domain of the IAP (*Cla*I, *Rsr*II) and in the 5′ flanking intronic domain (*Fsp*I, *Bst*BI). DNAs were restricted, in addition to these enzymes, with a methylation-insensitive enzyme (*Bgl*II) to generate a fragment of constant length that could be used as a reference to quantitate the extent of restriction by the methylation-sensitive enzyme. A detailed analysis of the methylation status as a function of mouse age was therefore performed, in parallel with the measurement reported above of the IAP transcripts levels. The results, given in Figure 3, disclose a marked decrease in the extent of methylation at the LTR level, compatible with the observed induction of expression with age, whereas almost no change can be observed for the outmost sites tested (i.e. *Fsp*I and *Rsr*II). Closer examination of the demethylation process upon aging further shows that the kinetics of demethylation are not strictly identical depending on the probed site. The *Hae*II and *Bsa*HI sites in the LTR are initially fully methylated and undergo a continous demethylation, with a sharp and early demethylation event before 2 months of age reproducibly observed for the *Bsa*HI site. The distantly located *Bst*BI and *Cla*I sites behave slightly differently, with a lag of ~5 months before demethylation proceeds. Interestingly, these demethylation features are reminiscent of the kinetics of the IAP-AR transcripts induction, with specific increases taking place between 1 and 2 months and at 5 months of age (Fig. 2C).

Analysis of the methylation status was similarly performed as a function of Zeitgeber time, by using the methylation-sensitive *Hae*II enzyme to probe the IAP LTR. As illustrated in Figure 4, no clear difference in the level of methylation can be observed between livers of young mice sacrificed at different Zeitgeber times, whereas IAP-AR transcripts levels, measured under identical experimental conditions, increased by 10-fold at dusk (ZT 16). As a comparison, a 15-fold increase in the level of IAP-AR transcripts between young and old mice is associated with a 10-fold decrease of methylation at the level of this site (Fig. 4). These results therefore indicate that, whereas the induction of the IAP-AR transcripts levels with age is associated with a demethylation process in the IAP LTR, its daily circadian induction is not, suggesting a lack of a strict correlation between the level of methylation and the level of gene inactivation for the daily process.

Related tissue specificities for circadian activation, age-dependent induction and demethylation of the *nocturnin*–IAP with age: a plausible model for gene induction by use

The analyses of IAP-AR transcriptional regulations reported above have been carried out on the liver, where strong age-dependent and circadian regulations can be observed. Yet we previously demonstrated that the *nocturnin* gene in which the IAP is inserted is expressed in almost all organs tested, with transcripts levels in some organs (brain and testis) higher than the maximal transcripts levels reached at dusk in the liver (12,14). An important issue was therefore to assess the tissue specificity of the age and daytime induction of the IAP-AR transcripts. Thus, a quantitative analysis of IAP-AR transcripts levels and methylation status was performed on several organs of mice, sacrificed at different times of the day and different ages (Fig. 5).

As illustrated in Figure 5A, strong circadian variations in the level of the IAP-AR transcripts are observed for the liver, as already shown in Figure 2A, whereas the levels of the IAP-AR transcripts in the kidney, testis, brain and lung remain close to background level, with no or only negligible variations. Similarly, induction with age of the IAP-AR transcripts is only observed in the liver, as illustrated in Figure 5B. Figure 5C shows that demethylation of the IAP LTR in the course of mouse aging, as assayed using the methylation-sensitive

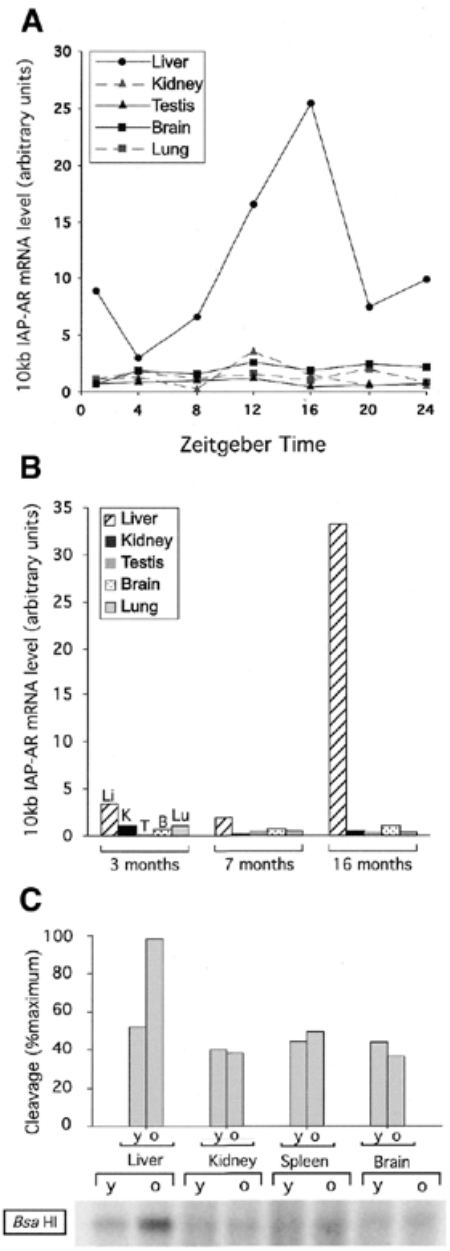

Figure 5. Circadian activation, and age-dependent induction and demethylation of the IAP LTR are liver-specific. (**A**) Real-time TaqMan quantitative RT–PCR analysis of the 10 kb IAP-AR transcript levels in several mouse organs at different Zeitgeber times. Same experimental conditions as in Figure 2, with the indicated organs of three mice (3-month-old) pooled. Same arbitrary units for all organs. (**B** and **C**) Real-time TaqMan RT–PCR analysis of the 10 kb IAP-AR transcript levels (B) and LTR methylation status (C) in several organs of aging mice. Mice were kept under standard light/dark conditions for at least 2 weeks before they were sacrificed, at the indicated ages, and the organs of three mice were pooled for each measurement. (B) Same arbitrary units for the IAP-AR transcripts levels of all organs, and same experimental conditions as in Figure 2. (C) Same experimental conditions as in Figure 3, using the methylation-sensitive *Bsa*HI enzyme.

*Bsa*HI enzyme (as in Fig. 3), also takes place exclusively in the liver. In conclusion, the circadian and age-dependent inductions of the IAP-AR transcripts are both specific to the liver, consistent with the hypothesis that the two phenomena are linked. Accordingly, a simple model can be proposed (Fig. 6 and see Discussion) in which it is the sole transient and repetitive activation of the IAP-AR which promotes its progressive demethylation and induction upon aging.

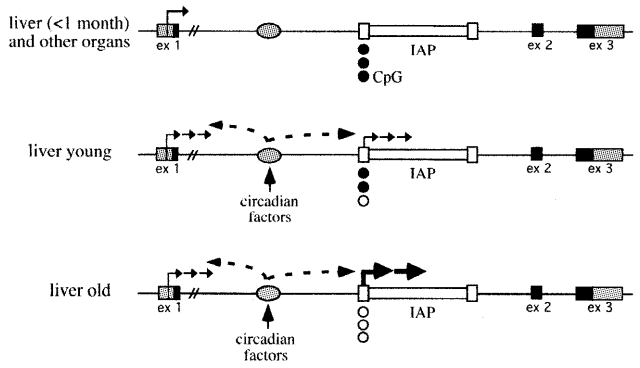

Figure 6. Daily, transient, liver-specific induction of the IAP is responsible for its progressive de-silencing: a plausible model for gene induction by use. The model states that it is the sole transient and repetitive activation of the IAP which promotes its progressive demethylation and induction upon aging ('teasing model' of gene derepression by use). In this model, the IAP would be inactive in most organs, as a result of strongly methylated LTR CpGs (schematized with filled circles). In the liver, circadian factors activate an enhancer element (schematized with an arbitrarily located gray oval) in the IAP-*nocturnin* locus, and induce circadian activation of both the IAP and the *m.nocturnin* promoters (repeated arrows). IAP transient and repetitive activation progressively triggers demethylation of LTR CpGs (schematized with empty circles) all through the animal's life, leading to enhanced expression in the liver of old mice.

DISCUSSION

In the present investigation, we have shown that a normally severely repressed IAP retrotransposon can escape its silenced state following a very slow kinetics—within months—in the course of mouse aging. The effect is exceedingly specific as it only concerns a given and well identified IAP copy among the ~1000 copies that are present in the mouse genome. The effect is associated with a progressive demethylation of the IAP promoter. Yet the copy itself has no specific sequence peculiarity which could account for this specificity, and previous experiments using demethylating agents such as 5-azacytidine (7,8) show that most probably each IAP sequence can be transcriptionally active once demethylated. Accordingly, the specific induction of the IAP most probably relies on its specific position within the active *m.nocturnin* circadian gene. This gene is active in a circadian manner all along the mouse life span, at a constant extent with age, and it triggers a circadian activation of the IAP promoter by an enhancer effect. We propose that IAP induction with age is the sole result of its repeated activation, which progressively triggers its demethylation and enhanced expression with age.

A model for promoter self-activation

Aging is a complex process, which involves numerous changes at the systemic level including, for instance, modulations in hormonal levels or metabolic activities, as well as at the cellular level, with genetic or epigenetic modulations of gene expression (for a review see 15). In this paper, the demonstration that IAP-AR expression is maintained *ex vivo*, in hepatocytes isolated from aged mice, strongly suggests that IAP-AR age induction is a cell-autonomous process. Among the

hypotheses that can be raised to account for IAP-AR induction with age, the simplest model would be that the IAP LTR activity is simply governed by the level of activity of the *m.nocturnin* gene in which the IAP is inserted. This model is rendered unlikely by the fact that organs where *m.nocturnin* displays the highest activity (e.g. brain and testis) (14) disclose no IAP-AR age-dependent demethylation and activation (Fig. 5B). Another model would predict that IAP-AR activation is triggered by the presence of an age-dependent enhancer sequence in the locus, which would be responsive to factors only present in the aging liver. The fact that the *m.nocturnin* transcripts level in the liver is not enhanced with age (Fig. 2B) and that we could not observe any age-dependent DNase I hypersensitive sites in the IAP-embedding *nocturnin* intron (data not shown) also argues against such a model. Finally, the model that we favor (Fig. 6) states that it is the sole transient and repetitive activation of the IAP-AR which promotes the passive, stochastic and progressive demethylation of the IAP LTR in the aging process, and its correlated derepression ('teasing model' of gene derepression by use). This model is consistent with the fact that the sole organ where an age-dependent induction of the IAP is detected is precisely the organ where a transient circadian activation of the IAP takes place (Fig. 5). It is also consistent with previous suggestions that gene inactivity would promote methylation and gene silencing (reviewed in 16,17). Accordingly, the observed age-dependent induction would simply be the consequence of gene 'use', with the very slow kinetics of the process (i.e. the animal life span) simply resulting from its transient, circadian, limited use.

The level of methylation versus level of activity paradigm

Analysis of the methylation status of the IAP LTR in the animal life timescale reveals a strong correlation between transcriptional silencing and methylation: the IAP LTR is heavily methylated in the liver of 1-month-old mice where the IAP-AR is not expressed, and then its progressive demethylation upon aging parallels the progressive increase in the IAP transcripts level. This is consistent with a number of *in vitro* and *in vivo* experiments showing that CpG methylation is a mechanism contributing to the repression of IAP LTR activity (6–9,18). However, and rather surprisingly, we observed that during the circadian cycle, IAP-AR transcripts levels can be enhanced (up to 10-fold) without significant demethylation of the IAP LTR. Although never reported for IAP elements, the ability of methylated genes to overcome repression has previously been described for tissue-specific genes (19,20), as well as for viruses, including the adenovirus (21) and the HIV retrovirus (22), a structure related to the presently studied retrovirus-like IAP elements. In the latter cases, the presence of the adenoviral E1A or the HIV tat transactivators reactivated, respectively, the adenovirus E2A and the HIV LTR promoters, under conditions where they were shown to remain methylated. Boyes and Bird (23) also showed that repression of some methylated genes can be overcome by the presence in *cis* of a strong enhancer. The molecular mechanism by which enhancer can trigger methylated promoter activity is unknown. One hypothesis is that blockade is overcome by the binding and activity of transcription factors that are not sensitive to methylation, such as Sp1 (24). In this respect, it is noteworthy that two putative Sp1 binding sites are present in the IAP LTR, and that LTR activity was reported not to be altered by the methylation of one of them (25); interestingly, it has also been shown that binding of the Sp1 factor can enhance demethylation of nearby sequences (26,27), a fact that in turn could account for the progressive demethylation of the IAP LTR and flanking sequence with age. Another hypothesis is that the Methyl-CpG-binding proteins (MeCP-1 and MeCP-2 proteins), which have been shown to interact specifically with methylated DNA and mediate transcriptional repression (28), are transiently displaced from the IAP LTR by factors with high affinity for the LTR sequence (an hypothesis formulated in ref. 23 for low density methyl-CpG promoters). Whatever the hypotheses, demethylation of the IAP LTR upon aging would be a consequence, rather than a cause, of transcriptional activation.

Finally, an interesting observation concerns the kinetics of demethylation of the IAP-AR locus, in relation to that of its transcriptional activation. As reported, a sharp induction of the IAP-AR takes place between 1 and 2 months of age—actually a period corresponding to the onset of circadian rhythmicity in mice—which correlates with a first demethylation event at the *Bsa*HI LTR site. The following lag, observed between 2 and 5 months of age for IAP-AR induction, is associated with a similar lag for demethylation of the IAP LTR locus, clearly visible at the level of the *Cla*I and *Bst*BI flanking sites. This lag could correspond to a period of passive and slow demethylation, at the end of which mCpG density would reach a threshold level and/or critical CpG residues would be demethylated (29), thus allowing re-initiation of the IAP-AR activation process and spreading of demethylation to distantly located sites.

Biological relevance

The proposed model for gene induction with age and/or use is likely to apply, first, to transposable elements, which are strongly repressed and methylated elements—most probably as a consequence of host defense mechanisms—and which often lie close to or within genes, due to their high copy number and mobility. Although additional mechanisms are likely to be involved in transposon induction, the model could account for the demethylation of some of the murine LINE transposable elements detected in the liver of old mice (30), and for the induction of endogenous MuLV and MMTV murine retroviruses, observed in the aging mice (31,32). If progressive induction takes place due to the presence of circadian 'inducer' genes, such as *m.nocturnin*, one can also imagine 'inducer' genes displaying constitutive expression, therefore leading to an induction too rapid to be detected. This could account, for instance, for the observed activity of a small number of transposable elements found to be transcribed in some tissues and physiological conditions (33–35). One can also imagine intermediate kinetics of gene induction, depending on the strength and periodicity (e.g. cell cycle, reproductive cycle) of the 'inducer' gene activity. The model could also apply, in principle, to X-linked inactivated genes, which display strong CpG methylation and share, for several of them, chromosomal positions in common with transcriptionally active genes that have escaped inactivation. Along this line, it is noteworthy that age-related reactivation of two X-linked genes has been reported (36,37).

In conclusion, the phenomenon reported in the present paper strengthens the complex interplay between transposable elements and their hosts: the latter have evolved strategies for repressing transposon propagation, through methylation-mediated

transcriptional inactivation and/or cosuppression (38,39), whereas the former are able, due to their high copy number and mobility, to take advantage of the host genome regulatory elements so that some of them stochastically escape repression and can still propagate. The observed phenomenon also exemplifies the possible involvement of circadian, daily clocks in the timing of processes taking place at a much larger timescale, i.e. that of animal life.

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