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Cloning and initial characterization of human and mouse Spot 14 genes

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Abstract The intricate regulation of Spot 14 expression in rat lipogenic tissues has provided a useful tool in studying nutritional and hormonal factors involved in transcription. To gain insight into its function and its possible involvement in human lipid disorders, we cloned human and mouse Spot 14 genes that shared with the rat gene a strong homology concerning the deduced amino acid sequence (81 and 94%, respectively) as well as the promoter region. The mouse promoter was characterized by transfection studies, while quantitative RT-PCR and in situ hybridization experiments showed that Spot 14 is expressed in human liver and, at a high level, in multiple symmetric lipomatosis nodules.

Key words: Spot 14; *Homo sapiens*; *Mus musculus*; Promoter; G12 protein; Multiple symmetric lipomatosis

1. Introduction

Since its initial characterization in rat liver as a thyroid hormone responsive gene [1], Spot 14 has been the focus of interest of many investigators because of the intricate regulation of its expression. Mainly expressed in tissues that synthesize triglycerides, the mRNA coding for Spot 14 has been shown to be increased in rat liver by insulin [2], dietary carbohydrates [3], glucose in hepatocyte culture medium [4], as well as thyroid hormone. In contrast, dietary fats [5] and polyunsaturated fatty acids [6], have been shown to decrease the amount of Spot 14 mRNA, while an elevated level of cAMP acts as a dominant negative factor [2]. In addition, liver-specific factors [7,8] or chromatin organization of the gene [9] have been shown to contribute to the regulation of its expression. The promoter region of rat Spot 14 has been previously cloned [1] and several sequences involved in the regulation of the transcription have been described such as thyroid hormone response elements (TRE) [10] or carbohydrate response element (CHORE) [11]. However, several subsequent studies have indicated that the mechanisms of interactions between the CHORE and the TREs are very complex [12,13].

Spot 14 expression has also been studied in adipose tissues (white as well as brown). In these tissues, insulin and cAMP [14], retinoic acid [15] and adipocyte-specific factors [16] seemed to play crucial roles in the control of the expression of the gene.

Despite this impressive wealth of studies, little is known about the function of the protein whose localization has been reported to be nuclear [17]. Nevertheless, the regulation of its expression, although slightly different in liver and in adipose tissues, featured Spot 14 as playing an important role in lipogenesis. To gain further insight into the regulation of its expression in the course of adipose differentiation process and thus the possible role of the protein, it seemed interesting to clone the mouse gene. Indeed, established adipocyte cell lines are derived from this animal species and moreover, the mouse gene is a requisite tool in transgenic studies. Similarly, cloning of the human homologue appears as a first step in the search for a possible involvement of Spot 14 in pathological processes related to lipid metabolism disorders. We present here the characterization of the human and mouse genes and show that Spot 14 is highly conserved between rodents and humans as well as being expressed in human lipogenic tissues such as liver and adipocytes.

2. Materials and methods

2.1. Cloning of mouse and human Spot 14 genes

A Balb c genomic library constructed in EMBL3 phage (Clontech) was screened according to standard protocol [18] with a *Pst*I-*Taq*I 789 bp rat genomic fragment that contains the whole coding sequence [19] as a probe. At every round of the screening procedure, each plaque giving a hybridization positive signal was checked for the presence of Spot 14 coding sequence by PCR amplification using a set of primers designed from the rat sequence. This protocol allowed us to reduce the time required for the isolation of the two Spot 14 containing clones (4011 and 4021) that were finally selected. Clone 4011 was digested by *Sac*I restriction enzyme and subcloned into the corresponding site of pBluescript (Stratagene) plasmid. One of these subclones, pM600, was shown by PCR procedure to contain the whole coding region. The restriction map of pM600 allowed us to sequence completely a 2044 bp fragment contained between a *Pst*I and an *Eae*I restriction site.

Considering the strong homology between mouse and rat coding sequences, we used a set of primers able to amplify both mouse and rat coding sequences. RT-PCR amplification from a human hepatic cDNA pool was carried out according to a previous report [20]. The amplified fragment was then sequenced and used to design two other sets of primers for walking upstream and downstream in uncloned human genomic DNA using a nested long distance PCR protocol (DNA walking kit, Clontech). Both upstream (about 800 bp) and downstream (about 2600 bp) amplified fragments were subcloned into PCR II vector (Invitrogen) and finally sequenced.

2.2. Study of the expression of the human and mouse genes

Two samples of human liver tissues (one of them producing extensive steatosis) in the vicinity of surgically removed hepatocarcinoma nodules as well as a sample of lipomatous nodule from the scapular region of a patient suffering from Launois-Bensaude's disease (multiple symmetric lipomatosis), were studied, with the informed consent

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Nucleotide sequences of the *Mus musculus* and *Homo sapiens* Spot 14 genes have been submitted to the EMBL Data Bank under the accession numbers X95279 and Y08409, respectively.

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of each patient. Samples were immediately frozen in liquid nitrogen and then stored at −80°C until use. HepG2 cells (human hepatoma cell line), FAO cells (rat hepatoma cells) and Huvec (human umbilical vein endothelial cells) were cultured in 35 mm diameter culture dishes with DMEM supplemented with appropriate antibiotics and 10% fetal calf serum according to standard protocols. Ob 17 cells, which derived from adult mouse periepididymal white adipose tissue [21], were cultured in 60 mm diameter dishes under the same conditions. After complete differentiation of the adipocyte cells, calf serum was withdrawn for 24 h and dexamethasone or retinoic acid was added. RNA extraction was performed according to a microscale method [22].

Amounts of mRNA encoding human and mouse Spot 14 proteins were assayed using a quantitative multistandard RT-PCR method that takes advantage of both Spot 14 and β-actin sequence conservation between animal species [20]. This protocol allowed us to normalize the amounts of Spot 14 mRNA with regard to that of β-actin mRNA in each sample. Briefly, total RNA samples extracted from human tissues or mouse cells were mixed with a constant amount of total RNA prepared from rat liver which brought both competitive rat β-actin and Spot 14 sequences and thus acted as an multistandard source. All RNA preparations were incubated in the presence of RNase-free DNase (Promega) in order to avoid amplification from possible contamination by genomic DNA. The mixture was reverse-transcribed using hexa random primers. Separate PCRs for Spot 14 and for β-actin amplification were then undertaken with oligonucleotide primers that are able to hybridize with rat, mouse and human sequences with the same efficiency. For Spot 14 amplification, the direct primer, SHR77, extended from nucleotide 99 to 122 and the reverse primer, SHR371, from nucleotide 402 to 379, according to the rat sequence [1]. For β-actin amplification, the direct primer (ACT53) extended from nucleotide 558 to 579 and the reverse primer (ACT33) from nucleotide 1126 to 1100, according to the human sequence [23]. Each amplification product was then distinguished by restriction site polymorphism: the human and mouse Spot 14 products were digested by *Pvu*II into two fragments (245 and 47 bp for human and 257 and 47 bp for mouse), while the rat product remained uncut. A similar difference in pattern of restriction sites was used to differentiate β-actin products: a single *Pvu*II restriction site (producing 475 and 94 bp fragments) was present in rat, but not in human or mouse. Quantification of each amplification product was performed after electrophoresis and analysis of ethidium bromide stained gels. In each human sample, the ratio of actual moles of Spot 14 mRNA vs. β-actin mRNA (sh/ah) is equal to $R \times c$, where R is the ratio of human Spot 14 product vs. rat Spot 14 product, normalized with regard to β-actin and c is the ratio of the actual amounts of Spot 14 mRNA vs. β-actin mRNA in the rat multistandard liver preparation [20]. The same quantification process was applied to mouse samples.

In order to perform in situ hybridization (ISH), human Spot 14 PCR product from genomic amplification was subcloned into the *Sma*I site of the polylinker of pBluescript (Stratagene) and sequenced to check for its orientation. Two PCRs were performed using this recombinant plasmid as a template and either T3 and SHR77 or T7 and SHR371 as primers. Products were purified from agarose gels after electrophoresis and used as matrices in an in vitro transcription protocol: 100 ng of each amplification product were used as matrices to produce 11-UTP-digoxigenin labeled sense and antisense cRNA probes in the presence of *T3* or *T7* RNA polymerase (Promega) respectively. Cryostat serial sections of human liver samples were fixed in paraformaldehyde, dehydrated, and stored at −80°C before being used in an ISH protocol with 10 μl of each probe at 50 ng/μl, as extensively described in a previous report [24]. In each experiment, controls included sections incubated with the sense probe, sections preincubated in the presence of RNase A (10 μg/ml) and sections without any probe. No significant labeling could be seen in these controls.

2.3. Study of mouse promoter region

Primer extension experiments were undertaken using a 5′ radiolabeled 46-mer oligonucleotide complementary to the mouse sequence that was hybridized against 50 μg of liver or adipocyte total RNA. Reverse transcription from annealed primer was achieved with 200 U Moloney Murine Leukemia Virus reverse transcriptase (Gibco-BRL). The length of the extended primer was evaluated by electrophoresis in a sequencing gel (5% polyacrylamide, 8 M urea).

In order to test the functionality of promoter sequence in various cells, two promoter sequences (−220/+19 in the case of pSAluc and −110/+19 in the case of pK1luc) were ligated in front of luciferase coding sequence in pGL2 vector (Promega). Three culture dishes of FAO cells (derived from rat hepatoma), or preadipocyte Ob17 cells or HepG2 cells were transfected with CsCl-purified DNA preparations of Spot 14 promoter luciferase expression plasmids, using lipofectamine (Gibco-BRL). Ob17 cells (60 mm diameter dishes) were transfected with 5 μg DNA, while HepG2 and FAO cells (35 mm diameter dishes) were transfected with 2 μg DNA. In both cases, the DNA/lipofectamine ratio was 1:5 (w/w). Cells were maintained in the presence of this mixture for 18 h, then washed and the luciferase activity was assayed 24 h later. As controls, pSV40luc and Basic (no promoter sequence) plasmids were transfected in parallel experiments. Luciferase activity was normalized in each dish according to the protein content.

3. Results

During the screening of the mouse genomic library, the use of a PCR amplification step greatly reduced the time necessary to clone the Spot 14 gene. We screened 0.5×10^6 clones and three hybridization rounds were necessary to purify two clones (4011 and 4021) which were shown to contain the coding sequence. Mouse clone pM600 presented an open reading frame of 450 bp whose translation led to a putative protein highly similar to that of the rat (see Fig. 1a). Since mouse and rat coding sequences shared a high degree of homology, we

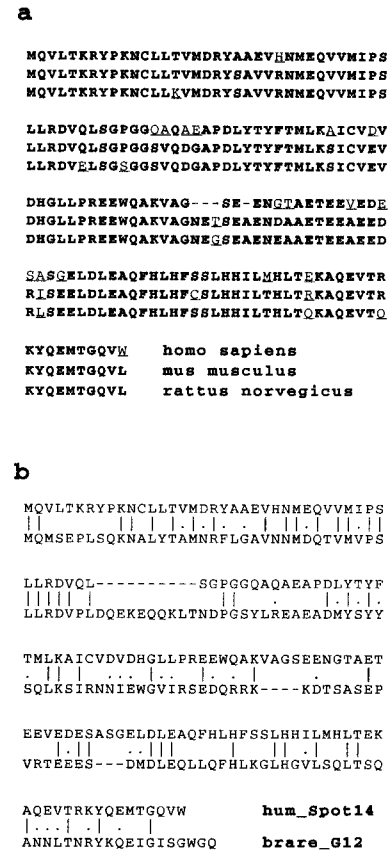

Fig. 1. (a) Alignment of human, mouse and rat Spot 14 amino acid sequences. The amino acids which are identical in human, mouse and rat Spot 14 are in bold. Non-conserved amino acids are underlined. (b) Amino acid sequence of human Spot 14 and comparison with zebrafish G12. Amino acids which are identical in both proteins are indicated by vertical lines. Conservative substitutions are dotted.

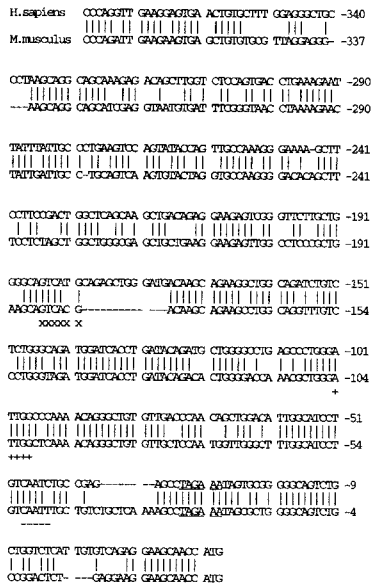

Fig. 2. Nucleotide sequence of human and mouse Spot 14 gene basal promoter regions. The TAGAA sequence is underlined. Relevant motifs CAAT box (-), MLTF (×), and NF1 (+) are shown. Conserved nucleotides are indicated by vertical lines.

hypothesized that a set of primers designed from these sequences could amplify the human homologue sequence from a supposed highly expressing tissue such as liver. This first RT-PCR allowed us to amplify a 300 bp fragment that was used to design two sets of nested primers and then to screen an uncloned human genomic DNA. The 800 bp upstream fragment and the 2.6 kb downstream fragment were subcloned and then sequenced. Obviously, the open reading frame deduced from these sequences was not interrupted by any intron. This allowed us to design another set of primers in order to amplify a 641 bp fragment which contained the supposed whole coding sequence. Three amplified products from genomic DNA of unrelated individuals produced the same sequence and confirmed that deduced from the sequencing of the library clones. The human 438 bp open reading frame was translated into a putative protein similar to those of mouse and rat (Fig. 1a). The rat Spot 14 amino acid sequence has recently been related to an acidic protein which is specifically expressed during zebrafish (*Brachydanio rerio*) development [25]. The amino acid sequence of this protein and its homology with the human Spot 14 sequence are shown in Fig. 1b.

Mouse and human promoters were strongly similar (Fig. 2). They also presented a strong homology with the formerly reported rat promoter sequence [1]. Interestingly, the TAGAA motif which is considered as the rat TATAA box element was found to be 100% similar in both mouse and human sequences. Analysis of the consensus signal sequences of promoters showed several potential regulatory elements. In Fig. 2, we marked some elements that can be considered as relevant: CTF/NF1 [26]; CCAAT box and MLTF [27]. It should be noted that the mouse −1370/−1401 sequence presents a strong homology (93%) with the rat −1457/−1448 fragment shown to contain the carbohydrate response element. Nevertheless, the mouse core motif itself differs from its rat counterpart by 1 nucleotide.

The expression of Spot 14 is well-documented in established cell lines such as 3T3-L1 [28,29] and 3T3-F442A [15,30]. We show here that Ob17 cells also express this gene and this expression is restrained to the fully differentiated cells. The level of mRNA increased when cells were incubated in the presence of 1 μM retinoic acid for 4 h or 100 nM dexamethasone for 72 h (Fig. 3a). Primer extension experiments are displayed in Fig. 3b. The extended strand from mRNA extracted from mouse adipocytes is of the same length as that obtained from rat liver mRNA and may be considered as 5′-complementary strands of mRNA whose transcription is under the control of the TAGAA motif. Furthermore, the mouse sequence, although it lacked a canonical TATAA box, produced an efficient promoter activity (Fig. 3c). This activity was stronger in mouse preadipocytes than in rat FAO cells or human HepG2 cells, when normalized with respect to pSV40 activities. Both pSAluc and pK1luc plasmids were equally efficient in Ob17 cells, but obviously weaker in human or rat cells. Furthermore, pK1luc turned out to be weaker than pSAluc in both hepatoma cell lines.

Human liver samples, HepG2 cell line and the Launois-Bensaude's disease nodule expressed the Spot 14 specific mRNA. When assayed using a quantitative RT-PCR method, the amount of human mRNA coding for Spot 14 with regard to cellular β-actin mRNA content, was markedly different from one sample to another (Fig. 4a). The amount of Spot14 mRNA was higher in the lipomatous tissue (20×10^{-3} mol of Spot 14 mRNA per mol of β-actin mRNA) than in control liver (14×10^{-3}), while it seemed weakly expressed in the steatosic liver (1.2×10^{-3}) and in HepG2 cell line (0.4×10^{-3}). In contrast, Huvec cell line and primary cultures of lipomatous undifferentiated adipocytes did not express Spot 14 mRNA (data not shown).

ISH experiments in liver sections confirmed these results and specified the cellular localization of Spot 14 mRNA (Fig. 4b). Blue-brown deposits within hepatocyte cytoplasm were present in control liver, while they were not detected in steatosic liver. Other liver cells remained unlabeled.

4. Discussion

Cloning of mouse and human genes allowed us to demonstrate that both of them share a similar organization, which is also similar to that of the rat, since the whole coding sequence is not interrupted by any intron. The three genes also display a high degree of conservation of their promoter sequences. Efficiency of the mouse promoter was tested in three different cell types: rat and human hepatoma cells (FAO and HepG2 cell lines, respectively) and Ob17 cells at the preadipocyte stage. When cloned in front of the coding sequence of luciferase, the mouse sequence was able to direct the expression of this gene in mouse preadipocytes as well as in rat and human hepatoma cells, although obviously this expression was weaker in these two last cases. Slight differences observed in sequences could explain the greater efficiency observed in mouse derived cells. It is also noteworthy that, if 3T3-L1 and 3T3-F442A cell lines are known to express actively the Spot 14 gene in culture, this expression is strictly restrained to cells fully differentiated into adipocytes [15,29]. Inasmuch as we revealed, in Ob17 preadipocytes, a marked luciferase activity when the expression of this gene is under the control of the proximal promoter sequence, it would be interesting to search for the existence of a silencing region – not present in our

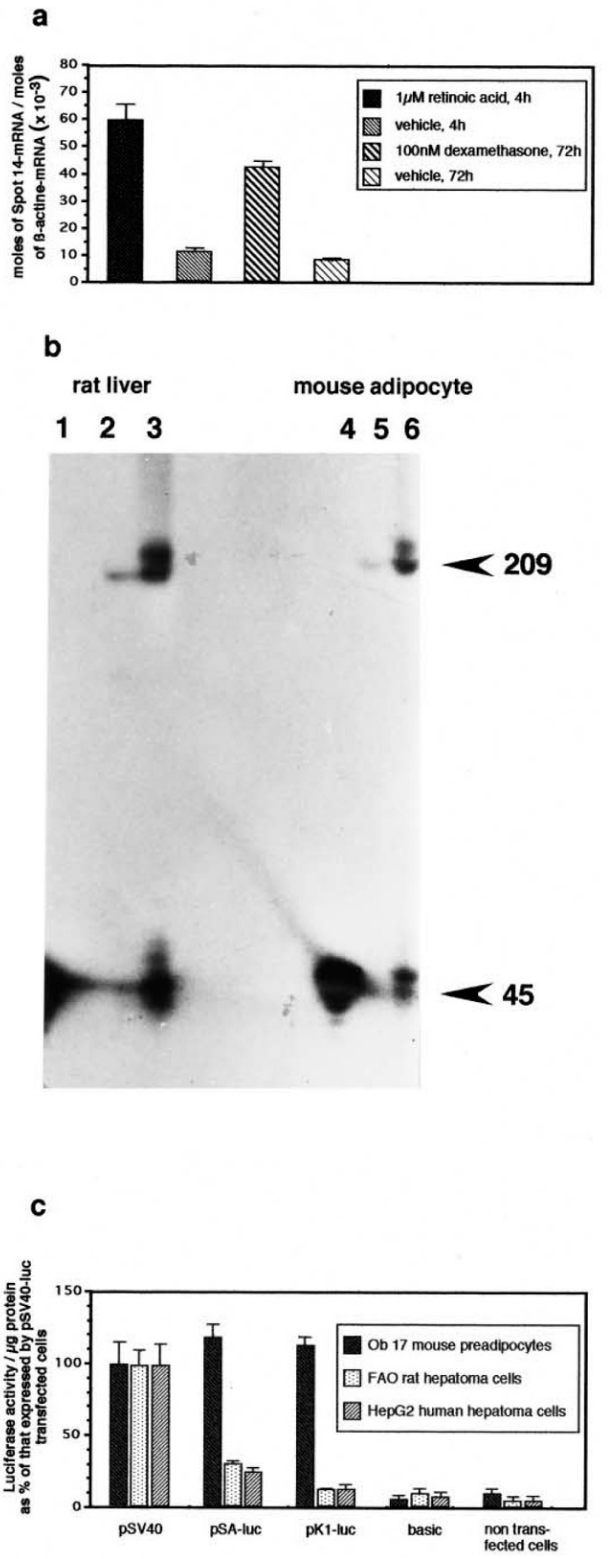

Fig. 3. (a) Expression of Spot 14 in Ob17 cells (fully differentiated adipocytes). Spot 14 mRNA levels were assayed by quantitative RT-PCR. Cells were incubated in the presence of agents or vehicles as indicated. Each data value is the mean of the values obtained from three independent experiments and bars indicate ±S.E.M. (b) Denaturing gel electrophoresis of 5′-extended strands using Spot 14 specific primer. Total RNA from rat liver or mouse Ob17 cells was hybridized against a radiolabeled 46-mer oligonucleotide at high (lanes 2,5) or low (lanes 3,6) stringency before reverse transcription. Lanes 1 and 4 refer to control run of labeled primer alone. Arrows indicate the size (in nt) of denatured strands. (c) Luciferase activity in various cell lines transfected with Spot 14 promoter-luciferase chimeric genes. 100% luciferase activity in cells transfected with pSV40-luciferase control plasmids corresponds to $16 \pm 1.5 \times 10^3$, $5.6 \pm 0.5 \times 10^3$ and $13 \pm 1.2 \times 10^3$ arbitrary units in Ob17, FAO and HepG2 hepatoma cells, respectively. The values shown are the mean of the values obtained from 3 independent transfections and bars indicate ±S.E.M.
←

constructions – able to repress the basal promoter at the preadipocyte stage.

Both rodent sequences are 150 amino acids long, while the human sequence is shorter by only 4 amino acids. The deduced amino acid sequences of mouse and rat proteins share a 94% homology. The human sequence presents 83 and 81% homology with regard to mouse and rat sequences, respectively. In a recent study, a 'gastrulation-specific' protein, named G12, cloned from Zebrafish, has been shown to share 33% homology with rat Spot 14 protein [25]. Conserved amino acids are regularly spaced along the primary structures and several clusters are mainly located at the N-terminal moiety of the proteins (see Fig. 1b). The highest degree of similarity (74%) is present in a region extending from amino acid 24 to 42 (Spot 14 sequence), which suggests that this region may fulfill a common function in these proteins. When matched to other sequences in the Genbank non- redundant database library, this 'spot-box' was present in some previously reported human sequences, *T68776, *T73661 and *R13768. The first two cloned from adult hepatic tissue library are obviously partial sequences of Spot 14. In contrast, *R13768, cloned from infant brain tissue library, encodes a putative peptide which is 100% similar to zebrafish G12 in its N-terminal part.

The Spot 14 amino acid sequence has never been related to any other known protein, but the recent cloning of the zebrafish G12-mRNA could help in elucidating its function. Spot 14 expression is known to be rapidly induced in response to agents such as thyroid hormone [31]. In addition, the protein has been shown to have a nuclear localization [17] and to be necessary in liver transcription process of proteins involved in lipogenesis [32]. Similarly, the G12 gene is expressed, for a short period, in the cells that will produce the enveloping layer.

We show here that mouse and human Spot 14 genes encode putative proteins whose p*I* values are 4.76 and 4.65, respectively. Rat protein presents a similar p*I* (4.62). It should be noted that most of the amino acid variations observed between rodent and human proteins, are located from amino acid 87 to 114 (rodent sequence) where, in the human sequence, a 4 amino acid gap as well as 8 substitutions can be found. Nevertheless, despite this relatively weak homology, this short region remains highly acidic (14 carboxyl-carrying residues/28 in rodents vs. 11/24 in human). Interestingly, G12 is also a small protein (152 amino acids), whose p*I* (4.94) is as low as that of Spot 14. In addition, G12 displays an acidic region similar to that of Spot 14 (from amino acid 95 to 114). Predictive analyses of the secondary structures [33] of these acidic regions reveal a highly probable amphipathic α-helix organization. Therefore, their low p*I* as well as the existence of this highly acidic and amphipathic region is certainly an important feature of Spot 14 and zebrafish G12. Indeed, this

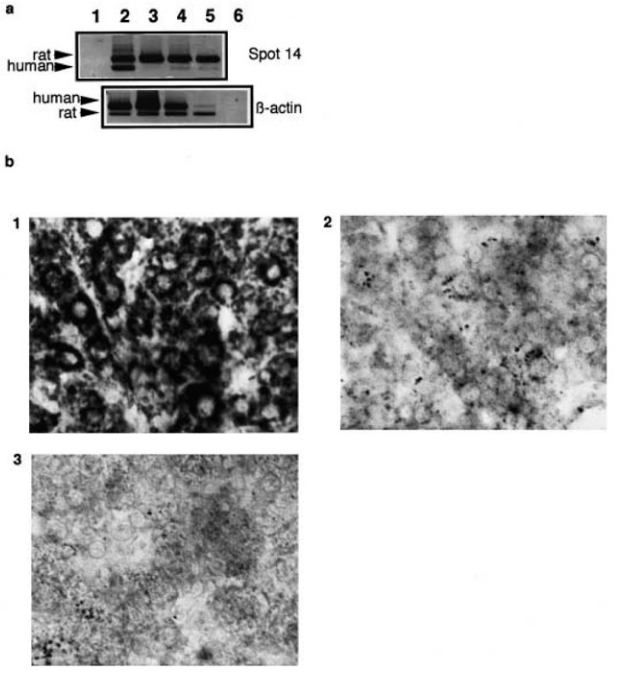

Fig. 4. (a) Quantification of Spot 14 mRNA with regard to β-actin content in human tissues. A mixture of total RNA from rat and human tissues was reverse transcribed and amplified separately for Spot 14 detection (upper gel) and for β-actin detection (lower gel). Multistandard was rat liver total RNA. Human total RNA was extracted from Launois-Bensaude nodule (lane 2), HepG2 cells (lane 3), steatosic human liver (lane 4) and human liver sample peripheral to hepatocarcinoma (lane 5). Lanes 1,6: control samples (without reverse transcribed RNA). When digested with *Pvu*II restriction enzyme, human Spot 14 amplification product produced a 245 bp fragment, while rat product remained uncut (292 bp). When digested by the same enzyme, human β-actin remained uncut (469 bp), while rat product displayed a 475 bp fragment. (b) In situ hybridization of human Spot 14 mRNA in liver tissues (magnification×320). (1) Hepatic tissue in the vicinity of hepatocarcinoma, hybridized with antisense probe. (2) Tissue section serial to previous one, hybridized with sense probe. (3) Steatosic tissue in the vicinity of hepatocarcinoma, hybridized with antisense probe.

brings to mind the secondary structure of some transcription factors such as GCN4 [34] or GAL4 [35]. Nevertheless, further studies will be necessary to specify the role that Spot 14 could play in a transcription process. Homology with G12 protein could be a helpful tool in this work.

Taking into account the regulation of Spot 14 expression in rodent liver and adipose tissues, expression of the human homologue gene was expected in lipogenic tissues. Indeed, we found that Spot 14 was expressed in hepatocytes, although at a lower level in hepatocytes from steatosic liver, in which lipid metabolism is deeply disturbed. Similarly, a high level of Spot 14 mRNA was demonstrated in nodules from a patient suffering from Launois-Bensaude's disease. This could be related to resistance to catecholamines described in this disease [36] and to the fact that cAMP is known to strongly decrease Spot 14 expression, at least in rodents tissues. Further investigations on lipid metabolism disorders in humans would be interesting to specify Spot 14 involvement in lipid metabolism.

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