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Human inter-α-inhibitor family in inflammation: simultaneous synthesis of positive and negative acute-phase proteins

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The inter-α-inhibitor (IαI) family encompasses four plasma proteins, namely free bikunin as well as IαI, pre-α-inhibitor (PαI) and inter-α-like inhibitor (IαLI). Each of the last three proteins is a distinct assembly of one bikunin chain with one or more unique heavy (H) chains designated H1, H2 and H3. The three H chains and the bikunin chain are encoded by four distinct mRNAs. These molecules and chains, as well as the corresponding mRNAs, were quantified in sera and liver biopsies from a series of patients with or without mild or severe acute infection. The decrease or increase observed for a given molecule or chain in the serum was in agreement with a similar change in the corresponding liver mRNA. In acute inflammation the H2 and bikunin chains are down-regulated and the relevant molecules (IαI, IαLI) behave as negative acute-phase proteins, whereas the H3 chain is up-regulated and the corresponding PαI molecule is a positive acute-phase protein. Also, PαI displays a higher-than-usual M_r; this is probably due to ligand binding. The H1 gene does not seem to be affected by the inflammatory condition. The quantitative changes in RNA levels seen *in vivo* were confirmed *in vitro* in the human hepatoma Hep3B cell line prior to or after induction with the acute-phase mediators interleukin-1 and/or -6. These results provide the first example in humans of positive and negative acute-phase proteins that are encoded by evolutionary related genes.

INTRODUCTION

The inter-α-inhibitor (IαI) family encompasses a set of four plasma proteinase inhibitors designated IαI, pre-α-inhibitor (PαI), inter-α-like inhibitor (IαLI) and bikunin. These molecules are synthesized in the liver and result from the assembly of four gene products, namely three evolutionary related heavy (H) chains designated H1, H2 and H3, and one chain designated bikunin after its tandem Kunitz-type proteinase inhibitory domains (reviewed in [1,2]). The protein IαI is made of three chains (H1 + H2 + bikunin), whereas IαLI is composed of two chains (H2 + bikunin) and PαI is also composed of two chains (H3 + bikunin); free bikunin molecules are also present in plasma [2]. The mature bikunin chain results from the processing of an α_1-microglobulin (A1M)/bikunin precursor polypeptide (AMBP) that splits into two components, i.e. bikunin and A1M [3]. A1M is present in plasma in two forms: (i) in a free state, and (ii) covalently bound to some IgA H chains (reviewed in [4]). A1M has never been found in mature IαI or related molecules and seems to be completely unrelated to proteinase inhibitors [5,6]. The covalent links between H chains and bikunin in IαI family members do not involve disulphide bonds, which is quite unusual for soluble blood proteins. Instead, the links are made of glycosaminoglycan (GAG) structures. The latter can be digested *in vitro* by hyaluronidase or chondroitinase, thereby allowing analyses of chain stoichiometry and assembly [7–9].

The functions of IαI family members remain largely unknown, and may or may not be related to their serine proteinase inhibitory activity [1,2,10,11]. The search for a physiological function is further complicated by the absence of any known IαI deficiency syndrome. Mammalian liver responds to acute systemic injury by dramatic increases or decreases in the synthesis of various plasma proteins, the so-called positive or negative acute-phase proteins (APPs). Accordingly, many laboratories have investigated the behaviour of IαI family members in inflammatory conditions in man as a step towards understanding the physiological and pathological importance of these molecules. Unfortunately, such studies have all been more or less hampered by the long-ignored existence of plasma PαI, the limited availability of antisera which could separately recognize H chains or bikunin chains, and the lack of cDNA probes needed to analyse all four chains mRNAs. Therefore, in the past, several laboratories have reported an inflammation-associated increase in free bikunin along with an unmodified level of IαI in plasma [10,12–15]. These results suggested that the bikunin-encoding AMBP gene is up-regulated in inflammation and synthesizes a positive APP, whereas the H chain genes remain unaffected [2,10].

By quantitative analyses of the molecules and chains of the IαI family in sera from patients suffering from acute inflammation, and performing quantifications of mRNA in liver biopsies from some of these patients, as well as mRNA studies in human hepatoma cultures, we now present a completely new view of the expression of IαI family genes in acute inflammatory states: the AMBP and H2 genes are down-regulated and the H3 gene is up-regulated, whereas the H1 gene activity remains unaffected.

MATERIALS AND METHODS

Patients and biological samples

Sera from 18 individuals, including patients presenting with an inflammatory condition resulting from an acute bacterial infection as well as control patients, were available for this study.

Abbreviations used: A1M, α_1-microglobulin; A1AGP, α_1-acid glycoprotein (also designated orosomucoid); A2HS, α_2-HS glycoprotein; AMBP, α_1-microglobulin/bikunin precursor; APP, acute-phase protein; CRP, C-reactive protein; GAR–HRP, horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins; H chain, heavy chain; IαI, inter-α-inhibitor; IαLI, inter-α-like inhibitor; PαI, pre-α-inhibitor; TBS, Tris-buffered saline; IL, interleukin; GAG, glycosaminoglycan.

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Table 1 Biological data for subjects classified according to their degree of inflammation

Subjects were classified according to CRP and A1AGP levels in the serum. The upper normal limit for CRP and the normal range for A1AGP and A2HS are given in parentheses.

Inflammation...	None	Mild	Severe
Age (years)	53.3 ± 23.0	50.7 ± 18.6	60.3 ± 22.1
n	6*	6†	6‡
male/female	3/3	3/3	3/3
CRP (μg/ml) (< 10)	4.0 ± 2.9	37.7 ± 24.2	187 ± 57
A1AGP (mg/ml) (0.55–1.40)	0.82 ± 0.20	1.58 ± 0.12	2.36 ± 0.76
A2HS (mg/ml) (0.33–0.68)	0.45 ± 0.07	0.39 ± 0.15	0.24 ± 0.06

* Includes patients 1–3, with liver biopsy.
† Includes patients 4 and 5, with liver biopsy.
‡ Includes patients 6–9, with liver biopsy.

Patients with chronic inflammation, cirrhosis or cancer were excluded. Among these 18 individuals, nine patients were included on the basis that part of a liver biopsy was available along with the serum sample. Liver tissue and serum were taken simultaneously. The liver tissues were immediately frozen in liquid nitrogen and stored at −80 °C until used. The sera from all patients were stored at −80 °C. The level of inflammation was assessed from the serum concentrations of two positive APPs [namely α_1-acid glycoprotein (A1AGP) and C-reactive protein (CRP)] determined by nephelometry, and one negative APP, [namely α_2-HS glycoprotein (A2HS)] determined by electroimmunoprecipitation. The patients were accordingly classified into three groups with no, mild or severe inflammation. Biological data and APP levels for each group are detailed in Table 1.

Materials

Chondroitinase ABC from *Proteus vulgaris* (EC 4.2.2.4) was from Sigma. Recombinant human interleukin-1β (IL-1) and recombinant human interleukin-6 (IL-6) were obtained from Ciba-Geigy and Genzyme respectively. Nitrocellulose BA85 was from Schleicher and Schuell.

cDNA probes

A full-length human AMBP (i.e. A1M and bikunin) cDNA probe and three partial H1, H2 and H3 cDNA probes were as described elsewhere [5,16]. Human A1AGP and A2HS cDNA probes were as previously described [17].

Antisera

Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins (GAR–HRP) were from Bio-Rad. Rabbit anti-(human A1M) antiserum was from Dako. A series of polyclonal antibodies directed against one or more of the IαI-family-related chains(s) were obtained in rabbits and rendered specific as described elsewhere [12,18]. They included the following: (i) anti-bikunin antibodies, (ii) anti-H2 antibodies, (iii) anti-H3 antibodies, (iv) anti-H1,2 antibodies, and (v) anti-(bikunin + H1,2,3) antibodies. The latter two antibodies recognize the H1 and H2 chains, and bikunin plus the H1, H2 and H3 chains respectively.

Enzymic deglycosylation

Chondroitinase ABC was diluted in a sodium acetate/NaCl buffer exactly as described in [7], with 5 mM of each of the proteinase inhibitors benzamidine hydrochloride, pepstatin A and leupeptin, and was reacted with human serum exactly as described in [7]: final enzyme concentration, 0.66 units/ml; final serum dilution, 1:2; 37 °C; 4 h. We verified that the pattern of IαI-related fragments resulting from overnight chondroitinase digestion did not differ from the one seen after a 4 h digest.

SDS/PAGE and Western immunoblotting

Polypeptides in sera were separated by SDS/12.5% PAGE in reducing conditions according to [20]. The M_r standards (from Bethesda Research Laboratories) were lysozyme (M_r 14300), β-lactoglobulin (M_r 18400), carbonic anhydrase (M_r 29000), ovalbumin (M_r 43000), BSA (M_r 68000), phosphorylase *b* (M_r 97400) and myosin H chain (M_r 200000). The separated polypeptides were electrophoretically transferred on to a nitrocellulose membrane. The free binding sites on nitrocellulose were blocked with 3% gelatin in 15 mM Tris/HCl buffer, pH 7.5, containing 500 mM NaCl (TBS) by gently agitating the solution on a shaker platform for 1 h at room temperature. The sheet was next incubated in the first antibody solution (antiserum usually diluted 1:500 in 1% gelatin in TBS) for 2–14 h and washed in 0.1% Tween 20 in TBS (four washes for 5–10 min each). The membrane was next incubated for 1 h in GAR–HRP diluted 1:1000 in 1% gelatin in TBS, and washed in Tween 20 in TBS as above followed by a final wash in TBS alone. The HRP colour reaction was carried out in a fresh solution of 30 mg of 4-chloro-1-naphthol (Bio-Rad) in 10 ml of methanol mixed with 30 μl of hydrogen peroxide in 50 ml of TBS, and stopped by rinsing in water. In some instances, after this staining step the blots were immediately photographed and kept wet until a further round of incubations with a first antibody followed by GAR–HRP was carried out.

The serum concentrations for a given IαI-family-related chain or molecule were quantified on the Western blots as follows. Various amounts (0.2, 0.5, 1 or 2 μl) of a serum used as an internal arbitrary standard were loaded on to the blots along with a fixed amount of each pathological serum under study. This fixed amount depended on the antibodies used for detection: 0.3 μl of serum with anti-A1M or anti-bikunin antisera; 0.5 μl with the anti-(bikunin + H1,2,3) antiserum; and 1 μl with all other antisera. After the immunoblotting steps, the band(s) detected were scanned with a Gelman DCD-16 gel scanner. Finally, the concentration for a given chain or molecule in each serum was expressed in arbitrary units/μl.

RNA purification and analysis by Northern blotting

Total RNAs from human liver were prepared by the guanidinium isothiocyanate procedure followed by centrifugation on to a CsCl cushion as described in [21]. Total RNAs from hepatoma cell cultures were obtained by a guanidinium thiocyanate/phenol/chloroform one-step protocol [22]. RNA integrity was controlled by visualization of the 18 S and 28 S rRNA bands after ethidium bromide staining in agarose gels. RNA concentrations were determined from the absorbance at 260 nm.

Samples of total RNAs were denatured for 15 min at 65 °C in 1 × Mops buffer (10 × Mops buffer is NaMops 200 mM, sodium

acetate 50 mM, Na_2EDTA 10 mM), pH 7.0, 2.2 M formaldehyde and 50% deionized formamide, then loaded on to a 0.8% agarose gel in 1 × Mops buffer/2.2 M formaldehyde, and separated by electrophoresis at 25–50 V for 14–20 h. Capillary blotting of RNAs on to Hybond N^+ nylon membranes (Amersham) was for 3 h in 50 mM NaOH as recommended by Amersham. The membrane was then neutralized briefly in 2 × SSPE (20 × SSPE is 3 M NaCl, 200 mM NaH_2PO_4, 20 mM Na_2EDTA, pH 7.4) and air-dried. The cDNA probes were labelled by the random oligo procedure at a specific radioactivity of (0.5–2) × 10^9 c.p.m./μg. Pre-hybridization (3–4 h) and hybridization (16–20 h) were carried out in 5 × SSPE, 50% formamide, 1% SDS, 5 × Denhart's solution (50 × Denhart's is 1% polyvinylpyrrolidone, 1% Ficoll, 1% BSA), 5% dextran sulphate and 100 μg/ml heat-denatured sonicated herring sperm DNA, at 42 °C in a hybridization oven with rotating glass tubes. Washings were at 65–68 °C in 2 × SSPE/0.5% SDS for 1 h and 1 × SSPE/0.5% SDS for 1 h. Exposure to Hyperfilm-MP (Amersham) was at −80 °C with intensifying screens for 0.5–10 days. In many instances, successive hybridizations were carried out by dehybridization of a nylon filter in boiling 0.1% SDS (4 × 5 min) and re-hybridization with another probe. When all required hybridizations were completed, the homogeneity in RNA loading and blotting between lanes was monitored by Coomassie Blue staining of 18 S and 28 S rRNAs on the nylon filter according to [23].

The relative amounts of a specific RNA were quantitatively compared between total RNA samples as follows. Various dilutions of an RNA preparation used as an internal standard were loaded on the agarose gel. These dilutions were blotted and hybridized along with a fixed amount of the total RNA under study (human liver RNAs, 10 μg; Hep3B RNAs, 20 μg) on to the same nylon filter. Quantification of autoradiographic bands was obtained by densitometric analysis on to a Shimadzu CS-930 gel scanner. Finally, the amount of a given mRNA within the fixed amount of electrophoresed total RNA in each sample was expressed in arbitrary units/μg of total RNA.

Hepatoma cell cultures

The human hepatoma cell line Hep3B has been extensively characterized elsewhere [24]. It was cultured as monolayers in 25 cm^2 flasks (Falcon) at 37 °C in a 5% CO_2 atmosphere and fed with 3 ml of RPMI 1640 medium (Boehringer), 10% heat-inactivated fetal calf serum (Boehringer), supplemented with Hepes buffer 10 mM, pH 7.0, 10 mM D(+)-glucose, 2 mM L-glutamine (Gibco-BRL), 250 units/ml penicillin, 250 units/ml streptomycin and 100 munits/ml insulin (Rapitard from MC-Novo, France). Fresh culture medium was added once a day until cell confluency. The cells were then trypsinized with a 0.5 mg/ml trypsin (Gibco-BRL) solution in 0.2 mg/ml Na_2EDTA and transferred into new flasks at about 30% initial confluency. After three or four such steps, the cells were grown with or without dexamethasone (1 μM) until they reached about 80% confluency and they were then stimulated for 24 h with IL-1 and/or IL-6 as detailed in the Results section. The supernatants were then recovered for control of IL induction (see the Results section) and the cells were extensively washed in 150 mM NaCl, 20 mM Na_2/NaH_2PO_4 buffer, pH 7.2, and finally lysed for RNA isolation as described above.

Statistical analyses

Comparisons of mean concentrations between groups were carried out by the non-parametric Mann–Whitney's U test. Comparisons for the presence or absence of inflammation-associated molecules in groups (see the Results section) were carried out by a Chi-square test or by Fisher's exact test for small numbers.

RESULTS

Biological data and groups of patients

In this study, nine patients with paired serum samples and liver biopsies were available. In the text, Table 1 and Figure 1, these patients are identified as numbers 1–9. Nine other patients whose serum only was available were also included in this study. Three sex- and age-matched groups were eventually obtained, which were based on the level of inflammation (none, mild or severe inflammation). The latter was assessed from the serum concentrations of known positive (A1AGP and CRP) and negative (A2HS) APPs, as indicated in Table 1. Our population of patients with an inflammatory condition had acute bacterial infections associated with septic haematoma, acute pancreatitis, peritonitis or pyocholecystitis.

Patterns of IαI family proteins on SDS/PAGE, and inflammation-associated changes

Inflammation-associated changes in the molecules and chains within the IαI family were investigated by SDS/PAGE and Western immunoblotting of sera from patients with various levels of inflammation. However, and given the current lack of a detailed SDS/PAGE pattern for the IαI family in serum as revealed with several molecule- or chain-specific antibodies, we had to first characterize such a reference pattern. Typical results obtained with serum from patients 1, 2, 4, 8 and 9 are presented in Figure 1. For each serum, the native molecules as well as the chains thereof released by chondroitinase treatment of serum were examined separately (panels Nat. and Ch. respectively). For the sake of clarity, all bands seen in one or more panel(s) are arbitrarily designated with a letter (A–L).

Figure 1(a) provides a general view of all IαI-related proteins that can be detected with our anti-(bikunin + H1,2,3) antibodies. In the native sera (panel Nat.), three major bands of M_r 240000 (A), 155000 (B) and 140000 (C) can be detected. In sera from the patients with severe inflammation (lanes 8 and 9) the 155000-M_r band (B) disappears, whereas a barely visible, fuzzy band of approx. M_r 165000 (D) appears. In the sera treated with chondroitinase (panel Ch.), five bands with M_r values of 170000 (E), 101000 (F), 88000 (G), 79000 (H) and 26000 (I) are seen.

The bands in panels Nat. were further identified as follows. Band A of M_r 240000 reacts with the anti-bikunin (Figure 1b), anti-H1,2 (Figure 1d) and anti-H2 (Figure 1e) antibodies. Therefore this band corresponds to IαI, comprising H1 + H2 + bikunin [8]. Bands B and D react with the anti-bikunin (Figure 1b) and anti-H3 (Figure 1f) antibodies. Therefore, given its M_r of 155000, band B is PαI, comprising H3 + bikunin [8], whereas band D, of M_r 165000, represents PαI bound to a ligand (see the Discussion section). Finally, band C reacts with the anti-bikunin (Figure 1b), anti-H1,2 (Figure 1d) and anti-H2 (Figure 1e) antibodies. Given its M_r of 140000, this band C is identified as IαLI, comprising H2 + bikunin [8].

The bands in panels Ch., released by chondroitinase treatment of the above-mentioned molecules, were further identified as follows. Band I of M_r 26000 reacts with the anti-bikunin antibodies only (Figure 1b) and therefore is bikunin. Band F of M_r 101000 reacts with the anti-H3 antibodies exclusively, and therefore is the H3 chain [8]. Both bands G and H are recognized by the anti-H1,2 antibodies, but only band G reacts with the

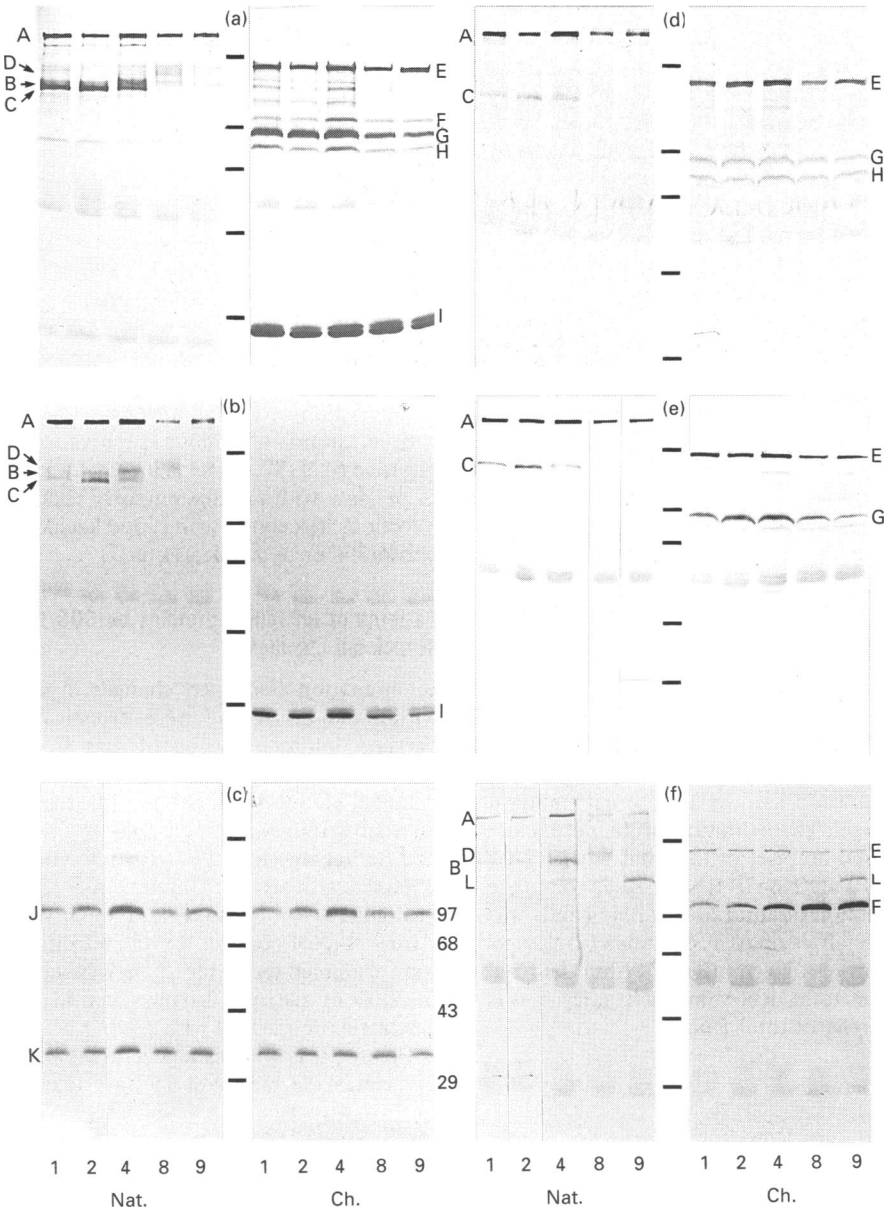

Figure 1 Immunoblotting analyses of IαI-related molecules or chains in sera from patients with a mild or severe inflammatory condition, and from controls

Sera are from patients nos. 1, 2, 4, 8 and 9, without an inflammatory state (lanes 1 and 2), or with acute, mild (lane 4) or acute, severe (lanes 8, 9) inflammation according to Table 1. SDS/PAGE in reducing conditions was performed with 0.5 μl (**a**) or 1 μl (**b–f**) of serum/lane. Note that the amount (1 μl) of serum loaded on the blots in (**b**) and (**c**) was not the amount eventually used for an optimized detection of quantitative changes (see the Materials and methods section). The native sera (panels Nat.) or sera digested with chondroitinase ABC (panels Ch.) were simultaneously run and transferred on to the same blot. After electrotransfer, the resulting blots (**a**)–(**f**) were treated for immunodetection with: (**a**) anti-(bikunin + H1,2,3) antibodies; (**b**) anti-bikunin antibodies; (**c**) anti-A1M antibodies; (**d**) anti-H1,2 antibodies; (**e**) anti-H2 antibodies; (**f**) anti-H3 antibodies. On each blot, the standard size markers are marked between panels Nat. and Ch. and the corresponding M_r values (200000, 97400, 68000, 43000, 29000) are indicated between panels (**c**) and (**f**). The various IαI-family-related bands detected on the blots are identified by an arbitrary one-letter code (A–L). Although bands B, C and D (M_r range 140000–165000) on the one hand, and bands F, G and H (M_r range 79000–101000) on the other, display sizes which are close to each other, unambiguous identification of each of the bands shown on the Figure was made by a second round of blot incubation with further antibodies (not shown; see the Materials and methods section).

anti-H2 antibodies. Therefore these bands G and H correspond to H2 and H1 chains respectively, which is in agreement with their relative M_rs of 88000 (G) and 79000 (H) [8]. Band E, of M_r 170000, reacts with the anti-H1,2 (Figure 1d) and anti-H2 (Figure 1e) antibodies. Given its M_r of 170000, we conclude that band E is made of residual assembled H1 and H2 chains. The occurrence of this band has been previously described and is accounted for by a chondroitinase-resistant GAG bond between some H1 and H2 chains [7,8].

Numerous inflammation-associated changes in the presence, mobility or concentration of IαI-related chains and molecules were observed. The major changes included a shift in electrophoretic mobility of PαI, the occurrence of a further H3-like chain, and increased or decreased contents of several

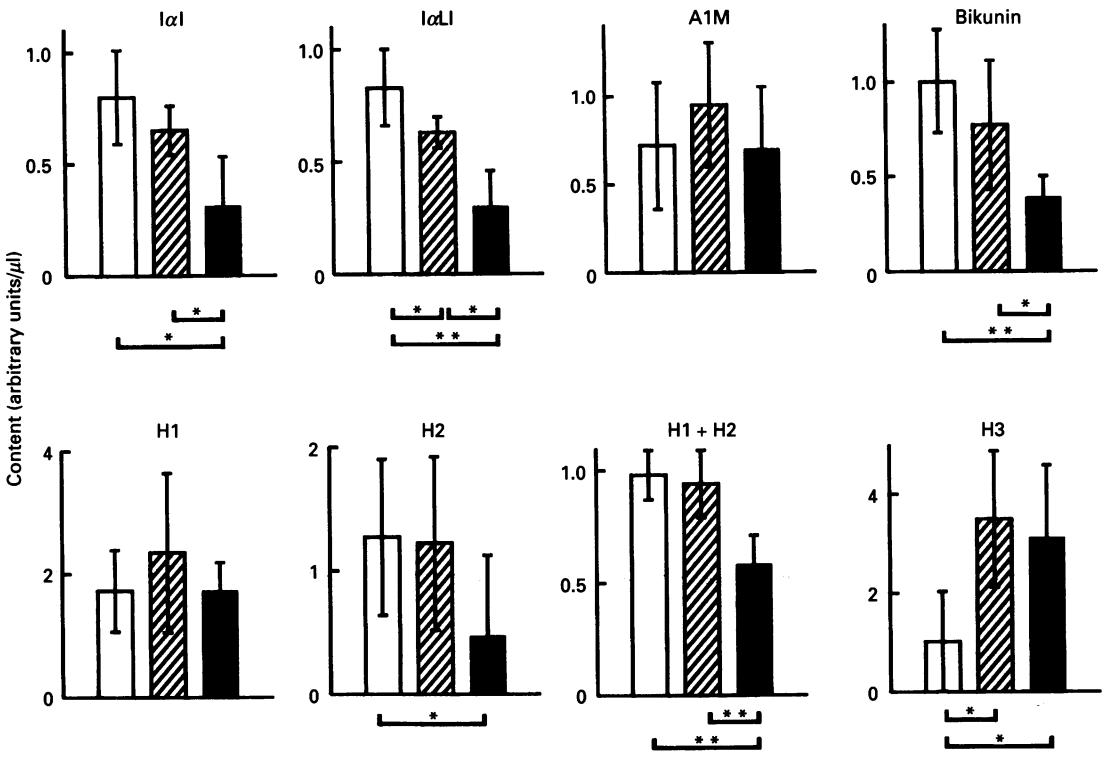

Figure 2 Inflammation-associated changes in the amounts of Iα-family-related molecules or chains in human serum

Sera from all patients listed in Table 1 were used. □, Control group; ▨, mild inflammation group; ■, severe inflammation group. Within each group, histograms are means ± S.D. ($n = 6$). *$P < 0.05$; **$P < 0.01$ (Mann-Whitney's non parametric test). H1, H2 and H3 are heavy chains 1, 2 and 3 respectively; H1 + H2 indicates residual H1 + H2 complex bound by a chondroitinase-resistant GAG bond.

molecules. These changes are illustrated in Figure 1 as follows. First, in sera from patients with inflammation, PαI (band B) is replaced by a heavier, as yet undescribed, PαI-like molecule (D) of M_r 165000 (Figures 1a and 1b, panels Nat., patients 8 and 9). Secondly, band L (Figure 1f), seen in both panels Nat. and Ch. (lanes 4–9), is a heavy H3-related chain since it reacts with the anti-H3 antibodies exclusively; this band L, found in sera from patients with inflammation (lanes 4–9), is as yet undescribed. Thirdly, quantitative changes seen in sera from patients with inflammation versus controls included: (i) a decrease in the content of IαI (A), IαLI (C), bikunin (I) (Figure 1b), H2 chain (G in Figure 1e) and residual H1 + H2 chains (E in Figure 1e), and (ii) an increase in the content of H3 chains (F in Figure 1f). All of these qualitative and quantitative changes were further analysed in the whole population of patients (see below).

Finally, Figure 1(c) depicts the A1M-related chains as revealed with anti-A1M antibodies. Regardless of whether the sera are native or chondroitinase-treated, two bands (J and K) are visible. In agreement with the fact that A1M is not part of any IαI family member [5,6], the sizes of these bands (K, M_r 31000; J, M_r 95000) are quite different from those of IαI, PαI and IαLI. These A1M bands K and J represent respectively free A1M and an A1M–IgA heavy chain complex associated by a reduction-resistant bond [25]. The serum content of either A1M form was not correlated with the inflammation level (see below).

Quantitative study of the inflammation-associated changes in Iαl family members in sera

Because the amounts of the PαI-like molecule of M_r 165000 (band D) were often below the linear range of our quantitative immunoblotting assay, the presence or absence of this molecule in sera was determined in an all-or-none fashion. Over the entire population of 18 patients (Table 1), the presence of this molecule in serum was associated with the inflammatory state ($\chi^2 = 10.5$; degrees of freedom = 2; $P = 0.005$) and was mostly found in the group with a severe inflammation (difference between patients without inflammation and with severe inflammation $P = 0.02$, Fisher's exact test).

For each patient within the three groups detailed in Table 1, the serum contents of the following molecules or chains was measured: IαI (A), IαLI (C), H1 (H), H2 (G), H1 + H2 (E), H3 (F), bikunin (I) and free A1M (K). The results are presented in Figure 2, where all of the statistically significant fluctuations between groups are in agreement with the observations presented in Figure 1. The concentrations of IαI and IαLI, as well as of H2 chains and residual H1 + H2 chains, were significantly decreased in the patients with acute inflammation. In the whole population of patients tested, each of these molecules or chains exhibited a serum concentration which correlated with the level of A2HS, a negative APP (IαI, $r = 0.58$, $P < 0.02$; IαLI, $r = 0.61$, $P < 0.01$; H2, $r = 0.51$, $P < 0.05$; H1 + H2, $r = 0.71$, $P < 0.01$). The serum content of H3 chain was significantly increased in both groups with inflammation versus controls (Figure 2), and in the whole population of patients tested, the serum contents of H3 and A1AGP (the latter being a positive APP) were correlated ($r = 0.68$, $P < 0.01$). The H1 chain content did not exhibit any difference between the control group and patients with inflammation, nor was it correlated with levels of A1AGP or A2HS. Finally, the serum content of bikunin decreased in severe inflammation, whereas that of A1M was not modified and did not correlate with levels of either bikunin or A2HS (Figure 2).

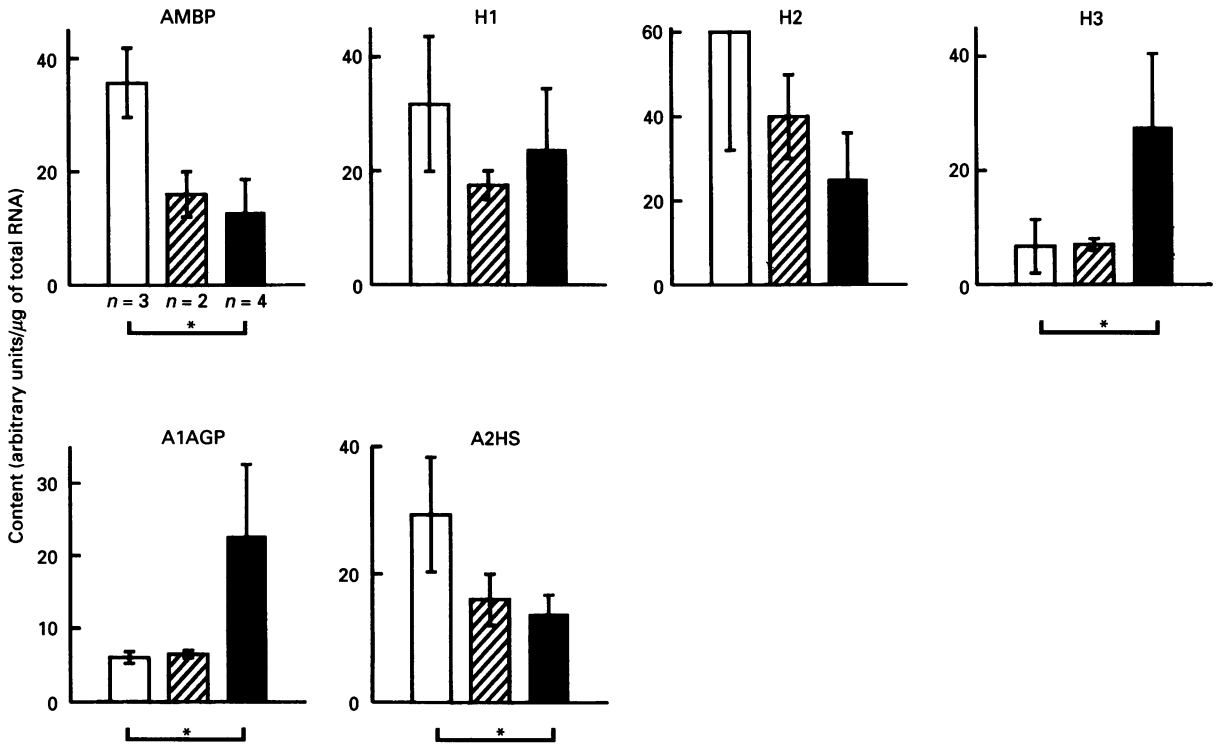

Figure 3 Inflammation-associated changes in the quantitative expression of IαI-related RNAs and other acute-phase RNAs in human liver

A fragment of liver biopsy from patients 1–9 (classified in Table 1) was used for RNA preparation. □, Control group; ▨, mild inflammation group; ■, severe inflammation group. Within each group, histograms are means ± S.D. *$P < 0.05$ (Mann-Whitney's non parametric test).

Expression of IαI family RNAs in livers from patients with an inflammatory condition versus controls

Each of the four IαI-family-related liver mRNAs, i.e. AMBP, H1, H2 and H3, was quantitatively compared between the patients 1–9 (included in Table 1) by Northern blot hybridization. Given the limited number of patients, the variation in average RNA concentration between groups was sometimes at borderline significance (e.g. H2 mRNA; see below). The H1 RNA level was not modified in the patients with inflammation (Figure 3). An inflammation-associated decrease in H2 and AMBP RNA levels was observed (Figure 3). A concomitant increase in H3 RNA level was also seen; this was restricted to the patients with severe inflammation (Figure 3).

We quantified the hybridization of total RNA from the same patients with A1AGP or A2HS cDNA probes, corresponding to well-known positive and negative APP genes respectively [26], and found a clear-cut inflammation-associated increase and decrease in the A1AGP and A2HS RNAs respectively (Figure 3), in a close agreement with our classification of the patients' level of inflammation in Table 1. Finally, within the whole population of nine patients, the levels of H2 and AMBP RNAs were correlated with the A2HS mRNA level ($r = 0.92$, $P < 0.01$; and $r = 0.85$, $P < 0.01$ respectively), whereas the H3 mRNA level was correlated with the A1AGP mRNA level ($r = 0.96$, $P < 0.01$).

IL-regulated expression of IαI family mRNAs in human hepatoma cell lines

In an attempt to clarify whether the above results obtained *in vivo* could correspond to an effect of some common inflammation-associated ILs, the human hepatoma cell line Hep3B was cultured in the presence or absence of dexamethasone and stimulated with IL-1 or IL-6, or a combination of the two (Figure 4). Dexamethasone alone did not affect the levels of any of the mRNAs. In the presence of dexamethasone, IL-6 alone down-regulated the levels of H2 and AMBP mRNAs (1.9- and 2.8-fold differences compared respectively with controls) and up-regulated the level of H3 mRNA, which was undetectable in the control cultures (Figure 4). These effects were enhanced when the culture was co-stimulated with both IL-1 and IL-6. In particular, the levels of the H2 and AMBP mRNAs were further decreased upon IL-1 + IL-6 induction, and exhibited 4.2- and 3.1-fold differences respectively compared with controls (Figure 4). H1 mRNA was not detectable in our Hep3B cultures even after extended autoradiographic exposure (results not shown). Probing the Hep3B Northern blots with A1AGP and A2HS cDNAs showed an IL-6- and IL-1 + IL-6-associated increase or decrease respectively in the corresponding mRNA (Figure 4), thereby confirming the efficiency and validity of the hepatoma inductions with IL-1 and/or IL-6. Furthermore, the effectiveness of IL-1 and/or IL-6 in inducing some well-known APP genes was further demonstrated by rocket electroimmunoassay of A1AGP released into the culture supernatants (results not shown). Finally, similar results were obtained with HepG2 hepatoma cells in terms of IL-1- and/or IL-6-associated expression of IαI family genes (results not shown).

DISCUSSION

In this study, inflammation-associated changes in levels of IαI-family-related mRNAs, proteins and chains were sought from

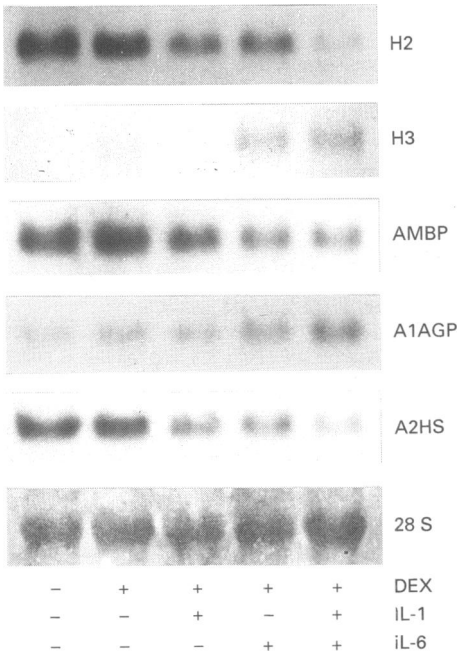

Figure 4 IL-regulated expression of Iαl family genes and other acute-phase genes in the Hep3B hepatoma cell line

Northern analysis of total RNAs (20 μg/lane) from Hep3B cells cultured in the presence (+) or absence (−) of 1 μM dexamethasone (DEX) and stimulated (+) or not (−) with IL-1 (100 units/ml) and/or IL-6 (100 units/ml). Hybridizations with various human cDNA probes are shown in separate panels. The panel 28 S is a control: the stained 28 S rRNAs indicate homogeneity in total RNA loading and blotting from lane to lane. The published sizes for human AMBP, H1-H2-H3, A1AGP and A2HS mRNAs are 1.3 kb, 3.0–3.2 kb, 0.85 kb and 1.5 kb respectively [2,3,36,37]. The H1 mRNA was not detected.

Northern blots of liver RNA and SDS/PAGE/immunoblotting patterns of serum proteins. Given the current lack of a detailed SDS/PAGE pattern for the IαI family, we have also established such a reference pattern as revealed with several molecule- or chain-specific antibodies. With our reagents, all immunochemically detected patterns are in a perfect agreement with what is currently known for the arrangement of chains within IαI family molecules [1,2,8]. In the IαI family, most of the chains are present on several molecules, and consequently an

Table 2 Summary of the major inflammation-associated changes in hepatic levels of the Iαl-related mRNAs and in serum levels of the corresponding molecules and chains

mRNA (liver)	Chain (serum)	Molecule (serum)
AMBP	A1M	
	Bikunin	IαI i.e. bikunin + H1 + H2
H1	H1	IαLI i.e. bikunin + H2
H2	H2	PαI i.e. bikunin + H3
H3	H3	

e.l.i.s.a. or rocket electroimmunoassay used for quantitative purposes would detect and quantify more than one molecule or chain. Therefore in this study we preferred the more reliable quantification of given bands that were first separated on blots. This complicated procedure forced us to limit the number of patients tested.

In acute inflammation the levels of H2 and AMBP mRNAs decreased. These findings were well correlated with a decrease in IαI and IαLI levels in native sera and with a decrease in the levels of bikunin and H2 chain in chondroitinase-treated samples (Figure 2). Concomitantly, the level of H3-encoding mRNA increased, which was correlated with a marked increase in the level of the H3 chain in chondroitinase-treated samples. The unmodified level of the H1 chain in inflammation is in agreement with the lack of change in the level of the corresponding mRNA. Overall, our results demonstrate that, in acute inflammation, H3 chain synthesis is up-regulated and is the basis for a positive APP, namely PαI, whereas bikunin and H2 chain syntheses are down-regulated and account for negative APPs, namely IαI and IαLI. A summary of the quantitative inflammation-associated changes seen in this study is given in Table 2. The presence of any IαI-family-related molecule in the serum depends upon the availability of bikunin chains, and a change in the amounts of bikunin could in itself modulate the level of each member of the IαI family. In practice, the amounts of IαI, PαI and IαLI in blood are quite different, with the latter two probably being present at concentrations that are at least one order of magnitude below that of IαI [2,8]. Therefore in inflammation it seems unlikely that the down-regulation of bikunin would severely affect the H3-chain-regulated increase in PαI that is concomitantly observed.

In the present study the serum A1M content did not correlate with acute inflammation; this is in agreement with previous reports [4,27], but in contrast with our observation of a decreased bikunin level in serum and a down-regulated AMBP mRNA in the same patients. Given this divergence of A1M and bikunin levels, the contents of both proteins were determined in sera from further patients with an acute bacterial inflammation (results not shown). This fully confirmed the lack of correlation between serum levels of A1M and bikunin in acute inflammation. Indeed, a comparison at a given time of the serum content of two mature proteins, i.e. A1M and bikunin, originating from a shared precursor does not take into account the possibly different half-lives of these mature proteins. Accordingly, a short half-life for bikunin along with a longer half-life for A1M would be consistent with our observations in acute inflammation, where the amounts of serum A1M persisting from the pre-inflammatory period would mask recent events, otherwise indicated by the decreased levels of serum bikunin and AMBP mRNA. In fact, it has been reported that in inflammatory states a decrease in the serum A1M level occurred later than that for other APPs such as albumin and fibrinogen [27].

In Western blot experiments, two heavier-than-normal H3-related bands, i.e. a heavy PαI-like molecule (band D) and a heavy H3-like chain (band L), were observed, mostly in sera from patients with inflammation (Figure 1 and Table 2). The PαI-like molecule (D) contains a bikunin chain and is cleaved by chondroitinase, which suggests that it may represent a complex of PαI with an as-yet unidentified ligand, for instance a proteinase. In this case, the bond(s) between PαI and its target would be resistant to the reducing conditions used in SDS/PAGE. The heavy H3-like chain (L) does not contain any bikunin chain, as indicated by (i) the lack of reactivity with an anti-bikunin antiserum (Figure 1b), and (ii) its size, which remains unmodified by chondroitinase treatment (Figure 1f). Its precise nature is still

unknown. However the three mature H chains in the IαI family originate from three large precursor polypeptides which are trimmed at their N- and C-termini [15,28]. Therefore, given the M_r of 107000 published for the unglycosylated H3 precursor [19], we speculate that this molecule of M_r 130000 (L) could represent a free glycosylated, H3 precursor which would reflect the high level of mRNA produced from the corresponding gene in inflammation. This hypothesis is currently under study.

Our quantitative results indicate similar inflammation-associated changes in the levels of IαI family protein molecules in sera and their corresponding liver mRNAs. To further confirm the effect of inflammation on mRNA levels, *in vitro* studies were carried out; these demonstrated that an IL-6 induction of cultured, human Hep3B hepatoma cells can mimic all of the quantitative mRNA changes seen in our patients. This is in agreement with the current concept that IL-6 is the major mediator of APP synthesis in mammalian hepatocytes [30,31]. Furthermore, in our hepatoma cultures IL-1 alone was a moderate inducer of the H2 and AMBP genes which were down-regulated; hence they probably belong to the so-called 'class I genes', a subset of IL-1-responsive acute-response genes [30,32], although an effect of IL-1 upon IL-6 synthesis may also account for this observation [33]. Although the presence of a physiological amount of dexamethasone in our hepatoma cultures did not in itself affect the level of any mRNA, this glucocorticoid was used because of a permissive effect on the induction of at least some acute phase genes [31,34,35]. However, a detailed account of the glucocorticoid-responsiveness of each IαI family gene, as well as an extensive study with other acute-phase response inducers such as tumour necrosis factor or transforming growth factor-β, were beyond the scope of this work. Whether the observed variations in mRNA levels reflect changes in gene transcription and/or mRNA stability also warrants further studies.

Although several laboratories are investigating in detail the biochemical structures, molecular genetics and *in vitro* functions in IαI family members, no significant correlation of IαI, IαLI or PαI abnormalities with any pathological condition have been observed so far, and the *in vivo* functions of these molecules still remain to be elucidated. Therefore the present report of a previously undescribed up- or down-regulated synthesis of the H or AMBP polypeptides that eventually give rise to these molecules is a first step towards understanding the physiological significance of the IαI family. Furthermore, the present observation of a simultaneous synthesis of positive and negative APPs encoded by evolutionarily related genes, namely the H1, H2 and H3 genes, is, to our knowledge, unprecedented in humans.

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REFERENCES

1 Gebhard, W., Hochstrasser, K., Fritz, H., Enghild, J. J., Pizzo, S. V. and Salvesen, G. (1990) Biol. Chem. Hoppe-Seyler **371** (Suppl.), 13–22
2 Salier, J. P. (1990) Trends Biochem. Sci. **15**, 435–439
3 Kaumeyer, J. F., Polazzi, J. O. and Kotick, M. P. (1986) Nucleic Acids Res. **14**, 7839–7850
4 Akerstrom, B. and Logdberg, L. (1990) Trends Biochem. Sci. **15**, 240–243
5 Salier, J. P., Diarra-Mehrpour, M., Sesboue, R., Bourguignon, J., Benarous, R., Ohkubo, I., Kurachi, S., Kurachi, K. and Martin, J. P. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 8272–8276
6 Schreitmuller, T., Hochstrasser, K., Reisinger, P. W. M., Wachter, E. and Gebhard, W. (1987) Biol. Chem. Hoppe-Seyler **368**, 963–970
7 Jessen, T. E., Faarvang, K. L. and Ploug, M. (1988) FEBS Lett. **230**, 195–200
8 Enghild, J. J., Thogersen, I. B., Pizzo, S. V. and Salvesen, G. (1989) J. Biol. Chem. **264**, 15975–15981
9 Enghild, J. J., Salvesen, G., Hefta, S. A., Thogersen, I. B., Rutherford, S. and Pizzo, S. V. (1991) J. Biol. Chem. **266**, 747–751
10 Gebhard, W. and Hochstrasser, K. (1986) in Proteinase Inhibitors (Barrett, A. J. and Salvesen G., eds.), pp. 389–401, Elsevier, Amsterdam
11 Chen, L., Mao, S. J. T. and Larsen, W. J. (1992) J. Biol. Chem. **267**, 12380–12386
12 Salier, J. P., Sesboue, R., Vercaigne, D., Bourguignon, J. and Martin, J. P. (1983) Anal. Biochem. **133**, 336–343
13 Sesboue, R., Basuyau, J. P. and Salier, J. P. (1983) Clin. Chim. Acta **135**, 23–33
14 Odum, L., Hansen-Nord, G. and Byrjalsen, I. (1987) Clin. Chim. Acta **162**, 189–198
15 Gebhard, W., Schreitmuller, T., Hochstrasser, K. and Wachter, E. (1989) Eur. J. Biochem. **181**, 571–576
16 Salier, J. P., Verga, V., Doly, J., Diarra-Mehrpour, M. and Erickson, R. P. (1992) Mamm. Genome **2**, 233–239
17 Daveau, M., Davrinche, C., Djelassi, N., Lemetayer, J., Julen, N., Hiron, M., Arnaud, P. and Lebreton, J. P. (1990) FEBS Lett. **273**, 79–81
18 Rouet, P., Daveau, M. and Salier J. P. (1992) Biol. Chem. Hoppe–Seyler **373**, 1019–1024
19 Diarra-Mehrpour, M., Bourguignon, J., Sesboue, R., Mattei, M. G., Passage, E., Salier, J. P. and Martin, J. P. (1989) Eur. J. Biochem. **179**, 147–154
20 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
21 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor
22 Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. **162**, 156–159
23 Wilkinson, M., Doskow, J. and Lindsey, S. (1991) Nucleic Acids Res. **19**, 679
24 Knowles, B. B., Howe, C. C. and Aden, D. P. (1980) Science **209**, 497–499
25 Grubb, A. O., Lopez, C., Tejler, L. and Mendez, E. (1983) J. Biol. Chem. **258**, 14698–14707
26 Daveau, M., Davrinche, C., Julen, N., Hiron, M., Arnaud, P. and Lebreton, J. P. (1988) FEBS Lett. **241**, 191–194
27 Itoh, Y., Enomoto, H., Takagi, K., Kawai, T. and Yamanaka, T. (1983) Digestion **27**, 75–80
28 Gebhard, W., Schreitmuller, T., Hoschstrasser, K. and Wachter, E. (1988) FEBS Lett. **229**, 63–67
29 Odum, L. (1990) Biol. Chem. Hoppe-Seyler **371**, 1153–1158
30 Castell, J. V., Gomez-Lechon, M. G., David, M., Andus, T., Geiger, T., Trullenque, R. Fabra, R. and Heinrich, P. C. (1989) FEBS Lett. **242**, 237–239
31 Castell, J. V., Gomez-Lechon, M. J., David, M., Fabra, R., Trullenque, R. and Heinrich, P. C. (1990) Hepatology **12**, 1179–1186
32 Mackiewicz, A., Speroff, T., Ganapathi, M. K. and Kushner, I. (1991) J. Immunol. **146**, 3032–3037
33 Heinrich, P. C., Castell, J. V. and Andus, T. (1990). Biochem. J. **265**, 621–636
34 Baumann, H., Richards, C. and Gauldie, J. (1987) J. Immunol. **139**, 4122–4128
35 Ganapathi, M. K., Rzewnicki, D., Samols, D., Jiang, S. L. and Kushner, I. (1991) J. Immunol. **147**, 1261–1265
36 Dente, L., Ciliberto, G. and Cortese, R. (1985) Nucleic Acids Res. **13**, 3941–3952
37 Lee, C. C., Bowman, B. H. and Yang, F. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 4403–4407