A specific distal promoter controls γ -glutamyl transpeptidase gene expression in undifferentiated rat transformed liver cells

Seiji NOMURA, Olivier LAHUNA, Takanobu SUZUKI, Arthur BROUILLET, Marie Nole CHOBERT and Yannick LAPERCHE*¹

Institut National de la Sant et de la Recherche Mdicale, Unit 99, Hpital Henri Mondor 94010, Crteil, France

In rat undifferentiated hepatoma cells, the γ -glutamyl transpeptidase (GGT) gene is transcribed into a 2.3 and a 2.6 kb mRNA which, in contrast with other rat GGT transcripts, are not detected in more differentiated liver cells or adult tissues. Analysis of the cDNA sequences obtained from H5 hepatoma cells reveals that these two transcripts differ from other GGT mRNAs by a 312-nt unique untranslated leader sequence; this sequence maps on the gene in a single exon 10 kb upstream from the GGT promoter IV transcription start site. We established that the 2.6 kb mRNA V-1 and the 2.3 kb GGT mRNA V-2 derive, by

alternate splicing, from a primary transcript initiated on a distal promoter on the rat GGT gene. This gene appears to be transcribed from five promoters, and the specific expression of this new distal promoter in undifferentiated hepatoma cells requires binding of activator protein-1 and hepatic nuclear factor 3 specific transcription factors to a composite *cis*-element in the proximal region of the promoter. The distal GGT promoter, specifically expressed in undifferentiated liver cells, might reflect the expression of that gene in liver precursor cells before they differentiate in the hepatocytic or biliary lineage.

INTRODUCTION

Tumour markers are valuable for permitting the early detection of liver transformed cells and to follow the histogenesis of hepatocarcinoma. Among these markers, γ -glutamyl transpeptidase (GGT) has been the most widely used to monitor liver-cell transformation in both *in vivo* and *in vitro* models [1,2]. In normal liver, GGT activity decreases sharply at birth, reaching an extremely low level in hepatocytes [3]. Its expression reappears in primary tumours as well as in early preneoplastic liver lesions induced by a number of diverse carcinogens [4]. *In vitro*, an increase in GGT activity or mRNA is associated with transformation of rat liver epithelial cells induced by oncogene expression or by repeated passages at confluency [2,5]. In the rat, the single-copy GGT gene is transcribed from at least four promoters (P1–P4) into multiple mRNAs, which are translated into the same GGT protein precursor [6–11]. The differential expression of these GGT transcripts [8–12] in liver transformed cells offers a suitable model to monitor the liver cell lineage in hepatocarcinogenesis. In addition, the study of the promoters expressed in transformed cells is also an interesting tool with which to investigate the molecular mechanisms that underly the phenotypic changes occurring at the onset of the transformation process.

We report here the characterization of two GGT mRNAs expressed exclusively in undifferentiated hepatoma cells. We established that these two GGT transcripts are initiated on a fifth distal promoter on the rat GGT gene. This promoter, extinguished in normal rat tissues, is very active in H5 undifferentiated hepatoma cells and is positively controlled by activator protein-1 (AP-1) and hepatic nuclear factor (HNF3).

EXPERIMENTAL

Materials

The rat liver genomic library constructed in λ phage charon 35A

was kindly provided by Dr. C. B. Kasper (MacArdle Laboratory, Madison, WI, U.S.A.). Rat tissues were obtained from female Wistar rats. The hepatoma cell lines H5, Fao, C2 and C2rev7 were derived from the clonal H4II EC3 (H4II) line established from the Reuber H-35 hepatoma [13], and HTC cells were derived from the chemically induced Morris hepatoma 7288 [14]. Cells were maintained in the modified Ham's F12 medium (Fd; Gibco, Cambridge, U.K.) containing 5% (v/v) fetal-calf serum, 200 units/ml penicillin, 50 μ g/ml streptomycin and 50 μ g/ml fungizone.

Methods

cDNA cloning

The procedure used was based on the rapid amplification of cDNA 5' ends ('5'-RACE') technique [15] with modification as previously described [9]. Briefly, RNA samples from H5 hepatoma cells were purified on a oligo-d(T) column and used as a template to synthesize the first cDNA strand. An oligo-d(T) sequence was added on the 3' end of the first strand, and the second cDNA strand was initiated from an anchor oligomer complementary to the poly-d(T) sequence and flanked on its 5' end by an *Eco*RI site (5'-CCAACAGAAATTC(A)₁₈-3'). The sequence between the anchor oligomer and the GGT specific oligomer complementary to residues –125 to –142 (oligomer 1) in the 5'-untranslated sequence, which is common to all the GGT mRNAs [16], was synthesized by PCR. The cDNA 3' ends were blunted by the *Escherichia coli* polymerase I fragment (Klenow fragment) and the 5' ends were phosphorylated by T4 polynucleotide kinase (Stratagene). The cDNA fragments were ligated into the *Sma*I site of pGEM-3Zf(+) which had been dephosphorylated by 10 units of calf intestine phosphatase for 1 h at 37 °C in 50 mM Tris/HCl (pH 9)/10 mM MgCl₂. The recombinant plasmids were introduced in the *E. coli* HB 101 strain and clones were screened for GGT recombinant plasmids using the

Abbreviations used: GGT, γ -glutamyl transpeptidase; HNF3, hepatic nuclear factor 3; AP-1, activator protein 1; AP-2, activator protein-2; CAT, chloramphenicol acetyltransferase; TTR, transthyretin; HNF-1, hepatic nuclear factor-1; poly(A)⁺, polyadenylated; TAT, tyrosine aminotransferase.

¹ To whom correspondence should be sent.

The genomic sequence reported in Figure 3 has been submitted to the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number AF000600.

³²P-labelled oligomer containing the residues –125 to –142 in the 5' untranslated GGT mRNA sequence. The recombinant cDNAs were sequenced using the Sequenase kit from United States Biochemical Corporation (Cleveland, OH, U.S.A.).

Northern- and Southern-blot analysis

Northern-blot analysis of polyadenylated [poly(A)⁺] RNA samples and Southern-blot analysis of phage and genomic DNA were performed as previously described [17]. Northern blots were hybridized to the RNA probe (cRNA 139A) complementary to the GGT mRNA sequence from –72 upstream the AUG to the nt +1557 in the coding region [12], a sequence common to all GGT mRNA species. A second hybridization was performed using the complementary RNA sequence (cRNA-V), which covers the 312 nt specific to GGT cDNA cloned from H5 cells. This probe was transcribed from the plasmid pGEM-3-V-2 that contains the cDNA V-2 cloned into pGEM-3Zf(+). This plasmid was linearized by *Xba*I and transcribed from the T7 promoter in conditions described elsewhere [12]. A third hybridization employed the 238 bp cDNA probe (cDNA-4-2) previously described [10]; it contains the sequence from –389 to –153 in the cDNA V-1 and covers also the second exon of the GGT mRNA IV. The Southern blot was hybridized to the cDNA V-2 labelled using the Megaprime labelling kit from Amersham.

Screening of rat genomic library and analysis of the rat genomic DNA

The GGT V-2 specific sequence was labelled by random priming and used as a probe to screen approximately three haploid genome equivalents (10⁶ plaques) of a rat genomic library as previously described [9]. The 6.3 kbp *Eco*RI fragment from the positive recombinant phage 8-2, which hybridized to the cDNA V-2 specific sequence, was subcloned into the corresponding site of pGEM-3Zf(+) to give the plasmid pGEM-G-V. This plasmid was used as a template for the sequencing reactions and S1-nuclease mapping analysis.

S1-nuclease mapping analysis

Single-strand cDNA probes that encompass the putative position for the mRNA V start site, were prepared from the plasmid pGEM-G-V. The oligomer (sn-9), complementary to the cDNA V-1 coding strand (–639 to –668) was labelled at its 5' end by the T4 DNA polymerase and [γ -³²P]ATP (3000 Ci/mmol) and annealed to the plasmid in 100 μ l of 10 mM Tris/HCl (pH 8)/10 mM MgCl₂ at 40 °C for 30 min [18]. The primer was then extended in the presence of DNA polymerase I (Klenow fragment) and 400 μ M of the four deoxyribonucleotides at 37 °C for 30 min. The synthesized DNA, digested with *Kpn*I or *Ava*II, yields fragments of 193 bp and 470 bp respectively. The labelled strands were purified on an alkaline-agarose gel. Aliquots of these probes (4 $\times$ 10⁵ c.p.m.) were hybridized to 5 μ g of poly(A)⁺ RNA at 42 °C overnight and digested with S1 nuclease as previously described [7]. The protected DNA fragments were analysed on a polyacrylamide denaturing gel along with a related sequence ladder.

Primer-extension analysis

The synthetic primer sn-9 complementary to the sequence from –639 to –668 in the cDNA V-1, was 5'-end-labelled by T4 polynucleotide kinase and [γ -³²P]ATP. An aliquot (5 $\times$ 10⁵ c.p.m.) was hybridized to 5 μ g of H5 poly(A)⁺ RNA in 30 μ l of 0.4 M NaCl/10 mM Pipes (pH 6.5)/1 mM EDTA and 80 % formamide at 42 °C overnight. After ethanol precipitation, the oligonucleo-

tide was extended as previously described [17]. The extended fragments were analysed on polyacrylamide denaturing gel along with a related sequence ladder.

Construction of luciferase plasmids

Genomic fragments from nt –3200, –207, –157, –106 and –64 upstream of the predominant transcription start site (+1) for the GGT mRNA V up to nt +19 were obtained by DNA amplification between two oligonucleotides derived from the genomic sequence. These oligonucleotides had been mutated on the underlined nucleotides to create a *Bgl*II site. The downstream complementary oligomer (sn 11: 5'-GCTAGATCTTGTCTTTGTGCTACTG-3') was designed from the sequence +2 to +26. The upstream oligomers were derived from the sequences 5'-CAAGGGTCTATGGAAGTCTGAAAC-3' at the 5' end of the 6.3 kb *Eco*RI GGT fragment in pGEM-G-V, 5'-GCAGATCTGGTCAGCATGTCTCC-3' from –207 to –184 (sn-12), 5'-ATCAGATCTTGCATGCCCTCTAGC-3' from –157 to –134, 5'-GAAAGATCTACCAGATGCTCCAC-3' from –106 to –83 and 5'-GTTAGATCTAGGGGCTGAGTAAAC-3' from –64 to –40. The amplified fragments, digested with *Bgl*II and inserted into the corresponding site of the promoterless pGL2 luciferase basic vector (Promega), yield the –3200+19, –202+19, –150+19, –98+19 and the –55+19 GGT-luc constructs. Four other constructs were derived from the –202+19 vector by introducing mutations in the HNF3 and/or AP-1 consensus sequences. These mutations were created by a double PCR experiment as follows: a coding primer and its complementary oligomer were mutated in the AP-1 and/or HNF-3 sites. The mutated coding and non-coding primers in combination with the sn-11 or sn-12 oligomer respectively, were used to amplify sequences from the –202+19 luc construct. The resulting fragments containing the designed mutations at their 5' and 3' ends respectively, were gel-purified and combined to the sn-11 and sn-12 primers for a second PCR. These fragments, digested with *Bgl*II were ligated into the *Bgl*II site of the pGL2 basic vector and the sequences of the mutated –202+19 GGT luc constructs verified.

Transient transfection

The rat hepatoma cell lines H5, Fao, C2 and C2Rev7 were seeded at a density of 3 $\times$ 10⁵ cells/6 cm dish, 24 h prior to transfection. Using a modified calcium phosphate method described previously [8], cells were co-transfected with 5 μ g of luciferase-plasmid and 1 μ g of pRSV-CAT (CAT = chloramphenicol acetyltransferase) plasmid [19] or 2 μ g of the pCH110 β -galactosidase eukaryotic vector (Pharmacia) to monitor variations in the transfection efficiency. The luciferase pGL2-control vector and pGL2 basic vector (Promega) were used as positive and negative controls respectively.

Luciferase, CAT and β -galactosidase assays

Luciferase activity was measured according to the protocol of Promega. Briefly, the transfected cells were lysed in 400 μ l of reporter lysis buffer (Promega). After centrifugation, 20 μ l of the supernatant were mixed with 100 μ l of luciferase assay reagent at room temperature. The luciferase activity was measured 25 s later in a LKB luminometer for 15 s.

CAT assays were performed as previously described [20]. In brief, 60 μ l of cellular extract heated at 65 °C for 10 min was incubated with 1 mM chloramphenicol, 30 μ M acetyl-CoA and 50 nCi of [³H]acetyl-CoA at 37 °C for 2 h before the addition of 4 ml of EconofluorTM (New England Nuclear). The radioactive

acetylated chloramphenicol was measured after a 30 min incubation.

The activity of β -galactosidase was assessed using the AuroraTM GAL-XE chemiluminescent reporter assay (ICN Pharmaceutical) in 20 μ l of cellular extracts as recommended by the supplier. Briefly, the extracts were heated at 48 °C for 50 min to inactivate endogenous β -galactosidase activity prior to a 60 min incubation at room temperature. Light emission was measured in an LKB luminometer immediately after addition of the light-emission accelerator.

DNase I footprinting and DNA protein-mobility-shift assay

The luciferase vector containing the GGT -202 +19 fragment was digested with *Xho*I and the 3' ends were labelled using the DNA polymerase I (Klenow fragment) and [α -³²P]ATP. A second digestion by *Hind*III generated the GGT insert labelled on the coding strand which was then purified on agarose gel. An aliquot of this probe (20000 c.p.m.) was incubated with nuclear extracts and analysed as described in [21]. Gel-shift assays were carried out by incubating a labelled double-stranded oligonucleotide (0.5 ng) with 8 μ g of nuclear extract under the conditions used for the DNase I footprinting.

RESULTS

Northern-blot analysis and cloning of the GGT mRNAs expressed in H5 hepatoma cells

Hybridization of poly(A)⁺ RNA from different tissues or hepatoma cell lines to the cRNA 139A GGT coding probe reveals GGT mRNA transcripts of different sizes ranging from 2.2 kb to 2.6 kb (Figure 1A), as previously reported [8,9,12]. The 2.6 kb and 2.3 kb GGT transcripts detected in H5 cells exhibit a different size than the 2.2 kb GGT mRNAs I and II characterized from the kidney [17], the 2.4 kb GGT mRNA III cloned from fetal liver and expressed in the mammary gland [9] or the 2.5 kb

GGT mRNA IV expressed in small intestine or HTC hepatoma cells [10].

Cloning of the GGT mRNA sequences from H5 cells resulted in the identification of two distinct GGT recombinant plasmids. These two plasmids contain GGT sequences of 583 nt (V-1) and 330 nt (V-2) shown in Figure 2A. The 272 nt sequence at the 3' end of the cDNA V-1 aligns with a 100 % identity with the sequence found in the GGT mRNA IV-1 [10] from -395 to -125 upstream of the initiation codon (+1) (Figure 2B). In the mRNA IV-1, this sequence corresponds to the second exon and the first 18 nt of the third exon which are common to all GGT mRNAs [10]. The 312 nt upstream of the position -395 in the V-1 cDNA, also present in the V-2 cDNA (Figure 2A), are specific to the GGT message cloned from H5 cells. The shorter cDNA (V-2) is a splicing variant that has lost the 251 nt corresponding to the second exon for the mRNA IV. In summary, we have identified two new GGT cDNAs from the H5 hepatoma cells which exhibit a 312 nt specific sequence at their 5' end.

A cRNA probe (cRNA-V), transcribed from the plasmid pGEM-3-V-2 (Figure 2A), was used to assess the expression of the GGT transcripts containing the 312 base specific sequence present at the 5' end of the cDNAs cloned from H5 cells. Hybridization of cRNA-V to H5 cells poly(A)⁺ RNA, reveals two bands corresponding to a 2.6 kb and a 2.3 kb transcript (Figure 1B). These two mRNA species (mRNAs V-1 and V-2) are detected in H5 or HTC hepatoma cells but not in other cell lines or tissues (Figure 1B) in which other GGT transcripts are expressed (Figure 1A). A third hybridization to the GGT cDNA 4-2, a sequence which maps in the cDNA V-1 (Figure 2A) and is absent in the cDNA V-2, identifies the 2.6 kb mRNA V-1 and not the 2.3 kb mRNA V-2 in H5 or HTC cells (Figure 1C). The cDNA 4-2 sequence, also present in the second exon of the GGT mRNA IV-1 [10], reveals also the 2.5 kb GGT mRNA IV-1 in the HTC cells, small intestine and epididymis (Figure 1C). In summary, the 2.6 kb GGT mRNA V-1 and 2.3 kb GGT mRNA V-2 transcripts detected only in H5 and HTC hepatoma cells share the same 5' end and are products of the same primary transcript by alternative splicing of the second exon.

Cloning and characterization of the GGT genomic sequence around the mRNA V start site

Screening of the rat genomic library with the 312 bp sequence at the 5' end of the cDNA V-2, resulted in the isolation of the 8-1, 8-2 and 16 recombinant phages (Figure 3). Southern-blot analysis of those phages, digested with *Eco*RI, *Bam*HI and *Xba*I, reveals overlapping fragments covering a 25 kb genomic sequence. The phage 8-1 contains a 17 kb genomic fragment that overlaps with the most 5' GGT genomic sequence previously reported in the region containing the transcription start site for the GGT mRNA IV-1 and its two leader exons [10]. The two other phages (8-2 and 16) extend more 5' in the GGT gene (Figure 3, upper panel) up to 21 kb upstream of the mRNA IV transcription start site. Hybridization of the restriction fragments with the cDNA V-2 maps this sequence into the 2.9, 6.3 and 7.2 kb *Eco*RI fragments of the phages 8-1, 8-2 and 16 respectively.

The 6.3 kb *Eco*RI fragment from phage 8-2 was ligated into the *Eco*RI site of pGEM-3 Zf(+), giving rise to the plasmid pGEM-G-V as shown in Figure 3 (upper panel). A partial sequence of this genomic fragment was obtained from oligonucleotides designed in the cDNA and the subsequent genomic sequence. Alignment of this sequence to the cDNA V-2 shows that the 312 nt at the 5' end of the H5 cDNAs, lie within a single exon whose 3' end is 9.7 kb upstream of the GGT promoter IV transcription start site. The H5 cDNA sequence from -398 to

Figure 1 Northern-blot analysis of GGT mRNAs

Poly(A)⁺ RNA (5 μ g) samples were separated on agarose denaturing gels and blotted on to a nylon membrane. The blots were hybridized to the cRNA-139A probe which hybridizes in the coding region of the GGT mRNAs (A), to the cRNA-V probe specific for the cDNA V-2 (B) and to the cDNA 4-2 probe specific to the sequence from -389 to -153 in the cDNA V-1 (C). Autoradiography was performed with two intensifying screens at -80 °C for 48 (A) or 24 h (B and C).

Figure 2 Sequence of the GGT cDNA cloned from H5 hepatoma cells

(A) Schematic representation of the two GGT cDNA sequences. Open boxes represent a sequence common to the previously characterized mRNAs, the chequered boxes correspond to the sequence found in the mRNA IV 1 [10] and the hatched boxes to the sequence specific for the cDNAs V-1 and V-2. The numbering is relative to the first base of the initiation codon (position +1). The cRNA-V probe was transcribed from the T7 promoter of the plasmid pGEM-V-2 as shown. The position of the cDNA-4-2 probe is mapped on the cDNA. (B) Nucleotide sequence of the cDNA V-1. Alignment of this sequence to the GGT mRNA IV-1 (lower-case letters) reveals a 100% similarity up to nt -395. The sequence of the oligomer 1, used in the cDNA synthesis, and oligomer sn-9 used in primer extension and S1 mapping analysis are underlined.

-145 (Figure 2) corresponds to a second exon which is common to the GGT mRNA IV-1 and mRNA V-1 and lies 10.2 kb downstream the mRNA V leader exon.

S1-nuclease protection analysis of two labelled genomic DNA probes of 193 and 470 nt, that encompass the 5' end of the cDNA V (Figure 4, far-left panel), was performed by hybridization of these probes to poly(A)⁺ RNA from H5 hepatoma cells, in order to map the GGT mRNA V transcription start site. Protection of these 193 and 470 nt probes results in an identical pattern of several bands or clusters of bands from 47 to 72 nt in size (Figure 4, lanes 2 and 3). The size of the protected fragments was obtained directly from the related sequence initiated from the sn-9 oligomer and loaded on the same gel (lanes CTAG). To identify in these 47–72 nt S1-protected fragments, the fragments whose 5' end maps at an mRNA start site, an extension of the sn-9 primer on H5 poly(A)⁺ RNA was performed in parallel. Analysis of the sequences extended from sn-9 reveals three fragments of 70, 60 and 58 bases (Figure 4, lane 1) which exhibit the size of three S1-protected fragments. We conclude that the 5' end of these fragments maps three distinct initiation sites on the gene for the GGT mRNA V-1 and V-2 synthesis in H5 cells. The A at the 5' end of the longest fragment maps (within one base) at the 5' end of the cloned cDNA V sequences and this nucleotide is referred to as +1 in the genomic sequence reported in Figure 3.

The DNA sequence upstream of the transcription start sites contains a TATA box sequence (TATAAA) at -29 and several motifs (Figure 3 lower) highly similar to the well-known activator protein-2 (AP-2) site [22] at -72 and AP-1 site [23] at -48; the sequence GTGGAAA at -169 is identical with the core sequence found in several viral enhancers [24]. The sequence from -48 to -39 (TGAGTAAACA) contains a consensus sequence for AP-

1 overlapping with a consensus HNF3-binding site [25]; a second motif from -33 to -27 matches (7/7) to the HNF3 consensus and overlaps with the TATAAA element.

Southern-blot analysis of rat kidney and H5 genomic DNA

Digestion of rat genomic DNA obtained from adult kidney or H5 cells and hybridization to the cDNA V-2 sequence results in two identical patterns of digestion (Figure 5). The *EcoRI*, *BamHI* and *XbaI* fragments recognized by the probe are in accordance with the restriction map obtained from the GGT recombinant phages isolated from the rat liver genomic library (Figure 3, upper panel). Therefore the DNA obtained from H5 cells displays the same banding pattern as the DNA prepared from normal adult rat tissue; thus, no major rearrangement was found in the H5 genome that could account for the specific GGT promoter V expression in this transformed cell line.

Promoter activity of the GGT sequence upstream from the transcription start sites for mRNA V

The sequence from nt -202 to nt +19 downstream from the major start site (+1), cloned into the pGL2 luciferase basic vector, drives a luciferase activity in H5 cells that represents 20% of the activity driven by the SV-40 pGL2 control vector (Figure 6). This latter construct exhibits a similar activity in Fao, C2 or C2Rev7 cell lines. The Fao cells transfected with the -202 to +19 GGT-pGL2 basic vector do not exhibit any luciferase activity that exceeds the background measured in cells transfected with the basic pGL2 vector. Extension of the GGT sequence up to the nt -3200, does not induce any activity in Fao cells, whereas this construct still exhibits a promoter activity in H5 cells (Figure 6). Transfection of these GGT constructs in C2 or C2 rev7 cells does

Figure 3 Cloning and structure of the 5' region of the rat GGT gene

Upper panel: schematic representation of the recombinant phages which hybridized to the cDNA V-2. The chequered boxes in phage 8-1 represent the two exons at the 5' end of the GGT mRNA IV-1 previously reported [10]. The most 3' one is present in the cDNA V-1; the arrow stands for the mRNA IV transcription start site. The hatched box maps the cDNA V-2 in the 2.9, 6.3 and 7.2 kb *EcoRI* fragments of the phages 8-1, 8-2 and 16 and in the 6.3 kb *EcoRI* fragment cloned in pGEM-G-V. B, E and $\times$ stand for *Bam*H1, *Eco*R1 and *Xba*I respectively. Lower panel: nucleotide sequence of the gene around the mRNA V transcription start sites. The arrow maps the major start site (nt numbered +1 in the sequence). Sequence in bold-face upper-case letters corresponds to the sequence in the mature mRNA V which maps in the hatched box (leader exon) and in the downstream chequered box. Nucleotides in lower-case letters are for sequences in the promoter region and in the introns. *Kpn*I and *Av*alI sites used to prepare S1 mapping probes are overlined. Consensus sequences for AP-1, AP-2 and HNF3 are boxed.

not induce significant luciferase activity (results not shown), as observed in Fao cells. In conclusion, the 202 nt genomic sequence upstream from the GGT mRNA V start sites, displays a cell-specific transcriptional activity which, together with S1 mapping, identifies a fifth promoter (promoter V) on the rat GGT gene.

Functional analysis of sequences upstream from the mRNA V transcription start sites

Footprinting analysis of the 202 bp sequence upstream from the GGT mRNA V start sites was performed to identify *cis*-acting elements that might be involved in the specific expression of the mRNAs V-1 and V-2 in H5 hepatoma cells. The addition of increasing amounts of crude nuclear extracts from H5 cells induces several changes in the DNase I digestion pattern (Figure 7). The sequences from -3 to -9 (A), -21 to -27 (B), -30 to -50 (C), -52 to -70 (D) and -72 to -101 (E) are protected from DNase I digestion by H5 nuclear proteins. This protection pattern exhibits major differences with those obtained with extracts from other cell lines. With Fao, C2 or C2Rev7 cell extracts, the protection is altered at the C site and totally disappears on the D and E sequences. In contrast, the F window (-100 to -121) appears more clearly. The DNase I-hypersensitive sites at -29 and -41, obtained only with H5 extracts, also reveal H5-cell-specific DNA-protein interactions. The differ-

Figure 4 Mapping of the GGT promoter V transcription initiation sites

The region of the gene which contains the leader exon for the GGT mRNAs V (hatched box) and the 5' flanking sequence is shown on the extreme left. The sn-9 oligomer and the 193 and 470 nt A and B probes extended from sn-9 to the *Kpn*I and *Av*alI sites are represented. Primer extension: the sn-9 primer labelled at its 5' end, was extended on 5 μ g of poly(A)⁺ RNA from H5 cells and the resulting cDNA fragments were analysed on a sequencing gel (lane 1) along with sequencing reaction products (CTAG) generated from the sn-9 oligomer. Arrows map the positions of the extended fragments. Autoradiography was performed for 3 days at -80 $^{\circ}$ C with two intensifying screens. S1 mapping: the 470 and 193 nt probes were hybridized to 5 μ g of poly(A)⁺ RNA from H5 cells. Hybrids were S1-nuclease-digested and resolved on a sequencing gel along with the related sequence ladder (CTAG). The upper band (arrowed A) in lane 3 corresponds to the 193 nt undigested A probe. Protected fragments obtained with the 470 or 193 nt B and A probes (lanes 2 and 3) are identical. The 5' ends of the protected fragments that match the 5' ends of the extension products are mapped * on the sequence.

Figure 5 Southern-blot analysis of rat genomic DNA

Genomic DNA (50 μ g) from H5 cells and rat kidney was digested and the fragments separated on an 0.8%-agarose gel. The blot was hybridized to the labelled cDNA V-2 and autoradiographed for 3 days at -80 $^{\circ}$ C with two intensifying screens.

Figure 6 Expression of luciferase activity in H5 cells transfected by GGT-luc plasmids

The $-3200+19$, $-202+19$, $-150+19$, $-98+19$, $-55+19$ GGT-luc sequences were obtained by PCR and cloned into the *Bgl*II site of the pGL2 basic luciferase vector as described in the method section. Luciferase activities, normalized to the CAT activity detected in the same lysate, are expressed as a percentage of the activity driven by the pGL2 control vector. Each value is the mean $\pm$ S.E.M. for at least five experiments, each performed in duplicate dishes.

DNA binding reactions were carried out with nuclear extracts from H5 (5, 20, 40, 60 and 80 μ g), FaO, C2, and C2Rev7 (40 and 60 μ g) cells. The $-202+19$ GGT DNA sequence was labelled on the sense strand. Chemical sequencing reaction products specific for G residues, were loaded in lane G. Control reaction without nuclear extract was run in lane 0. Positions of the footprints are indicated by the open vertical bars (A–E) on the left.

ences in DNA–protein interactions observed in H5 cells as compared with the other cell lines might be related to the cell-specific expression of the GGT mRNA V-1 and V-2 in H5 cells.

To determine whether the GGT sequences interacting with H5 proteins are critical for GGT promoter activity, three deletion mutants of the 202 bp proximal sequence were fused to the luciferase basic reporter vector and transfected in H5 and FaO, C2 and C2Rev7 cells. The two shortest constructs ($-55+19$ GGT-luc and $-98+19$ GGT-luc), which contain the A–C and A–E footprints respectively, drive a significant luciferase activity (10 times over the background) in H5 cells (Figure 6). Further extension of the GGT construct to the sequence that includes the F window ($-150+19$ GGT-luc), induces a 5-fold increase in the luciferase activity. None of these plasmids induces a luciferase activity in FaO (Figure 6), C2 or C2Rev7 cells (results not shown). These data indicate that the sequence from -150 to -98 contributes to the high level of GGT mRNA V expression in H5 cells, and that the 55 nt sequence adjacent to the predominant mRNA V transcription start site contains elements that control cell specificity of the GGT promoter V. H5 extracts generate a specific DNA protein interaction in this region characterized by the C footprint and two hypersensitive sites (Figure 7). This interaction is abolished by an excess of oligomer C covering the C footprint (Figure 8A) and is not affected by addition of an unrelated oligomer (results not shown). These results demonstrate the specificity of the interaction between proteins from H5 cells and the gene sequence from -50 to -30 upstream of the mRNA V start sites. The motifs 5'-ATAAACA-3' (from -27 to -33) and 5'-TGAGTAAACA (from -48 to -39) in the protected sequence display a high similarity to the consensus binding site for HNF3 [5'-(T/A)(A/G)(A/G)(G/A)(C/T)AAA(C/T)A-3'] [25]. The -48 to -39 element resembles a composite motif which also has the features of an AP-1-binding sequence (TGAGTAAA) [26]. The DNase-hypersensitive sites at -41 and -29 map in the middle of the HNF3 consensus sequences and are characteristic of HNF3 binding activity, as previously described for the tyrosine aminotransferase (TAT) gene [25].

Identification of proteins interacting with DNA at the C footprint

Interactions between proteins from H5 nuclear extracts and the proximal sequence of the GGT promoter V were further analysed

In order to confirm that the binding of AP-1- or HNF3-related proteins to the C sequence can generate C1 and C2, C_A and C_B shifted complexes, we mutated these sites in the oligomer C and tested the ability of these mutated sequences to interact with H5 extracts. The C oligomer mutated in the distal (d) and the proximal (p) HNF3 sites (C-HNF3d⁻p⁻) is no longer able to out-compete the C2, C_A or C_B complexes. The C oligomer mutated in the distal HNF3 site (C-HNF3d⁻) behaves as a weak competitor for C2, C_A or C_B complexes, since an 100-fold excess induces only a slight decrease in the intensity of the corresponding bands (Figure 9). The C oligomer, mutated in the proximal HNF3 site (C-HNF3p⁻), is not affected in its ability to compete for the C2, C_A or C_B complex formation (Figure 9). Gel-retardation assays using the C-HNF3d⁻p⁻ or the C-HNF3d⁻ mutated oligomers as probes, generate a single specific complex that comigrates with C1 (Figure 10A). The absence of the C2 major complex confirms the binding of HNF3-related proteins to the C sequence. The binding occurs only on the distal site, since the C-HNF3d⁻ probe does not generate any C2 retarded complex (Figure 10A). Therefore the proximal HNF3 consensus sequence does not display a significant affinity for HNF3 transcription

factors in gel-shift analysis, in spite of the DNase I-hypersensitive site detected at this position in the footprint.

The oligomer C, mutated on a single base in its AP-1 motif (C-AP-1⁻), cannot compete for the complex C1 (Figure 9). When used as a probe, this oligomer gives rise to the C2 major complex as well as the CA and CB minor ones previously observed, but does not interact with other proteins to form C1 (Figure 10B). Taken together, these data show that AP-1 transcription factors are involved in the C1 complex and that HNF3 factors interact strongly with the distal HNF3 site to form the C2, the CA and CB retarded complexes. Moreover, disruption of the HNF3 distal site in the C probe induces an increase in the level of the C1 complex (Figure 10A), suggesting a competition between HNF3 and AP-1 factors for binding to their overlapping sequences on the oligomer C. We also observed that the HNF3 sequence from the TAT gene (Figure 9), or AP-1 sequence from the T gene (Figures 9 and 10A), are poor competitors for C2 or C1 complexes respectively, in contrast with other similar consensus sequences. This suggests that the multiple AP-1 or HNF3 isoforms exhibit different affinities towards the AP-1- and HNF3-binding sites characterized in the GGT promoter V.

The oligomer-C-binding activity was also assessed with nuclear extracts from hepatoma cells that do not express the GGT promoter V. The gel-shift-assay patterns differ in several aspects from the pattern obtained with H5 extracts (Figure 11). The C1 complex does not appear in Fao cell extracts, whereas C2 cell or C2Rev7 cell extracts generate two minor complexes migrating as a doublet which are efficiently out-competed by an excess of AP1-Col or the C-HNF3d⁻p⁻ oligomer. The C2 complex, predominant with H5 extracts, is less abundant in Fao, and absent in C2 extracts. HNF3 complexes obtained with C2Rev7 extracts appear as a doublet of two minor bands which co-migrate with the C2 complex and are efficiently competed by an excess of HNF3-TTR oligomer, but not by an excess of C-HNF3d⁻p⁻ oligomer.

In summary, the oligomer C sequence, which maps from -56 to -23 in the GGT promoter V, is able to produce AP-1 and HNF3 complexes upon incubation with H5 cell extracts. The disappearance of at least one of these two major complexes and the appearance of new minor complexes in gel shifts obtained with Fao, C2 or C2Rev7 cell extracts reflect major quantitative and qualitative differences in the binding of HNF3 and AP-1 factors to the proximal sequence of the GGT promoter V, which might be related to the extinction of the GGT promoter V in these cell lines.

Promoter activity of the -202+19 GGT-luc construct mutated in HNF3 and AP-1 binding sites

Mutations in both the AP-1 and distal HNF3 sites (Figure 8A) are sufficient to abolish the luciferase activity driven by the -202+19 GGT luc-construct (Figure 12), suggesting that the HNF3 proximal sequence is not itself able to induce a promoter activity in H5 cells. However, mutation of this proximal HNF3 site results in a 35% inhibition in the promoter activity as compared with the wild type. Mutations in either the AP-1 or the HNF3 distal sites induce only a 40 and 25% inhibition respectively, an inhibition which can be further increased up to 58 and 62% by disruption of the proximal HNF3 consensus sequence. All these transfection experiments clearly establish that intact AP-1 and HNF-3 binding sites at positions -48 to -39 are required to obtain optimal GGT promoter V activity. These data also show that an interaction between proteins that bind to the AP-1/HNF3 composite site and the proximal HNF3

Figure 10 Gel retardation of GGT promoter V proximal sequence (-56 to -23) mutated in its HNF3- and AP-1-binding sites by H5 cell extracts

The oligomer C probes (wild-type and mutants) were incubated with H5 cell nuclear extracts in the presence or absence of a 50- or 100-fold molar excess of unlabelled competitors as described in Figure 9.

Figure 11 Gel retardation of GGT promoter V proximal sequence (-56 to -27) by H5, Fao, C2, and C2Rev7 extracts

The oligomer C probe (wild-type) was incubated with nuclear extracts prepared from H5, Fao, C2 and C2Rev7 cells in the presence or absence of a 50-fold molar excess of competitors as described in Figure 9.

site are necessary to obtain a maximal GGT promoter V activity in H5 hepatoma cells.

DISCUSSION

Cloning of GGT cDNAs from the rat H5 hepatoma cell line resulted in the identification of a new 312 nt sequence which corresponds to the leader exon for the 2.6 kb GGT mRNA V-1

Figure 12 Luciferase activity in H5 cells transfected with the $-202+19$ GGT luc constructs mutated in the HNF3 and/or AP-1 sites

Luciferase activities relative to β -galactosidase activities, assessed in the same extracts to correct for transfection efficiency, are expressed as the percentage of the activity driven by the $-202+19$ luc wild-type construct. Mutations (X) in AP-1 and/or HNF3 are those reported in Figure 8. Values are means $\pm$ S.E.M. from six experiments, each performed in duplicate dishes.

and the 2.3 kb GGT mRNA V-2. These two mRNAs are generated by alternative splicing of a primary transcript initiated on a fifth promoter on the rat GGT gene. This new promoter maps 10 kb upstream from the promoter IV previously characterized [10]. Expression of this promoter was detected by Northern-blot analysis in H5 and HTC hepatoma cells and not in other cell lines or tissues investigated. This cell-specific expression cannot be due to a major DNA rearrangement that might have occurred in transformed cells. In fact, Southern-blot analysis of the regulatory region of the GGT gene in H5 cells and in the adult rat kidney results in an identical pattern as expected from the restriction map of the genomic sequence isolated from the rat genomic library. Therefore the specificity of the GGT promoter V expression might result from transacting effects of factors or combination of factors present in undifferentiated hepatoma cells.

The 55 nt sequence immediately upstream from the major mRNA V transcription start site is sufficient to drive significant promoter activity in H5 cells. Extension of the GGT sequence up to nt -202 induces a 6-fold increase in the promoter activity, thus revealing the presence in this sequence of a *cis*-element acting positively on the GGT promoter V activity. In the proximal promoter, the -56 to -23 nt sequence binds nuclear proteins in a cell-type-dependent manner and may therefore be involved in the specific expression of the GGT promoter V in H5 cells. Mutations of AP-1 or HNF3 binding sites, which overlap in a composite element from -48 to -39 , prevent interactions of AP-1 or HNF3 factors at this site and inhibit promoter activity. More significant, association of these mutations results in a complete inhibition of the promoter activity. It can be concluded that binding of HNF3 or AP-1 transcription factors on the -48 to -39 composite element is absolutely required to obtain an expression of the GGT promoter V. In addition, the maximal expression of this promoter is observed when both AP-1 and HNF3 interact on this composite element.

Interactions between HNF3 and other transcription factors at overlapping sites have been described in several genes. In the aldolase B gene promoter, the binding of HNF3 decreases the accessibility for HNF1 and inhibits the promoter activity [30]. In the serum albumin gene, the nuclear factor-1 interacts with HNF3 and potentiates its positive transacting effect [31]. An interaction between specific members of the AP-1 family and

HNF3 α factors at a composite site is responsible for the expression of the CC-10 gene in the NCI-H441 adenocarcinoma cells, a gene which is silent in another lung adenocarcinoma cell line that express HNF3 β instead of HNF3 α [32].

Interactions between HNF3 and AP-1 factors on the GGT promoter V proximal sequence are essential for the expression of this promoter in H5 cells. The Fao, C2 and C2 Rev7 cells in which this promoter is silent are also known to express AP-1 and HNF3 DNA binding activities [28,33]. In these cell lines, however, we observed major differences in the AP-1 or HNF3 DNA binding patterns as compared with H5 cells. The heterogeneity in the oligomer C retardation patterns obtained with the different cell extracts indicates that the AP-1/HNF3 composite site in the GGT promoter V preferentially recognizes specific members of the AP-1 or HNF3 families which are expressed at different levels in these cell lines. Consensus sequences for AP-1 or HNF3 factors integrate indistinctly binding sites for AP-1 homodimers and heterodimers or α , β , γ HNF3 factors. Preferential recognition of a specific site by one type of these factors has already been reported [26,34]. Binding of specific members of the AP-1 or HNF3 families to the GGT promoter V in the H5 cell might explain the strong cell specificity of this promoter as reported above for the CC-10 gene expression in lung adenoma carcinoma cells [32]. Interactions of two AP-1 heterodimers (Fra-2/jun-D and Fra-2/c-jun) have also been shown to induce opposite effects on the expression of a reporter gene associated with the AP-1 binding site of the collagenase gene [23]. Therefore a precise definition of the AP-1 and HNF3 components that bind to the AP-1/HNF3 overlapping sequence at -48 to -39 of the GGT promoter V, as well as their cellular distribution, appear to be essential for the understanding of the mechanisms underlying GGT expression in undifferentiated liver cells.

The rat GGT gene appears now to be transcribed from five promoters into seven mRNAs with alternate 5' untranslated regions, but encoding the same polypeptidic chain. The reason why many promoters are needed to express the same protein remains unclear. A similar transcriptional organization has been reported for the rat cytochrome P-450 aromatase and the human insulin-like-growth-factor-II genes by which transcription from multiple promoters also gives rise to a unique polypeptidic chain [35,36]. Such a complex organization provides genes with the capacity to respond to various stimuli in a developmental stage or tissue-specific manner [37]. A fine tuning of gene activity can be achieved at a transcriptional as well as at a post-transcriptional level; in fact, alternate 5' untranslated sequences might contain a short open reading frame [38] or form stable secondary structures [39] that affect the stability or the translational efficiency of the transcript.

The organization of the GGT gene is well conserved between rat and mouse species, since the four promoters previously described in the rat gene have also been identified with highly conserved sequences [10] in the mouse gene [40], where they exhibit a comparable tissue specificity [41]. The new GGT promoter reported in this study has not been reported in the mouse gene, where the most distal promoter (promoter VI) shows sequence similarity to rat promoter IV. The use of several promoters in the same cell may be a way of achieving a higher amount of message; it should be noted that GGT promoters I, II and IV in the rat, and their mouse counterparts, are active in tissues like kidney, epididymis or small intestine, organs that express the highest GGT activity.

Rat GGT promoter V was identified in the distal part of the gene isolated from normal adult rat liver DNA; it is transcribed specifically in two undifferentiated cell lines derived from two different hepatomas and not in more differentiated cells or

tissues. Genes such as those coding for GGT, α -fetoprotein or glutathione S-transferase, whose expression is considered as a marker of liver transformed cells, are also active at the early stages of liver development. Expression of these oncofetal markers and the mixed hepatocytic and biliary phenotypes displayed in many rat or human hepatoma cells [42,43] support the concept of blocked ontogeny [44]. According to this concept, tumour cells arise from undifferentiated cells that deviate from the normal differentiation program; their phenotypic features are largely determined by the phenotype of the cells from which malignant clonal expansion occurred [45]. The expression of the GGT promoter V, limited to undifferentiated hepatoma cells, might also account for the high GGT activity detected early in liver embryogenesis [3] before the bipotent liver precursor cells undergo a differentiation in the hepatocytic or biliary lineage.

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REFERENCES

- Hanigan, M. and Pitot, H. (1983) *Carcinogenesis* **6**, 165–172.
- Huggett, A. C., Ellis, P. A., Ford, C. P., Hampton, L. L., Rimoldi, D. and Thorgeisson, S. S. (1991) *Cancer Res.* **51**, 5929–5936.
- Iannoccone, P. M. and Koziimi, J. (1983) *J. Histochem. Cytochem.* **31**, 1312.
- Pitot, H. C. (1990) *Annu. Rev. Pharmacol. Toxicol.* **30**, 465–500.
- Lafarge-Frayssinet, C., Estrade, C., Rosa-Loridon, R., Frayssinet, C. and Cassingena, R. (1984) *Cancer Lett.* **22**, 31–39.
- Rajagopalan, S., Park, J. H., Patel, P. D., Leibovitz, R. M. and Liebermann, M. W. (1990) *J. Biol. Chem.* **265**, 11721–11725.
- Kurauchi, O., Lahuna, O., Darbouy, M., Aggerbeck, M., Chobert, M. N. and Laperche, Y. (1991) *Biochemistry* **30**, 1618–1623.
- Lahuna, O., Brouillet, A., Chobert, M. N., Darbouy, M., Okamoto, T. and Laperche, Y. (1992) *Biochemistry* **30**, 9190–9196.
- Brouillet, A., Darbouy, M., Okamoto, T., Chobert, M. N., Lahuna, O., Garlatti, M., Goodspeed, D. and Laperche, Y. (1994) *J. Biol. Chem.* **269**, 14878–14884.
- Okamoto, T., Darbouy, M., Brouillet, A., Lahuna, O., Chobert, M. N. and Laperche, Y. (1994) *Biochemistry* **33**, 11537–11543.
- Griffiths, S. A., Good, V. M., Gordon, L. A., Hudson, E. A., Barrett, M. C., Munks, R. J. L. and Manson, M. (1995) *Mol. Carcinog.* **14**, 251–262.
- Darbouy, M., Chobert, M. N., Lahuna, O., Okamoto, T., Bonvalet, J. P., Farman, N. and Laperche, Y. (1991) *Am. J. Physiol.* **261**, C1130–C1137.
- Deschatrette, J., Moore, E. E., Dubois, M. and Weiss, M. C. (1980) *Cell* **19**, 1043–1051.
- Thomson, E. B., Tomkins, G. M. and Currare, J. F. (1966) *Proc. Natl. Acad. Sci. U.S.A.* **56**, 296–303.
- Frohman, M. A., Dush, M. K. and Martin, G. R. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8998–9002.
- Laperche, Y., Bulle, F., Aissani, T., Chobert, M. N., Aggerbeck, M., Hanoune, J. and Guellaen, G. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 937–941.
- Chobert, M. N., Lahuna, O., Lebarry, F., Kurauchi, O., Darbouy, M., Bernaudin, J. F., Guellaen, G., Barouki, R. and Laperche, Y. (1990) *J. Biol. Chem.* **265**, 2352–2357.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (eds.) (1987) *Current Protocols in Molecular Biology*, vol. 1, John Wiley and Sons, New York.
- Gorman, C. M., Merlino, G. T., Willingham, M. C., Pastan, I. and Howard, B. H. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6777–6781.
- Neuman, J. R., Morency, C. A. and Russian, K. O. (1987) *BioTechniques* **5**, 444–447.
- Garlatti, M., Tchesnokov, V., Daheshia, M., Feilleux-Duché, S., Hanoune, J., Aggerbeck, M. and Barouki, R. (1993) *J. Biol. Chem.* **268**, 6567–6574.
- Mitchell, P. J., Wang, C. and Tjian, R. (1991) *Cell* **50**, 847–861.
- Suzuki, T., Okuno, H., Yoshida, T., Endo, T., Nishina, N. and Iba, H. (1992) *Nucleic Acids. Res.* **19**, 5537–5542.
- Richardson, C. C. (1983) *Cell* **33**, 315–317.
- Roux, J., Pictet, R. and Grange, T. (1995) *DNA Cell Biol.* **14**, 385–396.
- Hirai, S. and Yaniv, M. (1989) *New Biol.* **1**, 181–191.
- Costa, R. H., Grayson, D. R. and Darnell, J. E. (1989) *Mol. Cell Biol.* **9**, 1415–1424.
- Kuo, C. J., Conley, P. B., Chen, L., Sladek, F. M., Darnell, Jr., J. E. and Crabtree, J. R. (1992) *Nature (London)* **355**, 457–461.
- Grange, T., Roux, J., Rigaud, G. and Pictet, R. (1991) *Nucleic Acids Res.* **19**, 131–139.
- Gregori, C., Kahn, A. and Pichard, A. L. (1993) *Nucleic Acids Res.* **21**, 897–903.
- Jackson, D. A., Rowader, K. E., Stevens, K., Jiang, C., Milos, P. and Zaret, K. S. (1993) *Mol. Cell Biol.* **13**, 2401–2410.
- Sawaya, P. L., Stripp, B. R., Whitsett, A. and Luse, D. S. (1993) *Mol. Cell Biol.* **13**, 3860–3871.
- Herbst, R. S., Nielsch, U., Sladek, F., Lai, E., Babiss, L. E. and Darnell, Jr., J. E. (1991) *New Biol.* **3**, 289–281.
- Lai, E., Prezioso, V. R., Tao, W., Chen, W. S. and Darnell, Jr., J. E. (1991) *Genes Dev.* **5**, 416–427.
- Van Dijk, M. A., Rodenburg, R. J., Holthuisen, P. and Sussenbach, J. S. (1992) *Nucleic Acids Res.* **20**, 3099–3104.
- Mahendroo, M. S., Mendelson, C. R. and Simpson, E. R. (1993) *J. Biol. Chem.* **268**, 19463–19470.
- Ayoubi, T. A. Y. and Van de Ven, W. J. M. (1996) *FASEB J.* **10**, 453–456.
- Ruan, H., Hill, J. R., Fatemie-Nainie, S. and Morris, D. R. (1994) *J. Biol. Chem.* **269**, 17905–17910.
- Guan, K. L. and Weiner, H. (1989) *J. Biol. Chem.* **264**, 17764–17769.
- Habib, G. M., Carter, Z. B., Sepulveda, A. R., Shi, Z. Z., Wan, D. F., Lebovitz, R. M. and Lieberman, M. (1995) *J. Biol. Chem.* **270**, 13711–13715.
- Habib, G. M., Rajagopalan, S., Godwin, A. K., Lebovitz, R. M. and Lieberman, M. (1992) *Mol. Carcinog.* **5**, 75–80.
- Marceau, N., Blouin, M. J., Germain, L. and Noel, M. (1989) *In Vitro Cell Dev. Biol.* **25**, 336–341.
- Van Eyken, P., Sciort, R., Paterson, A. C., Callea, F. and Kew, M. C. (1988) *Hum. Pathol.* **19**, 562–568.
- Sell, S. and Dunsford, H. A. (1989) *Am. J. Pathol.* **134**, 1347–1363.
- Sigal, S. H., Brill, S., Fiorino, A. S. and Reid, L. (1992) *Am. J. Physiol.* **263**, G139–G148.