Volume 282, number 2, 368–372 FEBS 09703 May 1991

ADONIS 001457939100410R

Differential regulation of the two mRNA species of the rodent negative acute phase protein α_1-inhibitor 3

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Received 14 February 1991; revised version received 15 March 1991

Screening of two rat liver cDNA libraries, one of which was constructed using an α_1-inhibitor 3 (α_1-I3) specific primer, yielded overlapping cDNA clones which correspond to the full length cDNA for α_1-I3 mRNA. On the basis of sequence microheterogeneity existing throughout the cDNA sequence we identified two α_1-I3 mRNA species whose sequences are so grossly different in their bait regions that the amino acid homology therein is only 30%. Using oligonucleotide probes derived from their respective bait regions we investigated the regulation of the two α_1I3 mRNA species and demonstrated that only one of them, α_1-I3 variant I, is regulated pretranslationally following experimentally induced inflammation.

Rodent proteinase-inhibitor; Signal peptide; α_1-Inhibitor3 mRNA; Differential regulation

1. INTRODUCTION

Proteinase inhibitors, responsible for keeping the activities of proteinases in check [1] are an essential component of the serum. The family of rat α-macroglobulins are proteinase inhibitors which respond positively, negatively or remain unaltered when the animal experiences trauma, e.g. inflammation [2–6]. An example of a positive acute phase response is the several hundred-fold rise in the serum concentration of α_2-macroglobulin [7–9]. On the other hand, the negative acute phase response in the rat is exemplified by the 70% reduction in the serum concentration of α_1-inhibitor 3 (α_1-I3) [4,5,10].

In spite of their different regulatory responses all members of the α-macroglobulin family are thought to inactivate proteinases by a similar, if not the same mechanism [11–14]. The point of attack on the proteinase inhibitor is the bait region where one finds the highest degree of sequence diversity between the α-macroglobulins [15]. The proteolytic attack of the scissile site(s) on this exposed region of the protein results in a conformational change of the protein molecule and causes the attacking proteinase to be sequestered by covalent binding to the thiol ester site of the proteinase inhibitor. The resulting proteinase/proteinase-inhibitor complex is removed from the serum by association with macrophages. This sequence of events has been shown for the human α_2-macroglubulin [11,12] but since all α-macroglobulins have a bait region and a thioester site other proteinase inhibitors may function according to the same principle.

To study the regulation of α_1-I3 at the molecular level we isolated corresponding cDNA clones for this proteinase inhibitor [6]. Sequencing revealed a significant number of nucleotide (nt) exchanges between the clones suggesting that there are at least two transcribed α_1I3 genes. Here we provide conclusive evidence for the existence of two α_1-I3 genes, only one of which encodes a negatively regulated acute phase protein.

2. MATERIALS AND METHODS

DNA and RNA preparations and manipulations including construction and screening of cDNA libraries were performed as described [6,16–18]. The cDNA sequence data generated were analyzed using the UWGCG sequence analysis software package, version 5 [19].

Inflammation was induced in 6-week-old Sprague-Dawley male rats (Ivanovas, Kissleg) by s.c. injection of 0.4 ml turpentine/100 g body weight. Throughout the experiment animals received water and normal lab chow Altromin Diet 1324 (4% crude fat by wt; Altrogge, Spezialfutterwerk, Lage) ad libitum. Livers from anaesthetized animals were placed immediately in liquid nitrogen and stored at −80°C.

For pBAIT I, two oligonucleotides corresponding to nt 2002–2034 (cf. Fig. 3, upper line) and its reverse complement were synthesized and so extended that upon annealing *SalI* and *PstI* restriction sites were created at their 5'- and 3'-ends respectively. This double stranded DNA fragment was ligated into the *PstI/SalI* restricted pSPT 19 vector (Boehringer, Mannheim). pBAIT II was constructed accordingly using the oligonucleotide corresponding to nt 2159–2179 of the

The complete cDNA sequence for rat α_1-I3 variant I has been assigned the accession number X52984 and the partial cDNA in pI3cDNA6 for α_1-I3 variant II the accession number M28297 in the EMBL Data Library.

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sequence in Fig. 3, lower line (5'-CCTTGGATTCTTCAACTGATA-3'). Successful cloning of the fragments was verified by sequencing.

The radioactive pBAIT I and pBAIT II riboprobes obtained from the *Bam*HI linearized plasmids according to standard procedures [20] were hybridized with nitrocellulose-bound total RNA isolated from the livers of control and turpentine-treated (19 h) rats. Hybridization was performed in 50% formamide, 6 × SSC, 5 × Denhardt, 0.1% SDS, 1 mg/ml herring sperm DNA for pBAIT I at 55°C and for pBAIT II at 42°C for 20 h. The filters were washed twice at room temperature, then twice at 57°C for pBAIT I and 47°C for pBAIT II in 2 × SSC, 0.1% SDS.

3. RESULTS AND DISCUSSION

3.1. *Cloning, sequencing and characterization of the cDNA for the two α_1I3 mRNA species*

We have previously described the isolation and characterization of cDNA clones representing 801 nucleotides before the poly (A) tail of the mRNA for the rodent negative acute phase protein α_1-I3 [6].

Based on their sequence microheterogeneity the twelve α_1-I3 cDNA clones described here can be divided into two groups (Fig. 1). The mRNA species from which the clones M7, M57, A22, B4, B12 and B21 have been derived has been named α_1-I3 variant I and the other which gave rise to pI3cDNA6 [6], M2, M34, M35, M22, B16 and B25 has been designated α_1-I3 variant II. The entire α_1-I3 variant I cDNA sequence is shown in Fig. 2. The majority of the nt exchanges throughout the total length of the cDNA are silent or cause the substitution of another aa with similar properties. However, the sequences of the two species differ pronouncedly about the middle of the reading frame. Comparing this region of α_1-I3 variants I and II as represented by A22 and B16 (cf. Fig. 1) revealed a 33 bp insertion (nt 2001–2034 in Fig. 3) followed 86 nt later by a deletion of three nt. Directly after this deletion the homology between A22 and B16 fell to approximately 60% over a length of 95 nt. In the following stretch the homology rose to the overall value of >96%. The sequence divergence in that part of α_1-I3 that we define as the bait region (nt 1996–2232, Fig. 3) together with the single nt exchanges throughout the cDNA support the hypothesis that there are two α_1-I3 mRNA species. The region of lowest homology corresponds to the two classes of peptides isolated and investigated by Sottrup-Jensen et al. [15] when analysing the complexes formed between α_1-I3 and various proteinases. The rodent proteinase inhibitors, α_1-I3, α_2-macroglobulin [9] and the human pregnancy zone protein (PZP) [21] are highly homologous at the aa level but peptide sequence analysis of the human PZP and the human α_2-macroglobulin [22,23] showed that the regions responsible for proteinase inactivation differ in length and aa sequence [15]. Our results suggests that a similar situation may exist for the two species of α_1-I3.

We find the N-terminus of mature α_1-I3 [3] is encoded by nt 124 to 169 of the cDNA sequence (Fig. 2). The

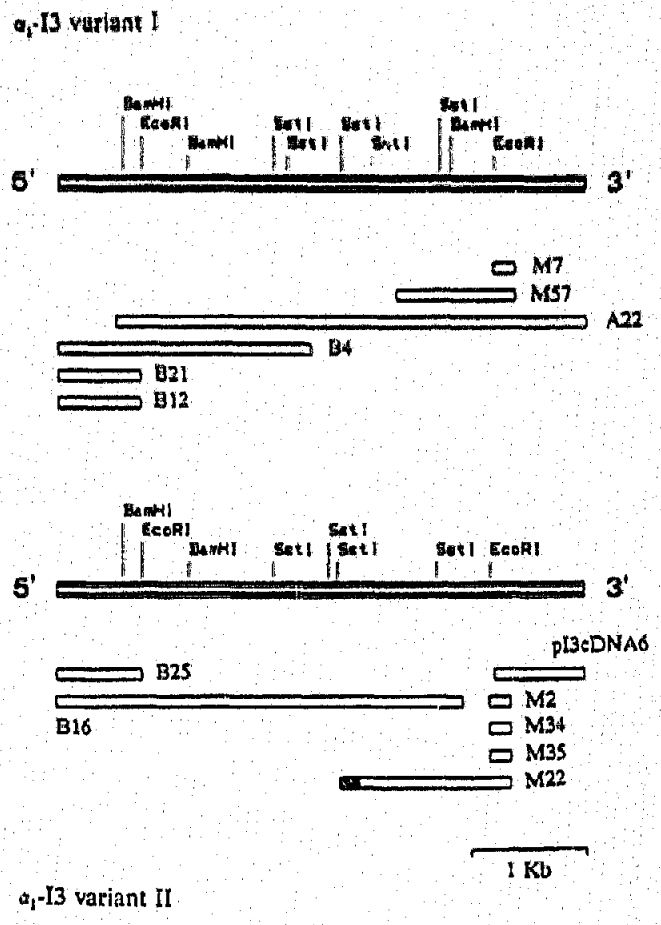

Fig. 1. Relative positions of the overlapping cDNA clones for α_1-I3 variants I and II. Continuous open bars represent the complete lengths and partial restriction maps of the cDNA for α_1-I3 variants I and II. Shown below each bar are the relative locations of their constituent cDNA clones. pI3cDNA6 has been described previously [6]. M7, M57, M2, M34, M35, M22 were obtained by screening the α_1-I3 specific library and sequenced over both strands in their entirety. The hatched region of M22 represents a cloning artefact. A22 was picked up by screening the commercially available library with the correct part of M22 and sequenced in both strands over its full length. The clones of the B-series were isolated by screening the same library with the 0.2 kb *Eco*RI fragment at the 5'-end of A22. Both strands of the *Eco*RI fragment at the 5'-end of B4 were sequenced, B12, B21 and B25 were sequenced over their lengths in one strand. Single strand sequences of B16 were obtained from the 5'-end to the second *Bam*HI site, its bait region and 400 bases at its 3'-end.

24 aa encoded by nt 52–123 in front of the N-terminus fit the overall pattern of a signal peptide in that the charged amino acids, e.g. arginine and lysine (positions -20, -22, -23 in Fig. 2), are followed by a number of hydrophobic residues. The residue at -3, alanine, is the most common at this position in various signal peptides [24]. The thiol ester binding site, diagnostic for α-macroglobulins, is conserved in both variants (aa 961–964 in Fig. 2). The receptor recognition sites for macrophages are located in the C-terminal region of the proteinase inhibitor [14,25] where the sequence

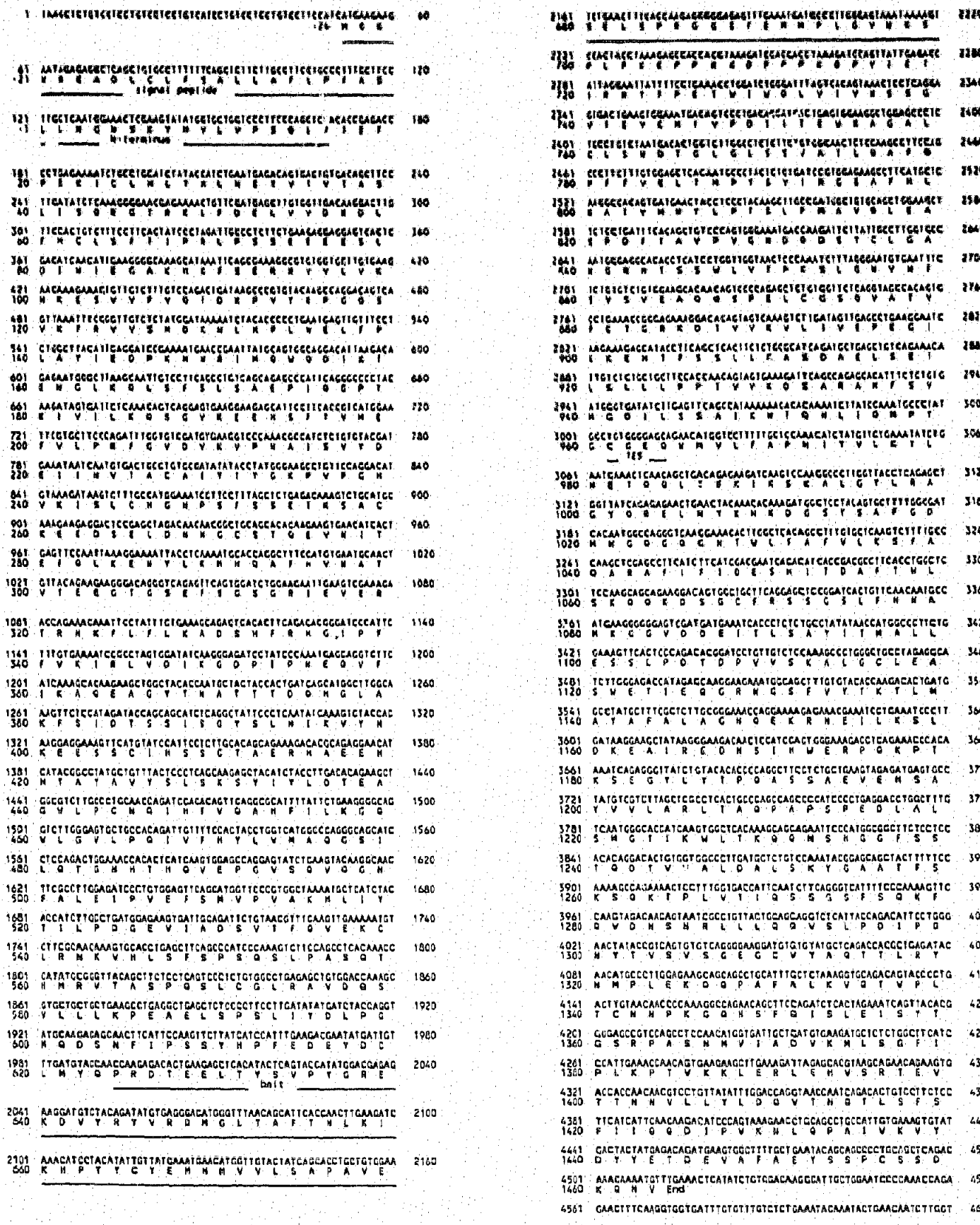

Fig. 2. The complete nucleotide sequence of the α_1-I3 variant I cDNA. The reading frame starts with ATG at nt 52–54 and ends with TGA at nt 4513–4515. The signal peptide, bait region and thiol ester binding site are labelled and underlined.

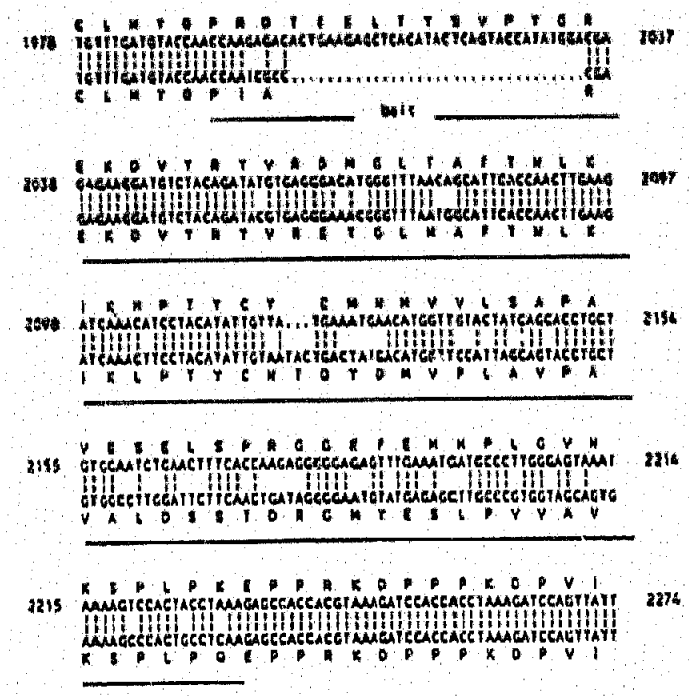

Fig. 3. Alignment of the bait regions from the α_1-I3 variants I and II. The portion of the nucleotide sequence of α_1-I3 cDNA variant I from nt 1978 to 2274 derived from sequencing of A22 (upper line) is compared with the corresponding nt sequence of α_1-I3 variant II obtained by sequencing the relevant part of B16 (lower line; cf. Fig. 1). The 33 bp deleted in α_1-I3 cDNA variant II and the three nt deleted in α_1-I3 cDNA variant I are indicated by dots.

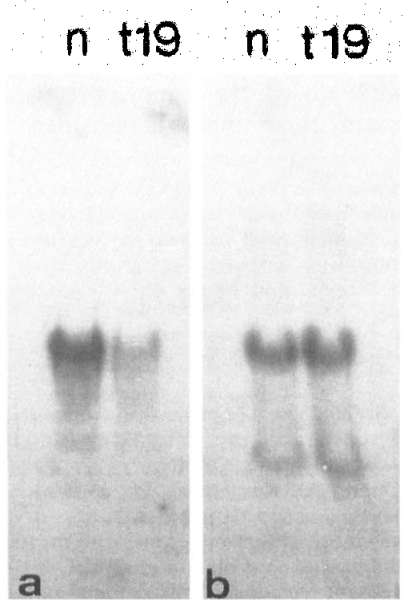

Fig. 4. Northern blot analysis of α_1-I3 mRNA. 20 μg of total RNA per lane prepared from the livers of control (n) and turpentine-treated (t19) rats were electrophoresed in 0.8% agarose/formaldehyde and transferred to nitrocellulose. One half of the filter (a) was hybridized with pBAIT I and the other (b) with pBAIT II.

homology found in the two α_1-I3 species also extends to α_2-macroglobulins of human [22,23] and rat [6,9]. It could be shown that the receptors are responsible for removing proteinase complexed with either α_1-I3 [5] or α_2-macroglobulin [13] from the serum.

Excluding the signal peptide α_1-I3 variant I has a calculated molecular mass of 162 631. The discrepancy between this and the published value of approximately 200 000 as determined by protein-chemical methods [4,5,10] may be accounted for by *N*-glycosylation at the 13 possible sites we found in the deduced amino acid sequence of α_1-I3. Taking into consideration an oligosaccharide content of 15% [10] we calculated a molecular mass of 191 331 for the mature α_1-I3 variant I.

3.2. *Regulation of the α_1-I3 variants I and II mRNA*

To determine whether or not the reduction in the amount of α_1-I3 mRNA which we documented previously [6] is due to a switching-off of one or other or both α_1-I3 mRNA species specific riboprobes were hybridized to filter-bound total RNA from the livers of normal and turpentine-treated rats. As seen in Fig. 4a the hybridization signal with pBAIT I in the t19 mRNA preparation is only minimal. However, hybridizing with pBAIT II we could detect no change in the amount of mRNA as a result of turpentine injection (Fig. 4b). Therefore, we postulate that of the two α_1-I3 mRNA species only variant I is regulated at the pretranslational level. Although we could find two transcription starts in normal mRNA corresponding to both α_1-I3 variants, the signal for variant I disappeared and that for variant II became blurred with RNA from the livers of turpentine-treated rats. It could be that the mRNA for α_1-I3 is more fragile following induction of the acute phase response. This may explain why only a full length cDNA for α_1-I3 variant II – the mRNA species least affected by the acute phase response – was found by screening a rat liver acute phase cDNA library with a hybridization probe for α_2-macroglobulin [26]. The other cDNA clones described seem to be scrambled up versions of α_1-I3 variant I since they all contain the sequence of variant I up to the bait region and one even contains the insertion of 33 nt characteristic of it. According to that part of the bait region which it contained the α_1-I3 clone isolated by Aiello et al. [27] is a variant I type. We feel confident in postulating that there are only two equally abundant α_1-I3 transcripts since we isolated 6 clones of variant I and 7 of variant II. Nevertheless, the possibility of other transcribed variants should not be dismissed since we have isolated 4 different classes of genomic clones for α_1-I3.

Somewhat surprisingly, the spectra of proteinases inactivated by variants I and II are not as different as one would expect from the differences in the bait regions [15]. The fact that variant II does not form a complex with thrombin correlates well with the regulatory properties of the variants I and II. Thrombin involved in

the blood clotting cascade [28] can be inactivated only by variant I [15]. The down-regulation of α_1-I3 variant I under conditions of trauma would ensure that the serum content of thrombin is maintained.

Acknowledgements: We thank Prof. E. Schweizer for laboratory facilities, G. Müller and J. Beck for oligonucleotide synthesis and K.-F. Beck and B. Rautenstrauß for helping with computer searches. This investigation was supported by grants from the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie to M.S.

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