Studies of translatable mRNA for rabbit C-reactive protein

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C-reactive protein (CRP) mRNA was assayed by cell-free translation of poly(A)-containing liver RNA isolated both from rabbits stimulated to undergo the acute-phase response and from unstimulated control rabbits. No CRP-related translation products were identified until the denaturant methylmercury hydroxide (CH_3HgOH) was added to the RNA before cell-free translation. In the presence of the denaturant, a 24000-Da translation product was synthesized which was immunochemically identifiable as the CRP primary translation product. It is likely that rabbit CRP mRNA can form a stable intramolecular duplex which interferes with its translatability *in vitro*. The 24000-Da CH_3HgOH -facilitated cell-free translation product was not detected in poly(A)-containing liver RNA from unstimulated animals, indicating that the concentration of translatable CRP mRNA was dramatically induced during the acute-phase response. On the basis of absorption experiments, the 24000-Da CRP primary translation product was immunochemically more closely related to denatured CRP than to native CRP.

In vertebrates, part of the systemic response to a wide variety of inflammatory stimuli is the increase in concentration of a group of serum proteins of hepatic origin, the acute-phase proteins (for review see Kushner, 1982). CRP was the first such protein to be discovered, and is prototypic of this group (Tillett & Francis, 1930). Although CRP is found in a number of phylogenetically diverse species, it does not always behave as an acute-phase reactant (Baltz *et al.*, 1982; Liu *et al.*, 1982). In rabbits and humans, CRP serum concentrations may increase 1000-fold or more over those found in uninfamed individuals. Circulating concentrations of the protein generally parallel the course of the inflammatory response (Kushner *et al.*, 1978; Pepys, 1981).

Both human and rabbit CRP are composed of five identical subunits, of about 21 000 Da (Gotschlich & Edelman, 1965). Although most acute-phase proteins are glycoproteins, CRP in rabbits and humans is non-glycosylated (Osmand *et al.*, 1977a,b). The dramatic increase in CRP serum concentration has been shown to be due to new synthesis of the protein by hepatocytes after an

inducing signal from an unknown mediator (Kushner & Feldmann, 1978; Macintyre *et al.*, 1983).

We sought to develop an assay for CRP mRNA to begin an analysis of the molecular events responsible for inducing and controlling CRP synthesis during the acute-phase response in rabbits. Increased amounts of mRNA and/or translatable mRNA for a number of glycosylated acute-phase proteins in mice and rats have been demonstrated: SAA (Morrow *et al.*, 1981), α_1 acid glycoprotein (Ricca *et al.*, 1981; Northemann *et al.*, 1983a), hemopexin (McLean & Taylor, 1983), α_2 -macroglobulin (Northemann *et al.*, 1983a,b), α_1 -proteinase inhibitor (Northemann *et al.*, 1983b), transferrin (Northemann *et al.*, 1983b) and fibrinogen (Princen *et al.*, 1981). We show here that the concentration of translatable rabbit hepatic CRP mRNA increases after an inflammatory stimulus and that rabbit CRP mRNA can form a stable secondary structure which either prevents or decreases its translatability *in vitro*.

Materials and methods

RNA isolation

The acute-phase response was induced in two New Zealand White rabbits (stimulated) by the

Abbreviations used: CRP, C-reactive protein; SDS, sodium dodecyl sulphate; CH_3HgOH , methylmercury hydroxide; SAA, serum amyloid A.

intramuscular injection of 0.5–1 ml of turpentine in each thigh 24 h before they were killed. Control (unstimulated) rabbits received no inflammatory stimulus. Blood samples were obtained from the marginal ear vein at the time of death, and CRP concentration was measured by radial immunodiffusion as described previously (Kushner & Somerville, 1971). Stimulated rabbits had CRP serum concentrations at the time of death of 60 and 70 µg/ml, whereas the unstimulated control had a serum concentration of 3 µg/ml. For RNA isolations, 5 g of fresh liver was immediately homogenized in guanidinium thiocyanate and RNA was prepared as described by Chirgwin *et al.* (1979). Poly(A)-containing RNA was isolated by chromatography on oligo(dT)-cellulose (Aviv & Leder, 1972). Finally, the RNA was precipitated with ethanol and dissolved in water at 0.5 mg/ml, stored in batches at -70°C until use.

Translation in vitro

RNA was translated either in wheat-germ extracts prepared as described by Roberts & Paterson (1973) or in nuclease-treated rabbit reticulocyte lysate (Pelham & Jackson, 1976) (New England Nuclear). [^{35}S]Methionine was purchased from New England Nuclear or Amersham. Each translation system was optimized for total [^{35}S]methionine incorporation by varying the RNA, K^+ and Mg^{2+} concentrations. All three RNA preparations had approximately the same optima. A typical reaction in the wheat-germ system was carried out in 30 µl containing 1 µg of RNA, 25 µCi of [^{35}S]methionine (3000 Ci/mmol), 135 mM-potassium acetate and 2.25 mM-magnesium acetate incubated at 25°C for 1 h. A typical reticulocyte-lysate assay was carried out in 25 µl containing 0.5 µg of RNA, 50 µCi of [^{35}S]methionine, 1 mM-magnesium acetate and 80 mM-potassium acetate incubated at 37°C for 1 h. Both wheat-germ and reticulocyte-lysate assays were stopped by freezing at -20°C . Samples were removed at the time of freezing for measurement of total ^{35}S incorporation and for analysis on polyacrylamide gels. For the latter, samples were mixed with an equal volume of sample buffer, boiled for 10 min, cooled and loaded on SDS/12.5%-polyacrylamide gels (Laemmli, 1970). Electrophoresis was carried out in 12 cm $\times$ 15 cm slab gels at 60 V for 6 h. Gels were dried directly or soaked in 1 M-sodium salicylate/1% glycerol (Chamberlain, 1979) and dried before autoradiography at -70°C with Kodak XAR-5 film. ^{125}I -labelled CRP was prepared as previously described (Macintyre *et al.*, 1983).

In translation assays in which CH_3HgOH (Alfa Products) was used (Payvar & Schimke, 1979), it was added from a freshly made 50 mM stock

solution to the appropriate concentration (usually 5 mM) directly in the RNA. After 5 min the CH_3HgOH was diluted 10-fold by the addition of reticulocyte lysate; the CH_3HgOH was kept in the assay during the 1 h incubation.

Immunoprecipitation

CRP immunoprecipitation of samples from translations *in vitro* or ^{125}I -labelled rabbit CRP was carried out with either a goat antiserum raised against rabbit CRP or a goat antiserum raised against human F-CRP (Potempa *et al.*, 1983), a variant produced by treating CRP with urea in the absence of Ca^{2+} . These antisera were generously given by Dr. H. Gewurz and Dr. L. Potempa (Department, of Immunology, Rush Medical College, Chicago, IL 60612, U.S.A.). The procedure used protein A (IgG Sorb; Enzyme Center, Cambridge, MA, U.S.A.) as previously described (Tucci *et al.*, 1983). Briefly, 5 µl of antiserum was incubated with 5 µl of cell-free translation reaction, diluted to 1 ml with phosphate-buffered saline (140 mM-NaCl, 3 mM-KCl, 8 mM- Na_2HPO_4 , 2 mM- KH_2PO_4 , pH 7.2) containing 1% Triton X-100, 0.5% sodium deoxycholate and 0.5% SDS (lysis buffer) and incubated overnight at 0°C . Then 50 µl of a washed 10% suspension of protein A was added in the same buffer with 0.5% bovine serum albumin and incubated for 1 h at 0°C . Pellets were collected by centrifugation for 1 min at 12000g, washed extensively with lysis buffer and finally resuspended in Laemmli sample buffer, boiled for 10 min and analysed by SDS/polyacrylamide-gel electrophoresis.

For competition experiments, 5 µg of purified rabbit CRP was incubated with 5 µl of antiserum in 100 µl of phosphate-buffered saline for 8 h at 0°C . Then 5 µl of cell-free translation reaction mixture or ^{125}I -labelled rabbit CRP was added to this preincubation, diluted to 1 ml with lysis buffer and incubated overnight at 0°C . Protein A suspension was added as described above.

Results

In initial attempts to assay for CRP mRNA we isolated poly(A)-containing RNA from livers from either stimulated or unstimulated rabbits, translated this RNA *in vitro* in either wheat-germ extracts or nuclease-treated rabbit reticulocyte lysates and fractionated the translation products by electrophoresis on SDS/polyacrylamide gels. Fig. 1 shows the autoradiographic pattern of translation products programmed by poly(A)-containing RNA isolated from the livers of two rabbits 24 h after an intramuscular injection of turpentine and from the liver of an unstimulated animal. No translation products were found in the 21000–

Fig. 1. Autoradiograph of SDS/polyacrylamide-gel electrophoresis of products of translation *in vitro* in a wheat-germ system

Reactions were programmed by poly(A)-containing liver RNA from two stimulated rabbits (stim) or poly(A)-containing RNA from an unstimulated rabbit (unstim). ^{125}I -labelled CRP serves as a M_r marker for the CRP subunit size of 21 000 Da.

25 000-Da region from stimulated rabbits which were not in equal or greater abundance in translations from the unstimulated rabbit. The illustrated experiment is representative of over 20 assays in which the CRP translation product could not be detected. In contrast, the most dramatic difference between the translation patterns for stimulated and unstimulated rabbit was a band at 15 000 Da, which was essentially absent from control translations. This band represented 12% of the total liver RNA translation products in inflamed rabbits, as determined by densitometry of appropriately exposed autoradiographs. Immunoprecipitation studies showed this band to be the precursor of the acute-phase reactant serum amyloid A (results not shown). In addition, a translation

Fig. 2. Effect of increasing concentration of CH_3HgOH on 20 000–26 000 Da products of translation *in vitro*

CH_3HgOH was added to poly(A)-containing liver RNA isolated from a stimulated rabbit, which was translated *in vitro* in a rabbit reticulocyte-lysate system. ^{35}S -labelled translation products were fractionated on SDS/polyacrylamide gels before autoradiography. CH_3HgOH concentration added: lane 1, none; lane 2, 1 mM; lane 3, 2 mM; lane 4, 5 mM; lane 5, 10 mM; lane 6, 15 mM; lane 7, 20 mM. ^{125}I -labelled CRP is a 21 000-Da standard (std). Arrow indicates the position of the 24 000-Da CH_3HgOH -facilitated band.

product at 40 000 Da, which we presume to be actin, increased in stimulated animals, consistent with the reported behaviour of this protein during the acute phase in mice (Morrow *et al.*, 1981). Albumin translation at 67 000 Da was decreased by about one-third in stimulated compared with unstimulated rabbits, consistent with observations in both rat (Aronsen *et al.*, 1972; Northemann *et al.*, 1983b) and mouse (Morrow *et al.*, 1981) that albumin protein synthesis and albumin mRNA content are decreased during the acute phase. Another marked and unexplained difference was an increase in a 60 000-Da band in translations of RNA from stimulated animals. We explored the possibility that this translation product was related to CRP, but the tendency of this peptide to precipitate spontaneously prevented us from arriving at a definitive conclusion. Finally, bands present at 25 000 Da and at 32 000 Da were found to be more abundant in translations of RNA from controls than in those from inflamed animals.

We next explored the possibility that treatment of liver poly(A)-containing RNA from inflamed animals with the denaturant CH_3HgOH before and during the assay might facilitate translation of the CRP primary translation product (Payvar & Schimke, 1979). At concentrations of CH_3HgOH above 2 mM, a band migrating at 24 000 Da was visible, which was absent without the denaturant (Fig. 2). Maximal amounts of the 24 000-Da band was seen at concentrations of 5 mM- CH_3HgOH and above; 24 000 Da is the size expected for the signal-peptide-containing primary translation product of CRP and is the size reported for the CRP primary translation product in humans (Tucci *et al.*, 1983).

In addition to facilitating the translation of the 24 000-Da peptide, the denaturant at 5–10 mM had a general 3-fold stimulatory effect on ^{35}S methionine incorporation in these cell-free translations.

Boiling and quick cooling on ice of the RNA samples before cell-free translation did not stimulate incorporation significantly, nor was the 24000-Da band visible after cell-free translation with boiled RNA. This argues that intramolecular duplex formation is responsible for the block in translation of the 24000-Da species and that denaturation is continuously required to permit translation.

Fig. 3 shows that the 24000-Da polypeptide is found only in translations of liver poly(A)-containing RNA from inflamed animals, as expected for the CRP primary translation product. CH_3HgOH treatment of liver poly(A)-containing RNA from controls (unstimulated) resulted in a

general 2–3-fold increase in [^{35}S]methionine incorporation and enhanced the presence of several peptides, including a 25000-Da translation product, but the 24000-Da band was not synthesized to any detectable extent in these preparations.

Evidence that the 24000-Da band is the primary translation product for CRP comes from immunoprecipitation and competition experiments shown in Figs. 4 and 5. Fig. 4 demonstrates that the 24000-Da product is immunoprecipitable with an antiserum directed against rabbit CRP. In this experiment, ^{35}S -labelled translation products were incubated with and without antiserum and then precipitated with protein A. The major precipitated translation product migrated at 24000 Da. This

Fig. 3. Autoradiograph of SDS/polyacrylamide-gel electrophoresis of products of translation *in vitro* in the rabbit reticulocyte-lysate system

The reactions were programmed with poly(A)-containing liver RNA from an unstimulated rabbit in the presence of 5 mM- CH_3HgOH (lane 1) or poly(A)-containing liver RNA from a stimulated rabbit in the absence (lane 3) or presence (lane 4) of 5 mM- CH_3HgOH . ^{125}I -labelled CRP is a 21000-Da standard (lane 2). Arrows indicate the position of the 24000-Da CH_3HgOH -facilitated band.

Fig. 4. Immunoprecipitation of the 24000-Da primary translation product

A rabbit reticulocyte-lysate translation reaction *in vitro* programmed with CH_3HgOH -treated poly(A)-containing RNA from a stimulated rabbit was immunoprecipitated with 5 μl of goat anti-(rabbit CRP) antiserum (lane 3) or without antiserum (lane 1) as described in the Materials and methods section. Lane 4, total translation products before antiserum treatment; lane 2, ^{125}I -labelled CRP 21000-Da standard. Arrow indicates the position of the 24000-Da CH_3HgOH -facilitated band.

Fig. 5. Effect of added CRP on the immunoprecipitation of the CRP primary translation product

A sample (5 μ g) of rabbit CRP was preincubated with goat anti-(rabbit CRP) antiserum before immunoprecipitation as described in the Materials and methods section. Lane 1, immunoprecipitation of 125 I-labelled CRP. Lane 2, same as lane 1 with added CRP as competitor. Lane 3, immunoprecipitation of a sample of products of a rabbit reticulocyte-lysate translation reaction *in vitro* programmed with poly(A)-containing liver RNA from a stimulated rabbit. Lane 4, same as lane 3, with added CRP as competitor.

product was not visible when comparable amounts of 35 S-labelled translation products were programmed by either liver RNA from uninfamed animals in the presence of CH_3HgOH or liver RNA from inflamed animals in the absence of CH_3HgOH . Antiserum directed against human F-CRP (Potempa *et al.*, 1983) was also able to immunoprecipitate the 24000-Da translation product.

Further evidence that the 24000-Da polypeptide is the CRP primary translation product is shown by the competition experiments in Fig. 5. Goat anti-(rabbit CRP) antiserum was preincubated with excess purified rabbit CRP, or with boiled CRP, for 8 h before the addition of 35 S-labelled translation products as described in the Materials and methods section. In parallel experiments 125 I-

Fig. 6. Effect of added boiled CRP on the immunoprecipitation of the CRP primary translation product

A sample (5 μ g) of boiled rabbit CRP was preincubated with goat anti-(rabbit CRP) antiserum before immunoprecipitation as described in the Materials and methods section. Lane 1, immunoprecipitation of 125 I-labelled CRP. Lane 2, same as lane 1, with added boiled CRP as competitor. Lane 3, immunoprecipitation of a sample of products of a rabbit reticulocyte lysate of a translation reaction *in vitro* programmed with poly(A)-containing CH_3HgOH -treated liver RNA from a stimulated rabbit, with added boiled CRP as competitor. Lane 4, same as lane 3, with no competitor. Lane 5, immunoprecipitation of a sample of products of translation *in vitro* programmed with poly(A)-containing CH_3HgOH -treated liver RNA from an unstimulated rabbit. Lanes 1 and 2 had equal amounts of 125 I-labelled CRP added; lanes 3 and 4 had equal amounts of 35 S-labelled translation products added. Lane 5 had 5 times as much 35 S-labelled translation products as was used in lane 4.

labelled CRP was substituted for translation products. Precipitation of the 24000-Da band was not abolished by incubation of antiserum with pure CRP (Fig. 5, lane 4). This treatment effectively abolished precipitation of the 125 I-CRP (Fig. 5, lane 2). However, when the purified rabbit CRP was boiled for 5 min before preincubation with antiserum, precipitation of the 24000-Da primary translation product was abolished (Fig. 6, lane 3),

establishing the relationship between CRP and the CH_3HgOH -sensitive 24000-Da polypeptide.

To clarify the immunochemical relationship between native and boiled CRP, the reciprocal experiment was performed. Boiled CRP was used as a competitor in an immunoprecipitation with ^{125}I -labelled native CRP. As shown in Fig. 6, the boiled competitor CRP did not effect the immunoprecipitation of native CRP, just as native CRP had no effect on immunoprecipitation of the primary cell-free translation product of liver RNA from inflamed-animal translations.

We explored the possibility that CRP mRNA might be stored in a non-translatable form in uninfamed hepatocytes, and that inflammatory stimuli might lead to an alteration of its secondary structure and convert the molecules into the translatable form. Even though five times more of the translation product of CH_3HgOH -treated unstimulated control RNA was used for immunoprecipitation than was used in parallel immunoprecipitation experiments with translation products of inflamed-animal RNA, Fig. 6 indicates that the 24000-Da primary CRP translation product was barely, if at all, detectable under these conditions. We conclude from this experiment that the increased synthesis of CRP after an inflammatory stimulus is due to an increase in accumulated translatable CRP mRNA and not due to modification of existing CRP mRNA.

Discussion

Tucci *et al.* (1983) have demonstrated that the primary translation product of human CRP had a molecular mass of 26000 Da and that it contained a signal peptide of 2000 Da. Their studies did not include a comparison of the amount of CRP mRNA in normal and stimulated individuals. In the present study, we found that the rabbit CRP primary translation product had a similar molecular size. In addition, we found that greater amounts of translatable CRP mRNA are found in livers of stimulated rabbits than in unstimulated rabbits. This observation has previously been reported for a number of acute-phase reactants studied in the mouse and the rat. There are several theoretically possible mechanisms that might lead to an increased amount of translationally active CRP mRNA. Increased transcription, as has been shown for the mouse acute-phase reactant SAA (Stearman *et al.*, 1982), increased stability of specific mRNA, or a combination of these two processes, might be responsible. Alternatively, structural modification of pre-existing mRNA so that it becomes more translatable might occur. In pursuit of this latter possibility, we determined whether denaturation of liver mRNA from

unstimulated rabbits might convert pre-existing but poorly translatable CRP mRNA into a more translatable form, and found no evidence to support this mechanism.

We encountered considerable difficulty in demonstrating translation of CRP mRNA when we used standard techniques in our initial cell-free translation efforts (Samols *et al.*, 1983). Only when RNA was treated with CH_3HgOH could we detect a translation product which migrated at the expected molecular size and was immunochemically identifiable as rabbit CRP. This difficulty contrasts with the apparent ease with which Tucci *et al.* (1983) were able to demonstrate the primary translation product of human CRP mRNA, suggesting differences in the secondary structures of the CRP mRNA molecules in these two species.

CH_3HgOH has previously been shown to be of general benefit in translation and reverse-transcription reactions *in vitro* (Payvar & Schimke, 1979). The simplest interpretation of our results is that in isolated RNA preparations rabbit CRP mRNA can form an intramolecular duplex and that this stable secondary structure either prevents or decreases the ability of CRP mRNA to be translated until it is disrupted by the denaturant. We have not determined the extent of denaturation by CH_3HgOH treatment. It may be that the secondary structure of CRP mRNA interferes with its ability to be translated to some extent even with CH_3HgOH present. Thus our results on the amount of translatable CRP mRNA are qualitative and may be an underestimate of the abundance of this mRNA in our RNA preparations. It must be pointed out that, even if rabbit CRP can form an intramolecular duplex in isolated RNA, we cannot conclude that this structure exists *in vivo*. If it does, it is uncertain whether modifications of such a structure might modulate, to some extent, the regulation of CRP synthesis.

It is of interest that absorption with native rabbit CRP did not eliminate reactivity of antisera raised against rabbit CRP or human F-CRP with the rabbit CRP primary translation product. This finding indicates that the CRP primary translation product in our system more closely resembles denatured than native molecules; immunoprecipitation of the translation product could be abolished with boiled CRP. We have recently isolated two cDNA clones for rabbit CRP mRNA (S. Miller, S.-I. Hu & D. Samols, unpublished work) for use in further studies of the mechanism by which CRP mRNA accumulates.

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