Biochem. J. (1992) 283, 705–712 (Printed in Great Britain)

Cis- and trans-acting elements required for constitutive and cytokine-regulated expression of the mouse complement C3 gene

Nobuaki KAWAMURA, Lori SINGER, Rick A. WETSEL and Harvey R. COLTEN
Edward Mallinckrodt Department of Pediatrics, Washington University School of Medicine, 400 South Kingshighway Blvd., St. Louis, MO 63110, U.S.A.

The third component of complement (C3) is an important mediator of inflammation. Murine and human genomic cosmid clones were isolated, characterized and sequenced 5′ to the complement C3 gene transcriptional initiation sites to determine cis elements that participate in constitutive and regulated C3 gene expression. The murine and human 5′ flanking regions are 51% identical overall, with positions −36 to −1 and −146 to −68 showing 80% identity. Four TATA boxes were identified upstream of the murine transcriptional initiation site, but deletion and transfection analysis using reporter gene constructs in HepG2 cells indicated that only the TATA element at position −30, together with sequences −395 to −111, are essential for constitutive expression of murine C3 in hepatocytes. Deletion analysis also suggested that sequences between −1457 and −800 contain regulatory elements that are involved in suppressing basal expression. Sequences between −90 to −41 confer both enhancer activity and interleukin-1/-6 (IL-1/IL-6)-responsiveness. Mutation analyses showed that both sequences between −88 and −83 and −77 to −72 are essential for enhancer activity and responsiveness to IL-1, but only sequences between −88 and −83 are necessary for IL-6-responsiveness. A gel-retardation assay showed that several nucleoproteins, perhaps of the C/EBP family, from HepG2 cells bound to sequences between −88 to −83. Collectively, these results localize cis-acting elements involved in constitutive and IL-1/IL-6-regulated murine C3 gene expression and provide evidence for specific transacting factors.

INTRODUCTION

The third component of complement (C3) is a substrate for the C3-cleaving enzymes of the classical and alternative pathways of the complement cascade, and is the source of biologically active fragments mediating immune cytolysis, phagocytosis and non-cytotoxic enzyme release, among many other complement-dependent reactions (Lambris, 1989). C3 is a heterodimer (Bokisch *et al.*, 1975; Tack & Prahl, 1976) (α-chain 110 kDa, β-chain 70 kDa), synthesized as a single-chain preproprotein (Brade *et al.*, 1977), which undergoes co- and post-synthetic modification involving cleavage of a signal peptide (Lundwall *et al.*, 1984), excision of an interchain-linking peptide (Goldberger *et al.*, 1981; Wetsel *et al.*, 1984) and glycosylation (Hase *et al.*, 1985; Tomana *et al.*, 1985). Generation of a functionally important thioester bridge proceeds via isomerization of a lactam to generate a thiolactone (Khan & Erickson, 1982), perhaps mediated by a specific enzyme (Iijima *et al.*, 1984). The C3 protein is programmed by an approx. 5.2 kb mature mRNA derived from a 42 kb gene (Fong *et al.*, 1990; Vik *et al.*, 1991) on human chromosome 19 (Whitehead *et al.*, 1982) and on murine chromosome 17 (Penalva-DaSilva *et al.*, 1978). Like many plasma proteins, C3 is synthesized in the liver (hepatocytes) (Alper *et al.*, 1969), but it is also expressed at extrahepatic sites: in mononuclear phagocytes (Stecher & Thorbecke, 1967; Einstein *et al.*, 1977), fibroblasts (Thorbecke *et al.*, 1965; Katz & Strunk, 1988), uterine epithelium (Sundstrom *et al.*, 1989), vascular endothelium (Warren *et al.*, 1987), type II alveolar cells (Strunk *et al.*, 1988) and astroglia (Levi-Strauss & Mallat, 1987).

Tissue injury or inflammation elicits an acute phase response which includes predictable changes in plasma protein concentrations. These primarily reflect changes in transcription of acute phase genes in liver which, for C3, results in a 2–3-fold increase in its concentration in plasma. Clinical (Ruddy & Colten, 1974) and experimental (Falus *et al.*, 1987; Passwell *et al.*, 1988) data indicate that acute phase stimuli also modulate complement C3 gene expression at extrahepatic sites, where the effects (up to 20-fold increases) often exceed the changes in hepatic expression. Stimuli for this response include bacterial products (e.g. endotoxin) among other exogenous moieties and endogenous mediators, including the cytokines interleukin-1 (IL-1), interleukin-6 (IL-6), tumour necrosis factor (TNF) and interferon-γ (IFN-γ) (Colten, 1992).

Efforts to ascertain the molecular mechanisms governing the regulated expression of the acute phase complement gene factor B tentatively defined 5′-flanking sequences necessary and sufficient to effect IL-1- and IFN-γ-induced increases in factor B transcription (Wu *et al.*, 1987; Nonaka *et al.*, 1989). In order to define the cis regulatory elements important for constitutive and regulated expression of C3, and to relate these to general mechanisms of regulated acute phase gene expression, we have isolated and characterized the 5′-flanking sequences of the murine and human C3 genes. Utilizing a series of mutant chimeric/reporter gene constructs we have defined enhancer and IL-1/IL-6-responsive elements juxtaposed to the C3 gene. Furthermore, we have identified DNA-binding proteins which bind to these regulatory regions. Evidence for a correspondence between nucleoprotein-binding sites within these regions and the functional data supports the role of these cis- and trans-acting elements in constitutive and regulated C3 gene expression.

EXPERIMENTAL

Materials

Except where noted, restriction enzymes and other molecular biology reagents were purchased from either Boehringer–

Abbreviations used: C3, the third component of complement; CAT, chloramphenicol acetyltransferase; IL-1, interleukin-1; IL-6, interleukin-6; IFN-γ, interferon-γ; NF, nuclear factor; ONPG, *O*-nitrophenyl-β-D-galactopyranoside; PMSF, phenylmethanesulphonyl fluoride; Tk, thymidine kinase; TNF, tumour necrosis factor.

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under accession numbers X62903 and X62904.

Mannheim Biochemicals (Indianapolis, IN, U.S.A.) or Promega Biotec (Madison, WI, U.S.A.), and were used according to the manufacturer's instructions. Acetyl-CoA and *O*-nitrophenyl-β-D-galactopyranoside (ONPG) were purchased from Boehringer–Mannheim Biochemicals. 1,10-Phenanthroline, 2,9-dimethyl-1,10-phenanthroline and cupric sulphate were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Recombinant IL-1β was a gift from Dr. John McKearn (Department of Biological Sciences, Monsanto, St. Louis, MO, U.S.A.). Recombinant IL-6 was purchased from Amgen Biologicals (Thousand Oaks, CA, U.S.A.). [^{14}C]-Chloramphenicol was purchased from ICN Biomedicals (Irvine, CA, U.S.A.).

Oligonucleotides

Oligonucleotides were synthesized using a PCR-Mate DNA synthesizer (Applied Biosystems, Foster City, CA, U.S.A.). Oligonucleotides were deprotected by incubation at 55 °C overnight with NH_4OH, then lyophilized and resuspended in sterile deionized/distilled water. Crude oligonucleotides were purified by electrophoresis on 16% polyacrylamide gels containing 7 M-urea, 90 mM-Tris/borate and 2 mM-EDTA, followed by NACS Pre-Pac column chromatography (Bethesda Research Laboratories, Gaitherberg, MD, U.S.A.). Oligonucleotides used in site-directed mutagenesis reactions were phosphorylated in a 20 μl volume containing 50 mM-Tris/HCl (pH 7.5), 10 mM-$MgCl_2$, 5 mM-dithiothreitol, 1 mM-ATP and 10 units of T4 polynucleotide kinase. Several oligonucleotides used for competitors of the gel-retardation assay were prepared: (1) CAGGAATTACGAAATGGAG [IL-6-dependent DNA binding protein (IL-6 DBP) binding site], (2) CACATTGCAA-TCTTAATAA [nuclear factor (NF)-IL-6 binding site], and (3) GGGGACTTTCC (NF-κB binding site).

Isolation of human and murine C3 genomic clones

Cosmid genomic clones containing the 5′-flanking region and most of the murine C3 gene were isolated from a mouse B10.D2/nSn liver library (Wetsel *et al.*, 1990). A 1.6 kb *Bam*HI fragment containing the 5′-flanking region and the first exon of the murine C3 gene was excised from a genomic clone and subcloned into pSP72 (Promega). Likewise, genomic clones containing the 5′-flanking region and most of the human C3 gene were isolated from a cosmid library constructed as described (Wetsel *et al.*, 1990) using high-molecular-mass DNA isolated from white blood cells of a healthy female donor. A 5.0 kb *Pst*I fragment containing the 5′-flanking region of the human C3 gene was excised from a genomic clone and subcloned into pBluescript II (Stratagene, La Jolla, CA, U.S.A.).

Chloramphenicol acetyltransferase (CAT)–(murine C3) fusion constructs

The translation initiation codon was deleted from the 1.6 *Bam*HI murine C3 subclone by linearization with *Sac*I, *Bal*31 digestion, addition of *Hin*dIII linkers, and re-ligation using T4 DNA ligase. The 1.5 kb *Hin*dIII fragment was subsequently excised from this plasmid and subcloned into pSVOCAT (Gorman *et al.*, 1982), generating the plasmid pC3-SVOCAT. The *Bam*HI fragment of pC3-SVOCAT was subcloned into pBluescript II (Stratagene), generating pC3-CAT. pBL-SV2CAT was generated by subcloning the *Acc*I/*Bam*HI fragment of pSV2CAT (Gorman *et al.*, 1982) into the *Sma*I site of pBluescript II-SK(+). The *Xba*I/*Sma*I fragment of the TKCAT gene from PTE2Δ*Sal*/*Nru* (Sullivan & Peterlin, 1987) was inserted between the *Xba*I and *Sma*I sites of pBluescript KS(+) (pBL-TKCAT). pC3-TKCAT was constructed by insertion of double-stranded synthetic oligonucleotides that corresponded to positions −90 to −41 of the murine C3 gene into the *Xba*I site of pBL-TKCAT. Oligonucleotides with mutations between positions −90 and −41 were synthesized by substitution to the *Hin*dIII site at positions −54 to −49 (M1), −77 to −72 (M2) or −88 to −83 (M3), and inserted into the *Xba*I site of pBL-TKCAT to generate pC3M1-TKCAT, pC3M2-TKCAT or pC3M3-TKCAT respectively. The orientation and copy number of inserts were defined by sequence analysis.

Deletion mutants and site-directed mutagenesis

The internal deletion mutants pM20, pM45, pM70, pM90, pM110, pM395 and pM606 were constructed by site-directed mutagenesis (Wang *et al.*, 1989) using oligonucleotides (42-mers) designed to delete positions −70 to −21, −70 to −46, −90 to −71, −110 to −91, −160 to −111, −445 to −396 and −655 to −607 respectively (see Fig. 1). These oligonucleotides were designed so that *Sph*I and *Bgl*II restriction sites were introduced 5′ and 3′ respectively to each deletion. Single-stranded template DNAs substituted with uracil were prepared from phagemid DNA recovered from the supernatant of pC3-CAT-containing *Escherichia coli* CJ236 after infection with R408 helper phage (Stratagene). After hybridization of the phosphorylated oligonucleotides to the uracil-containing template, *in vitro* DNA synthesis was performed in a volume of 100 μl containing 20 mM-Hepes (pH 7.8), 2 mM-dithiothreitol, 10 mM-$MgCl_2$, 500 μM-dNTPs, 1 mM-ATP, 4 units of T4 DNA polymerase and 4 units of T4 DNA ligase. DNA from mutagenesis reactions was used to transform competent *E. coli* XL-1-Blue (Stratagene). The long internal deletion mutants pM45S, pM110S, pM395S and pM606S were generated by subsequent removal of the *Sph*I fragment from pM45, pM110, pM395 and pM606 respectively. The 5′ deletion mutants pM45-NS, pM70-NS, pM90-NS, pM110-NS and pM395-NS were constructed by removal of the *Not*I/*Sph*I fragment from pM45, pM70, pM90, pM110 and pM395 respectively. The mutant pM280-NS was made by deletion of the *Xba*I fragment from pC3-CAT.

Cell culture and transfection

HepG2 cells were grown to 80% confluent monolayers (35 mm dishes) in Dulbecco's modified Eagle's medium (GIBCO, Gaithersburg, MD, U.S.A.) supplemented with 10% fetal calf serum for 24 h prior to transfection. The HepG2 cells were co-transfected by the calcium phosphate-mediated gene transfer procedure (Kingston, 1989) using 3.4 pmol of test plasmid and 0.6 pmol of the β-galactosidase expression vector pCH110 (Pharmacia). Precipitates were removed after 4 h, the cells were washed twice and fresh serum-free medium with or without cytokines was added and incubated with the cells for 20 h.

CAT assay

HepG2 cells (5×10^5 cells) were harvested and lysed by the freeze–thaw procedure in 120 μl of 0.25 M-Tris (pH 7.8). The β-galactosidase activity of cell lysates was determined as described (Herbomel *et al.*, 1984), and values were used to normalize variability in the efficiency of transfection. Briefly, 1 ml of 60 mM-Na_2HPO_4/40 mM-NaH_2PO_4/10 mM-KCl/1 mM-$MgCl_2$/50 mM-β-mercaptoethanol and 0.2 ml of ONPG (2 mg/ml in 60 mM-Na_2HPO_4/40 mM-NaH_2PO_4) were added to 20 μl of cell extract. The reactions were carried out at 37 °C until a yellow colour was obvious, and were stopped by adding 0.5 ml of 1 M-Na_2CO_3. Absorbance readings at 420 nm were measured to estimate β-galactosidase activity. The CAT assays were performed by the method of Gorman *et al.* (1982), with the following modifications. Cell extract (20 μl) was made up to 100 μl with 0.25 M-Tris/HCl (pH 7.8), 20 μl of 4 mM-acetyl-CoA and 0.3 μCi of [^{14}C]chloramphenicol. The reaction was carried out at 37 °C for 1 h. Then the reaction was stopped and the chloramphenicol was extracted

with 0.8 ml of ethyl acetate. The solvent was dried out, and the pellet was resuspended in 10 μl of ethyl acetate and spotted on a silica gel thin-layer plate to separate the native chloramphenicol from its acetylated derivatives. Migration was in chloroform/methanol (19:1, v/v) for 1 h. After autoradiography, the spots were cut out and radioactivity was counted to quantify the amount of chloramphenicol converted to the 3-acetyl forms.

Nuclear extracts and gel-retardation assays

Nuclear extracts were prepared essentially as described by Miskimins *et al.* (1985). Briefly, cells were collected with trypsin/EDTA solution (0.05%/0.02%), washed with phosphate-buffered saline (140 mM-NaCl, 2.7 mM-KCl, 15.3 mM-Na_2HPO_4, 1.5 mM-KH_2PO_4, pH 7.4) and resuspended in lysis buffer [10 mM-Hepes (pH 8.0), 50 mM-NaCl, 0.5 M-sucrose, 0.1 mM-EDTA, 0.5% Triton X-100, 1 mM-dithiothreitol, 5 mM-$MgCl_2$ and 1 mM-phenylmethanesulphonyl fluoride (PMSF)] at 3.5×10^7 cells/ml. After incubation on ice for 10 min, the nuclei were pelleted by centrifugation at 500 *g*. The pellet was washed twice in lysis buffer without Triton X-100, and resuspended in lysis buffer without Triton X-100 at 7×10^7 nuclei/ml. Spermidine was added to 5 mM and NaCl to 0.5 M. The suspension was incubated on ice for 1 h and then centrifuged at 12000 *g* for 30 min. The supernatant was dialysed against buffer [20 mM-Hepes (pH 8.0), 0.1 M-KCl, 0.2 mM-EDTA, 0.5 mM-PMSF, 0.5 mM-dithiothreitol and 20% (v/v) glycerol] overnight and then was divided into aliquots and stored at −70 °C. The gel-retardation assay was performed by the method of Fried & Crothers (1981). Briefly, double-stranded oligonucleotides corresponding to positions −90 to −41 of the murine C3 gene were 5′-end-labelled with T4 polynucleotide kinase and used as a probe. Nuclear extract (5 μg) from HepG2, 1×10^4 c.p.m. of labelled probe and 1.5 μg of poly(dI-dC)·poly(dI-dC) were incubated in the binding buffer [10 mM-Tris-HCl (pH 7.5), 50 mM-NaCl, 1 mM-dithiothreitol, 1 mM-EDTA and 50% (v/v) glycerol] for 30 min at room temperature. DNA–protein complexes were separated on a 5% polyacrylamide gel in TAE (7 mM-Tris/HCl, 3.3 mM-sodium acetate, 1 mM-EDTA, pH 7.5) buffer. The gel was dried and subjected to autoradiography.

Footprinting

Binding reactions, as described in gel-retardation assay, were carried out using 20 μg of nuclear extract, 6 μg of poly(dI-dC)·poly(dI-dC) and 1×10^5 c.p.m. of asymmetrically end-labelled, double-stranded, oligonucleotide probe. Free and bound DNA-containing complexes were excised from a 5% polyacrylamide gel after electrophoresis, and footprinting was performed according to the method of Kuwabara & Sigman (1987).

Briefly, excised bands were immersed in 100 μl of 50 mM-Tris/HCl (pH 8.0). A 10 μl volume of solution A (2 mM-1,10-phenanthroline and 0.45 mM-cupric sulphate) was added, followed by 10 μl of solution B (58 mM-3-mercaptopropionic acid). The digestion was carried out for 10 min at room temperature and then quenched by addition of 10 μl of 28 mM-2,9-dimethyl-1,10-phenanthroline and 270 μl of a solution containing 0.5 M-

Fig. 1. Murine and human 5′-flanking genomic sequences

The nucleotide sequences upstream of the murine and human C3 transcriptional initiation sites were aligned by the computer program GAP (Devereux *et al.*, 1984). Transcriptional start sites are at position +1. Human C3 has an additional transcriptional start site at T, two nucleotides downstream of the one indicated in the Figure. The TATA sequences are boxed and murine sequences protected in the footprinting analysis (see text) are shaded. Sequences with similarities to known nuclear binding sites and/or regulatory sequences are as follows. Murine: AP-2 (−115 to −122 and −129 to −136), SP-1 (−49 to −54, −72 to −77, −288 to −293, −659 to −664, −785 to −790, and −931 to −936), DEXAM-RE (−433 to −438), HP-1 (−172 to −184, −304 to −316, −453 to −465, and −578 to −590), IL-6 RE (−307 to −312, −656 to −661, −669 to −674, and −1144 to −1149), NF-κB (−67 to −76) and oestrogen (−1589 to −1601; results not shown). Human: AP-2 (−133 to −140), SP-1 (−53 to −58, −57 to −63, −93 to −98, −206 to −211, and −303 to −308), DEXAM-RE (−282 to −287), HP-1 (−274 to −286, −292 to −304, and −639 to −652), IL-6 (−219 to −224, −413 to −418, and −480 to −485) and NF-κB (−297 to −306, and −474 to −483).

ammonium acetate and 1 mM-EDTA. The DNA was then eluted overnight at 37 °C. The eluted DNA was ethanol-precipitated, resuspended in 80% (v/v) formamide, 10 mM-NaOH, 1 mM-EDTA, 0.1% Bromophenol Blue and 0.1% xylene cyanol, and loaded on a 10% sequencing gel.

RESULTS

Nucleotide sequences 5′ to the human and murine C3 genes

Murine (B10.D2/nSn strain) (Wetsel *et al.*, 1990) and human genomic libraries were screened with C3 cDNA probes that were derived from 5′ restriction fragments. Hybridizing clones were identified, and the corresponding cosmid DNA was isolated, digested with several restriction enzymes and subjectd to Southern analysis. A 1.6 kb murine *Bam*HI restriction fragment and a 5.0 kb human *Pst*I fragment were subsequently isolated, subcloned and sequenced on both strands. The human and murine C3 5′-flanking sequences were aligned starting from their major transcriptional initiation sites. As shown in Fig. 1, the murine *Bam*HI fragment extended 280 bp 5′ of the human *Pst*I fragment. The overall sequence identity between the murine and human C3 5′-flanking regions is 51%, with regional conservation of sequences of an even greater degree present. For example, the murine sequence is 80% identical to the human sequence from positions −36 to −1 and −146 to −68. The major transcriptional initiation site for murine C3 was determined to be 56 bp upstream of the ATG codon of prepro-C3 by RNAase protection and primer extension experiments using total liver RNA (results not shown). Two major transcriptional initiation sites were determined by primer extension analysis for human C3, using poly(A)$^+$ RNA obtained from adult human liver (results not shown). These sites were 61 and 59 bp upstream of the ATG translational start codon of human prepro-C3, and were in close agreement with the sites determined using HepG2 total RNA (Vik *et al.*, 1991). Four TATA boxes (at positions −30, −402, −613 and −1265) were identified upstream of the murine transcriptional initiation site. The most downstream murine TATA box and the human TATA box are identically placed, and are positioned upstream of their respective transcription initiation sites, as are most promoter TATA boxes in eukaryotic genes. However, the human sequence does not conform exactly to the consensus TATA box motif, i.e. the nucleotide at position −31 is a G rather than a T (see Fig. 1). It has been demonstrated recently that a wide variety of DNA sequences can functionally replace a TATA element for transcriptional activation in yeast (Singer *et al.*, 1990). Therefore it is possible that the same sequence flexibility can occur in mammalian promoter elements. Several other sequences with sequence similarity to previously recognized binding sites and/or potential 'regulator sequences' were also identified in the murine and human C3 5′-flanking regions. These include binding sites of AP-1, AP-2, SP-1 (Mitchell & Tjian, 1989), DEXAM (Kunz *et al.*, 1989), oestrogen (Martinez *et al.*, 1987), HP-1 (Ryffel *et al.*, 1989), IL-6 RE (Majello *et al.*, 1990) and NF-κB (Lenardo & Baltimore, 1989).

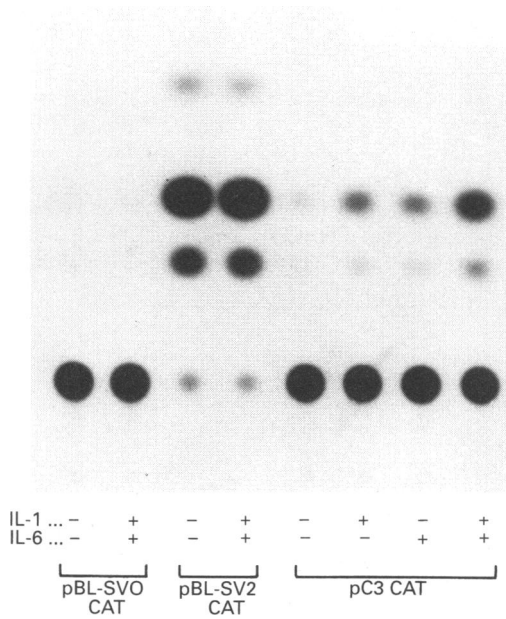

Fig. 2. Regulation of C3-CAT construct in HepG2 cells

HepG2 cells were transfected with pC3-CAT, which contained 1.5 kb of 5′-flanking region of the murine C3 gene, and treated with IL-1β (10 ng/ml) and/or IL-6 (10 ng/ml). pBL-SVO CAT and pBL-SV2 CAT were used for negative and positive controls respectively. The CAT assay was performed as described in the Experimental section. One of five independent experiments is shown here. Average induction by IL-1β, IL-6 and the combination of both cytokines was 5.0±1.17-, 3.8±0.42- and 11.6±2.25-fold respectively.

Table 1. Essential TATA box for murine C3 gene expression in HepG2 cells

The basal activity is the ratio of normalized CAT activity of each construct to that of pC3-CAT, in the absence of cytokines. The stimulation index is the ratio of normalized CAT activity from cells treated with cytokine to basal CAT activity. Means±S.D. of three independent experiments are shown.

Construct	Basal CAT activity	Stimulation index	
		IL-1	IL-6
pC3-CAT	1.00	5.2±1.3	3.6±0.4
pM20	0.03±0.01	0.9±0.1	1.6±0.2
pM395	1.13±0.26	5.0±0.4	4.1±0.3
pM606	0.96±0.04	5.6±0.5	4.3±0.3

Functional analysis of sequences flanking the C3 gene

A chimeric construct consisting of the 1.5 kb murine C3 5′-flanking sequence coupled to the reporter CAT gene was transfected into the human hepatoma HepG2 cell line. This cell line was chosen because previous studies established that it did not express either IL-1 or IL-6 (Perlmutter *et al.*, 1989), so that the effect of each cytokine could be tested independently. Following incubation in medium or medium containing various concentrations of IL-1, IL-6 or IL-1 plus IL-6, CAT activity was estimated and the data were normalized for copy number by assaying for the co-transfected β-galactosidase gene activity. Fig. 2 shows the result of one of five experiments in which optimal concentrations of IL-1 (10 ng/ml) and IL-6 (10 ng/ml) resulted in 5.0±1.2- and 3.8±0.42-fold inductions respectively over constitutive levels of expression. IL-1 plus IL-6 (each at 10 ng/ml) effected an 11.6±2.25-fold increase. Neither IL-1 nor IL-6 affected expression of negative (pBL-SVOCAT) or positive (pBL-SV2CAT) control transfectants. The minimal concentration of IL-1 or IL-6 that induced a significant up-regulation of this construct was 1 ng/ml (results not shown).

Promoter/enhancer elements flanking the C3 gene

Internal deletions of three of the four TATA boxes 5′ to the C3 transcriptional initiation site demonstrated that the element at −30 bp upstream is essential for constitutive expression, and that deletion of the elements at −402 and −613 had no effect on

Table 2. Regulatory region of the murine C3 gene

Basal CAT activity and stimulation index were determined as described in Table 1. Means ± S.D. of three to four independents experiments are shown.

(*a*) 5′ deletion mutants

5′ deletion mutants		Basal CAT activity	Stimulation index		
			IL-1	IL-6	IL-1+IL-6
−1457 +43	pC3-CAT	1.00	5.2±1.3	3.6±0.4	10.7±2.2
−395	pM395-NS	1.49±0.14	4.1±0.7	4.8±0.6	15.1±3.6
−280	pM280-NS	1.71±0.21	4.3±0.4	4.6±0.6	10.6±1.1
−110	pM110-NS	4.35±0.61	2.9±0.2	3.6±0.2	7.6±0.9
−90	pM90-NS	2.23±0.20	2.6±0.1	3.8±0.7	5.3±1.0
−70	pM70-NS	0.13±0.01	0.6±0.2	1.6±0.1	0.6±0.1
−45	pM45-NS	0.45±0.11	0.4±0.0	1.4±0.1	0.9±0.1

(*b*) Long internal deletion mutants

Internal deletion mutants		Basal CAT activity	Stimulation index		
			IL-1	IL-6	IL-1+IL-6
−1457 +43	pC3-CAT	1.00	5.2±1.3	3.6±0.4	10.7±2.2
−800 −606	pM606-S	1.00±0.05	4.1±0.8	3.6±0.2	9.9±2.0
−395	pM395-S	1.29±0.19	4.7±1.0	3.0±0.8	10.5±3.0
−110	pM110-S	0.37±0.05	3.0±0.8	2.9±0.4	7.0±1.5
−45	pM45-S	0.04±0.01	0.9±0.01	1.0±0.1	0.8±0.1

(*c*) Short internal deletion mutants

Internal deletion mutants		Basal CAT activity	Stimulation index	
			IL-1	IL-6
−1457 +43	pC3-CAT	1.00	5.2±1.3	3.6±0.4
−161 −110	pM110	1.53±0.07	4.1±0.9	2.9±0.3
−111 −90	pM90	1.21±0.10	5.7±1.1	3.3±0.3
−91 −70	pM70	0.03±0.01	0.8±0.1	1.1±0.1
−71 −45	pM45	0.04±0.00	1.1±0.2	1.2±0.1

constitutive or IL-1/IL-6-regulated expression (Table 1). Constitutive expression of a 5′ deletion mutant CAT construct which lacked the most upstream TATA box was similar to expression of the full-length construct.

A series of 5′ and internal deletion mutants was constructed and transfected into HepG2 cells to identify functionally one or more potential regulatory elements flanking the C3 gene (Table 2). In the 5′ deletion analysis (Table 2*a*), basal CAT activity was more than 4-fold greater than the activity of the intact 1.5 kb construct following deletion of sequences 5′ to −110; further deletions to −70 and −45 resulted in a marked decrease in basal CAT activity. IL-1 and IL-6 each induced an increase in CAT expression in all but the latter two deletion constructs. To refine this analysis, a series of internal deletions (Table 2*b*) was constructed. Deletion between −800 and −111 (pM110-S) resulted in a striking decrease in basal CAT activity, with little change in the capacity for IL-1/IL-6 induction. These data suggest that, in addition to the TATA box at −30, sequences between −395 and −111 are required for constitutive expression. Furthermore, a negative regulatory element between −1457 and −800 is likely, since the construct deleted 5′ to −110 is expressed at higher than constitutive levels and the internal deletion −800 to −111 at lower than constitutive levels. However, we cannot exclude the possibility that such an effect might be caused by

sequences in artificial constructs that do not necessarily have an effect *in vivo*. The further internal deletion of nucleotides to −46 [i.e. from −800 to −46 (pM45-S)] results in little basal CAT activity and no IL-1/IL-6 response. A further refinement using smaller internal deletion constructs (Table 2*c*) resulted in loss of constitutive and IL-1/IL-6 responsiveness with deletion of sequences between −90 and −71 and between −70 and −46.

Nucleoproteins bound to the enhancer and IL-1/IL-6 response region

Footprinting analysis using 1,10-phenanthroline–Cu^{2+} showed two protected regions after binding of nucleoproteins isolated from HepG2 cells with a radiolabelled fragment containing sequences from −90 to −41 of the murine C3 gene (Fig. 3). One of them (positions −89 to −69) is a strong footprint and includes sequences very similar to an IL-6-responsive element (Majello *et al.*, 1990), the binding sites of a C/EBP family [C/EBP (Ryden & Beemon, 1989), IL-6DBP (Poli *et al.*, 1990), and NF-IL6 (Akira *et al.*, 1990)] and the NF-κB-binding site (Lenardo & Baltimore, 1989). Another region (positions −56 to −48) is a weak footprint and contains sequences similar to the Sp1-binding site.

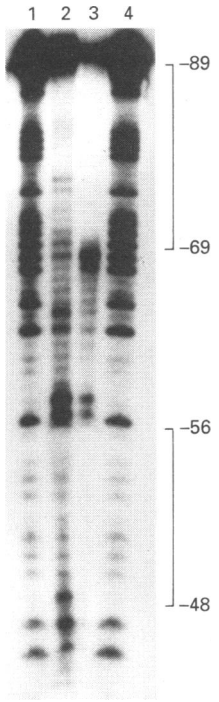

Fig. 3. Nuclear factor binding domains

Binding reactions and footprinting analyses were carried out on the non-coding strands as described in the Experimental section. Lanes 1 and 4 indicate the G+A reaction. Lane 2 indicates the free DNA. Lane 3 indicates the retarded complexes. Sequences protected from phenanthroline/copper treatment are indicated by brackets. Numbers refer to the positions relative to the start site of transcription (see Fig. 1).

Enhancer and IL-1/IL-6-responsive elements

To assess possible enhancer and cytokine-responsive elements in the region −90 to −41, CAT constructs containing the thymidine kinase (TK) promoter and C3 flanking sequences or mutant C3 sequences were constructed. As shown in Table 3, basal CAT activity was increased 2-fold by adding a single copy of the sequences from −90 to −41 of the C3 gene. Furthermore, basal CAT activity was increased 9-fold by three copies inserted upstream of the promoter. IL-1 decreased and IL-6 had no effect on expression of CAT in the construct with the TK promoter alone. The presence of the sequences from −90 to −41 conferred reproducible IL-1 and IL-6 responsiveness when compared with the effect of the cytokines on the TK promoter construct. Mutation between −88 to −83 (M3) caused loss of both IL-1/IL-6 responsiveness and enhancer activity. Mutation between −77 to −72 (M2) caused loss of IL-1 responsiveness and enhancer activity, but not of IL-6 response. Mutation between −54 to −49 (M1) had no effect on either IL-1/IL-6 responsiveness or enhancer activity. The M3 and M2 regions have high sequence similarities with the C/EBP-binding site and the NF-κB-binding site respectively.

These data suggest that enhancer activity and IL-1 responsiveness are very closely related and are mediated by several transcription factors similar to C/EBP or NF-κB. On the other hand, IL-6 and IL-1 responsiveness might be separable, although they are closely related to each other.

Characterization of nucleoproteins bound to the enhancer and IL-1/IL-6-responsive elements

Nucleoproteins isolated from non-stimulated HepG2 cells, when mixed with a radiolabelled fragment containing sequences from −90 to −41, showed at least four specific bands by the gel-retardation assay. The gel-shift patterns of cytokine-stimulated samples were different from that of an unstimulated sample, but

Table 3. Enhancer and IL-1/IL-6-responsive elements

The arrow indicates one copy of double-stranded synthetic oligonucleotide and its orientation. The X's indicate mutated sequences. Basal CAT activity means the ratio of normalized CAT activity without cytokine of the respective construct to that of pTK-CAT. Means ± S.D. of more than three independent experiments are shown.

TK-CAT constructs	Basal CAT activity	Stimulation index	
		IL-1	IL-6
TK	1.00	0.4±0.1	1.3±0.2
C3-TK ←	2.27±0.21	1.0±0.1	2.3±0.2
C3M1-TK ✱	2.11±0.01	1.1±0.1	2.3±0.1
C3M2-TK ✱	1.10±0.14	0.5±0.1	2.5±0.1
C3M3-TK ✱	0.71±0.10	0.4±0.1	1.5±0.2
C3(×3)-TK →→→	9.34±1.13	3.1±0.1	2.4±0.2
C3(×3)-TK ←←←	9.44±1.75	2.7±0.0	2.9±0.1
C3M1(×3)-TK ✱✱✱	12.50±0.81	2.5±0.2	2.1±0.1
C3M3(×3)-TK ✱✱✱	0.42±0.02	0.5±0.0	1.2±0.0

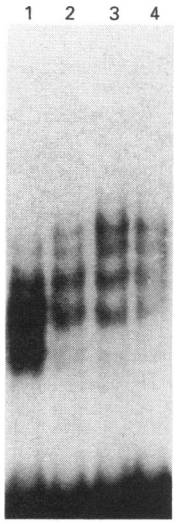

Fig. 4. Binding of nucleoproteins from HepG2 cells to enhancer and IL-1/IL-6 response regions flanking the C3 gene

The gel-retardation assay was carried out as described in the Experimental section. DNA fragment (1×10^4 c.p.m.) corresponding to positions -90 to -41 of the murine C3 gene was incubated with nuclear extracts from HepG2 cells. Gel-retardation assays are shown of nuclear extracts from HepG2 cells stimulated with no cytokines (lane 1), IL-1 (lane 2), IL-6 (lane 3) or IL-1 plus IL-6 (lane 4).

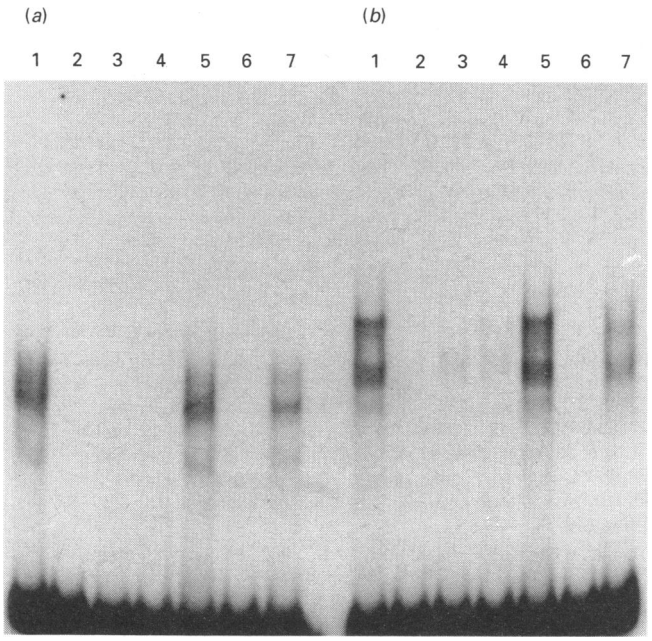

Fig. 5. Competition analysis of nucleoprotein binding to the enhancer and IL-1/IL-6-response region of the C3 gene

(*a*) Experiments performed with nuclear extracts prepared from non-stimulated HepG2 cells; (*b*) experiments performed with nuclear extracts prepared from HepG2 cells stimulated with IL-1 and IL-6. Lane 1 shows the gel-retardation pattern without competitors; others show competitions with murine C3 sequences (-90 to -41) (lane 2), IL-6DBP-binding sequences (lane 3), NF-IL6-binding sequences (lane 4), NF-κB-binding sequences (lane 5), M2 mutant (lane 6) and M3 mutant (lane 7).

we could not distinguish the patterns of stimulated samples from one another (Fig. 4).

Competition experiments with sequences corresponding to the C/EBP family or NF-κB-binding sites suggested that nucleoproteins bound to the enhancer and IL-1/IL-6-responsive region belong to the C/EBP family. Competition experiments with mutant C3 sequences (M2, M3) showed that these nucleoproteins bind to the M3 region, but not the M2 region (Fig. 5).

DISCUSSION

The molecular mechanisms governing expression of genes encoding the acute phase proteins, including serum amyloid A (Edbrooke *et al.*, 1989), C-reactive protein (Majello *et al.*, 1990), fibrinogen (Fowlkes *et al.*, 1984), α_1-antitrypsin (Rangan & Das, 1990), α_2-macroglobulin (Hattori *et al.*, 1990), acid glycoprotein (Dewey *et al.*, 1990) and the complement proteins (Colten, 1992) have been a subject of great interest in the past few years. Recent studies suggest that multiple, but overlapping, mechanisms account for the response of the positive acute phase reactants (increased expression follows an acute phase stimulus). Some of these genes are up-regulated only by IL-6 (e.g. α_1-antitrypsin, fibrinogen), others only by IFN-γ (e.g. C4), while several (e.g. factor B, C3) are up-regulated by multiple cytokines, including IL-1, TNF, IL-6 and IFN-γ. Evidence that the complement genes C3 and factor B are responsive to IL-1 and IL-6 was obtained in studies demonstrating a 10–100-fold induction of these genes by IL-1 in fibroblasts, but only a 2–3-fold increase of factor B and C3 in HepG2. The fibroblasts express IL-6, while the HepG2 cells used in the present study do not (Perlmutter *et al.*, 1989).

In the present study, we identified several cis- and trans-acting elements required for constitutive and cytokine (IL-1/IL-6)-regulated expression of the murine complement C3 gene. Multiple TATA boxes were identified upstream of the C3 transcriptional initiation site, though deletion of only one resulted in the loss of basal CAT activity in functional studies using HepG2, a well-differentiated human hepatoma-derived cell line. C3 is expressed constitutively in many tissues, and its regulated expression is tissue-specific. The possibility that one or more of the other TATA boxes is of functional importance and that alternative transcriptional initiation sites are utilized at extrahepatic sites cannot be excluded. Moreover, elements required for constitutive expression and negative regulatory elements were tentatively identified in the C3 gene upstream of this TATA box, at -395 to -111 and -1457 to -800 respectively. These elements might play an important role in the tissue specificity of C3 gene expression.

Mutation analyses revealed enhancer and IL-1/IL-6-responsive elements between positions -90 and -41 of the murine C3 gene. The sequences highly similar to C/EBP- or NF-κB-binding sites are located side by side in this region (TTGCGAAAT from -89 to -81 or GGGCAGTCCC from -77 to -68 respectively) and these sequences are highly conserved in both the human (-110 to -79) and the mouse (-89 to -68). Recently, Wilson *et al.* (1990) reported an analysis of human C3 gene regulation which reached similar conclusions. They suggested that TTGAGAAAT from -110 to -102 of the human C3 gene mediates IL-1-responsiveness, and CTGGGG from -101 to -96 confers IL-6-responsiveness, although they did not show any definitive functional data from which to draw such a conclusion. Their conclusion is different from ours. Our data indicated that IL-1-responsiveness required both sequences similar to C/EBP or NF-κB-binding sites and was closely related to enhancer activity, but only the sequences similar to C/EBP-binding site were necessary for IL-6-responsiveness (Table 3).

We could identify DNA-binding proteins bound to the C/EBP family binding consensus region which is important for enhancer

activity and IL-1/IL-6-responsiveness, but could not define any proteins bound to the NF-κB binding consensus region (Fig. 5). This should not be a result of problems with the experimental conditions, because we could detect NF-κB-like proteins under the same conditions by using the IL-1-responsive element of murine factor B gene as probe (results not shown). Brasier *et al.* (1990) demonstrated that a family of C/EBP-like proteins attenuated the binding of the IL-1-induced NF-κB to the acute phase response element of the angiotensinogen gene. This is not likely for the C3 gene, because the probe mutated at the C/EBP binding consensus region (M3 mutant) did not bind to any nucleoproteins, although the probe mutated at the NF-κB binding consensus region (M2 mutant) could bind to C/EBP-like proteins (results not shown).

More recently, Won & Baumann (1991) identified an IL-1-inducible and heat-labile factor (NF-AB) by Southwestern blot analysis which they could not detect by gel-retardation analysis or DNAase I protection assays. Such an approach might be helpful in investigating the interaction between DNAs and proteins. Another possibility is that some weak protein–protein interactions or bridging of two cis elements by a single nucleoprotein may be involved in the induction by cytokines. In any event, both the results of Wilson *et al.* (1990) and our data indicate that the enhancer activity and IL-1/IL-6-respoinsiveness of the C3 gene are closely related to one another and are regulated by some overlapping mechanisms.

We thank Barbara Pellerito for her assistance in preparation of this manuscript. This work was supported by United States Public Health Service Grants AI25011, AI24836, HL37591, HD17461 and AI24835. R.A.W. is the recipient of Research Career Development Award AI00919 from the National Institutes of Health.

REFERENCES

Akira, S., Isshiki, H., Sugita, T., Tanabe, O., Kinoshita, S., Nishio, Y., Nakajima, T., Hirano, T. & Kishimoto, T. (1990) EMBO J. **9**, 1897–1906

Alper, C. A., Johnson, A. M., Birtch, A. M. & Moore, F. D. (1969) Science **163**, 286–288

Bokisch, V. A., Dierich, M. P. & Muller-Eberhard, H. J. (1975) Proc. Natl. Acad. Sci. U.S.A. **72**, 1989–1993

Brade, V., Hall, R. E. & Colten, H. R. (1977) J. Exp. Med. **146**, 759–765

Brasier, A. R., Ron, D., Tate, J. E. & Habener, J. F. (1990) EMBO J. **9**, 3933–3944

Colten, H. R. (1992) J. Appl. Physiol. **72**, 1–7

Devereux, J., Haeberli, P. & Smithies, O. (1984) Nucleic Acids Res. **12**, 387–395

Dewey, M. J., Rheaume, C., Berger, F. G. & Baumann, H. (1990) J. Immunol. **144**, 4392–4398

Edbrooke, M. R., Burt, D. W., Cheshire, J. K. & Woo, P. (1989) Mol. Cell. Biol. **9**, 1908–1916

Einstein, L. P., Hansen, P. J., Ballow, M., Davis, A. E., Davis, J. S., Alper, C. A., Rosen, F. S. & Colten, H. R. (1977) J. Clin. Invest. **60**, 963–969

Falus, A., Beuscher, H. U., Auerbach, H. S. & Colten, H. R. (1987) J. Immunol. **138**, 856–860

Fong, K. Y., Botto, M., Walport, M. J. & So, A. K. (1990) Genomics **7**, 579–586

Fowlkes, D. M., Mullis, N. T., Comeau, C. M. & Crabtree, G. R. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 2313–2316

Fried, M. G. & Crothers, D. M. (1981) Nucleic Acids Res. **9**, 6505–6525

Goldberger, G., Thomas, M. L., Tack, B. F., Williams, J., Colten, H. R. & Abraham, G. N. (1981) J. Biol. Chem. **256**, 12617–12619

Gorman, C. M., Moffat, L. F. & Howard, B. H. (1982) Mol. Cell. Biol. **2**, 1044–1051

Hase, S., Kikuchi, N., Ikenake, T. & Inoue, K. (1985) J. Biochem. (Tokyo) **98**, 863–874

Hattori, M., Abraham, L. J., Northemann, W. & Fey, G. H. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 2364–2368

Herbomel, P., Bourachot, B. & Yaniv, M. (1984) Cell **39**, 653–662

Iijima, M., Tobe, T., Sakamoto, T. & Tomita, M. (1984) J. Biochem. (Tokyo) **96**, 1539–1546

Katz, Y. & Strunk, R. C. (1988) Arthritis Rheumat. **31**, 1365–1370

Khan, S. A. & Erickson, B. W. (1982) J. Biol. Chem. **257**, 11864–11867

Kingston, R. E. (1989) in: Current Protocols in Molecular Biology (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K., eds.), pp. 9.1.1–9.1.4, Green Publishing Associates and Wiley–Interscience, New York

Kunz, D., Zimmermann, R., Heisig, M. & Heinrich, P. C. (1989) Nucleic Acids Res. **17**, 1121–1138

Kuwabara, M. D. & Sigman, D. S. (1987) Biochemistry **26**, 7234–7238

Lambris, J. D. (ed.) (1989) Curr. Top. Microbiol. Immunol. **153**, 1–21

Lenardo, M. J. & Baltimore, D. (1989) Cell **58**, 227–229

Levi-Strauss, M. & Mallat, M. (1987) J. Immunol. **139**, 2361–2366

Lundwall, A. B., Wetsel, R. A., Domdey, H., Tack, B. F. & Fey, G. H. (1984) J. Biol. Chem. **259**, 13851–13856

Majello, B., Arcone, R., Toniatti, C. & Ciliberto, G. (1990) EMBO J. **9**, 457–465

Martinez, E., Givel, F. & Wahli, W. (1987) EMBO J. **6**, 3719–3727

Miskimins, W. K., Roberts, M. P., McClelland, A. & Ruyddle, F. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 6741–6744

Mitchell, P. J. & Tjian (1989) Science **24**, 371–378

Nonaka, M., Gitlin, J. D. & Colten, H. R. (1989) Mol. Cell. Biochem. **89**, 1–14

Passwell, J., Schreiner, G. F., Nonaka, M., Beuscher, H. U. & Colten, H. R. (1988) J. Clin. Invest. **82**, 1676–1684

Penalva-DaSilva, F., Hoecker, G. F., Day, N. K., Vienne, K. & Rubinstein, P. (1978) Proc. Natl. Acad. Sci. U.S.A. **75**, 963–965

Perlmutter, D. H., Colten, H. R., Adams, S. P., May, L. T., Sehgal, P. V. & Fallon, R. J. (1989) J. Biol. Chem. **264**, 7669–7674

Poli, V., Mancini, F. P. & Cortese, R. (1990) Cell **63**, 643–653

Rangan, V. S. & Das, G. C. (1990) J. Biol. Chem. **265**, 8874–8879

Ruddy, S. & Colten, H. R. (1974) N. Engl. J. Med. **290**, 1284–1288

Ryden, T. A. & Beemon, K. (1989) Mol. Cell. Biol. **9**, 1155–1164

Ryffel, G. U., Kugler, W., Wagner, U. & Kalig, M. (1989) Nucleic Acids Res. **17**, 939–953

Singer, V. L., Wobbe, C. R. & Struhl, K. (1990) Genes Dev. **4**, 636–645

Stecher, V. J. & Thorbecke, G. J. (1967) J. Immunol. **99**, 643–652

Strunk, R. C., Eidlen, D. M. & Mason, R. J. (1988) J. Clin. Invest. **81**, 1419–1426

Sullivan, K. E. & Peterlin, B. M. (1987) Mol. Cell. Biol. **7**, 3315–3319

Sundstrom, S. A., Komm, B. S., Ponce-de-Leon, H., Yi, Z., Teuscher, C. & Lyttle, C. R. (1989) J. Biol. Chem. **264**, 16941–16947

Tack, B. F. & Prahl, J. W. (1976) Biochemistry **15**, 4513–4521

Thorbecke, G. J., Hochwald, G. M., von Furth, L. R., Muller-Eberhard, H. J. & Jackson, E. B. (1965) Complement Ciba Found. Symp. 1964, 99–119

Tomana, M., Niemann, M., Gardner, C. & Volanakis, J. E. (1985) Mol. Immunol. **22**, 107–111

Vik, D. P., Amiguet, P., Moffat, G., Fey, M., Amiguet-Barras, F., Wetsel, R. A. & Tack, B. F. (1991) Biochemistry **30**, 1080–1085

Wang, L-M., Geihl, D. K., Choudhury, G. G., Minter, A., Martinez, L., Weber, D. K. & Sakaguchi, A. Y. (1989) BioTechniques **7**, 1000–1010

Warren, H. B., Pantazis, P. & Davies, P. F. (1987) Am. J. Pathol. **129**, 9–13

Wetsel, R. A., Lundwall, A., Davidson, F., Gibson, T., Tack, B. F. & Fey, G. H. (1984) J. Biol. Chem. **259**, 13857–13862

Wetsel, R. A., Fleischer, D. T. & Haviland, D. L. (1990) J. Biol. Chem. **265**, 2435–2440

Whitehead, A. S., Solomon, E., Chambers, S., Bodmer, W. F., Povey, S. & Fey, G. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 5021–5025

Wilson, D. R., Juan, T. S. C., Wilde, M. D., Fey, G. H. & Darlington, G. J. (1990) Mol. Cell. Biol. **10**, 6181–6191

Won, K. & Baumann, H. (1991) Mol. Cell. Biol. **11**, 3001–3008

Wu, L.-C., Morley, B. J. & Campbell, R. D. (1987) Cell **48**, 331–342

Received 13 August 1991/6 November 1991; accepted 12 November 1991