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Inducible and tissue-specific expression of human C-reactive protein in transgenic mice

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C-reactive protein (CRP) is a major acute phase reactant in man but not in mouse. It is synthesized in abundant quantities by human hepatocytes during the course of several diseases, mainly acute inflammations. To investigate the regulation of CRP expression, the human CRP gene was introduced into fertilized eggs by microinjection and transgenic mouse lines were derived. The CRP gene is exclusively transcribed in the liver and expression is strictly dependent on experimental inflammation. The kinetics of induction both for RNA and protein synthesis is very fast; RNA is first detectable after 2 h in the liver, the protein after 6 h in the serum. Human CRP levels in the sera of transgenic mice are comparable to those observed in human diseases. Nuclear run-on experiments indicate that regulation is primarily at the transcriptional level.

Key words: human C-reactive protein/acute phase response/transgenic mice/transcriptional regulation

Introduction

The acute phase response is characterized by a dramatic change in the metabolism of liver cells following stress, inflammation or infections of various origin (Kushner, 1982). As a consequence the serum concentration of several liver-specific proteins with a wide spectrum of functions is increased. These proteins are referred to as positive acute phase reactants. At the same time the serum levels of albumin and other proteins including lipoproteins show a 2- to 3-fold decrease. These changes are the consequence of the increased or decreased production of these proteins in the hepatocytes and the steady-state level of the mRNAs mirrors the changes in the quantity of protein synthesized (Ricca *et al.*, 1981; Crabtree and Kant, 1982; Baumann *et al.*, 1983; Whitehead *et al.*, 1983; Ramadori *et al.*, 1985; Perlmutter *et al.*, 1986).

One of the major acute phase reactants in man is C-reactive protein (CRP), which belongs to the pentraxin gene family together with the serum amyloid protein (SAP) and the female hamster protein (for a review, see Pepys and Baltz, 1983). During acute inflammation the serum level of human CRP rises from <1 mg/l to 300 mg/l (Kushner and Feldman, 1978) and this 1000-fold increase in concentration corresponds with the availability of the specific mRNA in liver cells (Whitehead *et al.*, 1983). Although the major site of CRP synthesis is the liver (Kushner and Feldmann, 1978), a complete analysis of CRP expression in all human tissues has not been carried out. More recently, however, Kuta and Baum (1986) have reported that some CRP synthesis also takes place in a subset of T lymphocytes with natural killer activity.

The molecular mechanisms governing the regulation of human CRP expression are widely unknown. The gene has been cloned and sequenced (Lei *et al.*, 1985; Woo *et al.*, 1985; Ciliberto *et al.*, 1987; Goldman *et al.*, 1987). It is located on chromosome 1 but no close linkage has been established with the homologous SAP gene, located on the same chromosome (Mantzouranis *et al.*, 1985). The different members of the pentraxin gene family show a peculiar pattern of gene activation during inflammation. CRP is highly induced in man but not in mice, where its serum levels never raise above 2–3 mg/l (Pepys, 1979). On the contrary, SAP is a major acute phase reactant only in mice but not in man (Pepys *et al.*, 1979). Several hypothesis have been postulated to explain this species-specific pattern of gene expression: one is that the structure of the *trans*-regulatory proteins responsible for gene activation during inflammation has undergone drastic changes during evolution from rodents to primates and therefore only the human transcription factors involved in the acute phase response are capable of interacting with the CRP gene. Alternatively, only the human CRP gene or the mouse SAP gene have evolved those *cis*-regulatory sequences necessary for the interaction with evolutionary-conserved *trans*-acting factors.

Transgenic mice provide a powerful tool to study the regulation and the tissue-specificity of gene expression. The newly introduced genes at different positions in the mouse genome often exhibit a pattern of developmental and tissue-specific control of gene expression, which is identical to that shown in the natural environment from which they have been isolated (Palmiter and Brinster, 1986; Wagner and Stewart, 1986). In a few cases transgenic mice have allowed a precise definition of the *cis*-regulatory elements necessary for the developmental switch in the expression of a certain gene or the determination of sequences required for tissue or cell-specific expression (Ornitz *et al.*, 1985; Hammer *et al.*, 1987; Pinkert *et al.*, 1987).

We have started to analyse the regulation of human CRP in transgenic mice. Unlike the mouse counterpart, human CRP behaves as a major acute phase reactant when introduced into the mouse genome. The gene is quantitatively expressed in the liver only upon induction and expression is regulated at the transcriptional level.

Results

Generation of transgenic mice carrying the human CRP gene

We have previously characterized a cosmid clone carrying the human gene for CRP (pCOS-CRP1) (Ciliberto *et al.*, 1987). A 30-kb *Cla*I DNA fragment from pCOS-CRP1 was isolated which eliminates most of the vector sequences except for 300 bp at the 3′ end. The CRP gene is located in the centre with >16 kb of 5′ flanking sequence and ~ 10 kb of 3′ flanking sequence. This fragment was injected into the pronucleus of eggs of F_2 (C57/BL6 × SJL) hybrids. Four DNA-positive animals were identified by Southern blot analysis. Of these only two (292-9 and 292-10) were able to transmit the human CRP gene to offspring and those were chosen for further analysis.

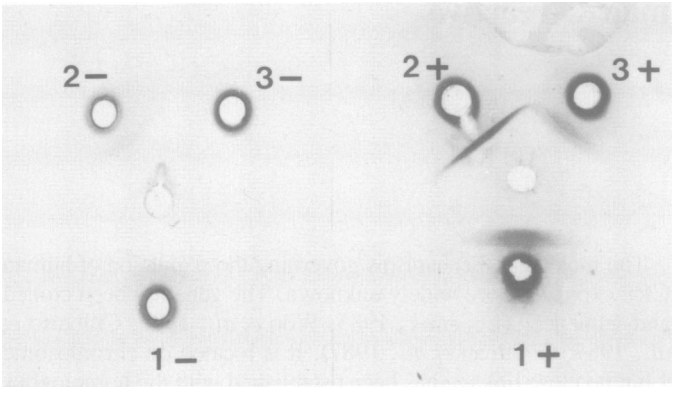

Fig. 1. CRP induction by different irritants. The result of a double immunodiffusion assay is shown. Three mice of family 292-10 were bled and afterwards injected with 100 μg of *Escherichia coli* lipopolysaccharide (LPS) (1), 200 μl of a 1% croton oil solution (2) or with 500 μl supernatant of induced human monocytes (3). The sera obtained before (−) and after 18 h induction (+) were reacted with antibodies against human CRP.

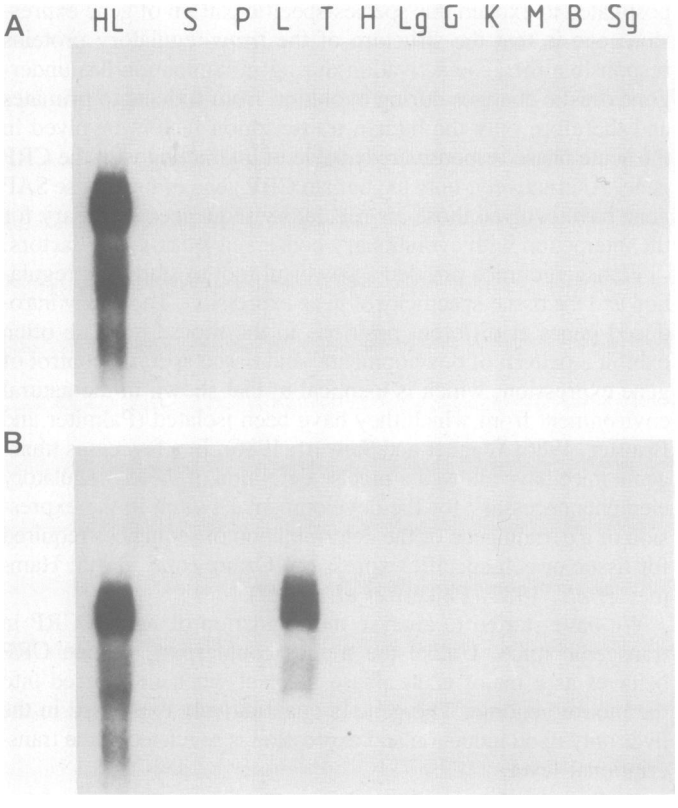

Fig. 2. Expression profile of CRP mRNA in transgenic mice. RNA from a transgenic mouse (292-10) was extracted from various organs before (**A**) or after induction with 100 μg LPS (**B**). Identical amounts of total RNA (20 μg) were subjected to agarose−gel electrophoresis and blotted to nylon membranes. A human CRP-specific cDNA probe was used for hybridization. Abbreviations are: HL, human liver; S, spleen; P, pancreas; L, liver; T, thymus; H, heart; Lg, lungs; G, gonads; K, kidneys; M, skeletal muscle; B, brain; Sg, salivary glands.

Human CRP behaves as a major acute phase reactant in transgenic mice

Initially we established the presence of the human CRP protein in the sera of mice from the two transgenic families before and after induction with several irritating agents such as bacterial lipopolysaccharide (LPS), croton oil or conditioned medium from human peripheral monocytes stimulated with LPS (Darlington *et al.*, 1986). This is possible since the endogenous CRP levels in mice are very low and antibodies raised against human CRP do not detect mouse CRP in the sera of uninduced or induced animals. Blood was taken from the experimental mice at time 0 and 18 h after injection of the inducer. The results from an immunodiffusion test on three members of family 292-10, each one treated with a different irritant, are shown in Figure 1. Human CRP is highly increased in the serum of the animals after exposure to different inflammatory stimuli. The same response has also been observed in mice from the second family 292-9 and in the two founder animals which did not transmit the gene to their offspring (data not shown). The faint ring-like precipitation which

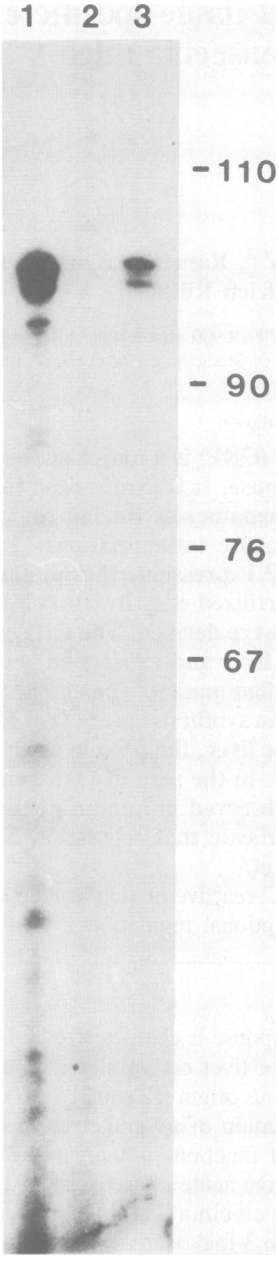

Fig. 3. Primer elongation analysis on CRP mRNA in transgenic mice. The primer used was a synthetic oligonucleotide complementary to CRP mRNA from nucleotide 73 to nucleotide 97. **Lane 1**, 20 μg human liver RNA in acute phase; **lane 2**, 20 μg liver RNA from a CRP transgenic mouse; **lane 3**, 20 μg liver RNA from CRP transgenic mouse stimulated for 24 h with 100 μg LPS.

can be seen in wells 1 and 2 of the uninduced mice is probably due to some unspecific reaction of the antibody rather than to a basal level of CRP production. As shown by a more sensitive ELISA assay (see below) the CRP protein was never detected in the serum of uninduced animals. It is of interest to note that the level of inducibility of the CRP gene is strain dependent. The SJL strain is known to be defective for the expression of certain acute phase genes (Yamamoto *et al.*, 1986). For our initial breeding F_1 (C57/BL6 × SJL) mice were used. These crosses have therefore resulted in a segregation of the genetic background which might explain the fact that responding and non-responding offspring in both families were observed. Therefore, we backcrossed mice from both families to the C57/BL6 strain and found comparable levels of induction in almost all offspring.

CRP transcription is liver specific

We have performed an analysis of human CRP mRNA expression in 11 different tissues before and after injection of LPS. The results are shown in Figure 2. CRP mRNA is not detectable without inflammation, but an mRNA with identical migration and abundance as human CRP mRNA is produced in liver after experimentally induced inflammation. In addition, there is a precise correlation between the appearance of mRNA in the liver and the appearance of the protein in sera (see below). We could not detect any CRP mRNA in other tisuses such as thymus or spleen, which are rich in lymphocytes; therefore we can conclude that, if the gene is transcribed in these cells, the rate of transcription must be very low compared with that in hepatocytes.

To investigate the initiation site of CRP transcription in transgenic mice we have performed a primer elongation analysis on total RNA from uninduced and induced liver cells making use of a synthetic oligonucleotide complementary to CRP mRNA from nucleotide 73 to nucleotide 97 (Lei *et al.*, 1985). The result is shown in Figure 3, which confirms and extends the results of the Northern analysis: CRP mRNA is produced only after exposure to LPS and the RNA produced in transgenic mice starts at the same nucleotide as in humans.

Time course of CRP mRNA and protein synthesis

Since CRP is one of the acute phase reactants whose synthesis is undetectable in the absence of an inflammatory status, the induction of expression can easily be studied. The kinetics of appearance of CRP mRNA in the liver of transgenic animals was examined after LPS injection. Mice which showed high levels of protein secretion after induction were used for these experiments. One week after the first induction, these mice were injected again with LPS, and RNA was extracted from the livers at different times. RNA is already detectable after 2 h; the peak of RNA production is reached 9–10 h after LPS injection but the RNA disappears rapidly and is almost undetectable after 24 h (Figure 4A). It is not possible to determine the half-life of CRP mRNA in the whole animal but CRP mRNA seems to be rather unstable; at 18 h only about one-tenth of the RNA detected after 9 h is still present in the liver tissue. This is also suggested by the presence of breakdown products in the total RNA preparations which do not contain breakdown products for the endogenous α_1-acid glycoprotein or albumin mRNAs (not shown).

In addition to liver-specific RNA synthesis we have also determined the kinetics of CRP appearance in the blood. Serum samples were collected at different times and CRP concentration was determined by an ELISA test. In all cases CRP is not detected in uninduced transgenic mice and the highest level of CRP protein in the sera is reached 15 h after LPS injection with a decline to basal levels within 48 h (Figure 4B). The maximal

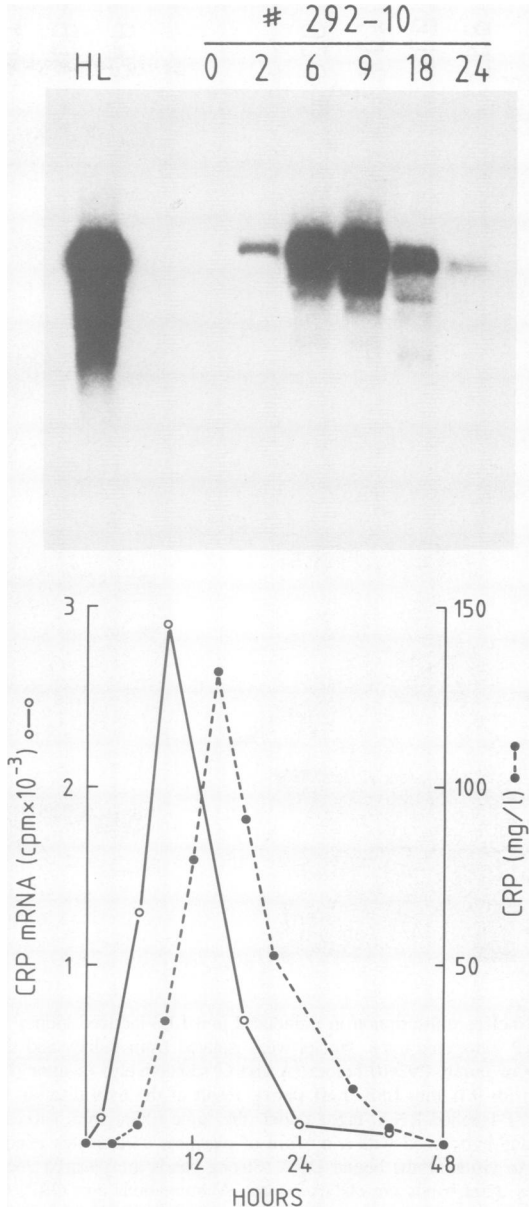

Fig. 4. Kinetics of CRP mRNA and protein synthesis. (**A**) Liver RNA was isolated after 0, 2, 6, 9, 18 or 24 h after induction with 100 μg LPS and analysed by Northern blotting as described in Figure 2. (**B**). The kinetics of RNA accumulation shown in (**A**) has been quantitated by cutting out the positions of the bands on the membrane and measuring the bound radioactivity. In addition CRP protein production was measured in the serum at different time points. Presented is the average from two animals induced with 100 μg LPS. The quantitation of the protein in the sera was carried out as described in Materials and methods.

levels vary by a factor of 5 from animal to animal, being on average ~130 mg/l. We have also observed, in some cases of induction with other irritants, such as azocasein or croton oil, maximum values of up to 350 mg/l, which are similar to the maximal values observed during the course of several human diseases.

Transcriptional control of CRP expression

To establish whether the regulation of CRP expression takes place at the transcriptional level or post-transcriptionally via stabilization of the mRNA we used the nuclear run-on assay (McKnight and Palmiter, 1979). *In vitro* transcription in purified nuclei essen-

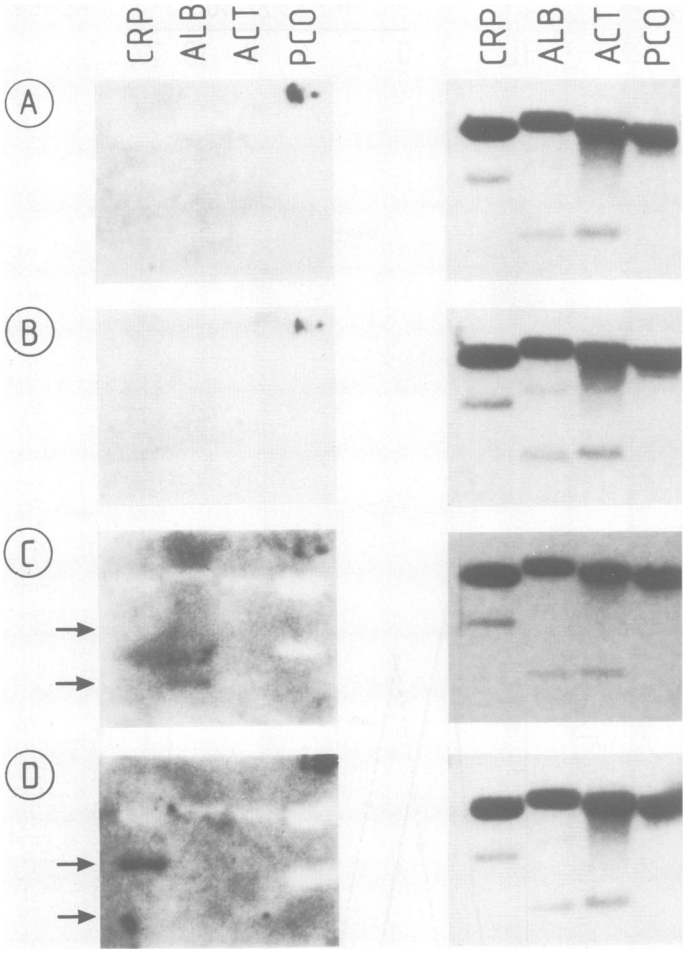

Fig. 5. Nuclear transcription in uninduced and LPS-induced kidney and liver from CRP transgenic mice. Nuclei were isolated from kidney and liver of offspring of family 292-10 before (**A** and **C** respectively) or after (**B** and **D**) induction for 9 h with LPS. **Left panel**: result of the hybridization to nascent ^{32}P-labelled RNA; **right panel**: the same filters were successively washed and hybridized with a mixture of plasmids used in this experiment in order to visualize the bound DNA. Strong bands are plasmid vector sequences, faint bands are cDNA inserts. Abbreviations are: CRP, C-reactive protein cDNA clone; ALB, mouse albumin cDNA clone; ACT, human β-actin cDNA clone; PCO, plasmid control.

tially measures the rate of gene transcription *in vivo*, because under these conditions RNA polymerase II is only able to elongate nascent RNA chains but not to initiate new rounds of RNA synthesis. We purified nuclei from liver or kidney (as a negative control) before and 9 h after LPS injection and transcribed RNA *in vitro*. The labelled RNA was hybridized to Southern blots carrying different DNA fragments. The results are shown in Figure 5. Kidney cells (panels A and B) are not actively transcribing CRP or albumin either before or after LPS induction; on the other hand, liver nuclei transcribe albumin but not CRP in the absence of LPS (panel C). As expected, the rate of transcription of the albumin gene drops after LPS administration (panel D). At the same time the rate of transcription of the CRP gene is greatly enhanced. Hybridization to the actin fragment was not detected by any of the four nuclear run-on experiments. Therefore we cannot exclude a basal level of expression of CRP equivalent to that of the actin gene. Nevertheless, the results support the conclusion that CRP gene expression is regulated at the transcriptional level.

Discussion

In this paper aspects concerning the regulation of human CRP gene expression in mice are described. Whereas CRP is highly expressed during inflammation in man, no induction of the endogenous gene is observed in the mouse. However, the human gene, when transferred into mice behaves as it does in man: its expression is highly inducible and tissue specific. It appears that the human regulation signals are correctly recognized by the transcription factor in mice and accurately initiate RNA synthesis. In addition, nuclear run-on experiments show that gene expression is predominantly regulated at the transcriptional level.

An interesting aspect of the acute phase response is the species specificity of individual acute phase proteins. For instance, α_2-macroglobulin (α_2-MG) is a major acute phase reactant in rats but not in mice or man (Kushner, 1982); SAP is induced several-fold during inflammation in mice but not in man; the reverse being true for CRP (Pepys and Baltz, 1983). Our results with the CRP gene suggest that the species specificity is exclusively due to the properties of the individual genes and to the way *cis*-acting regulatory sequences located in them are able to interact with widespread and evolutionary conserved *trans*-acting regulatory factors. During the course of evolution, the promoter or the enhancer regions of the genes encoding the acute phase reactants have probably undergone more or less dramatic changes leading to a better or poorer affinity for general transcription factors which mediate the response to inflammation. These changes have been extreme in the case of CRP, α_2-MG or SAP and less pronounced for the minor reactants; they are probably the consequence of a selective pressure based on the need for each individual species to present a certain pattern of serum protein production in order to better counteract the effects of stress, trauma or inflammation.

The CRP levels reached in the sera of the transgenic animals in conditions of experimental inflammation are similar to those reported during the course of various diseases in humans. This means that there is a proper interaction between the gene and the mouse factors responsible for gene activation. Several inducers are able to elicit CRP induction, including conditioned media from LPS-stimulated human peripheral monocytes. This medium contains hepatocyte stimulating factors (HSFs) responsible for the induction of CRP expression in a cultured human hepatoma cell line Hep3B (Darlington *et al.*, 1986). Human HSFs are, therefore, able to interact with the appropriate receptors on the surface of mouse hepatocytes.

CRP induction is rapid and the response can be considered as extinguished after 24 h. In the case of LPS injection the peak of protein secretion in the serum is reached at 15 h, preceded by a peak of mRNA produced in the liver. The two kinetics are shifted by ~5 h. While RNA is already detectable in the liver after 2 h, the protein is not yet present in the serum. The maximal RNA values are reached after 9 h, when the protein starts to be secreted. This rapid response stresses the importance of CRP in the first hours after the onset of inflammation as a general ligand for phospholipids and nucleic acids released from damaged tissues (Narkates and Volanakis, 1982; Robey *et al.*, 1984). Our data indicate that, among the acute phase reactants, CRP has probably the most rapid induction kinetic. For example, in rats and in mice α_2-MG mRNA peaks 18 h after turpentine injection (Gehring *et al.*, 1987); in mice SAP peaks 24 h after injection of LPS or croton oil (Pepys *et al.*, 1979; Le *et al.*, 1982). The peak of hemopexin synthesis is reached 2 days after turpentine injection in chicken (Grieninger *et al.*, 1986). In the case of mouse serum amyloid A protein (SAA), the three genes are

turned on rapidly and the steady-state RNA levels for SAA1 and SAA2 remain elevated from 12 to 36 h after LPS injection (Lowell *et al.*, 1986). RNA transcription of the SAA genes is also turned on rapidly but declines quickly and long before the decline in mRNA accumulation. The authors have therefore postulated the existence of a mechanism of mRNA stabilization in order to justify the presence of high SAA1 and SAA2 mRNA levels in the liver long after RNA transcription has ceased.

From our run-on experiments we conclude that CRP expression is mainly regulated at the transcriptional level. Even though we have not determined the kinetics of gene transcription, the very rapid decline of steady-state RNA in the liver argues against involvement of CRP mRNA stabilization during the acute-phase response. Finally, transfection experiments using the human hepatoma cell line Hep3B stimulated with conditioned medium from peripheral monocytes have shown that the human CRP 5′ flanking region is able to induce the expression of a linked reporter gene (R.Arcone, G.Gualandi and G.Ciliberto, in preparation). Birch and Schreiber (1986) postulated the existence of two classes of genes whose expression is increased during acute-phase. α_1-acid glycoprotein (α_1AGP) and α_2-MG belong to the first class where an increased transcription rate is accompanied by increased mRNA stability. Fibrinogen and the α_1 major acute phase protein belong to the other class where increased gene expression is attributable to an increased rate of transcription. According to the results shown in this paper CRP belongs to this second class of reactants. In this respect the activation of CRP gene expression closely resembles gene induction by glucocorticoids, metal ions or heat shock (Ringold *et al.*, 1977; Pelham, 1982; Karin *et al.*, 1984). The information reaching the hepatocytes in the form of HSFs is rapidly transferred to the nucleus where transcription is turned on, but the association of the relevant transcription factors with the gene is not permanent and does not result in a prolonged stimulation of gene expression. One possible explanation is that CRP gene induction is dependent on the modification of a pre-existing transcription factor brought about by HSFs. This modification must be either transient or the protein is very unstable to explain the rapid extinction of the response in the absence of a continuous stimulus.

Another important aspect of CRP expression is the tight regulation of this gene. Several groups have already reported on accurate tissue specificity of genes introduced in transgenic mice (Palmiter and Brinster, 1986; Wagner and Stewart, 1986). In these cases, however, the genes are constitutively expressed in the cells of the specific organ or tissue. This is the first report on a transgene whose expression is limited to one tissue, the liver cell, but is also strictly dependent on a specific stimulus. In our studies we were never able to detect mRNA or protein in the absence of inflammation. Other inducible promoters, like mouse or human metallothionein promoters have been found to be leaky and are functional in several tissues in transgenic mice (Palmiter *et al.*, 1983; Hammer *et al.*, 1985).

Liver-specific gene expression has already been studied in transgenic mice (Babinet *et al.*, 1985; Pinkert *et al.*, 1987); e.g. for the mouse albumin gene an enhancer like element has been localized 10 kb upstream from the cap site (Pinkert *et al.*, 1987). In the case of the CRP gene, it should be possible to introduce 5′ deletion mutants into mice, which will allow determination of the regulatory elements important for the tissue-specific and inducible expression. The identification of these elements will be of importance in defining problems concerning liver organogenesis. Fusion between the CRP promoter and oncogenes, for example, will allow both growth and differentiation of liver cells to be influenced in a regulatable fashion. Proper manipulation of such a system in transgenic mice will offer the opportunity to study the molecular biology of liver development and regeneration.

Materials and methods

Mice and microinjection

F_1 (C57/BL6 × SJL) mice were bred in the mouse colony at the EMBL and the eggs for microinjection were isolated from F_1 crosses after superovulation. All the manipulations were performed as summarized in Hogan *et al.* (1986).

RNA analysis and primer elongation

Total RNA was isolated according to Chirgwin *et al.* (1979). RNA was separated in formaldehyde-containing agarose gels, transferred to nylon membranes and hybridized to either nick-translated or oligo-labelled probes. As probe we used the full-length human cDNA for CRP from clone pCRP5 (Woo *et al.*, 1985). Primer elongation experiments were performed according to Bensi *et al.* (1985) using as a primer a 25-base-long synthetic oligonucleotide labelled at the 5′ end, which is complementary to nucleotides 73–97 of the CRP mRNA.

Protein analysis

Double immunodiffusion assays were performed in 1% agarose in phosphate-buffered saline. The central well contained 1 μl of rabbit antiserum to human CRP (DaKopatts GmbH). The peripheral wells contained 2 μl each of transgenic mice sera. Human CRP concentration with sera of transgenic mice was determined with an ELISA assay (Sopazyme-RP test, LBL, Hamburg).

Nuclear run-on transcription

Measurements of transcription in isolated nuclei were as described by Vannice *et al.* (1984). Fifty milligrams of liver and kidney tissues from CRP transgenic mice before and 9 h after 100 μg LPS injection were homogenized in each experiment and 50 μl of freshly isolated nuclei at the concentration of 10^8 nuclei/ml were used for each *in vitro* transcription reaction. Labelled RNA was extracted using the trichloroacetic acid precipitation method (Groudine *et al.*, 1981) and hybridized to Southern blots which were prepared as follows: 5 μg of plasmid DNA was digested with various restriction enzymes, separated by electrophoresis in 1% agarose gels and transferred to nylon membranes as described (Church and Gilbert, 1981). RNA was allowed to hybridize for 72 h and filters were washed for 5 h in 40 mM NaPi pH 7.2, 1% SDS at 65°C. The plasmids were pCRP5, a full length CRP cDNA clone (Woo *et al.*, 1985), pAlb2, a 700-bp mouse albumin clone (kindly provided by Shirley Tilghman) and pH A-3′ UT, a human β-actin clone (Ponte *et al.*, 1983).

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