Participation of c-*myc* Protein in DNA Synthesis of Human Cells

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The protein product of oncogene c-*myc* is believed to be important in regulation of the cell cycle. However, its direct role in DNA synthesis has not been explored. Experiments presented here show that the addition of affinity-purified antibodies against the human c-*myc* protein to nuclei isolated from several types of human cells reversibly inhibited DNA synthesis and DNA polymerase activity of these nuclei. This suggests that c-*myc* encodes a protein that is functionally involved in DNA synthesis.

THE ONCOGENE C-*myc* WAS ONE OF the first cellular genes to be linked to neoplastic growth (*1*), and is rearranged and abnormally expressed in a number of human and animal cancers (*2, 3*). Its highly conserved structure in cells of vertebrate species suggests that it serves a critical function in these cells, and it has been proposed that c-*myc* is involved in the regulation of cell proliferation (*4*). The mechanism by which this regulation may be achieved is not understood. We demonstrate that the addition of affinity-purified polyclonal or monoclonal antibodies against the human c-*myc* protein to nuclei isolated from several types of human cells inhibited DNA synthesis and DNA polymerase activity, but not the transcription of RNA. This suggests that c-*myc* encodes a protein that participates in DNA replication.

Isolated nuclei supplied with appropriate substrates are capable of elongating nascent DNA chains (*5, 6*) and of transcribing RNA (*7*). Addition of antibodies to non-nuclear proteins did not appreciably affect DNA or RNA synthesis in nuclei isolated from human leukemic cells (HL60 or ML-1), while the affinity-purified polyclonal antibody to the human c-*myc* protein (anti-myc) (*8*) markedly inhibited DNA, but not RNA, synthesis (Table 1). Similar results were obtained with monoclonal antibodies B3 and F5, which specifically recognize the c-*myc* protein (*9*), confirming that the inhibition is due to interaction with *myc* protein (Table 2). As further controls, we used antibodies to DNA polymerase α and to two nuclear proteins that have a role in RNA synthesis: RNA polymerase II and actin, with actin serving as an abundant nuclear protein (*10*) that has been implicated in RNA transcription (*11*). Antibodies to the latter two proteins inhibited RNA synthesis but not DNA synthesis (Table 1), showing that formation of antibody-protein complexes in these nuclei does not disrupt all nuclear functions nonspecifically. Conversely, a monoclonal antibody to DNA polymerase α gave results similar to the effects of the anti-myc on nucleic acid synthesis in isolated nuclei (Table 1). Treatment of the nuclei with drugs that specifically inhibit DNA and RNA synthesis [aphidicolin, which inhibits DNA polymerase α (*12*), the polymerase primarily responsible for replicative DNA synthesis (*13*); and α-amanitin, which selectively inhibits RNA polymerase II (*14*)] resulted in a similar degree of inhibition to that observed after incubation with the antibodies.

The c-*myc* gene is amplified (*2, 15*) and is overexpressed approximately tenfold in HL60 cells (*16*). The resultant high levels of c-*myc* protein in these cells might suggest that it serves a special function, and that the effects of anti-myc may be limited to HL60 cells and similar leukemic cells. The generality of the inhibitory effect of these antibodies is shown by the finding that anti-myc also inhibited DNA synthesis in nuclei from normal human liver, spleen, and cultured fibroblasts (Table 3).

The inhibition of DNA synthesis by anti-myc became almost complete when the concentration of the antibody incubated with the nuclei was increased (Fig. 1A), or when the duration of the incubation was prolonged (Table 4). The concentration of antibody that resulted in an 80% inhibition of DNA synthesis was an approximately tenfold excess over the amount of c-*myc* protein in the assay (*17*). As discussed below, the residual incorporation of DNA precursors in the presence of high concentrations of the antibody may be due to repair-type DNA synthesis. Addition of recombinant c-*myc* protein prevented the inhibition of DNA synthesis in a concentration-dependent manner (Fig. 1, A and B). When the protein was added to the nuclei after a short incuba-

Table 1. Effect of antibodies on DNA and RNA synthesis in nuclei isolated from human cells. HL60 cells and ML-1 cells are established cell lines of human leukemic cells, and were cultured as described previously (*24, 25*). Nuclei were obtained by disruption of cells, swollen in a hypotonic buffer, through a syringe and needle (*6*).

Addition*	HL60		ML-1	
	DNA synthesis (%)†	RNA synthesis (%)‡	DNA synthesis (%)†	RNA synthesis (%)‡
Buffer (control)	100	100	100	100
PRS	98 ± 6	93 ± 14	91 ± 6	91 ± 16
PRS + c-*myc* protein	109 ± 5	103 ± 7	94 ± 4	95 ± 2
Polyclonal anti-myc	19 ± 3	102 ± 2	17 ± 4	99 ± 13
Polyclonal anti-myc + c-*myc* protein	90 ± 9	94 ± 18	82 ± 15	102 ± 3
Polyclonal heated anti-myc§	107 ± 9	92 ± 8	102 ± 13	98 ± 8
Anti–human DNA polymerase α	29 ± 3	95 ± 9	26 ± 2	94 ± 8
Anti–human RNA polymerase II	95 ± 2	35 ± 4	88 ± 5	31 ± 1
Anti–β actin (40 μg/ml)	100 ± 1	66 ± 2	96 ± 2	73 ± 6
Anti–β actin (160 μg/ml)	98 ± 2	39 ± 1	90 ± 8	58 ± 2
Anti–mouse spleen ferritin	100 ± 11	88 ± 10	110 ± 10	105 ± 6
Anti–mouse brain	96 ± 10	95 ± 7	105 ± 10	98 ± 6
OKT3 (human)	98 ± 8	89 ± 12	109 ± 12	96 ± 7
OKM1 (human)	109 ± 6	98 ± 12	101 ± 13	95 ± 12
Aphidicolin (300 μ*M*)	9 ± 2	99 ± 8	9 ± 1	99 ± 10
α-Amanitin (2 μg/ml)	98 ± 2	28 ± 7	91 ± 6	24 ± 6

*All antibodies and preimmune rabbit serum (PRS) were added to a final concentration of 40 μg/ml, except where indicated otherwise, and the c-*myc* protein at 100 μg/ml. All compounds were added to isolated nuclei 30 minutes before adding the precursors for DNA or RNA synthesis. The samples were kept on ice and the reaction was initiated by placing the tubes at 37°C and adding nucleoside triphosphates. †DNA synthesis was measured by the incorporation of [α-^{32}P]dCTP, in the presence of other deoxyribonucleoside triphosphates, into the acid-insoluble fraction of 2 × 10^6 isolated nuclei incubated in 20 μl for 5 minutes at 37°C, as described previously (*6*). The values are means ± SEM of four determinations, expressed as percentage of buffer control. The actual counts per minute for the buffer controls were 10,219 ± 125 and 11,600 ± 145 for HL60 and ML-1 cells, respectively. ‡RNA synthesis was measured by the incorporation of [α-^{32}P]UTP in a system analogous to the one used for determination of DNA synthesis (*25*). The values are means ± SEM of four determinations expressed as percentage of buffer controls and performed in parallel with the measurement of DNA synthesis. The actual counts per minute for the buffer controls were 16,939 ± 160 and 14,302 ± 674 for HL60 and ML-1 cells, respectively. §Placed at 60°C for 1 hour.

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Table 2. Inhibition of DNA synthesis in HL60 nuclei by monoclonal antibodies (B3 and F5) to c-*myc* protein. Procedures were as described in Table 1.

Antibody concentration (μg/ml)	DNA synthesis (%)*	
	B3	F5
None	100	100
5	25.8 ± 2.7	20.2 ± 0.5
10	16.7 ± 0.7	10.3 ± 0.5
20	9.5 ± 2.0	9.1 ± 0.8

*Actual counts per minute in the absence of antibody was 9038 ± 324.

tion with, and subsequent removal of, the antibody, an almost complete reversal of the inhibition of DNA synthesis was seen (Fig. 1C).

DNA synthesis observed in this system appeared to be essentially replicative rather than repair-type. First, the proportion of labeled nuclei determined by autoradiography after incubation with [^{3}H]thymidine triphosphate in the DNA synthesis system approximated the proportion of labeled nuclei when intact cells were labeled with [^{3}H]thymidine. Second, the incorporation of precursors was inhibited approximately 90% by aphidicolin (Table 1), and required the presence of all four deoxyribonucleoside triphosphates (Table 3), which are needed for replicative DNA synthesis but not for repair-type synthesis. Finally, synthesis of the DNA of several individual genes was demonstrated by a DNA "run-on" assay (Fig. 2). When aphidicolin was included in the nuclear DNA synthesis system, only a small amount of residual, probably repair-type, DNA synthesis was apparent (Fig. 2A, column a), showing again that DNA chain elongation was the principal mode of DNA synthesis in the absence of this inhibitor. Addition of anti-myc to this system reduced DNA synthesis of both *myc* and *ras* genes to approximately 20% of the rate seen in its absence, as determined by densitometric scanning of the autoradiograms (Fig. 2A, columns b and c). In an analogous nuclear RNA "run-on" assay (*18*) no effect of anti-myc on the transcription of the genes studied could be observed (Fig. 2B). As expected from the amplification of c-*myc* in HL60 cells (*2, 15*), this gene was replicated and transcribed more rapidly than the c-Ha-*ras* gene, which is a single-copy gene in HL60 cells. Anti-myc also inhibited the DNA synthesis of these and several other genes (c-*myb*, c-*fos*, c-*fms*, c-*fes*, and histone H4) to a similar extent, suggesting that the inhibition occurred uniformly throughout the genome.

A clue that the function of one of the targets of anti-myc may be related to the DNA replicative complex, consisting of polymerase α and associated proteins (*19*), was provided by data shown in Table 4. DNA polymerase activity of isolated nuclei was assayed with an exogenous template, and this activity was markedly reduced by the addition of anti-myc to the nuclei. In a complementary experiment, the nuclei preincubated with anti-myc provided as good a template for an exogenous DNA polymerase as the control nuclei. Thus, the endogenous DNA polymerase but not the endogenous template appears to be inhibited by anti-myc.

The inhibition of DNA synthesis by anti-myc was accompanied by the accumulation of the antibody in the nuclei, shown by immunofluorescence (Fig. 3A), but when the antibody was blocked by preincubation with the recombinant c-*myc* protein, it could not be visualized in the nuclei. The penetration of the antibodies into isolated nuclei

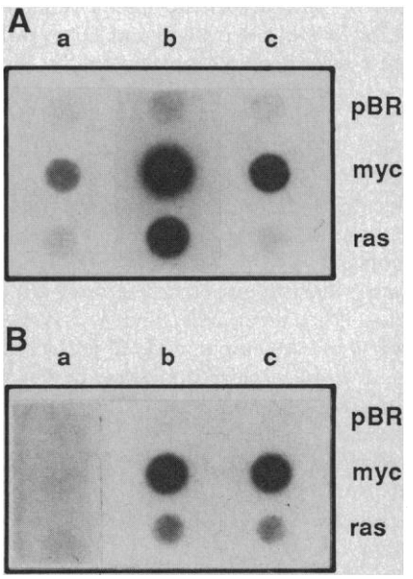

Fig. 2. Effect of anti-myc on DNA synthesis (panel A) and RNA transcription (panel B) of individual genes in nuclei isolated from HL60 cells (*6*). Gene probes were the plasmid pBR322 (pBR) as background control, the Pst I insert (the complementary DNA of the second and third exons of c-*myc* gene) of plasmid pMyc 7.4, and the 6.2-kb Bam HI fragment from plasmid pc-ras. The vertical columns represent (a) controls [panel A, 200 μ*M* aphidicolin to inhibit replicative DNA synthesis (*13*); panel B, α-amanitin at 2 μg/ml to inhibit messenger RNA synthesis (*14*)]; (b) nuclei incubated with PRS (40 μg/ml) as a control for the anti-myc; (c) nuclei incubated with the affinity-purified anti-myc (*8*) at 40 μg/ml. (Panel A) Nuclei from 2 × 10^6 HL60-AB26 cells (*24, 25*) were mixed with the indicated compounds for 30 minutes on ice, and incubated at 37°C for 30 minutes in a total volume of 200 μl. The reaction mixture included 10 μCi [α-^{32}P]-dCTP (specific activity approximately 3000 Ci/mmol), 0.1 m*M* each of dTTP, dGTP, and dATP, 5 m*M* adenosine triphosphate, 50 m*M* tris-HCl (*p*H 7.2), 10 m*M* $MgSO_4$, 0.1 m*M* dithiothreitol, and bovine serum albumin (50 μg/ml). After the incubation, high molecular weight DNA was extracted as described (*26*), and digested to completion with Eco RI. DNA (50 μg) was hybridized to 10 μg of probe DNA that had been blotted on "Biotrans" nylon membranes by means of a Schleicher & Schuell manifold apparatus. The filters containing the probes were previously baked and prehybridized for 4 hours at 45°C. Filters were washed three times with 1× SSC for 2 hours each at 50°C, dried, and exposed to X-AR2 film with intensifying screen for 8 days. (Panel B) Demonstration of RNA transcription from genes c-*myc* and c-Ha-*ras* in an experiment analogous to the one described above (*25*).

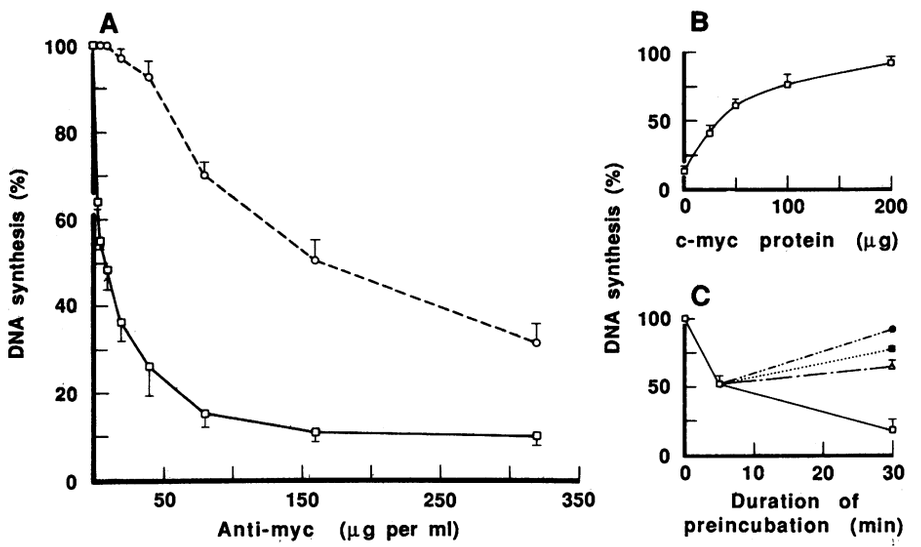

Fig. 1. DNA synthesis in nuclei isolated from HL60 cells in the presence of (A) Varying amounts of anti-myc (□); varying amounts of antibody plus c-*myc* protein (100 μg/ml) (○). (B) Anti-myc (80 μg/ml) plus the indicated amounts of c-*myc* protein per milliliter of incubation mixture. (C) Anti-myc (40 μg/ml) was present for 5 minutes, then the antibody was removed from the incubation mixture by pelleting the nuclei, and the nuclei were divided into four groups and incubated for a further 25 minutes with anti-myc (40 μg/ml) (□); or c-*myc* protein at 100 (△), 200 (■), or 400 (●) μg/ml. Means ± SEM of three experiments.

Table 3. Inhibition of DNA synthesis by polyclonal anti-myc in nuclei isolated from human tissues and cell lines. DNA synthesis was measured as described in Table 1.

Addition	DNA synthesis (%)*			
	Liver tissue	Splenic tissue	K562†	AG2804†
None	100	100	100	100
PRS	90 ± 6	95 ± 4	90 ± 8	91 ± 4
Anti-myc	20 ± 2	17 ± 5	21 ± 4	25 ± 3
−3 dNTP's‡	14 ± 5	8 ± 2	8 ± 3	11 ± 2

*The actual counts per minute for liver tissue, splenic tissue, K562, and AG2804 were 7779 ± 231, 5740 ± 172, 7444 ± 221, and 7305 ± 291, respectively. †K562 is a human leukemic cell line; AG2804 cells are human embryonic lung fibroblasts transformed by SV40. ‡dNTP's, deoxyribonucleoside triphosphates. Incorporation of a precursor in the absence of the other three precursors is taken to represent short gap repair.

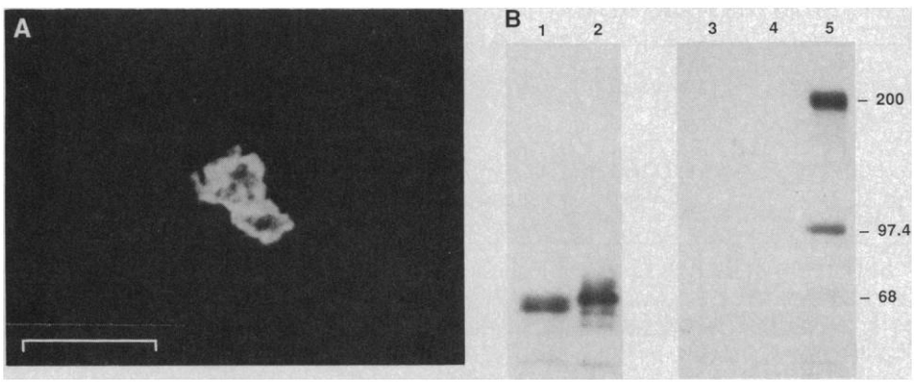

Fig. 3. (A) Demonstration of the localization of anti-myc in nuclei of HL60 cells by immunofluorescence. A total of 2×10^6 isolated nuclei (*6*) in 200 μl were mixed with anti-myc at 40 μg/ml (*8, 27*) and placed on ice for 30 minutes. The nuclei were then centrifuged, and the pellet diluted with phosphate-buffered saline (PBS), *p*H 7.4, smeared on glass slides, rinsed with PBS, and incubated at room temperature (RT) for 60 minutes with goat anti-rabbit IgG-FITC (Hy-Clone Laboratories, Logan, Utah) diluted with PBS at 1:100. Cover slips were placed on slides with a nonfluorescent mounting medium, and viewed and photographed under epifluorescent illumination. No immunofluorescence was observed in nuclei incubated with antibody that had been preabsorbed with c-*myc* protein at 100 μg/ml. Nuclei incubated with anti-myc alone showed immunofluorescence in the nucleoplasm but not in nucleoli. Bar = 10 μm. (B) Immunoblot with affinity-purified anti-myc. Samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis; transferred to nitrocellulose, and incubated with anti-myc (lanes 1 and 2) or anti-myc blocked by preincubation with recombinant c-*myc* protein (lanes 3–5), and then treated with ^{125}I-labeled protein A. Lanes 1 and 3, lysate of HL60 cells (2×10^5 cells); lanes 2 and 4, purified recombinant c-*myc* protein (20 ng); lane 5, [^{14}C]methylated protein markers.

Table 4. Effect of preincubation with polyclonal anti-myc on DNA synthesis in HL60 nuclei and possible targets of *myc* protein action. The antibody or the PRS were added at the final concentration of 40 μg of protein per milliliter. All values are means ± SEM of four determinations of the incorporation of [α^{32}P]dCTP by nuclei that had been preincubated at 0°C for 30 minutes (unless otherwise stated) with PRS, or the affinity-purified anti-myc. The + sign indicates an increase over the incorporation of isotope seen with no addition of exogenous template or DNA polymerase.

Protocol		DNA synthesis (count/min)	
		PRS	Anti-myc
		Preincubation	
Time (minutes):	5	11,597 ± 696	5,334 ± 931
	30	10,669 ± 464	1,855 ± 235
	240	9,973 ± 927	1,162 ± 701
		*Exogenous template**	
No exogenous template		11,286 ± 100	4,113 ± 233
Activated DNA (micrograms):	5	+1,265 ± 34	+593 ± 56
	10	+3,090 ± 50	+1,453 ± 39
	25	+5,514 ± 172	+2,188 ± 182
		Exogenous DNA polymerase†	
No exogenous polymerase		11,286 ± 100	4,113 ± 233
Polymerase (units):	10	+2,039 ± 302	+2,331 ± 187
	20	+3,917 ± 83	+3,841 ± 234
	40	+5,605 ± 63	+5,499 ± 212

*Calf thymus DNA was activated with pancreatic deoxyribonuclease I as described (*6*). The concentration of deoxyribonucleoside triphosphates in the incubation mix was doubled in this experiment. †Kornberg *Escherichia coli* polymerase I was obtained from Boehringer Mannheim, Indianapolis, IN. The concentration of deoxyribonucleoside triphosphates was twice that described (*6*).

probably occurs through tears in the nuclear membrane (*20*), which occur during preparation of the nuclei.

It is important to demonstrate that the inhibition of DNA synthesis is due to the specific interaction of anti-myc with the c-*myc* protein. We have presented several lines of evidence to support this: (i) the specificity of binding of the affinity-purified anti-myc is demonstrated by the immunoblot in Fig. 3B, which shows that anti-myc recognizes the c-*myc* protein in a lysate of HL60 cells, and that this recognition is blocked by preincubating anti-myc with recombinant c-*myc* protein, (ii) monoclonal antibodies that specifically recognize *myc* protein cause similar inhibition of DNA synthesis, and (iii) the inhibition can be reversed in a concentration-dependent manner by adding *myc* protein to the assay.

Previous studies have shown that the v-*myc* gene product is located in the nucleus and that it is a DNA-binding protein, as determined by in vitro assays (*21*). Microinjection of the human counterpart, obtained by recombinant DNA technology (*8*), into nuclei of mouse fibroblasts induces DNA synthesis if the cells are later exposed to platelet-poor plasma (*22*). We have now extended these studies by showing that the function of the human c-*myc* protein is required for DNA synthesis in nuclei of human cells. This function appears to be required at least for the elongation of nascent DNA chains, since initiation does not take place in isolated nuclei (*5*). These data are intriguing in light of concurrent studies indicating an association of c-*myc* with small nuclear ribonucleoprotein particles (*23*). These results are not inconsistent with each other and certainly require further investigation, since the c-*myc* protein may have more than one function, either direct or indirect, in the metabolism of the cell. In any case, if this protein is ordinarily present in limiting amounts in normal cells, its increased or inappropriate expression might contribute to the selective growth advantage of neoplastic cells.

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Shock and Tissue Injury Induced by Recombinant Human Cachectin

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Cachectin (tumor necrosis factor), a protein produced in large quantities by endotoxin-activated macrophages, has been implicated as an important mediator of the lethal effect of endotoxin. Recombinant human cachectin was infused into rats in an effort to determine whether cachectin, by itself, can elicit the derangements of host physiology caused by administration of endotoxin. When administered in quantities similar to those produced endogenously in response to endotoxin, cachectin causes hypotension, metabolic acidosis, hemoconcentration, and death within minutes to hours, as a result of respiratory arrest. Hyperglycemia and hyperkalemia were also observed after infusion. At necropsy, diffuse pulmonary inflammation and hemorrhage were apparent on gross and histopathologic examination, along with ischemic and hemorrhagic lesions of the gastrointestinal tract, and acute renal tubular necrosis. Thus, it appears that a single protein mediator (cachectin) is capable of inducing many of the deleterious effects of endotoxin.

BACTERIAL ENDOTOXIN (LIPOPOLYSACCHARIDE, LPS) is highly toxic to most mammals. When administered intravenously, it evokes a "shock" state, characterized by fever, hypotension, and multi–organ system failure (*1–5*). Fatality is associated with injuries involving the lungs (shock lung syndrome), kidneys (acute tubular necrosis), and gastrointestinal tract (mesenteric ischemia) (*1, 2, 4, 6*). This syndrome frequently occurs in the course of invasive Gram-negative infections and is associated with a high mortality (*2, 5, 7*).

Recently, it has become clear that endotoxin does not injure host tissues directly, but does so through the action of an endogenous mediator or mediators. C3H/HeJ mice, which are highly resistant to the lethal effect of endotoxin as the result of a genetic lesion (the *lps*^{d} allele on chromosome 4) (*8*), are rendered endotoxin-sensitive if subjected to total body irradiation and transplanted with marrow obtained from mice of the closely related, endotoxin-sensitive strain C3H/HeN (*9*). Similarly, irradiated C3H/HeN mice reconstituted with C3H/HeJ marrow are endotoxin resistant (*9*). Thus, a host factor expressed by cells of hematopoietic origin appears to confer endotoxin sensitivity.

The macrophage appears to be the principal cell involved in mediating the effects of endotoxin. Infectious agents capable of stimulating reticuloendothelial hyperplasia render animals exquisitely sensitive to the effects of endotoxin (*10*). Moreover, macrophages activated by endotoxin in vitro produce a soluble factor that is capable of killing endotoxin-resistant animals (*11*).

Cachectin (*12, 13*) is a macrophage-derived polypeptide hormone known for its ability to modulate adipocyte metabolism (*12, 14*), lyse tumor cells in vitro (*15, 16*), and induce hemorrhagic necrosis of certain transplantable tumors in vivo (*15*). It constitutes between 1 and 2% of the total secretory protein produced by endotoxin-activated macrophages in vitro (*12*). Copious quantities of the protein are also produced in vivo in response to endotoxin. Approximately 5.5 mg of cachectin are present per liter of rabbit serum 1.5 hours after endotoxin administration (*17*). With consideration given to the half-life of the hormone in vivo (*18*), this would suggest the net production of milligram quantities of cachectin per kilogram of body mass.

Recently, Beutler *et al.* (*19*) suggested that cachectin itself might initiate the tissue injury elicited by endotoxin. This suggestion was based on the observation that animals passively immunized against cachectin were protected against the lethal effect of endotoxin (*19*). However, direct analysis of cachectin as a critical mediator of organ injury in sepsis required the isolation of large amounts of purified hormone, devoid of pharmacologically active quantities of endotoxin.

Accordingly, an artificial gene, encoding the amino acid sequence of cachectin, was constructed by means of an Applied Biosystems model 380A gene synthesizer. The gene was synthesized in 25 overlapping segments, which were annealed and ligated in a single reaction. Codons were chosen so as to optimize expression of the protein product in yeast. At the 5′ end, additional codons were added to specify the signal sequence of yeast α-factor, in order to obtain a secreted product (*20*). The synthetic

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