C-Reactive Protein (CRP) of the Syrian Hamster[†,‡]

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ABSTRACT: Complementary and genomic clones encoding the mRNA and gene for a protein in the Syrian hamster that is highly homologous to C-reactive protein (CRP) have been isolated and studied. Coding sequence of the genomic clone is identical with that of the cDNA clone and predicts a mature protein of 206 amino acids and a 19 amino acid signal peptide. The single intron is 217 base pairs long and contains a short repetitive $(GT)_n$ motif. RNA blot analysis demonstrates that mRNA for hamster CRP is ~2.0 kb long, and unlike the closely related pentraxin female protein (FP), expression of this mRNA is not affected by the gender of the animal and accumulates equally in males and females during inflammation. In vivo administration of interleukin 1, interleukin 6, and tumor necrosis factor induces accumulation of hepatic CRP mRNA, and the acute-phase alterations in CRP mRNA levels arise as a result of enhanced gene transcription.

The systemic response to inflammation includes fever, neutrophilia, and hemodynamic alterations as well as metabolic and biochemical adjustments. Accompanying these changes are alterations in the concentrations of several serum proteins—the acute-phase plasma proteins. These proteins are a structurally and functionally heterogeneous group and the prototypic major human acute phase plasma protein is C-reactive protein (CRP). The plasma concentration of human and rabbit CRP increases 100–1000-fold during tissue necrosis due to enhanced gene transcription and faster transit of pre-CRP through the cytosol from the Golgi apparatus (Kushner, 1982; MacIntyre et al., 1985; Goldberger et al., 1987).

CRP is one of two phylogenetically conserved pentraxins found in the serum of many mammals, elasmobranchs, marine teleosts, and the primitive invertebrate *Limulus polyphemus* (Robey & Liu, 1981; Baltz et al., 1982). Pentraxins are named for the cyclic pentameric configuration assumed in the circulation since each assembled oligomer is composed of five identical noncovalently bound monomers. CRP may be distinguished from the other member of the pentraxin protein family, serum amyloid P component (SAP), by ligand binding specificity, glycosylation, and the extent of alteration in serum concentration during tissue injury as well as structural and immunologic properties. While CRP is a major acute-phase reactant in man, the serum concentration of human SAP does not alter significantly following an inflammatory stimulus. In mice SAP is an acute-phase reactant and the murine serum concentration of SAP is genetically determined in inbred strains (Pepys et al., 1979). Other differences between CRP and SAP include the lack of glycosylation of CRP and specific binding for phosphocholine moieties by CRP and agarose by human SAP via the 4,6-pyruvate acetal moiety of β-D-galactopyranose (Hind et al., 1984; Baltz et al., 1982). SAP has been immunochemically localized in glomerular basement membranes, perivascular elastic tissues, and all deposits of amyloidosis, independently of the nature of the origin of the fibril protein (Dyck et al., 1980; Breathnach et al., 1981).

The serum of the golden Syrian hamster (*Mesocricetus auratus*) contains a unique pentraxin that was originally termed female protein (FP) (Coe, 1977). FP is found in deposits of reactive amyloidosis in hamsters, and the expression of the FP gene is gender dependent (Coe & Ross, 1985, 1983). Resting levels of FP in the serum of male hamsters are low, while in females the serum concentration is high. The direction of alteration of serum FP concentration during inflammation in Syrian hamsters is divergent with a decrease observed in females and an increase in males. These serum levels are also modulated by sex steroids and are reflected in content of hepatic FP mRNA (Dowton et al., 1985). Mature FP monomers share 72% amino acid sequence identity with human SAP, while 50% of residues are shared with human CRP. Although hamster FP appears to more closely resemble human SAP structurally, chromatographic studies show that FP binds phosphocholine groups in a manner similar to human CRP. Earlier data suggests that FP is encoded at a single locus and that the regulation of FP biosynthesis occurs at a pretranslational level (Dowton et al. 1985).

Understanding the molecular mechanisms regulating expression of pentraxin genes in this unique animal will further define the importance of CRP and SAP in the host response to injury and during amyloidogenesis. Accordingly, as an adjunct to studies of FP biosynthesis, we have characterized a gene and a transcription product in the Syrian hamster that are homologous to CRP genes and mRNAs from other species. In addition, we have examined the expression of CRP-like mRNA during inflammation and have identified several cytokines that modulate the expression of the corresponding gene.

Experimental Procedures

Animals and Reagents. Randomly outbred golden Syrian hamsters (*M. auratus*) were obtained from Charles River Laboratories (Wilmington, MA) or from a colony maintained at Washington University Medical School. The cDNA synthesis kit was purchased from Bethesda Research Laboratories, and λZAP vector was from Stratagene. *Eco*RI linkers were obtained from New England Biolabs, and restriction endonucleases were from Promega Biotec and Boehringer-Mannheim. Nucleotide sequence analysis was performed with Sequenase and reagents from USB. Radiochemicals were sup-

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plied by New England Nuclear. Reagents and *Taq*I polymerase for the polymerase chain reaction were purchased from Cetus/Perkin Elmer. Chemical reagents were supplied by Sigma and Fluka. Oligonucleotides were synthesized on an Applied Biosystems 360B synthesizer.

Acute-phase reactions were induced in male and female hamsters by administration of turpentine (0.5 cm^3 subcutaneously) or lipopolysaccharide (LPS; derived from *Escherichia coli* serotype 0111:B4) @ 200 μg/kg intraperitoneally.

For kinetic studies cytokines were administered to adult male Syrian hamsters by single intraperitoneal injection as follows: interleukin 1 (IL-1) 2 ng/g of body weight; interleukin 6 (IL-6), 20 ng/g of body weight; and tumor necrosis factor (TNF), 2.5 ng/g of body weight. Responses to varying doses of IL-6 and TNF were examined by single intraperitoneal injection to male animals. All cytokine preparations were purchased from Amgen Inc. and diluted prior to use in sterile water. Sham animals were given equivalent volumes of intraperitoneal injections of sterile water.

cDNA Library Construction and Screening. A cDNA library was constructed from acute-phase Syrian hamster hepatic RNA isolated as detailed below. First-strand cDNA was synthesized by MMTV reverse transcriptase and was followed by second-strand DNA production by *E. coli* DNA polymerase I and ribonuclease H (Gubler & Hoffman, 1983). After addition of *Eco*RI linkers, the cDNA fragments were ligated into alkaline phosphatase treated *Eco*RI-cleaved λZAP arms. Recombinant clones were packaged with a Gigapack Gold in vitro packaging kit and transformed into competent XL1-B cells. The cDNA library was screened by plaque lift hybridization and radiolabeled pCRP5, a cDNA insert encoding human CRP (Whitehead et al., 1983). Prehybridization and hybridization were performed in 0.75 M sodium chloride, 0.075 M sodium citrate, 1× Denhardt's solution, and 100 μg/mL denatured salmon sperm DNA at 65 °C, each for 16 h. Posthybridization washes were completed in 0.15 M sodium chloride, 0.015 M sodium citrate, and 0.1% sodium dodecyl sulfate at 55 °C. Hybridizing signals were visualized by autoradiography on Kodak XAR-5 film.

Candidate plaques were purified and recombinant phagemids were released by autoexcision with addition of R408 helper phage. Insert sizes were studied by restriction enzyme digestion and gel electrophoresis. The clone containing the longest insert (phCRP1) was selected for further study.

5′ cDNA Characterization. The 5′ end of the hamster CRP-like cDNA was isolated by a modification of the rapid amplification of cDNA ends (RACE) protocol of Frohman et al. (1988). After synthesis of a CRP-specific first-strand cDNA from a 23-mer oligonucleotide complementary to the sequence at the 5′ end of phCRP1 (CCTACCAAAGACTGGTTTGCATC), the products were tailed with dATP by using terminal deoxynucleotidyltransferase. For amplification by the polymerase chain reaction, the 5′ adaptor and adaptor–$(dT)_{17}$ primers were GACTCGGGATCCGAATTC and GACTCGGGATCCGAATTC–$(dT)_{17}$, respectively, while the 3′ internal primer, specific for hamster CRP, was TCGATCGAATTCGGATTCATATGAGTCCTGCTC. *Eco*RI and *Bam*HI recognition sequences were included in the 5′ decamer of each PCR primer to facilitate subsequent cloning. Products of the PCR reaction were purified by phenol–chloroform extraction and ethanol precipitation prior to cleavage with *Eco*RI, gel purification, and ligation into EcoRI-digested, alkaline phosphatase treated pBS-M13$^-$. The nucleotide sequence for this insert (phCRP–[5′]) was determined as described below.

Genomic Library Construction. High molecular weight DNA was isolated from the liver of a male Syrian hamster by homogenization in 12 mL of 1× SET buffer (0.15 M sodium chloride, 0.001 M EDTA, and 0.02 M Tris, pH 7.8) in a motor-driven Teflon/glass homogenizer, followed by centrifugation at 3000*g* for 10 min at 4 °C. After resuspension in 12 mL of 1× SET buffer, the nuclear pellet was gently extracted for 2 h in phenol–chloroform (1:1) with 0.5 M sodium perchlorate and 0.3% sodium dodecyl sulfate. The aqueous phase was reextracted for 30 min with chloroform–isoamyl alcohol (24:1) after centrifugation at 7000*g* for 10 min at 15 °C. The aqueous phase was again recovered by centrifugation, and after addition of cesium chloride (1.2 g/mL), the preparation was subjected to density gradient ultracentrifugation at 40000*g* for 20 h at 15 °C in a Beckman VTi80 rotor. Following recovery from the gradient, the viscous high molecular weight DNA was dialyzed against 0.1× SET buffer for 16 h.

Partial digestion of the high molecular genomic hamster DNA with *Sau*3AI was performed, followed by fractionation on a 5–25% sucrose density gradient at 22500*g* for 16 h at 20 °C in a SW28 rotor. Aliquots of fractions from the gradient were analyzed by electrophoresis, and DNA from fractions containing fragments >30 kb were recovered by ethanol precipitation in the presence of 0.3 M sodium acetate. Genomic DNA fragments were ligated into cosmid (pTCF) arms prepared by restriction digestion of independent aliquots with *Cla*I and *Hpa*I followed by alkaline phosphatase treatment and *Bam*HI cleavage prior to final gel purification (Grosveld et al., 1982). Recombinant cosmids were packaged with Gigapack Gold prior to transformation of competent *E. coli* strain 490A$^-$. Colonies (3×10^6) were screened on nitrocellulose filters under conditions described above and the radiolabeled phCRP1. Two clones were identified, colony purified, and studied by restriction mapping, and one clone, ghCRP1, was subjected to nucleotide sequence analysis.

Nucleotide Sequence Analysis. The dideoxynucleotide chain-termination method was used for sequencing on both strands of the selected cDNA and genomic clones with Sequenase and α-^{35}S-dATP. Nucleotide sequence analyses were performed as described (Devereux et al., 1984).

Isolation and Analysis of RNA. Total cellular RNA was isolated from hamster livers by using guanidinium isothiocyanate as described (Sambrook et al., 1989), and the polyadenylated poly(A)$^+$ fraction, to be used in cDNA library construction, was purified by affinity chromatography on oligo(dT)–cellulose (Aviv & Leder, 1972). For blot analyses, total cellular RNA was size fractionated by agarose–formaldehyde gel electrophoresis and transferred to a nylon membrane. Prehybridization and hybridization were performed for 12 h each at 65 °C in 0.75 M sodium chloride, 0.075 M sodium citrate, 5× Denhardt's solution, 0.1% pyrophosphate, and 100 μg/mL denatured salmon sperm DNA. Hybridizing signals were revealed by autoradiography after blots were washed in 0.15 M sodium chloride and 0.015 M sodium citrate with 0.1% sodium dodecyl sulfate. The 1369-bp CRP-like insert was labeled by nick translation with [^{32}P]dCTP (3000 Ci/mmol) (Sambrook et al., 1989) and used as a hybridization probe to determine the size of CRP-like mRNA and to study the induction of expression of the corresponding gene following administration of turpentine, LPS, IL-1, IL-6, and TNF. Samples following turpentine administration were studied by dot blots, while RNA isolated from animals exposed to LPS and cytokines were analyzed by RNA blots following gel electrophoresis. The resting hepatic CRP-like mRNA levels

were also studied in male and female hamsters by RNA blot studies. After autoradiographic exposure, hybridizing signals were analyzed densitometrically with a Beckman DU64 spectrophotometer. For comparison with FP mRNA, the blots were rehybridized with pFP (Dowton et al., 1985).

Uniformity of sample application to the gel was checked by visualization of ethidium bromide stained RNA under ultraviolet light. In addition, the effects of turpentine, LPS, and cytokines on hepatic mRNA populations in general were studied by stripping blots and rehybridizing with a cross-reacting actin cDNA probe, pAC269 (Schwartz et al., 1980).

Transcription Studies. Following induction of inflammation by turpentine as described above, nuclei were isolated from cells of hamster livers by initial homogenization in a sucrose-containing solution followed by sequential centrifugations over solutions of increasing sucrose density as described (Lowell et al., 1986). Nuclei were resuspended and frozen at −70 °C in storage buffer (50% glycerol, 0.02 M Tris, pH 8, 0.075 M sodium chloride, 0.5 mM EDTA, 0.85 mM dithiothreitol, 0.125 mM phenylmethanesulfonyl fluoride, and 3 mM magnesium chloride). A total of 4×10^7 nuclei were used per transcription reaction in 25% glycerol, 0.02 M Tris, pH 8, 0.15 M potassium chloride, 0.002 M dithiothreitol, 0.004 M manganese chloride, 0.003 M magnesium chloride, 0.1 mM EDTA, 0.003 M EGTA, 0.01 M creatine phosphate, 0.1 mM phenylmethanesulfonyl fluoride, 0.001 M each CTP, ATP, and GTP, 80 units of RNasin, and [α-^{32}P]UTP(3000 Ci/mmol). The elongation reaction proceeded for 30 min at room temperature and was terminated by using RQ1 DNase. After addition of 100 μg of yeast tRNA as a carrier, radiolabeled nascent transcripts were recovered by extraction in guanidinium isothiocyanate and centrifugation over a cesium chloride cushion as described (Sambrook et al., 1989). Labeled transcripts were hybridized for 48 h with insert isolated from phCRP1 in 50% formamide, 0.9 M sodium chloride, 0.05 M Tris, pH 7.5, 0.005 M EDTA, 0.2% sodium dodecyl sulfate, 2× Denhardt's solution, and 50 μg/mL denatured salmon sperm DNA. The phCRP1 insert was denatured and immobilized on a nitrocellulose membrane as described (Goldberger et al., 1987). Posthybridization washes were performed with 0.9 M sodium chloride, 0.09 M sodium citrate, and 0.1% sodium dodecyl sulfate for 10 min at room temperature. Signals were viewed by autoradiography with Kodak-XAR5 film and a 48-h exposure period at −70 °C. Also included in each strip were dots containing denatured cDNA inserts for hamster SAA (phSAA2) (Webb et al., 1989), another acute-phase reactant, and actin (pAC269) (Schwartz et al., 1980) and pBR322 as experimental and hybridization controls, respectively.

Results

Nucleotide and Amino Acid Sequences Derived from CRP-like cDNA. The complete nucleotide sequences were determined for the inserts of phCRP1 and the PCR-generated clone phCRP-[5′] (Figure 1). phCRP1 contains 1369 base pairs including 1094 base pairs of 3′ untranslated region extending from the stop codon to the poly(A) tail. A polyadenylation signal consensus sequence (AATAAA) begins at nucleotide 1983, 17 residues prior to a short poly(A) tail. A second consensus polyadenylation signal (AATAAAAA) is noted slightly further upstream beginning at residue 1944, although this may not be functional since this segment is followed by 54 nucleotides of 3′ untranslated sequence prior to the poly(A) tail. The nucleotide sequence of phCRP1 begins with a codon representing residue 121 (nucleotide 638) in the mature polypeptide sequence. The PCR-generated clone, phCRP-[5′], contains 572 base pairs and overlaps with 68 bases in the 5′ end of the phCRP1 sequence. The RACE-generated PCR clone contained 86 base pairs of the 5′ untranslated region as well as the 5′ end of the coding sequence including 57 base pairs encoding the signal peptide and 429 base pairs encoding the mature protein. The complete cDNA sequence for the hamster CRP-like transcript predicts a 206 amino acid residue mature polypeptide and a leader peptide of 19 amino acids.

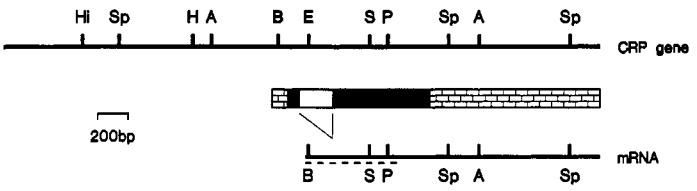
Structure and Nucleotide Sequence for CRP Genomic Clone. Two genomic clones containing inserts of ~34 and ~37 kb, respectively, were isolated from the partial *Sau*3AI cosmid library by using the insert of phCRP1 as a hybridization probe. Studies of these clones by restriction endonuclease digestion, Southern blotting, and hybridization with radiolabeled phCRP1 insert reveal that each contain identical segments of genomic DNA in the region of interest but that the clones do not completely overlap in portions flanking the CRP gene (data not shown). Two overlapping fragments (3.0-kb *Bam*HI and 2.0-kb *Eco*RI) of the first cosmid clone, ghCRP1, were subcloned into pBS-M13$^-$ to facilitate nucleotide sequence analysis. The organization of the hamster CRP-like gene, determined by these studies, is shown in Figure 1A and the nucleotide sequence of the genomic clone concurs completely with matching portions of the cDNA sequence (Figure 1B). Notable features of the hamster CRP-like gene include a TATA box at position −116 and 57 base pairs encoding the leader sequence. A single intron, 217 base pairs long, begins at the codon specifying the third amino acid residue in the mature polypeptide (i.e., nucleotide position 64 in Figure 1B) and is bounded by the consensus sequences AGGTAGGAT and TCTCACAG for donor and acceptor splicing sites. The polyadenylation signal is present in the genomic clone and is followed by 17 base pairs shared with the cDNA clone.

The 3′ untranslated region of the hamster CRP-like gene and mRNA contains a $(AT)_{22}$ repeat. A similar segment has been identified in the mouse CRP 3′ untranslated region, and a possible role for such repeats has been suggested in the differential regulation of CRP genes between and within species (Whitehead et al., 1990).

In the 5′ flanking region a consensus polyadenylation signal (AATAAA) was identified commencing at position −253, in a position similar to one recognized in the sequence for the human CRP gene. The sequence of an additional 1630 base pairs was determined 5′ to this potential polyadenylation signal and neither open reading frames, potential initiation sites, regulatory sequences, nor exon–intron splice junctions were recognized in this additional sequence data (data not shown).

Expression of CRP mRNA. RNA blotting studies demonstrate that hamster CRP-like transcript is ~2.0 kb long and is inducible in male and female hamsters upon administration of turpentine or lipopolysaccharide (Figure 2A,C,D). No significant gender difference was observed in the resting levels of CRP-like mRNA (Figure 2B), and transcripts for CRP-like mRNA accumulate rapidly following onset of inflammatory stimulation with peak levels of hepatic CRP-like mRNA occurring approximately 18 h after administration of LPS (Figure 2C). Figure 2B contrasts the expression of hamster CRP-like mRNA with that for hamster FP, showing that while there is no significant difference in expression of the CRP-like gene between males and females, the level of FP mRNA in male hamsters is 10% of that observed in female hamsters.

In vivo administration of cytokines involved in modulation of host responses to inflammation resulted in accumulation

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-261 agtttgtaataaataacctacattgatttctctgttccagaatgattttggcttcccttcctttaagtggtctgatgtttaccccaagagacagtgttag
-161 gaaatcatttacaaagtggtacagcctctccttctggtatagttataaatacaaggttgggctgggactgaagca  AGGGATCCAGAACTTCTCATCGT
 -63 GATTTTTCAGACAAAACTGAGTTTTCCTAATGTGAAAATCCAGGTCCAGCAGCATCAATAGCC  ATG GAG AAG CTG CTG TGG TGC TCA CTG
                                                                      M  E  K  L  L  W  C  S  L
  28 GTC ATG ATC GGC TTC TCT CAG GCT TTT GCC CAG AAA G   gtaggatctattataaagatctgatctgggggagacgggtgagagcc
     V  M  I  G  F  S  Q  A  F  A  Q  K
 114 taatttcttacatatggaattcaagaaacagcatcaggtacagatgtgtgtttgtgtaacctgggaaaaggccacatgcctcttgatcctaaactctact
 214 tgtaagtgagggatgatagatctttgttatctgagaaactactcactttcttcgtttcctgtctcacagAC ATG TCT AAA ACA GCC TTC GTA
                                                                          D  M  S  K  T  A  F  V
 306 TTT CCC AAA GAG TCA GCC AAT TCC TAT GTA TCC CTG GAA GCA CAG TCA AAG AAG ACA TTG AAA GCC TTC ACT GTG
     F  P  K  E  S  A  N  S  Y  V  S  L  E  A  Q  S  K  K  T  L  K  A  F  T  V
 381 TGT CTC CAC ATC TTC ACG GAA CTG AGC ACA ACC CGC AGC TTC AGT ATT TTC TCT TAT GCT ACC AAG AAC AGC CCT
     C  L  H  I  F  T  E  L  S  T  T  R  S  F  S  I  F  S  Y  A  T  K  N  S  P
 456 AAC GAA ATC CTC ATA TTT TGG TCT AAG GAT AGA GGG TAT GCT TTT GGA GTG GGT GGG CCT GAA GTA CTA TTC AAG
     N  E  I  L  I  F  W  S  K  D  R  G  Y  A  F  G  V  G  G  P  E  V  L  F  K
 531 GCT TCT GAA ATT CCT GAA GTT CCA ACA CAC ATC TGT GCC AGC TGG GAG TCT GCT ACA GGG ATT GCA GAG TTA TGG
     A  S  E  I  P  E  V  P  T  H  I  C  A  S  W  E  S  A  T  G  I  A  E  L  W
 606 GTT GAT GGG AAA CCC AAG GTG CGG AAA ATT CTG CAG AAG GGC TAC ACT GTG GGG ACA GAT GCA AGC ATC ATC CTG
     V  D  G  K  P  K  V  R  K  I  L  Q  K  G  Y  T  V  G  T  D  A  S  I  I  L
 681 GGG CAG GAG CAG GAC TCA TAT GGT GGT GGT TTT GAT GCA AAC CAG TCT TTG GTA GGA GAC ATT GGA GAT GTG AAC
     G  Q  E  Q  D  S  Y  G  G  G  F  D  A  N  Q  S  L  V  G  D  I  G  D  V  N
 756 ATG TGG GAC ATT GTG CTG TCT CCA GAA CAG ATA AAC ACA GTT TGT GTT GGT GGG ACA CTA GAC CCC AGT GTT TTA
     M  W  D  I  V  L  S  P  E  Q  I  N  T  V  C  V  G  G  T  D  L  P  S  V  L
 831 AAC TGG CAG GCA CTG AAG TAT AAA GTA CAG GGT GAT GTG TTT ATT AAG CCC CAG CTG TGG CCC TGA CCTACTGTTGTG
     N  W  Q  A  L  K  Y  K  V  Q  G  D  V  F  I  K  P  Q  L  W  P
 981 AGCCCTGAGACAACCCTTGGGATTACATTGTCTTCCTTGTCTCTGGTTATAGACCTTTTAACCCCAACAGATGCTGTAGTTCTATTCTGTGAATATGGTC
1009 TTTCACTTCCCTGCTCTGCCTTTCCTCAGCACCAGAGCATGCAATTTAAGTGTATGGCTTGGCAGTATGTACATCATATTAGGAATATTTATCAAAGAGA
1109 ATCCTAATTACATGTTTTTGTTTATATATACACACTATAAGTATATATATATATATATATATATATATATATATATATATATATATATATTTCCAGGTGTAAC
1209 AGCCCCGGCTGACCTGGAACTTGCTGTGTAGACCAGGGCTGACCTCAAACTCACTGAGATCCACCTGCCTCTGCCTCCCAAGTGCTGGGATTAAGGGCCTG
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1709 AGGGTTGGTCACTTGGGACTTGGATAATCTATTAGATGGCACACCCCTGAAGAAAACGGACTCTCCTTTTCTCAGCAGCCATTGATTGCCTGTAGCTCTT
1809 CATATAGGGGTGGGGCTTTGTGAAATTTCTTCTGTCCATGTTGCATGCTAGTGTTGTCATTATGTAGGTCTTATTTAGGCAACCCAGACTGATGGGGGAC
1909 CGACTATACTGGGCTCAGAGTTAGTCCTTTTCTGGAATAAAAATCTGTATCTGAACTTCCTGGGTCCATGAGTCAATAAAGCTACCTTTGGTTTGGAtga
2009 attggagttattgcatgattcattttattatcaaccctctggaataaatagtctgaagttcaggctgactgtcacttccctgca
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FIGURE 1: Organization and structure of the hamster CRP gene and mRNA. (A, top) Restriction map of hamster CRP gene derived from genomic clone ghCRP1 and mRNA. Hi, *Hin*cII; Sp, *Sph*I; H, *Hin*dIII; B, *Bam*HI; E, *Eco*RI; S, *Sca*I; P, *Pst*I; A, *Acc*I. The center bar diagram shows coding sequence (solid), the single intron (open), and untranslated regions (hatched). Nucleotide data were determined for both sequences as indicated by the solid lines. The dashed line represents the area of cDNA sequence derived from the PCR fragment phCRP[5′], generated by a modified RACE protocol. The 5′ end of phCRP1 begins at the *Pst*I site. (B, bottom) Nucleotide sequence of the hamster CRP genomic (lower and upper case) and cDNA clones (upper case). The numbering system begins arbitrarily at the start of sequence encoding signal peptide. Amino acids for coding sequence are shown below the corresponding codons. The intron begins at nucleotide number 65. Consensus TATA and polyadenylation signals are underlined.

of CRP-like mRNA in hamsters (Figure 3). These studies were performed by isolating hepatic RNA after intraperitoneal administration of IL-1, IL-6, or TNF. The time course of accumulation of CRP-like mRNA in this experiment is shown in Figure 3A and demonstrates that maximal levels occurred 8 h after administration of each cytokine. The dosage–response plots in Figure 3B,C show increased accumulation of CRP-like mRNA in response to increasing doses of IL-6 and TNF. No similar effect was noted in the expression of actin.

Studies of CRP Gene Transcription. In order to determine the basis for accumulation of CRP-like mRNA during inflammation, an in vitro transcript elongation assay was performed. Hepatic nuclei isolated from hamsters at 0, 12, 24, and 44 after creation of a sterile abscess with turpentine were incubated with [α-^{32}P]UTP, followed by isolation of radiolabeled nascent transcripts and hybridization with immobilized phCRP1 insert. Autoradiographic results are displayed in Figure 4 and show an increase in the transcription of the hamster CRP-like gene following administration of turpentine. Nascent transcripts for SAA, another acute-phase reactant, were also noted to accumulate, while there was no alteration in the signal intensity when dots of pAC269 were cohybridized with the labeled transcripts (data not shown) (Dowton & Colten, 1986; Schwartz et al., 1980). Similarly, as a control for nonspecific hybridization, no signal was detected when dots containing denatured pBR322 DNA were cohybridized with the transcripts (data not shown).

Discussion

This report describes the isolation and characterization of complete sequences for genomic and cDNA clones encoding a protein in the golden Syrian hamster resembling C-reactive protein of other species. The hamster CRP gene is structurally similar to all reported pentraxin genes including those for CRP in man, mouse, and rabbit as well as murine and human SAP genes. All mammalian pentraxin genes have a single intron beginning within 10 residues after the 5′ end of DNA encoding the mature polypeptide sequences (Woo et al., 1985; Hu et al., 1986; Lei et al., 1985; Paska et al., 1986). The position of the pentraxin gene intron, in contiguity with the junction between the leader sequence and mature protein sequence, is a feature shared by mouse λ_1 immunoglobulin genes (Woo et al., 1985). The length of the intron of the hamster CRP-like gene, 217 base pairs, is shorter than the size reported for corresponding regions of the human and lagomorph CRP genes, where the intron lengths are 278 and 252 base pairs, respectively, while the murine CRP gene is of similar length, 213 base pairs. In the introns of human and rabbit CRP genes, attention has been drawn to the presence of almost identical repeated $(GT)_n$ elements, which are capable of assuming the left-handed Z-DNA conformation and may be implicated in activation of chromatin domains (Woo et al., 1985; Nordheim

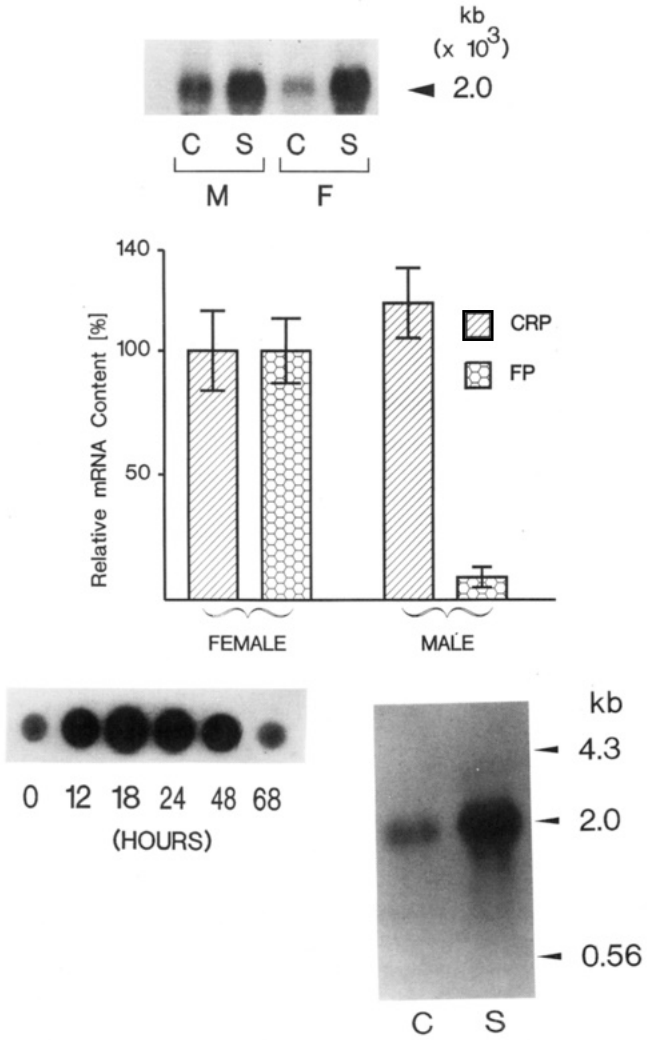

FIGURE 2: Hamster CRP mRNA expression. (A, top) Hamster CRP mRNA expression during inflammation. Autoradiograph of RNA blot of hepatic RNA from male and female hamsters hybridized with phCRP1. Inflammatory stimulation (S) was incited by subcutaneous turpentine injection. Saline was administered to control animals (C). (B, middle) Expression of hamster CRP mRNA in male and female hamsters compared with levels of FP mRNA. Hepatic RNA was isolated from adult hamsters (five male and five female) and for each sample three separate aliquots (5, 10, and 15 μg) were subjected to blot analysis. Results are depicted as a percentage of the signal intensity for female samples determined by densitometry of autoradiographs. Error bars indicate 1 standard error of mean. (C, bottom left) Time course of induction of CRP mRNA in hamster livers after administration of LPS. Autoradiograph of dot blot of hamster liver RNA samples (5 μg) isolated and hybridized at varying time points after administration of LPS (200 μg/kg). (D, bottom right) RNA blot demonstrating size of CRP transcript. Hepatic cellular RNA (C, control; S, turpentine stimulated) from male hamsters was electrophoresed and blotted prior to hybridization with radiolabeled phCRP1. Molecular size was determined by coelectrophoresing radiolabeled λ *Hin*dIII.

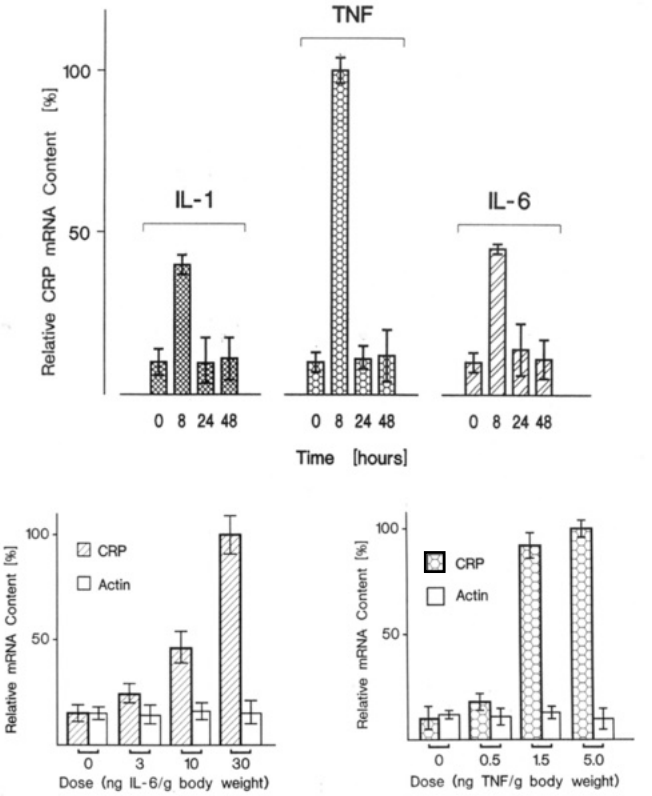

FIGURE 3: Induction of CRP mRNA in hamster livers by cytokines. (A, top) Time course of CRP mRNA accumulation after cytokine administration. RNA was isolated from livers of adult male hamsters after administration of a single dose of cytokines: IL-1,2 ng/g of body weight; TNF, 2.5 ng/g of body weight; IL-6, 20 ng/g of body weight. Three animals were used for each point, and RNA from individual animals was subjected to blot studies. Results are displayed as a percentage of the maximal CRP mRNA signal determined by densitometry of autoradiographs. (Bottom) Dose response for accumulation of CRP mRNA after dosage with IL-6 (B, bottom left) or TNF (C, bottom right). RNA was isolated 8 h after intraperitoneal administration of IL-6 or TNF at indicated doses. Results are displayed as for Figure 5A. Control data for actin are shown by open bars and were obtained by rehybridizing the blots with pAC269.

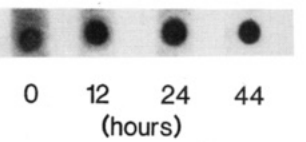

FIGURE 4: Transcription of hamster CRP gene studied by in vitro transcription reactions. phCRP1 insert was deposited on a membrane and individual dots were hybridized with radiolabeled nascent transcripts generated in nuclei isolated at varying time points after administration of subcutaneous turpentine (0.5 cm^3).

& Rich, 1983). While no such repetitive sequence is evident in the intron of the murine CRP gene, a similar but abbreviated form is present in the corresponding position in hamster DNA. This dodecameric sequence, GTGTGTTTGTGT, occurs between residues 159 and 171 in the hamster CRP-like gene and aligns the beginning of the $(GT)_{15}G(GT)_3$ region in the human gene. In the hamster, this region is of unknown importance and may simply represent evolutionary diversity. The unusual segment of 16 adenines in the intron of the human CRP gene seems peculiar to man since this repetitive element has not been observed in mouse, rabbit, or hamster and hence remains of unknown significance.

The overall identities of the intron of the CRP-like gene in hamster compared with those for mouse, human, and rabbit CRP introns are 79%, 58%, and 54%, respectively. Analysis of these intronic sequences fails to show any nucleotide string longer than a 5-mer that is identical in all species. However, in general, greater identity is observed in the 3′ half of the intron, distal to the $(GT)_n$ area, than in the first 100 bases of the hamster intron, including a segment 31 residues in length in which 90% of nucleotides are shared with mouse, 80% with human, and 70% with rabbit introns, respectively (nucleotides 235–266 in hamster). Intron sequences are generally not highly conserved between mammalian species, although several exeptions have been documented. No homology of the conserved region of the CRP-like intron is seen with the intronless genomic sequences of CRP genes from *Limulus*, perhaps suggesting that this conserved region has evolved to assume

some functional significance in mammalian CRP genes that remains to be elucidated (Nguyen et al., 1986b).

The 3′ untranslated region of the hamster CRP-like transcript is 1040 base pairs long, intermediate in length between similar regions of human (~1200 bases), rabbit (1550 bases), and mouse (858 bases) mRNAs and longer than similar regions of SAP genes from any species. Historically, CRP has been defined as a pentraxin that binds the pneumococcal C-polysaccharide in a Ca^{2+}-dependent manner. Such binding is to the phosphocholine moiety, and while affinity chromatography on PC fails to yield a second product binding FP, the data of the gene structure suggest that the CRP-like gene and transcript reported herein more likely have evolved from a CRP lineage than an SAP lineage. Hence, the structures isolated and characterized in this report will be designated as hamster CRP. Greatest overall nucleotide sequence identity in the 3′ untranslated region is shared with murine CRP untranslated mRNA sequence (77%), although shorter segments of considerable homology in this area are observed between hamster and human sequences; e.g., of the 38 residues between 1372 and 1410 in hamster, 35 are shared with a corresponding segment of human CRP. The length of the 3′ untranslated region in CRP genes has been implicated in interspecific variation in inducibility of CRP gene expression during inflammation, but clearly further studies are warranted to resolve these questions.

Several DNA segments that may be important in regulation of gene expression are identified upstream of the hamster CRP-like coding sequence. Two potential attachment sites for RNA polymerase II are identified in close proximity to one another, TATAAA beginning at residue −116 and TATA at position −122, but a CAAT sequence is not recognized in proximity to the TATA box. An octamerlike box (ATTTACAA) begins at residue −155 and is in a similar position with respect to the TATA box noted in the human CRP gene. Although studies of this element in the CRP gene by site-specific mutagenesis originally suggested that it was not involved in induction of CRP gene expression in man (Arcone et al., 1988), it now appears clear that this octameric segment is part of a longer consensus sequence for the IL-6-responsive β region of the acute-phase response promoter element of the human CRP gene (Toniatti et al., 1990). The consensus binding site for the HNF-1 transcription factor that interacts with this element is quite conserved in the corresponding region of the hamster CRP gene (residues −164 to −148). In downstream contiguity with this β region is a decameric sequence similar to α-related sequences of the promoter regions of IL-6-responsive human hepatic genes. However, in this segment of the hamster CRP gene, $^{-147}$AGTGGTACAG^{-138}, the seventh and tenth residues are not conserved with similar elements consistently identified in human haptoglobin, hemopexin, and CRP (Oliviero et al., 1987; Majello et al., 1990). In addition, upstream sequence in the hamster CRP gene does not have a segment that resembles the γ sequence consensus binding site for HNF-1 noted in albumin and CRP genes of man. Studies similar to those performed for the human promoter elements are in progress in hamster to define the sequence elements that interact with nuclear transcription factors inducible by administration of cytokines.

Enhanced transcriptional rate is responsible for many changes in serum concentration of acute-phase proteins. An increase in transcription coupled with alterations in the kinetics of posttranslational processing has been documented for the rabbit CRP gene during inflammation (MacIntyre et al., 1985). The runoff experiments in this report show that transcription of the hamster CRP-like gene is induced during inflammation. This is in contrast to observations for the FP gene, in which no transcriptional difference is evident between sexes or during inflammation (data not shown). The relative magnitude of increase in transcription of the hamster CRP-like gene is apparently less than the accumulation of the corresponding mRNA as determined by blot analyses and may imply additional mechanisms of regulation including alterations in mRNA stability.

The effect of monokines upon synthesis of acute phase reactants has been explored by in vivo and in vitro methods (McAdam et al., 1982). Experiments employing human primary hepatocyte cultures demonstrated that IL-6 enhances synthesis of CRP (Moshage et al., 1988; Castell et al., 1988), and subsequent studies in the human hepatoma cell line Hep3B and NPLC/PRF/5 show that induction of CRP is completely inhibited by antibodies to recombinant IL-6 (Kushner et al., 1989). IL-6 alone induces CRP synthesis in a concentration-dependent manner in NPLC cells, while it is only with the addition of IL-1 and Il-6 in Hep3B cells that stimulation of CRP production is observed (Kushner et al., 1989). Other data have been derived by studying the effects of monokines on promoter elements in constructs linking 5′ elements of human CRP gene to a reporter gene (Arcone et al., 1988; Toniatti et al., 1990). Although a modest effect of IL-6 alone was documented, maximal stimulation of CRP-like mRNA expression appears to require coordinated involvement of IL-1 and IL-6. In those studies, elements of the CRP gene responsive to IL-6 correspond to the α, β, and γ regions discussed above.

In this report the hamster CRP-like mRNA accumulates rapidly after in vivo administration of IL-1, IL-6, and TNF. Maximal response for the doses used is noted for TNF. These observations do not necessarily imply a direct effect of each mediator upon CRP gene expression since there is a complex interplay between cytokines during inflammation including, for example, induction of IL-6 release from stromal cells and activated T lymphocytes by IL-1 (Dinarello & Mier, 1987; Philip & Epstein, 1986). TNF has been considered to have a more restricted spectrum of activity in inducing acute-phase plasma protein gene expression (Perlmutter et al., 1985; Darlington et al., 1986), and these observations in hamster represent the first demonstration, albeit perhaps indirect, that a CRP-like gene may respond to TNF. Further definition of this property is being explored by using murine L-cells transfected with the hamster CRP-like gene.

Pentraxin proteins have been extensively conserved throughout evolution and the predicted polypeptide sequence for hamster CRP shares extensive identity with human, murine, and rabbit CRPs (Figure 5). The predicted hamster and murine CRP monomers share 79% sequence identity, whereas 72% and 70% of amino acids are shared with human and rabbit CRP, respectively (Woo et al., 1985; Whitehead et al., 1990). Sequence data for 46 carboxyl-terminal residues of rat CRP are available and 31 amino acids are identical with hamster CRP (Taylor et al., 1984). Hamster CRP and FP share 48% of predicted amino acid residues, with greater identity occurring in the carboxyl-terminal half of the molecule (Dowton et al., 1985). CRP isolated from the hemolymph of the horseshoe crab (*Limulus polyphemus*) has been characterized as a hexameric phosphocholine-binding protein which also binds C-polysaccharide of pneumococci (Robey & Liu, 1981; Hu et al., 1986). Although the concentration of CRP in the hemolymph of *Limulus* markedly exceeds the maximal concentration of human CRP, the notion that mammalian

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              -19                 1                                                          59
Hamster CRP   MEKLLWCSLVMIGFSQAFAQKDMSKTAFVFPKESANSYVSLEAQSKKTLKAFTVCLHIFTELSTTRSFSIFSYATKNS
Mouse CRP     -------L-I--S--RT-GHE--F-K--------DT-------E---P-NT------FY-A---V----V------KN
Rabbit CRP    -------F-TLVS--NMSD-AG-H-K--------D------N-----P--------YFY-D--M--GY-------RRQ
Human CRP     ----- -F--LTSL-H--G-T---RK--------DT-----K-PLT-P---------FY-E--S--GY--------RQ
Hamster FP                       -t-1tgkv----rq-etd--k-iprld-p-qn----fraysd--  -ph-1---n-eyg

                                                                                          135
Hamster CRP   PNEILIFWSKDR GYAFGVGGPEVLFKASEIPEV PTHICASWESATGIAELWVDGKPKVRKILQKGYTVGTDASIIL
Mouse CRP     S-D-----N--K Q-T-----A--R-MV-----A --------------V-F-I--------S-H------P------
Rabbit CRP    F----L-----I --S-S---D-II--V-D--VD ---L------S------------M---S-K---IL-PE-----
Human CRP     D----------I --S-T---S-I--EVP-VTVA -V--T-----S--V-F-V----R---S-K------AE -----
Hamster FP    e--1--  y-eri-eyelyi-nqgtkvhgveefas-v-f-t----ss----f--n---w-k-g-------knkp----

                                                                            206
Hamster CRP   GQEQDSYGGGFDANQSLVGDIGDVNMWDIVLSPEQINTVCVGGTLDPSVLNWQALKYKVQGDVFIKPQLWP
Mouse CRP     ---------D---K-------------F----------Y-----S-N----R--N--A-----------S
Rabbit CRP   ------F--S-EKQ--------N-----YA----E---IYA---FS-N--D-RE-T-Q-R-E-HV------
Human CRP     ------F--N-EGS--------N-----F-----E---IY----FS-N----R-- Y ---E-- ------
Rat CRP                                ---F--*-----A-Y--RVFS-N----R--FYETH-----------
Hamster FP    ----n------ny--f--e---1----s--t--e-ks-yqgvp-e-ni-d----n-emn-yavirprcvalssynkis
```

FIGURE 5: Comparison of predicted hamster CRP protein sequence with hamster SAP sequence and CRP from other animals. Predicted polypeptide sequence for hamster CRP primary translation product is shown on top line. Residues of sequence identity for murine, rabbit, rat, and human CRPs (upper case) as well as hamster FP (lower case) are shown below, with a dash indicating residue identity with hamster CRP. An asterisk represents an unidentified residue (Taylor et al., 1984). Sequences have been aligned to provide maximum identity.

CRP may have evolved from the homologous protein in the invertebrate *Limulus* is supported not only by the calcium-dependent ligand binding specificity but also by sequence similarities. Although there is overall 25% identity of human and *Limulus* CRP genes, two conserved regions of sequence have been identified, leading to speculation upon the nature of the phosphocholine-binding domain. Residues 52–66 in human CRP (^{52}F-S-Y-A-T-K-R-Q-D-N-E-I-L-I-F^{66}) and 53–67 in *Limulus* CRP (^{53}F-S-Y-A-T-A-K-K-D-N-E-L-L-T-F^{67}) are quite similar, including juxtaposition of dibasic residues in both (^{58}Lys-Arg in human and ^{59}Lys-Lys in *Limulus*), putatively providing a strong interaction with the polar phosphate group (Nguyen et al., 1986a). Similarly, Nguyen et al. have reasoned that Glu^{62} and Glu^{63} of human and *Limulus* CRP, respectively, may enhance binding of the cationic ammonium moiety in phosphocholine. Comparison of this region with the corresponding segments of hamster CRP (^{52}F-S-Y-A-T-K-N-S-P-N-E-I-L^{64}) and FP (F-S-Y-N-T-E-Y-G-E-N-E-L-L-I) suggests that comparison of primary sequence data alone does not allow prediction of possible phosphocholine binding sites. Hamster FP, which binds phosphocholine, has no duplication of dibasic amino acids in this region and there are, in fact, no arginine, lysine, or histidine residues in the conserved sequence. In contrast, ^{57}Lys is conserved in the putative hamster CRP but is adjacent to an asparagine, and preliminary phosphocholine affinity studies in this laboratory have failed to identify a second serum protein in Syrian hamster serum besides FP that will bind phosphocholine (data not shown). The glutamate residue postulated to provide a locus for the binding of phosphocholine's ammonium moiety is conserved in both pentraxins in hamsters. Hence prediction of the phosphocholine binding site may not be possible by considering the primary sequence alone. This notion is supported by the recent observation that CRP phosphocholine binding is in fact determined by the polar and acidic residues in the regions discussed above (^{57}Lys-Arg^{58} and ^{60}Glu-Asn-Asp^{62} respectively, in human CRP) (Swanson et al., 1991). Indeed, studies of binding of murine myeloma proteins to phosphocholine occur independently of availability of Ca^{2+} ions and may occur due to contiguity of peptide bands rather than presence of particular primary structural motifs. A third previously unrecognized short segment of homology is noted between positions 103 and 108 in hamster CRP (Figure 5). Amino acid substitutions in this hexameric peptide in other mammals and *Limulus* are all neutral in charge. Eight residues are shared between positions 132 and 139 in all CRP-like pentraxins studied and remain of unknown significance.

This report documents that hamster CRP is encoded by a 2.0-kb mRNA. Uniform length CRP transcripts have been reported for CRP in all animals studied as well as for human, murine, and rat SAP. This observation is in sharp contrast to the report of multiple transcript lengths for hamster FP, in which four transcripts are noted (Dowton et al., 1985). This report documents the structure and expression of the hamster CRP gene. Study of CRP and FP in the Syrian hamster presents an unusual opportunity to study the differential regulation of genes that have most likely evolved from a common ancestral gene. The characterization of the hamster CRP gene and inducible mRNA suggests that although PC binding is not a property of SAP in any other species, hamster FP should be redesignated as the genuine SAP of that species. This is supported by the convincing description that FP is deposited in amyloidosis (Coe & Ross, 1985), after which SAP was originally named. Ultimate proof of this thesis will require investigation of translatability of the CRP-like mRNA described in this report as well as isolation and characterization of the structure and ligand-binding properties of a mature translation product.

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