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Transcription of *unr* (upstream of N-*ras*) down-modulates N-*ras* expression in vivo

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Abstract The ras proteins (Harvey, Kirsten and N-ras) are key regulators of signal transduction and a perturbation of their GDP/GTP cycle is frequently observed in tumors. In mammals, N-*ras* constitutes with *unr* (upstream of N-ras) a tightly linked tandem of ubiquitously expressed genes. Although *unr* and N-*ras* appear to be involved in distinct functions, this unusual genetic organization could be important for the regulation of N-*ras* expression. Specifically, transcription of *unr* could negatively regulate that of N-*ras* by transcriptional interference. To investigate this possibility, we have deleted the *unr* promoter by homologous recombination in murine embryonic stem cells. Analysis of tissues of heterozygous mice revealed an increase in N-*ras* mRNA accumulation ranging between 20 and 65%, in agreement with the suppression of a transcriptional interference.

Key words: Gene tandem; Promoter deletion; Transcriptional interference

1. Introduction

Studies of the N-*ras* gene in mammals have led to the characterization of a gene located immediately upstream of N-*ras* designated *unr* (upstream of N-ras) [1–3]. A striking feature of this genetic organization (Fig. 1) is the short 'intergenic' distance between these two genes as 150 nucleotides separate the predominantly used polyadenylation site of *unr* (A3, Fig. 1) from the transcription initiation sites of N-*ras* [3]. Although it is increasingly apparent that gene density varies significantly along chromosomes [4], this structure is unusually compact. Head to tail gene tandems are often the result of gene duplication and the corresponding clusters usually constitute functional and regulatory units. Well characterized examples of this type of organization are the β-globin locus [5] and the Hox clusters [6]. *unr* and N-*ras*, however, do not fit in this scheme as the corresponding genes and proteins present no homology. N-ras is involved in signal transduction and belongs to a large family of small GTPase while unr contains five cold shock domains [7–9]. Thus the *unr*/N-*ras* gene tandem differs from others by the clustering of two genes of unrelated functions and the smaller intergenic distance.

The presence of N-*ras* within this unusual genetic structure could reflect the existence of a novel level of regulation which could contribute to the control of N-*ras* expression. Specifically, the head to tail tandem organization of *unr* and N-*ras* creates the opportunity for *unr* to negatively regulate N-*ras* expression via a transcriptional interference or promoter occlusion. This phenomenon, which has been initially observed in prokaryotes [10,11], reflects the possibility that within a tandem of genes the polymerases which transcribe the upstream gene could interfere with transcription initiation at the downstream promoter. In eukaryotes, it has been proposed that a transcriptional interference was responsible for the repression of internal promoters in integrated retroviruses [12], the adenovirus gene IX [13], the Adh gene in *Drosophila* [14] and the actin gene in yeast [15]. For cellular genes in higher eukaryotes, the available data come from transient transfection experiments with plasmids containing duplicated genes or gene tandem. In these experiments, abrogation of the transcription of the upstream gene led to a 30–300% increase in expression of the downstream one [16,17]. Yet, the importance of transcriptional interference has not been evaluated so far for genes in their chromosomal context.

In mammals, transcription by RNA polymerase II generally extends well beyond the polyadenylation site [18] reflecting that transcription termination requires another signal, such as a pause site for polymerases, in addition to polyadenylation [19–21]. Thus a transcriptional interference can occur even when the mature mRNAs do not overlap, as is the case for *unr* and N-*ras*. Indeed, the existence of nuclear poly(A)-transcripts which contain the intergenic region and extent up to N-*ras* exon 2 supports that the polymerases which transcribe *unr* enter the N-*ras* transcription unit (O.B. and F.D., manuscript in preparation). As the density of RNA polymerase II is probably insufficient to sterically hinder transcription of the

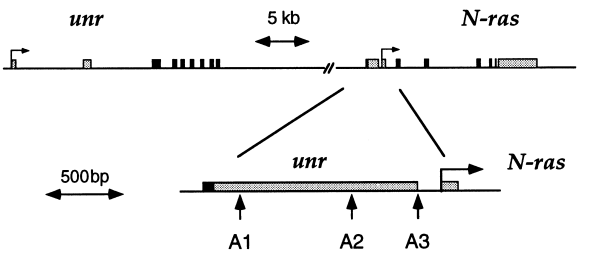

Fig. 1. The *unr*/N-*ras* gene tandem. Genomic organization of *unr* and N-*ras*. Top: Presentation of the known genomic structure of *unr* and N-*ras*. Exons are indicated by boxes (shaded for non-coding exons and solid for coding exons) and transcription initiation sites by arrows. Bottom: Enlargement of the *unr*/N-*ras* junction with the indication of the three polyadenylation sites of *unr* (A1, A2 and A3). This figure combines results on the organization of the N-*ras* gene [39,40] and the *unr*/N-*ras* junction [1–3] and the genomic organization of the *unr* gene (H.J.-S. and O.B., unpublished results).

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downstream gene, it is more likely that transcriptional interference results from the displacement by polymerases of proteins bound to the downstream promoter [22,23]. In this context it is worth noting that the analysis of the N-*ras* promoter has revealed the presence of regulatory sequences within the last exon of *unr* [24].

unr and N-*ras* are ubiquitously expressed, *unr* mRNA accumulating to higher levels than those of N-*ras* [3]. Thus if a transcriptional interference takes place between these two genes it does not lead to a complete occlusion of the N-*ras* promoter. While, in vitro, cells in culture exhibit a very limited range of *unr* and N-*ras* expression, significant differences can be observed in vivo. To assess the contribution of a transcriptional interference to the expression pattern of N-*ras* in vivo, we have inactivated *unr* transcription by gene targeting and analyzed the expression of N-*ras* in tissues of heterozygous mice.

2. Materials and methods

2.1. Targeting vector

The 5′ end of the murine *unr* gene was isolated from a 129/sv genomic library using a human *unr* exon 1 probe. The 16-kb insert was subcloned into pSL1190 (Pharmacia) and characterized by restriction mapping, hybridization with probes for the human exons 1 and 2 and partial sequencing. In the targeting vector (Fig. 2A), an 8.5-kb *Nru*I-*Pst*I internal fragment corresponding to positions −300 to +8200 with respect to *unr* transcription initiation sites, was replaced by the 1.2-kb *Sal*I-*Xho*I fragment of the pMC1neoPA plasmid [25] and a 49-bp oligonucleotide containing the synthetic rabbit β-globin polyadenylation site [26]. The synthetic rabbit β-globin polyadenylation site is in the same orientation as *unr*, while the *neo* cassette is in the opposite one. A Herpes simplex thymidine kinase gene (HSV-tk, the 1.8-kb *Bam*HI-*Hin*dIII fragment of pMC1tk) was added outside of the short arm of homology for negative selection against random integrations.

2.2. Selection of recombinant ES cell clones

Targeting vectors (30 μg), linearized at the unique *Spe*I site located within the plasmid sequences, were electroporated in the R1 embryonic stem cells (10^7, kindly provided by A. Nagy). Colonies were selected with G418 (300 μg/ml for 10 days) and Gancyclovir (2 μM for 3 days) and screened by the polymerase chain reaction (PCR) on cell lysates as previously described [27] with the primers unr1 (5′-GAAAGTACACCAGGATGAGAATG-3′) and neo1 (5′-CTTGACGAGTTCTTCTGAGG-3′). PCR reactions consisted of 40 cycles (2′ at 94°C, 45″ at 65°C and 2′ at 72°C). The genomic organization of the clones was further analyzed by Southern blot with probes for *unr* and *neo*.

2.3. Generation of mutant mice

C57/Bl6 blastocysts were microinjected with 8 to 12 R1 ES cells and implanted into pseudopregnant B6/CBA foster mothers to obtain chimeric progeny. Chimeras with a predominantly agouti coat color were mated with C57/Bl6 mice and the agouti F1 were screened for the presence of the mutation by Southern blot analysis of tail DNA.

2.4. RNA extraction and analysis

Cellular RNA were extracted by lysis in guanidinium thiocyanate and centrifugation over a cesium chloride cushion [28]. For adult mice tissues, samples were frozen in liquid nitrogen, ground into a fine powder and homogenized in guanidinium thiocyanate with an ultraturax blender prior to centrifugation. For RNase protection analysis 10 μg of RNA were hybridized with 0.5 ng of RNA probe, labeled with [α-^{32}P]UTP to a specific activity of 6×10^7 cpm/μg, for 16 h at 55°C, digested with RNase A and T1, and electrophoresed through a 5% acrylamide/urea gel according to Neel et al. [29]. Quantification of the results was performed with a Fuji Bioimager (BAS 1000), taking into account the number of labeled residues in each protected fragment. The murine UKN probe (*unr*, Ki-*ras*, N-*ras*) was constructed by the ligation into the plasmid Bluescript (Stratagene) of a 180-nt fragment of a *unr* cDNA (generated by PCR with the primers UKN1 5′-GATCTCTAGAGGAGAGGGGGCGCTGAGCTG-3′ and UKN2 5′-CTAGTCTAGAGAGTAGGCACAAACTTCTTGTTTCAG-3′, located in exons 1 and 2, respectively), a *Hin*dIII-*Nsi*I fragment of a Ki-*ras* cDNA containing 148 nt of exons 1 and 2 and a *Pvu*I-*Eco*RI fragment of an N-*ras* cDNA containing 243 nt of exons 2 and 3.

3. Results

3.1. Deletion of the unr promoter

To inactivate *unr* transcription we have deleted its promoter by homologous recombination in murine embryonic stem cells. We first isolated from a 129/sv genomic library a phage containing the 5′ end of the murine *unr* gene. Sequence analysis revealed the presence of a domain with a high similarity to the human proximal promoter and exon 1 (99% identity from −200 to +100, using the numbering of the human promoter [30]). Further analysis by primer extension and RNase protection established that transcription initiation occurred at homologous nucleotides in both species (O.B., unpublished results). The targeting vector was designed to create an 8.5-kb deletion extending from −300 to +8200 with respect to the major transcription initiation site (Fig. 2A). This deletion encompasses the minimal *unr* promoter as defined by transfection experiments [30] as well as *unr* exon 1 and intron 1. In addition, an efficient poly(A) site [26] was introduced in order to quench transcripts which could come from promoters located upstream of the bona fide *unr* promoter [20]. To minimize the possibility that the promoter of the *neo* selection marker could generate novel *unr* transcripts, this transcription unit was introduced in the opposite orientation of that of *unr*.

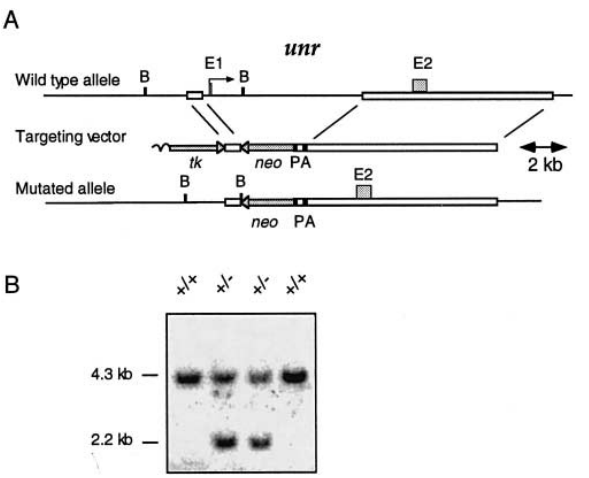

Fig. 2. Deletion of the *unr* promoter. A: Deletion of the *unr* promoter by homologous recombination. Top: Schematic representation of the 5′ end of the wild type *unr* allele. *unr* exons are indicated by shaded boxes, transcription initiation by an arrow and the homology regions present in the targeting vector by open boxes. Middle: Targeting vector. The t*k* and *neo* expression cassettes are represented by shaded arrows indicating the transcription orientation, and the synthetic β-globin poly(A) site (PA) by a stippled box. Bottom: Mutated allele resulting from the integration of the targeting vector by homologous recombination. The *Bam*HI restriction sites relevant to the Southern analysis are indicated by B. B: Southern blot analysis of ES clones. Following digestion with *Bam*HI, DNA samples were analyzed by Southern blot and hybridized with a *unr* probe derived from the short homology region present in the targeting vector. The predicted size of the wild type and mutated alleles are indicated on the left.

Following selection with G418 and Gancyclovir, clones of ES cells containing the modified *unr* allele were isolated at a frequency of 1/100, as illustrated by the Southern blot analysis of Fig. 2B. The absence of other integrations of the targeting vector and of rearrangement of the locus was confirmed by further Southern blot analysis with *unr* and *neo* probes (data not shown). Following injection of *unr*+/− cells into host blastocysts, chimeras where obtained which transmitted the mutation to their offspring. No viable homozygous mutant mice were observed in crosses between heterozygotes, indicating that the mutation is lethal.

3.2. Analysis of unr and N-ras expression in heterozygous mice

We used an RNase protection assay to develop a quantitative analysis of *unr* and N-*ras* mRNA accumulation in murine tissues. Fig. 3A presents the structure of the probe used; it contains 180 nt from *unr* exons 1 and 2, 243 nt from N-*ras* exons 2 and 3 and 148 nt from Ki-*ras* exons 1 and 2 as an internal control. This probe does not encompass any known alternative splice site [3,31,32] and therefore, for each of these genes, should detect all the messages with an equal efficiency. To evaluate the reproducibility of this analysis and the variability between animals, we analyzed total RNA preparations derived from the brain of three wild type mice. The autoradiograph from an experiment in which each sample was analyzed three times is presented in Fig. 3B. Protected fragments with the expected size for N-*ras*, *unr* and Ki-*ras* were observed. Repeated analysis of the same sample yielded results which differed by about 10% as could be expected for the RNase protection assay [29]. When samples from different animals were analyzed, results differed by about 20% and these variations were reduced to 10% when the Ki-*ras* signal was used to normalize the N-*ras* and *unr* signals. Thus, the variability of *unr* and N-*ras* mRNA accumulation between animals is comparable with the precision of the RNase protection assay.

In the absence of *unr*−/− mice, we analyzed *unr* and N-*ras*

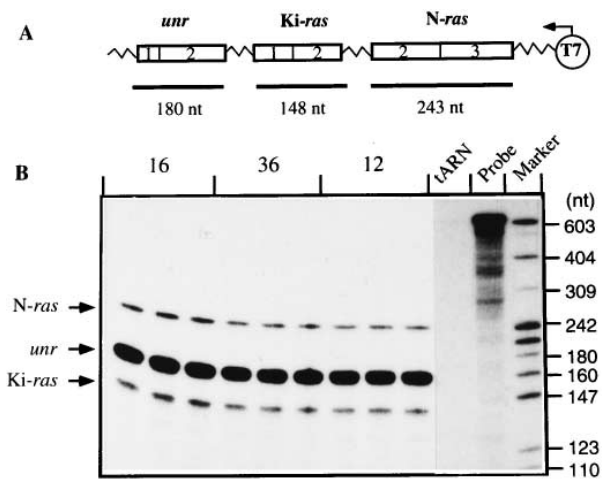

Fig. 3. *unr* and N-*ras* RNA accumulation in the brain of wild type mice. A: Schematic representation of the probe used in the RNase protection assay with the the size of the protected fragments. B: Autoradiogram of an RNase protection assay performed on brain RNA samples from three wild type mice (animals 16, 36 and 12). Each sample was analyzed in triplicate with 10 μg of total RNA. Lane tRNA, 10 μg of tRNA; lane Probe, 16 pg of undigested probe; lane Marker, molecular weight markers (*Msp*I-digested pBR322). The expected migrations of the protected fragments are indicated on the left.

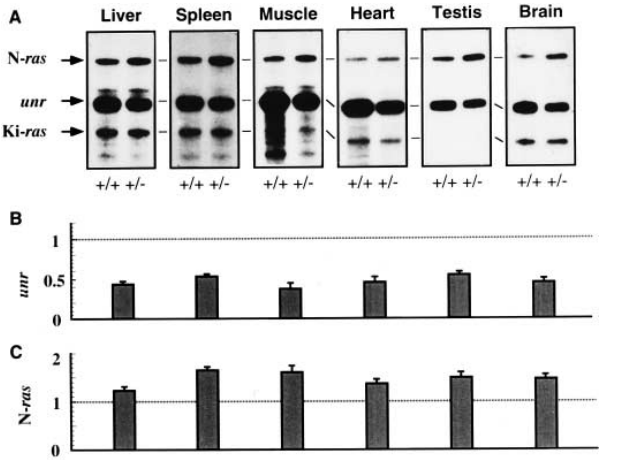

Fig. 4. *unr* and N-*ras* RNA accumulation in tissues of *unr*+/+ and *unr*+/− mice. A: Representative autoradiograms for each tissue of RNase protection assays performed as in Fig. 2, the wild type sample is on the left and the heterozygous sample on the right. Exposure times were adjusted for the N-*ras* protection to be visible. The expected migrations of the protected fragments are indicated on the left. B: Quantification of the relative accumulation of *unr* RNA in heterozygous and wild type samples. Data from at least four independent experiments for each tissue were used to determined the mean and S.E.M. of the ratio of the accumulation in heterozygous samples vs. that in wild type controls. C: Quantification of the relative accumulation of N-*ras* RNA in heterozygous and wild type samples. Data from at least four independent experiments for each tissue were used to determine the mean and S.E.M. of the ratio of the accumulation in heterozygous samples vs. that in wild type controls.

mRNA accumulation in a panel of tissues (liver, spleen, muscle, heart, testis, brain) from wild type and heterozygous mice (*unr*+/−) with the RNase protection assay of Fig. 3. We observed no protection of *unr* exon 2 alone (Fig. 4A), indicating that there is no residual transcription of the mutated *unr* allele. In addition, Northern blot analysis did not reveal the presence of abnormal *unr* or N-*ras* transcripts in heterozygous samples (data not shown). Four determinations were performed for each tissue and the signals were quantitated with a Fuji Bio-imager. Figs. 4B and 4C present for *unr* and N-*ras* the ratio of the expression in *unr*+/− and *unr*+/+ samples. In the six tissues analyzed, *unr* mRNA accumulation in *unr*+/− samples was about one half of that in *unr*+/+ samples (Fig. 4B, see Table 1 for the actual numbers). Thus, *unr* expression obeys a simple gene dosage. In the same tissues, N-*ras* mRNA accumulation increased in *unr*+/− samples by 20 to 65% (Fig. 3C and Table 1). In all cases the observed increase in mRNA accumulation was statistically significant (P ranging from 0.01 to 0.001). In tissues in which the level of Ki-*ras* mRNA accumulation could be accurately determined (liver, spleen, brain), no difference between *unr*+/+ and *unr*+/− samples was observed for this gene. Thus, in *unr*+/− samples *unr* expression was reduced by 50% while that of N-*ras* was increased by 20 to 65%.

To investigate whether the influence of *unr* on N-*ras* expression was dependent on its expression level, the results of the experiments of Fig. 4 were compiled and expressed using a single arbitrary unit, *unr* mRNA accumulation in liver being taken as 100 (Table 1). In wild type tissues, *unr* expression varied 7-fold between liver (100) and testis (700). In the corresponding heterozygous samples, the increase in N-*ras* ex-

Table 1
unr and N-*ras* mRNA accumulation in tissues of wild type and heterozygous mice

	Expression in wild type		Change in expression in heterozygotes	
	unr	N-*ras*	*unr*	N-*ras*
Liver	100	17	−57% ± 5	+23% ± 7
Brain	200	40	−55% ± 6	+45% ± 10
Spleen	250	62	−47% ± 3	+65% ± 6
Heart	450	24	−55% ± 7	+36% ± 9
Muscle	500	17	−62% ± 10	+61% ± 15
Testis	700	230	−46% ± 3	+49% ± 10

Results of the RNase protection assays of Fig. 3 were compiled, using internal standards to allow the comparison of the expression levels between tissues. *unr* and N-*ras* mRNA accumulation is expressed with the same arbitrary unit, *unr* mRNA accumulation in the liver being taken as 100.

pression was unrelated to *unr* expression level, with the possible exception of liver which had the smallest increase in N-*ras* and the lowest expression of *unr*. Thus, beyond a possible threshold, the impact of *unr* on N-*ras* expression is independent of its expression level.

4. Discussion

The short 'intergenic' distance between *unr* and N-*ras* is unexpected in view of the large size of mammalian genomes. Moreover, neither *unr* nor N-*ras* have a compact organization indicating that the overall gene density is not particularly high in this chromosomal region. To investigate whether the transcription of *unr* had an impact on N-*ras* expression, we have deleted *unr* promoter by gene targeting. This mutation induces no overt phenotype in heterozygotes, but leads to an embryonic lethality in homozygotes (manuscript in preparation). Using a quantitative analysis of *unr* and N-*ras* mRNA accumulation in tissues of wild type and heterozygous mice, we observed that *unr* expression closely follows the gene dosage while that of N-*ras* increased in all the tissues examined. This increase in N-*ras* mRNA accumulation varied between 20% in liver and 65% in spleen. No modification of expression was observed for the closely related Kirsten-*ras* gene as expected if *unr* transcription modulates N-*ras* expression in *cis*. Thus, if only the N-*ras* allele in *cis* of the *unr* mutation is affected, its expression is increased by 40 to 130%.

This study was performed to evaluate in vivo the importance of *unr* transcription for N-*ras* expression. Although the experimental strategy was designed to directly assess the importance of a transcriptional interference, other mechanisms could participate to the observed effects on N-*ras* expression. Specifically, the introduced mutation involves the removal of *unr* regulatory sequences and their replacement by a *neo* expression cassette. While *unr* and N-*ras* promoters are more than 30 kb apart, we cannot exclude the possibility of long range interactions between them. However, in cases where similar mutations have been observed to alter the expression of adjacent genes (see [33] for a review), a specific mechanism has been implicated. It involves an interaction between the *neo* selection marker and a locus control region [34,35] [36] which usually leads to a pattern of expression of the *neo* marker which is typical of the targeted locus. It is very unlikely that such a mechanism is acting here as (i) we have used an MC1neo cassette which in contrast with the PGKneo cassette has not been associated with altered expression of the adjacent gene [33], (ii) all of the reported examples occurred in clusters of functionally homologous genes (Hox, globin) with some type of locus regulation, (iii) we have failed to detect the expression of the *neo* gene in adult tissues of unr+/− mice (data not shown) and (iv) in all the reported cases, the *neo* cassette excludes the promoter of the adjacent gene from the interaction with regulatory sequences and suppresses rather than activates its expression.

The increased N-*ras* expression in all heterozygous tissues substantiates the existence of a transcriptional interference between these two genes. The amplitude of this effect is comparable with what has been observed in transient expression assays with constructs containing duplicated genes [16,17]. Our results on this specific gene tandem, however, do not support the proposal that transcriptional interference could be much more severe between genes in their chromosomal context. Experiments with model systems on plasmids have revealed that in some [12,15], but not all, cases there was a correlation between the expression level of the upstream gene and the strength of the transcriptional interference. Our results indicate that, for *unr* and N-*ras*, the expression level of *unr* was not a major determinant of the transcriptional interference at least when the expression level is greater than in liver. The increase in N-*ras* expression is smaller than the more than 10-fold variation of N-*ras* expression among different tissues indicating that N-*ras* expression is primarily controlled by its own promoter and that *unr* transcription acts as a modulator of this expression.

In terms of N-*ras* the *unr*+/− mice should be somewhat similar to mice carrying a third N-*ras* allele. Since the ras proteins act as molecular switches in signal transduction, their biological activity should be sensitive to their expression level. This is clearly illustrated by vulva development in *C. elegans* which is sensitive to the dosage of *let-60*, a *C. elegans ras* homologue [37]. By contrast, we did not observe any overt phenotype in heterozygous *unr*+/− mice. This probably reflects the existence in mammals of three *ras* genes with overlapping functions as suggested by the lack of phenotype of mice homozygous for the inactivation of N-*ras* [38]. Nevertheless, the increase in N-*ras* expression could lead to a phenotype within the appropriate genetic background, e.g. in the presence of mutations which increase the activity of the ras signaling pathway.

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