

The Structure of an Alternate Form of Complement C3 that Displays Costimulatory Growth Factor Activity for B Lymphocytes

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Summary

In this study, the structure of a novel 1.9-kb transcript coding for complement component 3 (C3) is described. This alternate C3 is identical to the 3' end of the C3 message beginning at position 3300 of the C3 cDNA. Its transcription appears to be driven by an alternate promoter located within intron 8 of the C3 gene. This alternate C3 message contains an open reading frame that may encode a 536-amino acid-long protein identical to the 3' part of the C3 α chain. The resulting protein contains the complement receptor CR2 binding site. The suggested 5' end of coding region of the alternate C3 includes information for a potential hydrophobic leader peptide that would allow secretion of the protein. In vitro assays with macrophage-depleted mouse splenic B cells indicate that an activity is secreted from cell lines transfected with the alternate C3 cDNA. Together with Sepharose-bound immunoglobulin M-specific monoclonal antibodies and interleukin 2, it costimulates the proliferation of B cells. Implications for possible in vivo functions are discussed.

The third component of complement (C3)¹ and the receptors that bind its activated fragments have been recognized as regulators of humoral immune responses. In humans, guinea pigs, and dogs, genetic deficiencies in specific components of the classical pathway (C1-C4) are associated with diminished primary antibody responses to T-dependent and -independent antigens (1-3) and low levels of certain Ig isotypes (4). Treatment of mice with cobra venom factor, which leads to C3 depletion by activating the alternative complement pathway, results in suppression of the antibody response to T-dependent (5) and -independent antigens (6). Furthermore, these mice fail to localize immune complexes on follicular dendritic cells in germinal centers and, thus, generate an impaired memory response to T-dependent antigens (7, 8).

Our own previous experiments have provided further evidence for the role of C3 in B cell proliferation and its action through the CR2 receptor. Proliferation of purified LPS-activated splenic B lymphocytes was found to be controlled by three restriction points in the cell cycle (9). The first, occurring immediately after mitosis, could be overcome by in-

teractions of Sepharose-bound IgM-specific antibodies with surface IgM. The second restriction point, observed in the G1 phase, appeared to be influenced by factors contained in macrophage cell line-conditioned media. These factors were termed α -factors. The third restriction point, occurring in the G2 phase of the cell cycle, was found to be controlled by factors secreted by helper T cells. Experiments have identified the latter activities as IL-2 or IL-5 (10). In addition, it was determined that macrophage-derived α -factor could be replaced by the human C3 fragments C3b and C3d (11). It is interesting to note that the C3 fragments had to be presented to B cells in polymerized or Sepharose-bound form to be costimulatory. Soluble fragments were found to be inhibitory for B cell proliferation. This activity was apparently mediated via the CR2 (CD21, C3d/EBV-receptor) since a peptide covering the CR2 binding site of C3 specifically inhibited the stimulation (10, 12). It has been proposed that a proteolytic cleavage product of the C3 protein, iC3i, might regulate B cell proliferation (13).

Further evidence of the biological role for C3, its fragments, and CR2, comes from various approaches. In vivo studies involving mAbs directed against murine CR2 have resulted in a suppression of the primary antibody response (14-16). A soluble chimeric protein formed with the ligand binding site of CR2 and murine IgG1 has been shown to suppress a primary in vivo antibody response in mice (17).

¹ Abbreviations used in this paper: α p, α -promoter; CAT, chloramphenicol acetyl transferase; C3, third component of complement; NF-IL-6, nuclear factor IL-6; ORF, open reading frame; RACE, rapid amplification of cDNA ends.

In contrast, F(ab')₂ fragments of rabbit anti-CR2 have been shown to synergize with T cell-derived factors, inducing human B cell proliferation (18). Anti-CR2 antibodies or multivalent C3d augment human B lymphocyte activation in the presence of PMA (19). Cross-linking of CR2 increases cytoplasmic Ca²⁺ influx induced by anti-IgM stimulation of human B cells (20–22). Interaction between ligand-loaded CR2 and cross-linked surface Ig was demonstrated by cocapping on murine B lymphocytes (23, 24). Furthermore, CR2 is a component of a signal transduction complex containing CD19 and several other components, and this complex is distinct from the membrane IgM complex (22). B cell activation by the IgM complex can be synergistically enhanced by the CR2–CD19 complex (22).

It has generally been accepted that the ligand for CR2 is generated by proteolytic processing of C3. On the other hand, preliminary experiments from our laboratory have detected a 1.9-kb RNA that crosshybridized with C3 gene probes (25). Furthermore, cell lines such as the plasmacytoma X63 and the T cell hybridoma A32-26, which produce and secrete α -factor activities, were found to express the 1.9-kb RNA. In contrast, cell lines that do not produce α -factor activities, such as the B cell lines, J558 and Sp2/0, the T cell line BW5147, and the fibroblast line Ltk⁻, did not express the 1.9-kb RNA. These preliminary experiments indicated that a correlation might exist between α -factor production and the expression of a 1.9-kb RNA hybridizing to C3 gene probes.

In the present report, the structure of this novel, 1.9-kb, C3-related transcript is described. Cell lines, which by themselves did not produce α -factor activities, were transfected with cDNA corresponding to the 1.9-kb RNA. The products made and secreted by these transfectants indicated that α -factor activity can be expressed from the 1.9-kb RNA.

Materials and Methods

Tissue Culture Conditions. The murine T cell hybridoma A32-26 (26), clones C4, B5, and B6, and the murine plasmacytomas X63/Ag8-653 (27) and J558 (28), were cultured in Iscove's modified Dulbecco's modified Eagles medium supplemented with 5% FCS, antibiotics, and 5 × 10⁻⁵ M 2-ME. The L cell fibroblasts were cultured under the same conditions as above except that the 2-ME was omitted.

RNA Preparations and cDNA Libraries. Total RNA and poly(A)⁺ RNA were prepared according to previous reports (29). cDNA libraries were made as before (29) and cloned with EcoRI linkers in λ gt11 (Stratagene, La Jolla, CA).

Rapid Amplification of cDNA Ends. For location of oligonucleotides used, see Fig. 1. A specific primer within the known sequence (No. 1005, position 4000–4220 or No. CR2, position 3754–3785) (30) was used to make the first strand cDNA on poly(A)⁺ mRNA; this was then tailed with poly(A). The second strand was primed with oligo dT linked to an adaptor for restriction enzyme sites. PCR amplification was performed between the adapter primer and a second C3-specific primer (No. 974, position 3940–3960 or No. 315 in case of CR2-primed first strand cDNA) (30) within the known sequence to increase the specificity of the desired product.

DNA Constructs. All DNA constructs were made using conventional DNA techniques (29). PCR reactions were performed

according to the manufacturer's specifications (Perkin Elmer Cetus, Norwalk, CT). Intron 8 was PCR amplified using 28 nucleotide primers (starting position 3226 and 3326, respectively) (30) in exons 8 and 9, including restriction sites, and thereafter cloned into the pBluescript SK⁺ vector (Stratagene) and named pSK α in8. The insert was sequenced according to the manufacturer's specifications (Sequenase, Cleveland, OH). The ~400-bp fragment α p promoter used in the transfection vector was made by digesting the plasmid pSK α in8 with BamHI and EcoRI (in the polylinker). The fragment was filled in by Klenow and then ligated into the unique 5' HindIII site (Klenow fill in) of the pUC-CAT and pUC-CAT-E_μ vectors (31).

Transfection and Chloramphenicol Acetyl Transferase Assay. Cells were transiently transfected using DEAE-dextran as described elsewhere (31) except that 2.5 × 10⁶ cells per transfection sample was used. Cell extracts were prepared 40–46 h after transfection and tested in chloramphenicol acetyl transferase (CAT) assay as described elsewhere (31). The TLC plates were measured using a phosphor imager (Compaq; Molecular Dynamics, Inc., Sunnyvale, CA).

Expression Vectors. The open reading frame (ORF) (starting at position +143 [see Fig. 2], i.e., position 3443 to the 3' end of the normal C3 cDNA; 30) of the alternate C3 was amplified by PCR and cloned into the PSCT/TC vector (PSCT/TC-ORF) for in vitro transcription/translation. The insert was in vitro transcribed using T7 RNA polymerase followed by in vitro translation (reticulocyte lysates) in the presence of [³⁵S]methionine (Amersham Corp., Arlington Heights, IL) according to the manufacturer's specifications (Promega, Madison, WI). The in vitro translated products were analysed by SDS-PAGE gels. They were also used for immunoprecipitations with two different goat anti-C3 antisera. The immunoprecipitated products were analysed by SDS-PAGE gels.

The ORF was also cloned into the BCMGS-Neo vector (BCMGS-Neo-ORF) which has a CMV promoter/enhancer element, rabbit β -globin splice site, polyadenylation signal, SV40 origin of replication, and the gene encoding neomycin resistance. This vector was introduced by electroporation into J558 plasmacytoma cells and Ltk⁻ cells. After selection in G418-containing medium, resistant transfectants were analysed by dot blot hybridizations for the presence of RNA corresponding to the insert (alternate C3). Positive cells were then transferred to serum-free media without G418 and 2–4 d later, supernatants were collected and analyzed in the proliferation assay (see below). Over 95% of the transfected cells remained viable after the 2–4-d incubation period.

The B Cell Assay System. The B cell test system for α -factor activity, originally described as macrophage conditioned medium, was performed as described elsewhere (9, 32). Briefly, small resting B cells were prepared from C57BL/6 *nu/nu* spleens depleted of residual T cells by anti-Thy1.2 and complement. The cells were separated according to their size by velocity sedimentation for 5 h and the fraction of small resting B cells was collected. These cells were depleted of macrophages by anti-Mac1 binding followed by magnetic cell sorting (MACS) according to the manufacturer's specifications (Miltenyi Biotec, Cologne, Germany). Purified B cells were cultured at 1.5 × 10⁵ cells/ml in serum-free medium in the presence of Sepharose-bound, anti-IgM-specific mAbs and purified recombinant IL-2 (concentrations according to figure legends). Serum-free supernatants of the BCMGS-Neo-ORF transfected clones to be tested were added at 25%, unless indicated otherwise.

Results

Cloning and Sequencing of the 1.9-kb RNA Species Hybridizing with the C3 Gene. Previous experiments from our labora-

tory have detected a 1.9-kb RNA that crosshybridizes with C3 gene probes (30). A correlation between α -factor production and the expression of this 1.9-kb RNA was found (25, and Cahen-Kramer, Y., unpublished observations). We therefore set out to clone and characterize this 1.9-kb RNA species. Several cDNA libraries were constructed from the α -producing A32-26 T cell line. cDNA clones hybridizing to the C3 cDNA probe were analyzed. Fig. 1 B shows the sequenced parts of seven individual cDNA clones, as indicated by the arrows. All clones obtained showed sequence identity with the 3' end of the full-length C3 cDNA. Since none of the sequences appeared to contain the full-length of the 1.9-kb RNA, the rapid amplification of cDNA ends (RACE [33]) was used to clone and sequence the 5' terminal part of 10 additional cDNA (Fig. 1 C). The 5' end was further determined by primer extension analyses showing a result similar to the RACE clones (Fig. 1 C). These results suggest that the short C3 message is not generated by alternative splicing, since no additional sequences were found in the cDNA clones that could have been spliced into the 5' end of the 1.9-kb message from another part of the gene.

PCR experiments were performed on A32-26 cDNA using two oligonucleotides derived from the first C3 exon in conjunction with an oligonucleotide located within exon 9 of the cloned cDNA. No specific PCR amplification products were found here when either of the two oligonucleotides from the first C3 exon was used (data not shown). This made it more improbable that the alternate C3 message was generated by alternative splicing. In summary, a total of 1.8 kb cDNA was cloned and sequenced. All sequences were found to be identical with those of normal C3. With an expected additional 100–200 bp poly(A) tail, this would correspond well to the length of a 1.8–1.9-kb mRNA. Furthermore, the experiments described above make it likely that the 1.9-kb C3 mRNA initiates within the C3 gene around position 3300

of the full-length C3 cDNA (30) and therefore represents a continuous 5' truncated version of the normal C3 mRNA.

Genomic Sequences Upstream of the Transcription Start Site of the Alternate 1.9-kb mRNA. The 1.9-kb alternate C3 RNA starts at position 3300 in the normal C3 cDNA. Information about intron/exon junctions is not available for the mouse C3 gene but has been determined for the human C3 gene (34). Comparison of human and mouse C3 cDNA sequences reveals a conserved basic organization of this gene. By analogy to human C3 intron/exon junctions, the 5' end of the alternate 1.9-kb transcript seems to be located 11 nucleotides into exon 9. There was no evidence for the 1.9-kb C3 mRNA being derived from alternative splicing of the normal C3 gene, and the initiation site appeared to be very close to an intron/exon boundary. We therefore attempted to clone the 5' sequences including intron 8 to look for possible *cis*-elements regulating transcription of the 1.9-kb alternate C3 mRNA. The whole intron 8 has a length of 274 bp and the sequence revealed a putative TATA box (35) at position -26 and a CAAT box (36) at position -51 relative to the presumed start site (Fig. 2). Other regulatory sequence motifs were also found. Particularly striking among them are an Sp1 consensus sequence in opposite orientation at position -20 (37, 38) and a nuclear factor IL-6 (NF-IL-6) recognition site at position -139 (39).

Promoter Activity Conferred by Intron 8 of the C3 Gene. To test if the sequences 5' of the ORF in exon 9 could act as a promoter, the 400-bp α promoter sequence (αp) was cloned upstream of the reporter gene, *CAT*, in the absence (αp -*CAT*) or presence (αp -*CAT*-*E*) of the IgH chain, E_{μ} , intron enhancer (Fig. 3 A). The constructs were transiently transfected into X63 plasmacytoma cells shown to produce the 1.9-kb C3 transcript. As seen in Fig. 3, B and C, the αp -*CAT* vector showed no activity. However, in the presence of the E_{μ} enhancer, i.e., αp -*CAT*- E_{μ} , the *CAT* activity was increased 10-

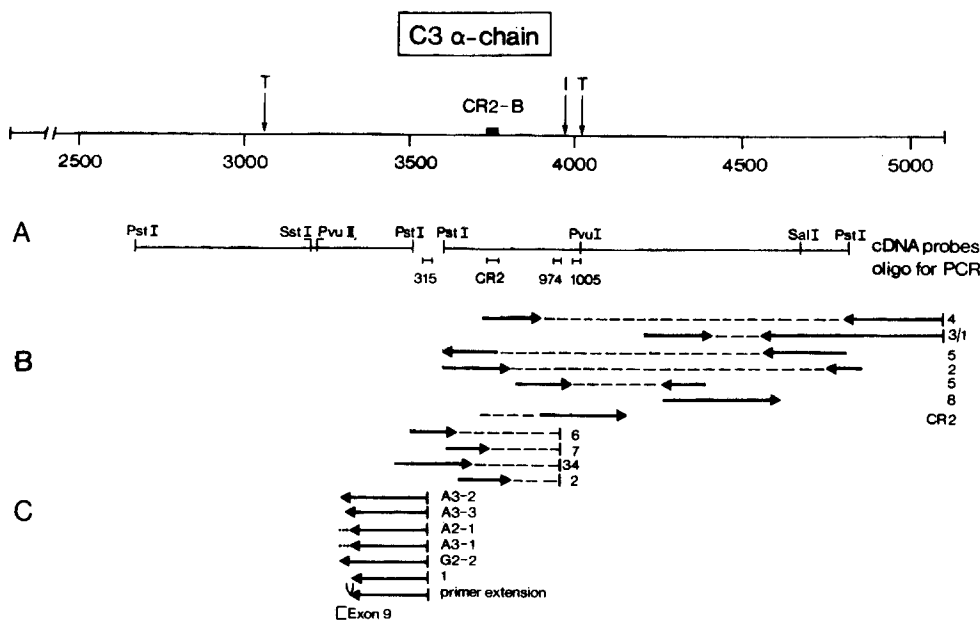


Figure 1. (A) Structure of the 3' end of the cDNA encoding the C3 α chain and obtained cDNA clones from the alternate 1.9-kb C3 mRNA. The scale is in base pairs. (T) Trypsin; (I) Factor I cleavage sites. (CR2-B) CR2 receptor binding site in the corresponding C3 protein. The C3 cDNA probes (*Pst* I fragments) were derived from pML C3/4 (59). (315, CR2, 974, and 1005) Oligonucleotides used for PCR amplification by the 5' RACE method. (B) cDNA clones obtained from the A32-26 cDNA libraries; (arrows) sequenced parts. (C) Alternate C3 cDNA clones obtained by the 5' RACE method; transcription start site as determined by primer extension.

Sequence upstream of the translational start codon for the alternate C3

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-348 GCTGGCTTCA AACAGCCAG CTCTGCCTAT GCTGCCTCA ACAACCGGCC CCCCAGCACC
      exon 8
-288 TGG GTAGCGG GTTGTGAGCT CTGTCCCTC TGCCTCAACA TCCAGTGAG CAAAGCCTGA
      Intron 8
-228 TTCCCCACCA GTGGTGGTCT GGCCTCTCTC TGTCAAGGCT GCAGGAGCTG AATGAGCCTT

-168 AGAGTCCTTT AAGCACCAGC TTTATGCGGC TTTGAATA AAAATCCATA ACTGAGGGCT
      NF-IL6
-108 CTGCACCAGG CCCTCTCTGG TCATTGGTGG GTGAAGATGT CAATCTATCT ACTAAACCA
      CAAT
-48 ATCGAGTCTC AGCTGGTGT C CCATAACTC CGCCCC AGCT GACAGCCTAC +1 GTGGTCAAGG
      TATA Sp1 exon 9
+13 TCTTCTCTCT AGTGCCAAAC CTCATCGCCA TCGACTCTCA CG TCCTGTGT GGGGCTGTTA

+73 AATGGTGTAT TCTGAGAAA CAGAAGCCGG ATGGTGTCT TCAGGAGGAT GGGCCCGTGA

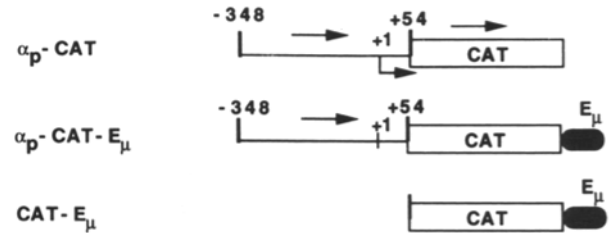
+133 TTCACCAAGA AATG
      Met
  
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Figure 2. Sequence of 3' end of exon 8, intron 8 (274 bp) and 5' end of exon 9 of mouse C3 α chain. Oligonucleotides derived from the mouse C3 cDNA which correspond to intron/exon boundaries in the human gene, were used to PCR amplify mouse C3 intron 8 from genomic DNA (see Materials and Methods). The transcription start site determined by primer extension is set to +1. Intron 8 starts at position -285. The exon 8/intron 8/exon 9 boundaries are shown. The ORF starts at position +143. Underlined are TATA element, CAAT box, Sp1 site, and a potential NF-IL6 site. These sequence data are available from EMBL/GenBank/DBJ under accession number Z37998.

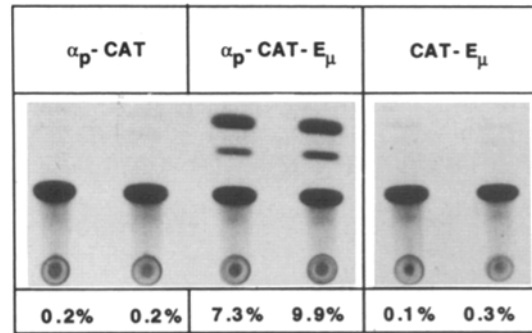
fold whereas a control construct (*CAT-E μ*) showed only background activities. These data suggest that the sequences 5' of the ORF in exon 9 of the C3 gene can serve as a promoter.

In Vitro Translation of the Alternative C3 cDNA. The longest ORF in the 1.9-kb transcript potentially encodes a 536-amino acid protein initiating with an in-frame, relative to C3, AUG. This AUG is located 143 nucleotides downstream from the putative 5' end of the transcript (Fig. 2). For cloning into expression vectors, the ORF of the 1.9-kb transcript was amplified by PCR from the A32-26 cDNA. The PCR product was cloned in the PSCT/TC vector for in vitro translation and in the BCMGS-Neo vector for stable expression in eucaryotic cells. Fig. 4 A shows the result of an in vitro translation experiment in which the products were analyzed on a polyacrylamide gel. Lane 1 correspond to the control translation product. Lanes 2-4 correspond to three different clones containing the ORF cDNA after 15 min of in vitro translation (30 and 60 min showed similar results, data not shown). The size of the putative alternate C3 protein could be 55-60 kD (arrow Fig. 4 A). The in vitro translated proteins were also tested in immunoprecipitations for the expression of antigenic determinants of C3 using different anti-mouse C3 polyclonal antibodies (Fig. 4 B). Purified goat anti-mouse C3c (Fig. 4 B, lane 1) and a goat anti-C3 antiserum (Fig. 4 B, lane 2) were both able to precipitate an in vitro translated alternative C3 protein with a molecular mass of 55-60 kD, whereas normal goat serum (Fig. 4 B, lane 3) did not precipitate any protein. This shows that a 55-60-kD protein can be made in vitro from the vector containing the alternate

A) Transfection vectors



B) CAT assay: X63/0 myeloma cells



C) CAT activities in X63 myeloma cells

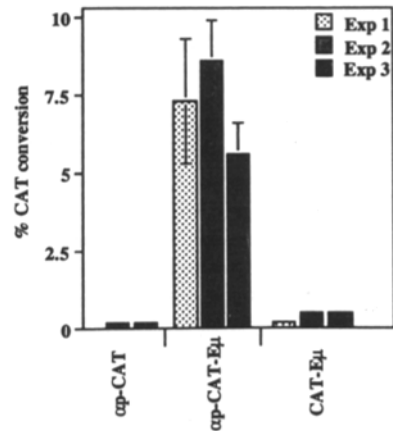


Figure 3. (A) A 400-bp fragment containing the potential α promoter sequence (see Fig. 2; position -348 to +54) including the potential cap site, TATA box, CAAT box, and the NF-IL6 site was cloned upstream of the reporter gene CAT, either in the absence (*α_p -CAT*) or presence (*α_p -CAT- E_μ*) of the IgH intron enhancer (E_μ). CAT- E_μ correspond to vector without promoter. The CAT structural gene contains its own translation start codon. (B) The constructs in A were transiently transfected into X63/0 myeloma cells shown to produce the 1.9-kb C3 transcript. After 2 d, the amount of CAT enzyme produced was measured in a CAT assay. One representative experiment (duplicate) is shown including actual percent CAT activity. (C) Three independent experiments as in B with different plasmid preparations.

C3 cDNA and this protein can be recognized by anti-C3-specific antibodies.

B Lymphocyte Costimulatory Activity Secreted by Transformants Expressing the Alternate C3 mRNA. The BCMGS Neo plasmid, carrying the ORF of the alternate C3 cDNA, was introduced into the J558 mouse plasmacytoma and Ltk-

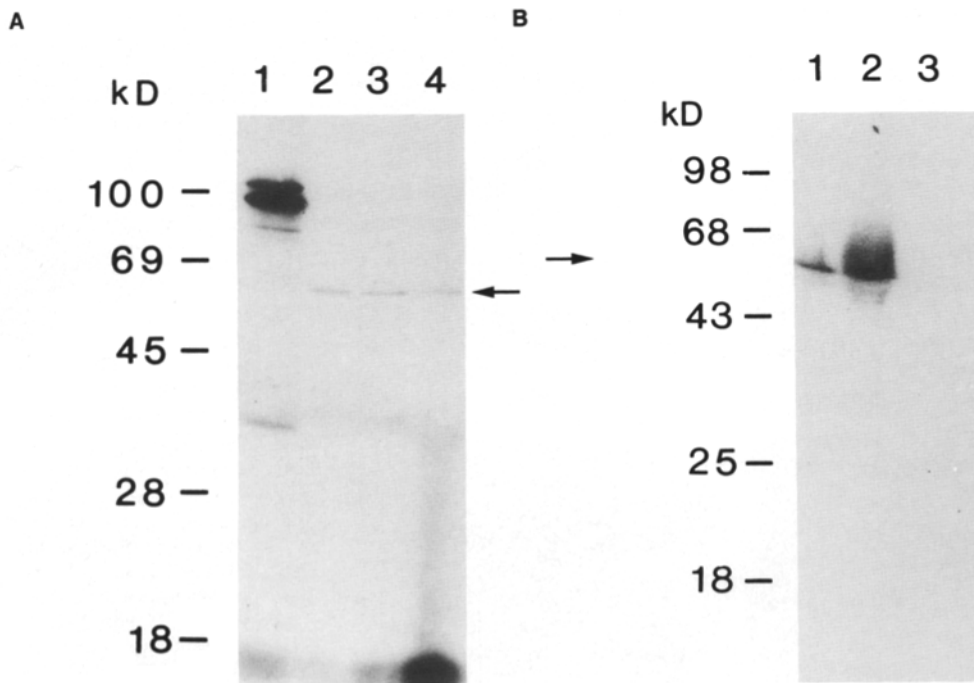


Figure 4. (A) PAGE gel after 15 min in vitro translation of: a positive control (lane 1) and three individual samples (lanes 2–4) containing the alternate C3 cDNA in the PSCT/TC vector. (B) Immunoprecipitation of the in vitro translated products in A with: purified goat anti-mouse C3c (lane 1), goat anti-mouse C3 serum (lane 2) and normal goat serum (lane 3). (Arrows, A and B) Position of the potential alternate C3 protein.

fibroblast cell lines. Both are negative for endogenous C3 RNA. G418-resistant transformants were tested by RNA dot blot analysis for the presence of the transfected message using a C3 cDNA probe (data not shown). The supernatants of 11 positive J558 and 10 positive Ltk⁻ transformants, as determined by the dot blot assay, were chosen to be tested for α -factor activity. Figure 5:1 shows the result of such an experiment. When the resting B cells were cultured in medium alone (Fig. 5 A) in the presence of anti-Ig antibodies (Fig. 5 B) or in the presence of anti-Ig antibodies, IL-2 and conditioned medium from nontransfected L cells (Fig. 5 C), they did not proliferate. In fact, initially as a source of rIL-2, supernatants from L cells transfected with BMCGS/Neo/IL-2 were used and in the absence of α -factor, scored negative in the above assay. However, if the B cells were cultured in the presence of anti-Ig antibodies, IL-2 and conditioned medium from L cells transfected with the alternative C3 cDNA, they did proliferate (Fig. 5 D). Experiments with supernatants from J558 transformants showed similar results (data not shown).

In another set of experiments, different concentrations of L cell-conditioned medium (as source of α -factor) and of rIL-2 were tested. The titration curve in Fig. 6 (left) suggests that at 25% of L cell-conditioned medium, maximum proliferation appears to be reached. Experiments with supernatants from J558 transformants showed similar results (data not shown). In case of rIL-2 (Fig. 6, middle) a plateau is reached at 100 U/ml. When the increase in the number of B220⁺ cells in the culture was monitored with time (Fig. 6 right), the cells were found to divide continuously for 6 d. Thereafter, the cells began to die, i.e., the cell numbers decreased (data not shown). At this stage, they could not be rescued even in fresh medium containing all stimulatory factors, similar to earlier studies with anti-IgM- or LPS-stimulated splenic

B cell cultures. Thus, L cells and J558 cells transfected with a cDNA coding for the longest ORF in the alternate 1.9-kb C3 transcript produce and secrete α -factor activity. The activity is costimulatory with IL-2 and anti-Ig and is able to promote B cell proliferation for at least five to six divisions in vitro.

Discussion

In this paper we have elucidated the structure of a variant 1.9-kb transcript of the mouse C3 gene initiated from an internal promoter. It encodes a 60-kD protein that acts as a costimulator in the proliferation of mature B lymphocytes, together with IgM-specific antibodies and rIL-2. These findings raise a series of intriguing questions. Is the alternate transcript produced by normal cells and which cells make it? Is its production regulated by external stimuli such as IL-6 and how is this transcription regulated on the level of the internal promoter? How is this transcription made constitutive in many cell lines? Does the three-dimensional structure of the alternate C3 protein differ from normal C3 and its proteolytic degradation products? Can it be secreted? Which developmental stage(s) of B cell development is (are) the target(s) of this protein? What is the physiological role of alternate C3 in vivo?

In previous preliminary experiments, a variant 1.9-kb transcript of the C3 gene has been detected in the cell lines of mouse and human T and B lymphocytes, monocytes, macrophages, and fibroblasts. However, it appeared to be absent in normal resting and activated mouse B lymphocytes (25, and Lernhardt, W., personal communication). These observations are in line with the data shown in this paper where normal B cells do not proliferate in the presence of IgM-specific

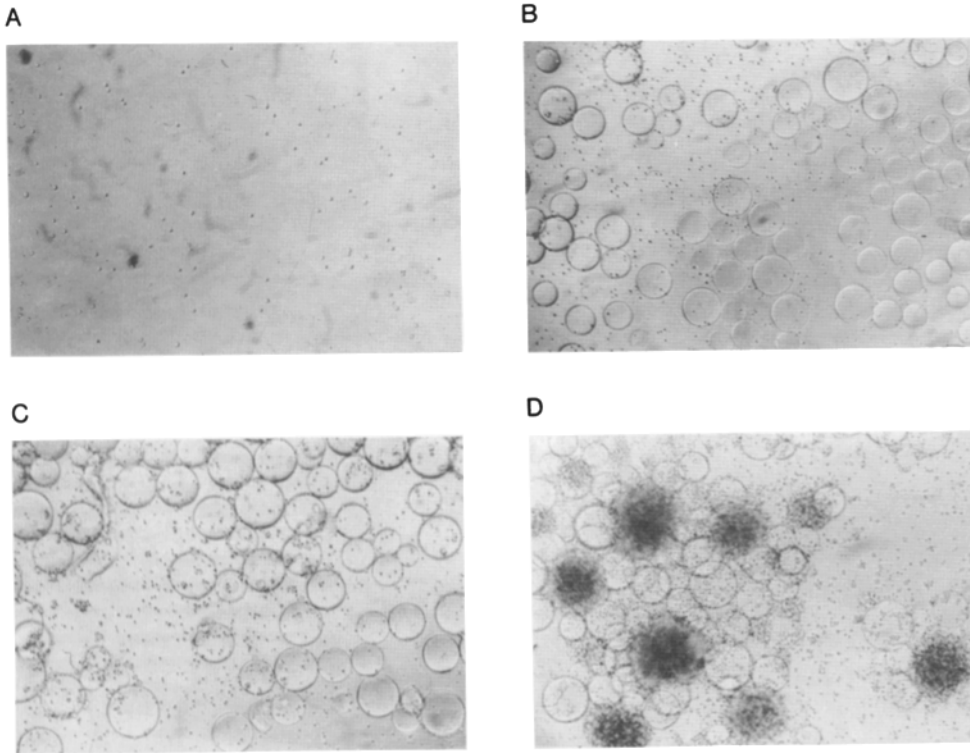


Figure 5. Proliferation of macrophage and T cell-depleted splenic B cells after 5 d in culture: (A) in serum-free medium alone; (B) in the presence of Sepharose-bound anti-IgM antibodies; (C) in the presence of Sepharose-bound anti-IgM antibodies, 500 U rIL-2, and 10% medium conditioned for 2 d with non-transfected L cells; and (D) in the presence of Sepharose-bound anti-IgM antibodies, 500 U rIL-2, and 10% medium conditioned for 2 d with L cells transfected with the alternate C3 cDNA.

antibodies and rIL-2 alone. Since small numbers of normal macrophages can provide α -factor activity in B cell proliferation assays (32), they might in fact transcribe a 1.9-kb alternate transcript and translate and secrete the alternate C3 protein from it. Detection of the alternate C3 transcript has so far been hampered by our inability to purify sufficient quantities of normal macrophages producing α -factor activity to allow a Northern blot analysis. Such analysis cannot be replaced by RNA PCR analysis since it would not be able to distinguish a normal 5.6-kb C3 transcript from the alternate 1.9-kb form. The detection of the protein product of the alternate C3 transcript would best be done with specific mAbs able to distinguish this protein from normal C3 and its proteo-

lytic degradation products (iC3, C3b, and C3d), but such mAbs are not yet known.

An alternate 1.9-kb C3 transcript could, in principle, arise either from alternative splicing, alternative initiation of transcription, or from a second, truncated C3 gene in the genome. We have been unable to detect any nucleotide sequences at the 5' end of the 1.9-kb mRNA that are not contiguous with the published sequence of the 3' half of the C3 gene. This makes it unlikely that a region further upstream, e.g., from the 5' promoter region of the C3 gene, would be spliced into the 1.9-kb mRNA. Whereas our results show that the 1.9-kb transcript can be derived from an alternative internal transcription start site, we have also searched for the possible

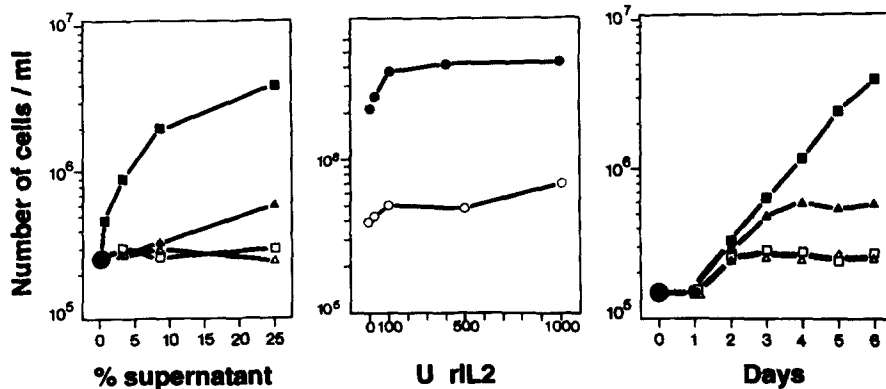


Figure 6. Proliferation of macrophage and T cell-depleted splenic B cells. Different culture conditions were analyzed and the number of cells/ml determined. (Left) Titration of serum-free supernatants of α -factor-producing L cells in the presence of anti-Ig with or without 500 U IL-2. Open symbols are supernatants of nontransfected cells with (□) or without (Δ) IL-2; closed symbols are supernatants of cells transfected with the alternate C3 cDNA with (■) or without (▲) IL-2. Assayed after 5 d in culture. (Middle) Titration of IL-2 in the presence of anti-Ig with (●) and without (○) 25% α -supernatant. Assayed after 5 d in culture. (Right) Growth kinetics (Days) of in vitro proliferating B cells. Open symbols are cell numbers/ml in the presence of anti-Ig and

nontransfected cell supernatants with (□) or without 500 U IL-2 (Δ). Closed symbols are cell numbers in the presence of anti-Ig and transfected cell supernatants with (■) or without 500 U IL-2 (▲).

existence of a second, truncated C3 gene. The mouse C3 gene has been mapped to chromosome 17, outside the MHC class III gene cluster (40) and has been estimated to be ~24 kb in size (41). No C3-related or pseudo genes have been seen in these studies. We searched for C3 pseudo genes that could encode the truncated C3 mRNA in Southern blots of genomic DNA from A32-26 cells and from the inbred mouse strains from which the cell line was generated. All the DNA preparations, digested with several restriction enzymes, showed a hybridization pattern compatible with the existence of a single copy gene (data not shown). This also makes it unlikely that the transformed A32-26 cell line, from which the alternate C3 transcript was sequenced, carries a chromosome aberration at the site of the C3 gene.

An analogous 1.9-kb C3 transcript has been found in the human B cell line Raji (Lernhardt, W., personal communication). By comparison of C3 protein sequences of different species it becomes evident that the AUG codon found at the beginning of the long ORF of the alternative C3 transcript is conserved among all available sequences, i.e., mouse (30), human (42), pig (43), and rabbit (44). This suggests the possibility that the alternate C3 product could be a B cell-costimulatory factor in all these species.

The fact that intron 8 was active in the transcription assay suggests that it can act as a promoter. Without an enhancer, it has low promoter activity. This is similar to the normal C3 promoter which shows low activity in itself, an activity that can be upregulated with IL-1 plus IL-6 (45). Inspection of the intron sequences 5' of the putative transcription start site of the alternate C3 reveals several sequences with homologies to known *cis*-acting elements of other promoters. At position -26 to -20 (Fig. 2) a TATA box is found (35) and at position -51 to -47 there is a CAAT box known to bind the CTF/NF-1 transcription factor (36). Downstream of the TATA box is a GC-rich sequence CCGCCCC, which matches the consensus motif for the Sp1 transcription factor binding site (GGGGCGG) on the opposite strand (37). A NF-IL-6 recognition site is found at nucleotide -139 to -130. NF-IL-6 has been reported to be a member of the C/EBP family of leucine zipper proteins (39). Its binding site has not only been found in the promoter of the IL-6 gene but in several other cytokine and various acute-phase protein genes (39). NF-IL-6 mRNA is induced by LPS, IL-6, and IL-1, along with TNF and glucocorticoids, all well-known mediators of an acute phase response (46-48). It is therefore conceivable that in an acute phase reaction, an activation by LPS or IL-6 could directly induce expression of the short C3 gene product. It remains to be investigated whether the NF-IL-6 motif binds the corresponding NF in cells that can regulate the transcription of the alternate C3. This cannot be done with the cell lines expressing the alternate C3 that we have tested so far, since they are not responsive to LPS or IL-6 (data not shown). Normal macrophages might be a more likely source of cells for an investigation as to whether IL-6 or an inducer of IL-6 expression have direct or indirect effects on the alternative C3 expression.

We have not yet undertaken a detailed study of the mode by which the alternate C3 protein may be released from the

cells. It is unlikely that death of the transfected cells would be the mechanism by which B cell costimulatory activity conditions the supernatant medium used in our proliferation assays. Furthermore, the structure of the alternate C3 protein offers the possibility for its active secretion. A hydropathy plot (49) of the deduced amino acid sequence of the alternate, truncated C3 predicts a hydrophobic region at the NH₂ terminus. This hydrophobic region is found between residues 13 and 23 and may represent a signal sequence active in secretion of the protein from the cells (50, 51).

The alternate C3 protein lacks the thiolester group that is involved in rapid covalent binding of the activated C3 molecule (C3b) to cell surfaces, complex carbohydrates, or immune complexes. However, it includes the CR2 binding site (12). Due to its shorter length, it has a free cysteine residue which, in the normal C3 molecule, is involved in a disulphide bridge. This raises the possibility that the alternative C3 product may be different in three-dimensional structure and may, under certain conditions, even form a homodimer or a heterodimer. Previous experiments with C3d have indicated that CR2 had to be cross-linked to costimulate proliferation of B cells. Monovalent ligands, by contrast, were shown to suppress B lymphocyte activation and proliferation (11, 19, 24, 52). If the alternate C3 protein can form dimers, then CR2 could either be cross-linked with itself or with another receptor. Other experiments (22) have suggested that association of CR2 with CD19 is needed for B cell proliferation, suggesting that such heterocross-linking could be as effective as homocross-linking (of CR2) in B cell stimulation. The apparent strong hydrophobicity of the alternate C3 protein has so far prevented the characterization of mAbs that might specifically recognize it, as well as binding experiments that could determine whether it really interacts with CR2.

Our assay system has previously defined three restriction points in the cell cycle of primary murine B lymphocytes. The three synergistically acting ligands are anti-Ig specific antibodies acting early in G1 of the cell cycle, macrophage-derived α -factors, or C3d in insoluble form controlling the entry to S phase, and IL-2 or IL-5 as T helper-derived β -factors in G2 phase. All other recombinant interleukins so far tested (IL-1, -3, -4, and -6), at concentrations as high as 1-10 μ g/ml, were not able to replace any of these factors (10). We show here that the alternate C3 protein appears to act in place of α -factor or insolubilized C3d in the cell cycle. The strong hydrophobicity of the alternate C3 protein has so far prevented absorption experiments with polyclonal C3-specific antibodies to test whether the growth-promoting activity for B lymphocytes is indeed conveyed by this protein. Therefore, it remains a rather unlikely possibility that the alternate C3 induces, in fibroblasts and other transfected cells, the production of a (yet unknown) B cell stimulatory factor.

We would like to propose a model for a possible *in vivo* activity of the alternative C3 which acts either in the early phase of an immune response or as an additional cofactor, released by macrophages in an acute phase response, to trigger B cell proliferation. The model uses the following two observations as its basis: (a) The alternate C3 might be produced *in vivo* by monocytes and macrophages (11, 32); (b)

The transcription of the truncated message might be regulated by NF-IL-6 which is involved in the regulation of genes in acute phase and inflammation. The interaction between macrophages and primary B cells may occur in the microenvironment of a localized immune response, i.e., at the site of inflammation caused by tissue injury, bacterial or viral infection, parasites, or neoplasia. C3 expression, including that of the alternate C3, might be upregulated by these external influences. Upregulation could also occur within lymphoid organs at the sites of first encounter with the antigen presumed to be in splenic red pulp or in the paracortical T-zone of lymph nodes (53).

There is other evidence for a role of C3 (and possibly of the alternate C3 protein) in primary B cell responses, especially in those mediated by LPS. LPS or lipid A stimulation of either peripheral blood monocytes or the human monocyte line U937 increases C3 synthesis 5–30-fold (54, 55). Fibroblasts are induced to synthesize C3 and Factor B with 15- and 40-fold increases, respectively, by IL-6 and IL-1, and

it is suggested that LPS could act, at least in part, through induction of IL-6 and possibly IL-1 (47, 48). The changes in synthesis are achieved through elevated mRNA levels (47, 55, 56). As these results are significantly different from the 50% increase in serum C3 concentration during the acute phase (57), it was suggested that tissue-specific regulation of local C3 concentration plays a very important role in the local inflammatory response. Possibly the C3 product derived from the alternative C3 mRNA accounts for some of these effects and could lead to the activation of primary B cells.

It has been proposed previously that complement is involved in long-term maintenance and generation of B cell memory by mediating uptake of antigen by follicular dendritic cells that also express CR2 (58). Our model shifts the primary role of C3, in the form of the alternate C3, to an earlier part of the B cell response. However, the two models are not mutually exclusive if alternate C3 acts early and full-length C3 acts later in the response.

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