Expression of human α 1-acid glycoprotein genes in cultured cells and in transgenic mice

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The human genome contains three α 1-glycoprotein genes (AGP-A, AGP-B, and AGP-B') encoding for slightly different forms of the protein. The major component of human α 1-acid glycoprotein found in plasma is coded by AGP-A, which is expressed in liver and in hepatoma cell lines and is induced by inflammatory stimuli. We have studied the regulation of the cloned AGP-A gene by transfection into cell lines of hepatic and nonhepatic origin. Unlike any other liver-specific gene investigated so far, every AGP construct tested was expressed with comparable efficiency in hepatoma and HeLa cells. In contrast, identical constructs in transgenic mice are expressed in a tissue-specific manner and are regulated by acute-phase stimuli. Transgenic mice carrying the cluster of three AGP genes secrete the human protein in the serum, and the corresponding mRNA is mainly derived from the AGP-A gene. The mRNA is liver specific, and its concentration increases several fold following experimentally induced inflammation. Additional transgenic lines carrying only the AGP-A gene showed that sufficient information for tissue-specific and regulated expression is contained within a 6.6-kb segment comprising the whole coding region plus 1.2-kb 5'-flanking and 2-kb 3'-flanking DNA.

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A large number of genes are exclusively or preferentially expressed in hepatocytes. It is not known how liver-specific gene expression is established, maintained, and regulated in response to different stimuli such as acute infection, toxic agents, or dietary uptake (Chan 1982; Greengard 1971; Kushner 1982). Considerable information has been accumulated on the signals required in *cis* to direct hepatocyte-specific transcription and several *trans*-acting factors have been identified. Several genes coding for liver-specific proteins have been characterized, and their expression has been studied in various experimental systems: (1) by transfection of the cloned genes into cultured cells (Ott et al. 1984; Ciliberto et al. 1985; D'Onofrio et al. 1985; Colantuoni et al. 1987; De Simone et al. 1987; Heard et al. 1987; Oliviero et al. 1987); (2) more recently in cell-free nuclear extracts (Gorski et al. 1986); and (3) by microinjection of DNA constructs into fertilized eggs to generate transgenic mice (Palmiter and Brinster 1986; Wagner and Stewart 1986). The immediate conclusion from these studies is that there is no obvious structural or functional feature that is common to all liver-specific genes studied so far. Therefore, it is important to extend the investigation to several other liver-specific genes.

α 1-acid glycoprotein (AGP) is a plasmaprotein of unknown function, which is synthesized in the liver and

secreted into the blood. Its serum concentration increases several fold in many pathological conditions. Therefore, AGP is included among the positive 'acute-phase reactants' (Kushner 1982). The induction of AGP during the acute-phase reaction has been studied in various mammalian species. An increase in the mRNA concentration of about 100-fold has been observed in the rat (Ricca et al. 1981). Direct measurements of the acute-phase response of AGP mRNA in human liver have not been performed. However a 10- to 20-fold induction of AGP mRNA has been observed in Hep3B, a hepatoma cell line, when the cells were treated with conditioned medium from human peripheral monocytes, stimulated with bacterial lipopolysaccharide (LPS) (Darlington et al. 1986; Dente et al. 1987).

We have previously characterized the genes coding for AGP in human: there are three genes (AGP-A, AGP-B, and AGP-B') clustered in one locus and coding for two slightly different proteins. The product of gene A is the major component of plasma AGP; AGP-B and AGP-B' are identical, probably as a result of a recent duplication event. In our previous study we showed that only AGP-A can be induced by acute-phase stimuli in cultured hepatoma cells (Dente et al. 1987). Here we report an initial study to characterize the cell-specific expression and acute-phase induction of cloned human AGP

genes both in cell lines in vitro and in transgenic mice in vivo.

Results

Expression of the cloned human AGP-A gene in human cell lines

To characterize the regions responsible for tissue-specific expression of the AGP-A gene, whose product is the major component of plasma AGP, we first tested the ability of its promoter and 5'-flanking region to confer this property to a linked reporter gene. This strategy has previously been used for other liver-specific genes in several laboratories (Ott et al. 1984; Ciliberto et al. 1985; Colantuoni et al. 1987; De Simone et al. 1987; Heard et al. 1987; Oliviero et al. 1987). As the expression system, we used the human hepatoma cell line Hep3B, which expresses the AGP-A gene; as a nonhepatic control cell line, we used HeLa cells, in which the AGP-A gene is not active. The overall AGP gene organization and structure is identical in both cell lines as determined by Southern blot analysis (data not shown). We constructed a chimeric gene carrying 1228 bp of the 5'-flanking region and 17 bp of the coding region of the AGP-A gene linked to the coding sequence of the bacterial gene chloramphenicol acetyl transferase (CAT). In Figure 1a a schematic representation of the clone AGP+17CAT is shown; it is derived from *Bal31* treatment of plasmid HS1700, as previously described for other fusion plasmids of the same series (Dente et al. 1987). This construct was tested for transient expression by transfection in Hep3B and HeLa cell lines. As negative control AGP Δ 50CAT was used, a promoterless construction previously described (Dente et al. 1987). In contrast to similar constructs from other liver-specific genes studied so far, AGP+17CAT efficiently produced CAT enzyme in both cell types (Fig. 1b).

This result was confirmed at the RNA level by S1 mapping experiments. AGP+17CAT was co-transfected with RSV-CAT, in which the CAT gene is driven by the RSV enhancer-promoter cassette, and is used here as a non-tissue-specific internal marker (G. Morrone and L. Monaco, unpubl.). The results are shown in Figure 1c. Bands of protected RNA are indicated with arrows; band a, 150 bases long, corresponds to the CAT coding region of RSV-CAT transcript; band b, 201 bases long, corresponds to the major AGP transcript; band c, 223 bases long, corresponds to one of the minor AGP transcripts already observed in the endogenous AGP gene (Dente et al. 1987). AGP-CAT is transcribed with comparable efficiency in Hep3B and in HeLa cells, suggesting that the information for cell-specific expression is not located in the 1228 bases 5'-flanking the coding sequence, unlike all other liver-specific genes studied so far (Ott et al. 1984; Ciliberto et al. 1985; D'Onofrio et al. 1985; Colantuoni et al. 1987; De Simone et al. 1987; Heard et al. 1987; Oliviero et al. 1987).

Cell-specific signals could obviously be located else-

Figure 1. Expression of the human AGP promoter in Hep3B and HeLa cells. (a) Schematic representation of the plasmids used in the expression studies by transient transfection into HeLa and Hep3B cells. AGP+17CAT contains 1228 bp of the 5'-flanking region and 17 bp of the coding region of the AGP-A gene linked to the coding sequence of the bacterial gene chloramphenicol acetyl transferase (CAT). RSV-CAT carries 500 bp of RSV-LTR from pRSV-CAT (Gorman et al. 1982a). (b) Assay of the CAT enzymatic activity after transfection of AGP+17CAT and AGP Δ 50CAT. (c) S1 mapping experiment on total RNA extracted from HeLa and Hep3B cells, transfected with plasmids AGP+17CAT and RSV-CAT, using a uniformly labeled single-stranded probe synthesized in vitro by primer extension on PVU-AGP-CAT. Arrows indicate bands of protection (see text).

where in the AGP gene. To explore this possibility we constructed a plasmid, pHBB, carrying 1228 bases of 5'-flanking sequence and the complete AGP-A gene with about 2000 bp of 3'-flanking region (Fig. 2a). This construct was transfected in Hep3B and HeLa cells, and after 48 hr the RNA was extracted and subjected to Northern blot analysis using as probe AGP cDNA (Dente et al. 1985). The results, described in Figure 3, show that the transfected gene is efficiently transcribed in HeLa cells, yielding an mRNA indistinguishable from that synthesized in Hep3B cells. In these cells we assume that the increase in the amount of AGP mRNA observed in transfected Hep3B cells is due to the presence of transcripts from the transfected gene, comigrating with the endogenous transcript.

These data indicate that *trans*-acting factors capable of activating the transfected AGP gene are present in HeLa

Figure 2. Schematic representation of plasmid pHBB, carrying the truncated AGP-A gene, and of pCOS1, carrying all three AGP genes, previously characterized (Dente et al. 1987). Filled boxes represent exons; stippled lines represent vectors sequences. (H) *HindIII*; (B) *BamHI*; (C) *ClaI*; (S) *SalI*.

cells, whereas the resident chromosomal AGP gene is not expressed. This suggests that either the cell-specific controlling elements are located far away from the AGP-coding region and are not present in plasmid pHBB, or the mechanism responsible for cell-specific control of AGP gene expression is different from that operating on the other liver-specific genes studied so far. Rather than transfecting longer AGP DNA fragments in the search for putative controlling elements, we decided to investigate the expression of the human AGP gene in transgenic mice, thereby expecting that in this experimental system the specificity of expression of the cloned genes might more closely resemble that observed *in vivo*.

Generation of a transgenic mouse line carrying the human AGP gene cluster

It has clearly been shown in several reports that foreign genes inserted into the mouse genome by microinjection into fertilized eggs are efficiently expressed in the resulting transgenic mice. They often display a 'physiological' pattern of tissue-specific transcription and developmental regulation (Palmiter and Brinster 1986). We decided to investigate the behavior of cloned fragments of the human AGP genes in transgenic mice. For this purpose, we first used a 40-kb DNA fragment, containing the three AGP genes, that had previously been characterized (Dente et al. 1987) and is schematically shown in Figure 2b. The 40-kb insert was isolated as a *ClaI-SalI* fragment and microinjected into fertilized eggs of C3H

inbred mice. One AGP-transgenic mouse line (No. 221-6) was derived: the founder male carried intact copies of the complete cluster of AGP genes and was able to transmit it to his offspring in a Mendelian way.

Expression of human AGP in the plasma of AGP-transgenic mice

AGP-transgenic mice secrete human AGP into the blood, as shown by Western blot analysis using as probe a specific anti-human AGP antibody (Fig. 4). The broad shape of the bands shown in Figure 4 is a consequence of the heterogeneity in posttranslational modifications described by Schmid (1975). Quantitative analysis, performed by immunoassay, showed that the concentration in the serum of the transgenic mouse was comparable to that found in normal human serum (data not shown).

Transcription of the human AGP genes in the tissues of AGP-transgenic mice

Expression analysis was performed on total RNA extracted from various organs and tissues of transgenic mice by Northern blot assay using as probe human AGP cDNA (Fig. 5a), which does not cross-hybridize with the endogenous mouse AGP (lane M). An RNA species with the same size as human AGP mRNA was mainly found in the liver of transgenic mice. Very low expression was found in other organs (about 100-fold less than in liver), and it was detectable only after longer exposure of the

Figure 3. Northern blot analysis of total RNA extracted from Hep3B and HeLa cells transfected with plasmid pHBB and the vector pEMBL8 (mock).

autoradiographs (data not shown). Hybridization with a rat AGP cDNA probe (Baumann et al. 1986), which does not hybridize with human AGP (lane H), showed an identical pattern of expression for the endogenous mouse gene (Fig. 5c).

Acute-phase response of the human AGP gene in transgenic mice

The concentration of several plasma proteins increases during acute infections or in response to other pathological stimuli (Kushner 1982). This phenomenon observed in many mammals is called 'acute-phase reaction'. In all cases studied so far, the increment of plasma protein concentration appears to be a consequence of the increase in the corresponding mRNAs in the liver.

We investigated the effect of experimentally induced

Figure 4. Western blot analysis on sera from the AGP-transgenic mouse (TM), control mouse (M), and human (H).

Figure 5. Northern blot analysis of total RNA from various organs of (a, b) AGP-transgenic mice 221-6, using as probe human AGP cDNA and of (c, d) control mice using as probe rat AGP cDNA. Lanes: (M) RNA from liver of control mouse; (H) RNA from HepG2, a hepatoma cell line that produces AGP RNA (Dente et al. 1987). Numbers indicate different organs (3) pancreas; (4) liver; (5) thymus; (6) heart; (7) lungs; (8) gonads; (11) kidneys; (12) muscles; (13) brain; (14) salivary glands. (–) and (+) indicate mice untreated and treated, respectively, with 100 μ g LPS for 16–18 hr.

inflammation on transcription of human AGP genes in the transgenic mice. Mice were injected with different irritating agents such as turpentine or LPS. After 24 hr the animals were killed and AGP mRNA levels were determined by Northern blots of RNA extracted from various organs (Fig. 5b). We observe an approximately 10-fold induction of human AGP mRNA in liver, which is comparable with that observed for the endogenous mouse AGP RNA (Fig. 5d). On overexposing the autoradiograph shown in Figure 5b, we found a low level of induction in some other organs of the induced mice,

such as kidney or brain, suggesting that the acute-phase response of the AGP gene is not strictly a liver-specific phenomenon (data not shown). We conclude that the human DNA fragment incorporated in the mouse genome contains the information necessary for the acute-phase response.

Differential expression of AGP genes in transgenic mouse liver

In humans only AGP-A gene is efficiently expressed (Dente et al. 1987). To test the accuracy of transcription in the transgenic mice we analyzed the relative levels of expression of AGP-A and AGP-B/B' genes. We have previously shown that it is possible to discriminate between the product of the different genes using primer extension experiments with synthetic oligonucleotides corresponding to regions where they diverge (Dente et al. 1987). In the experiment shown in Figure 6 we used oligonucleotides o13A and o13B, which are complementary to exon 3 of AGP-A and AGP-B/B' genes, respectively, and which differ in six positions. After primer extension of transgenic liver RNA they give rise to a 323-base-long cDNA. The result also shows that in the

Figure 6. Primer elongation of liver RNA from AGP-transgenic mice untreated (–) and treated with LPS (+), using oligonucleotides o13A and o13B as primers. Lane [M] marker.

Figure 7. Northern analysis of total RNA from various organs of untreated (–) and from the liver of LPS-treated (4+) AGP-A transgenic mice of line no. 358-3. Organ numbers are identical to those in Fig. 5.

context of the mouse genome, AGP-A is preferentially expressed, and following experimentally induced inflammation it is mainly responsible for the observed induced level, as previously shown in the human hepatoma cell line (Dente et al. 1987). This result also confirms that the start site used in the transgenic mice is identical to that used in human liver and in human hepatoma cell lines (Dente et al. 1987).

Transgenic mice carrying only the human AGP-A gene

In light of the results obtained with the entire gene cluster in vivo, which confirmed the accurate, tissue-specific and regulated expression of the human genes, we decided to generate transgenic mice containing only the human AGP-A gene that was used for transfection in cultured cells (Fig. 2a). This gene was transcribed with comparable efficiency, when transfected in Hep3B and in HeLa cells (Fig. 3).

The DNA to be injected was isolated as a 7600-bp *Clal*–*SmaI* fragment, carrying all the human insert plus a region of the pEMBL8 plasmid corresponding to the F1 origin (Fig. 2). Two independent transgenic mouse lines (No. 358-3 and 382-5) were derived, both carrying intact copies of the human gene.

RNA analysis from various organs of the two transgenic mice showed AGP mRNA in liver, even though its basal level is somewhat lower than that observed in the mouse line 221-6, carrying the complete cluster (Fig. 7). However, the level of human AGP mRNA in the liver increases several fold following experimental inflammation, reaching approximately the same levels observed in induced transgenic mice carrying the complete cluster. In the transgenic mice the human AGP-A gene is clearly tissue-specifically expressed; this result is in striking contrast with the transfection data, which show a similar level of expression of this gene in cells of hepatic and nonhepatic origin.

Discussion

Cultured cells of hepatic or nonhepatic origin have been useful to define some of the putative mechanisms involved in liver-specific gene expression. Both human and rat hepatoma cell lines have been used successfully as recipients for cloned DNA fragments derived from genes coding for albumin, α 1-antitrypsin, retinol-binding protein, and haptoglobin (Ciliberto et al. 1985; Colantuoni et al. 1987; De Simone et al. 1987; Heard et al. 1987; Oliviero et al. 1987). It has been generally observed that relatively short DNA fragments located in the 5'-flanking region of these genes are capable of directing transcription in hepatoma cell lines, but not in cell lines of different origin. Furthermore there is good correlation between the expression of the transfected fragment of DNA and that of the resident chromosomal gene. This is most clearly shown in the case of the rat albumin gene, in which transcription of the transfected gene follows closely the expression of the endogenous gene in a series of differentiated and dedifferentiated hepatoma cell lines (Heard et al. 1987). In the case of the human haptoglobin gene, cell-specific and acute-phase-dependent transcription parallels that of the chromosomal gene in every human cell line tested (Oliviero et al. 1987). Therefore, cultured cells offered a suitable and convenient experimental system for the study of liver-specific gene expression. This appears not to be the case for human AGP gene expression.

We were surprised to observe that the 5'-flanking region of the AGP gene is capable of directing transcription not only in hepatoma cell lines but also in HeLa cells (Fig. 1). The 5'-flanking region of all other liver-specific genes contains sufficient information for restricting transcription to cell lines of hepatic origin. We believe that this phenomenon is not due to lack of regulatory signals in the fragment of DNA used for transfection because the same construct is expressed in a liver-specific manner in transgenic mice (Fig. 7). The frequent observation that DNA fragments from cell-specific genes are expressed in a cell-specific manner when introduced in the appropriate cell types can be taken as evidence that diffusible cell-specific *trans*-acting factors play a role in the maintenance of differential gene expression. In the case of AGP the discrepancy between the specificity of expression of the chromosomal gene *in vivo* and the lack of specificity with the transfected DNA *in vitro* indicates that other mechanisms may be involved.

The HeLa cell transcriptional apparatus is capable of recognizing the AGP promoter, nevertheless the chromosomal gene is silent in these cells. This observation might be related to the phenomenon of 'extinction,' where cell-specific gene expression is negatively regulated in nonexpressing cells (Killary and Fournier 1984; Petit et al. 1986; Chin and Fournier 1987). Perhaps the gene is repressed by a factor whose concentration is limiting and therefore insufficient to repress the many copies of the gene introduced by transfection. Alternatively the gene might be inaccessible to positive *trans*-acting factors because of its chromatin conformation or

because of methylation at crucial residues. Negative control, gene inaccessibility, or both might also play a role in the lack of expression of the AGP gene in tissues other than liver of the transgenic mice. It is of course well established that methylation of DNA represses several genes: many observations correlating methylation with repression have been reported, both for housekeeping and cell-specific genes (Adams and Burdon 1985; Jones 1985). Inaccessibility to *trans*-acting factors was postulated to explain some of these results: for instance in mouse myoblast-HeLa cells heterokaryons, the human cell surface antigen 5.1H11 and the creatine kinase genes were turned on only if the HeLa cells had been treated with aza-cytidine prior to fusion with myoblasts (Chiu and Blau 1985). Recently Becker et al. (1987) have reported that the TAT gene is inaccessible *in vivo* to *trans*-acting factors that are present in the cell and are capable of binding to naked DNA. The authors also present convincing evidence that DNA methylation might be responsible for these phenomena (Becker et al. 1987). It will be interesting to investigate the 'status' of the AGP gene by DNase I sensitivity, methylation, and *in vivo* footprint experiments using expressing and non-expressing cell lines and the various tissues of transgenic mice.

We have reported previously that in human liver and in human hepatoma cell lines the first gene of the cluster, AGP-A, is preferentially expressed. The same phenomenon is observed in the transgenic mouse carrying the human 40-kb fragment in which all three AGP genes are included (Fig. 6). The DNA sequence of AGP-B and -B' does not indicate any obvious reason for their lack of transcription: In fact their 5'-flanking region shows a 99% homology with that of AGP-A. The reciprocal position in the cluster might be important for expression: It will be interesting to investigate the expression of AGP-B or -B' after removal of the AGP-A gene.

In the transgenic mouse liver, as in human liver or in human hepatoma cell lines, AGP-A gene is induced several fold during experimentally induced inflammation. From our data we can conclude that sufficient information for tissue-specific expression and acute-phase induction of the human AGP-A gene is contained in the 6.6-kb fragment, comprising the whole coding region, with 1228 bp of 5'-flanking and about 2000 bp of 3'-flanking sequences. With respect to induction, this observation is in contrast with the data on the rat AGP gene (H. Baumann, pers. comm.). There a DNA sequence has been identified, which is located about 5000 bp upstream of the transcriptional start point and which is essential for acute-phase induction of the rat gene transfected in human hepatoma cell lines. It is possible that the relevant DNA sequences are located in different positions in the rat and the human gene and/or the inductions in the hepatoma cell line and in the mouse are based on different mechanisms.

The work presented in this paper underscores the importance of *in vivo* studies. While the specificity of AGP gene expression could not be reproduced in cultured cells, the pattern of expression in transgenic mice ap-

pears to be 'physiological': it closely resembles the pattern of expression of the endogenous mouse gene, and it reflects what is known about the regulation of the human gene in human cells. Similar findings were obtained with other human liver-specific genes using transgenic mice. Three reports describe the correct expression of the human α 1-antitrypsin gene (Kelsey et al. 1987; Rüther et al. 1987; Sifers et al. 1987); recently Ciliberto et al. (1987) showed that the human C-reactive protein gene is properly regulated in mice and that a high level of induction was obtained following acute-phase inflammation. All these studies clearly demonstrate the usefulness of the mouse as a system to analyze the regulation of human genes.

Materials and methods

Bacterial strains and plasmids

Escherichia coli K12 (Strain 71/18 and K514) was used for transformation. Plasmids pEMBL8 and TG131 (Dente et al. 1983; Kieny et al. 1983) were used for cloning. The rat AGP cDNA clone was a gift from H. Baumann.

Enzymes and chemicals

Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, DNA polymerase I holoenzyme, Klenow fragment, and S1 enzyme were purchased from BRL (Neu Isenburg, FRG) and New England Biolabs (Schwalbach, FRG). AMV reverse transcriptase of Seikagaku was a gift from C. Schneider. 32 P-labeled compounds were purchased from Amersham (Braunschweig, FRG); 14 C-labeled chloramphenicol was from NEN (Dreieich, FRG) and acetyl-coenzyme A from Sigma (St. Louis, Missouri).

Blot hybridization, S1 mapping, and primer extension

Northern blot analysis was performed as previously described (Dente et al. 1985). The S1 mapping experiment was carried out as previously described (Ciliberto et al. 1985). The probe was isolated from plasmid PVU-AGP-CAT, a subclone of AGP+17CAT including 502 bp of AGP, 36 bp of linker sequence, and 150 bp of CAT sequence.

Primer elongation experiments with specific oligonucleotides were carried out as described by D'Onofrio et al. (1985) with a few modifications to improve the specificity of hybridization: 10 μ g of total RNA were annealed with 0.1 pmole of oligonucleotides at 55°C for 1 hr. After isopropanol precipitation to eliminate unannealed oligonucleotides, polymerization by reverse transcriptase was performed at 42°C.

Cell culture, DNA transfections, and CAT assays

Human hepatoma cell lines Hep3B (Knowles et al. 1980) and HeLa cells were cultured as monolayers in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

DNA transfections were performed by the calcium phosphate precipitation technique (Graham and van der Eb 1973). Cells were transfected with 5 μ g (5-cm dishes) or 15 μ g (10-cm dishes) of plasmid DNA. Transfected cells were harvested 48 hr after transfection either for CAT assay or for RNA extraction by the guanidine thiocyanate procedure (Chirgwin et al. 1979). The CAT assays were performed according to Gorman et al. (1982b).

Microinjection

In principle all the manipulations were performed as described in Hogan et al. (1986).

Electrophoresis, electroblotting, and immunological reactions

Slab gel electrophoresis was done according to Laemmli (1970) with 12% acrylamide for the running gel and 4% acrylamide for the stacking gel. Electrotransfer of proteins to nitrocellulose membranes was performed as in Towbin (1979). Immunological reactions were performed in the presence of 5% mouse serum, 0.5% Triton X-100 using rabbit anti-human α 1-AGP and swine peroxidase conjugated anti-rabbit IgG antibodies from Dakopatts (Denmark).

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