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Isolation and characterization of a mouse cDNA clone that expresses mast-cell growth-factor activity in monkey cells

(inducer T cell/interleukin 3/gene cloning/transient expression/DNA sequence analysis)

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ABSTRACT **A cDNA sequence coding for mouse mast-cell growth-factor (MCGF) has been cloned from a cDNA library prepared from mRNA derived from a concanavalin A-activated mouse T-cell clone. Cloning was achieved using the pcD vector that permits expression of cDNA inserts in mammalian cells. The DNA sequence codes for a polypeptide of 166 amino acid residues including a putative signal peptide. The supernatant fluid obtained after transfection of COS-7 monkey cells with the pcD-MCGF plasmid had mouse MCGF activity. The MCGF amino acid sequence between amino acids 33 and 41, deduced from the nucleotide sequence of its cDNA, is identical to the NH_2-terminal sequence of the mouse lymphokine, interleukin 3, suggesting that MCGF is related to interleukin 3.**

T lymphocytes may regulate the growth and differentiation of certain lymphopoietic and hematopoietic cells through the secretion of soluble protein factors. One population of murine T lymphocytes, termed helper or inducer cells, can be identified by the surface phenotype, Lyt-1^+, Lyt-2^-,3^- (1). These cells can be activated by antigen or concanavalin A (Con A) to produce several factors that activate target cells to divide or differentiate *in vitro* (1–4). We have described a clone, Cl.Ly1^+2^-/9, that displays multiple biologic activities (5, 6) including (*i*) activation of B cells to secrete immunoglobulin, (*ii*) induction of hematopoietic precursor cells to produce colonies of monocytes and granulocytes, (*iii*) enhanced proliferation of T-cell clones, and (*iv*) stimulation of cloned mast-cell proliferation (7). Further definition of the activities of the relevant inducer molecules requires the availability of the polypeptides in biochemically pure form. One approach to resolving the proteins responsible for the various biological activities is to clone and express their genetic coding sequences separately. In addition to providing materials for biochemical study, this approach should facilitate studies of the corresponding genes and their regulation.

In this paper, we describe the first of these efforts—the cloning of a nearly full-length mast-cell growth-factor (MCGF) cDNA and its expression in cultured monkey cells. The MCGF cDNA was obtained from a clone library prepared in the pcD expression vector (8) with mRNA from the mouse T-cell clone Ly1^+2^-/9, which produces MCGF after Con A activation. Cloned plasmids containing MCGF cDNA were detected by their ability to anneal mRNA from Con A-stimulated cells, which was translated in *Xenopus laevis* oocytes to biologically active MCGF. Clones containing nearly full-length cDNA segments were identified by their ability to direct the synthesis of MCGF production after transfection into COS-7 monkey cells. The amino acid sequence deduced from the nucleotide sequence of the cDNA reveals a region of homology with a sequence previously described for the lymphokine, interleukin 3 (IL3) (9–12).

MATERIALS AND METHODS

Cell Lines and Tissue Culture. Cl.Ly1^+2^-/9, a cloned T-cell line derived from C57BL/6 mice (5), was maintained in Dulbecco's modified Eagle's (DME) medium supplemented with 50 μM 2-mercaptoethanol/2 mM glutamine/10% heat-inactivated fetal calf serum and spleen conditioned medium as described (6). MC/9, a cloned mast-cell line, was grown in DME medium supplemented with 5% supernatant from Con A-activated Cl.Ly1^+2^-/9 cells, as described (7). To prepare Con A-stimulated Cl.Ly1^+2^-/9 cells, 5×10^5 T cells per ml were cultured in DME medium supplemented with 50 μM 2-mercaptoethanol/2 mM glutamine/4% fetal calf serum/2 μg of Con A per ml. Cells were harvested after 12–14 hr.

Biological Assays for MCGF. MCGF activity was determined by [^{3}H]thymidine incorporation (7) or a colorimetric assay (13) using MC/9 mast cells. Briefly, MC/9 cells were cultured in flat-bottom Falcon microtiter trays (10^4 cells per well) in DME medium supplemented with 4% fetal calf serum/50 μM 2-mercaptoethanol/2 mM glutamine/nonessential amino acids, essential vitamins, and varied concentrations of tested supernatant in a final volume of 0.1 ml.

For the radioactive assay, 0.5 μCi [^{3}H]thymidine (1 Ci = 37 GBq) was added to each culture for the last 4 hr of the 24-hr incubation period, the cells were harvested onto glass filters, and the radioactivity was measured in a liquid scintillation spectrometer. For the colorimetric assay, 50 μg of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (Sigma) in 10 μl of phosphate-buffered saline was added to each cell culture after a 20 hr incubation. Four hours later, 0.1 ml of 0.04 M HCl in isopropanol was added to solubilize the colored formazan reaction product. The absorbance at 570 nm (reference 630 nm) was measured on a Dynatech Micro Elisa reader.

Isolation and Size Fractionation of mRNA. Total cellular mRNA was isolated using the guanidinium-thiocyanate extraction procedure of Chirgwin *et al.* (14). Approximately 100 μg of poly(A)$^+$ mRNA, selected on columns of oligo(dT) cellulose, were obtained from $1–2 \times 10^8$ cells. The mRNA was fractionated according to size as follows: 100 μg of poly(A)$^+$ mRNA was layered on a 10-ml 5–25% sucrose gradient (10 mM Tris·HCl, pH 7.4/100 mM NaCl/1 mM EDTA), and centrifuged for 19 hr at 26,000 rpm in a Beckman SW41 rotor; 450-μl fractions were collected and the RNA was precipitated with 2 vol of ethanol.

Hybrid Selection and Microinjection of *X. laevis* Oocytes. Filter hybridizations were carried out essentially as described by Parnes *et al.* (15). Aliquots of the eluted mRNA

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Abbreviations: bp, base pairs; Con A, concanavalin A; IL3, interleukin 3; kb, kilobase(s); MCGF, mast-cell growth-factor; SV40, simian virus 40.

were injected into individual *X. laevis* oocytes and the supernatants from viable oocytes were collected after 48 hr, pooled, and assayed for MCGF activity.

Construction of cDNA Library. The cDNA library was constructed using the pcDV1 vector-primer and the pL1 linker fragment according to the procedure of Okayama and Berg (8, 16). The plasmid vector, which contains the simian virus 40 (SV40) early region promoter and SV40 RNA processing signals, is designed to promote expression of the cloned cDNA segment in mammalian cells. The cyclized vector–cDNA preparation was used to transform competent *Escherichia coli* MC1061 (17) cells using calcium chloride (18). About 5 μg of poly(A)$^+$ RNA from Con A-stimulated Cl.Lyl$^+$2$^-$/9 cells yielded 1.5 × 10^6 independent transformants. A collection of 10^4 independent clones were picked and propagated in wells of microtiter plates containing 200 μl of L broth, ampicillin at 50 μg/ml, and 7% dimethyl sulfoxide. In addition, sublibraries based on the size of the cDNA insert, were prepared from the total cDNA library as described (8). Briefly, pooled plasmid DNA was digested separately with either *Sal* I, *Cla* I, or *Hin*dIII endonucleases and electrophoresed in a 1% agarose gel. The ethidium bromide-stained gel was sliced into seven sections corresponding to cDNA insert sizes of 0–1, 1–2, 2–3, 3–4, 4–5, 5–6, and >6 kilobases (kb); after extraction of the DNA from each gel slice, it was recyclized with T4 DNA ligase and used to transform MC1061.

Preparation of Subtracted cDNA Probe. To enrich the ^{32}P-labeled cDNA probe for mRNAs induced by Con A, ≈2 μg of mRNA from a sucrose gradient fraction that produced MCGF activity in the oocyte assay was used to prepare cDNA with reverse transcriptase and oligo(dT)$_{12-18}$ primers. After removal of the RNA with alkali, the ^{32}P-labeled cDNA was annealed with a mixture of 20 μg of mRNA from WEHI-231, a B-cell line, and 20 μg of mRNA from a B-cell hybridoma at 68°C for 14 hr (C_0t = 5000). This removes cDNA sequences that are shared with B cells (19). The remaining cDNA was separated from the cDNA·RNA hybrids by chromatography on hydroxylapatite and was then annealed with 10 μg of mRNA (C_0t = 1100) from uninduced Cl.Lyl$^+$2$^-$/9 cells and the DNA·RNA hybrids were removed as described above. The residual single-stranded ^{32}P-labeled cDNA, which is enriched for Con A-induced T-cell mRNA sequences, constitutes 1–2% of the starting material; this fraction was used to screen the cDNA clones by colony hybridization (20).

DNA Transfections of Monkey Cells. Approximately 10^6 COS-7 monkey cells were seeded onto 60-mm plates one day prior to transfection. Transfections were carried out with 15 μg of plasmid DNA in 1.5 ml of DME medium containing 50 mM Tris·HCl (pH 7.4) and 400 μg of DEAE-Dextran per ml (Pharmacia) (21). After 4 hr the solution was removed and replaced with 2.0 ml of DME medium and 4% fetal calf serum. Seventy-two hours later the medium was collected and assayed for MCGF activity.

DNA Sequence Analysis. The nucleotide sequence of the cDNA inserts of pcD–MCGF clones was determined according to the modified procedure of Maxam and Gilbert (22, 23).

RESULTS

Assay of MCGF mRNA. After exposure of Cl.Lyl$^+$2$^-$/9 cells to Con A, the cell supernatant stimulates the proliferation of a mast-cell clone as measured by incorporation of [^{3}H]thymidine (7) (Table 1). To investigate whether the Con A induction of MCGF activity occurred at the level of transcription, poly(A)$^+$ mRNA from induced and uninduced cells was injected into *X. laevis* oocytes, and the supernatants from the oocytes were assayed for MCGF activity. Only supernatants from oocytes injected with mRNA from Con A-induced cells containing MCGF activity, suggesting the MCGF mRNA is Con A inducible (Table 1). This result shows that *Xenopus* oocytes can produce MCGF from injected mRNA and export at least some portion of it into the medium.

Table 1. Production of MCGF by Cl.Lyl$^+$2$^-$/9

	Cell supernatant, units/ml	Injected mRNA, units/ml
Without Con A	50	40
With Con A	26,383	1,403

Supernatants collected from untreated or from Con A-stimulated Cl.Lyl$^+$2$^-$/9 cells were assayed for MCGF with the MC/9 mast-cell line (7). Incorporation of [^{3}H]thymidine by the target cells was determined. Poly(A)$^+$ mRNA isolated from Con A-treated and untreated cells was injected into 25–30 *X. laevis* oocytes (≈20 ng of RNA per oocyte), and the oocyte incubation medium was collected after 48 hr and assayed in the same manner. Titration of all samples and the reference standard was done in triplicate. One unit of MCGF causes 15% of the maximal level of [^{3}H]thymidine incorporation obtained with a Cl.Lyl$^+$2$^-$/9 supernatant.

Enrichment of MCGF mRNA. Using the assay described above, it was possible to fractionate the Con A-induced mRNA and enrich for the MCGF mRNA. Poly(A)$^+$ mRNA was centrifuged in a sucrose density gradient and aliquots were assayed for MCGF activity using the *Xenopus* oocyte translation system. The peak of MCGF mRNA activity sedimented slower than 18 S (Fig. 1). These fractions were enriched ≈10-fold for MCGF mRNA and were subsequently used for the preparation of a ^{32}P-labeled cDNA probe.

Screening the cDNA Library by Hybrid Selection. The pcD vector was used to construct the cDNA library because we anticipated that the expression of the cloned cDNAs in mammalian cells would be necessary to identify the MCGF cDNA. However, because the assay for MCGF is rapid and sensitive, the cDNA library was initially screened by annealing pooled cDNA clones with poly(A)$^+$ RNA from Con A-

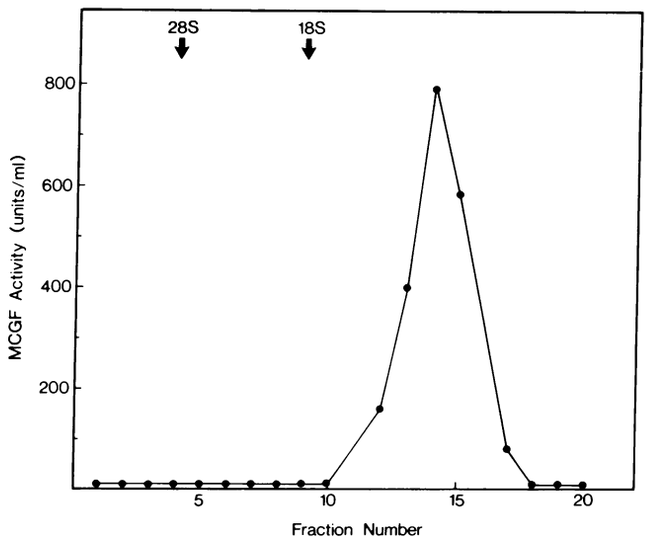

FIG. 1. Sedimentation of mouse MCGF mRNA. Poly(A)$^+$ mRNA (100 μg) isolated from Con A-induced Cl.Lyl$^+$2$^-$/9 cells were fractionated on a 10-ml 10–25% sucrose gradient. After concentration of the mRNA by ethanol precipitation, aliquots of each fraction were injected into *X. laevis* oocytes. The oocyte incubation medium was then assayed for MCGF activity using the colorimetric assay (13). ^{3}H-labeled RNA markers were run in a parallel gradient, and the locations of 18S and 28S rRNA peaks are indicated. One unit of MCGF is the amount of factor that results in 15% of the maximal absorbance obtained with Cl.Lyl$^+$2$^-$/9 supernatant.

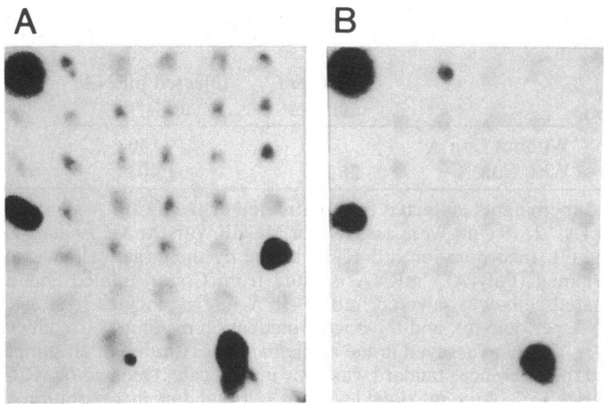

FIG. 2. Colony hybridization of cDNA clones with the enriched ^{32}P-labeled cDNA probe. Individual clones from a subpool of 48 cDNA clones, which was positive by hybrid selection, were grown in wells of a 96-well microtiter dish. Colonies were replicated onto duplicate nitrocellulose filters, and the DNA was immobilized on the filters (20). The ^{32}P-labeled cDNA probe was synthesized using as the template mRNA from a sucrose gradient fraction containing MCGF activity as determined by translation in oocytes (see Fig. 1). The initial ^{32}P-labeled DNA was enriched by two cycles of subtractive hybridization. The first subtraction was carried out using a mixture of mRNA from the B-cell line WEHI 231 and a B-cell hybridoma. The unhybridized cDNA (T-cell specific probe) was used to probe one filter (*A*). After a second subtraction with mRNA from uninduced Cl.Ly1$^+$2$^-$/9 cells, the single-stranded ^{32}P-labeled cDNA (Con A-induction-specific probe) was used to probe a second filter (*B*).

induced Cl.Ly1$^+$2$^-$/9 cells and assaying the hybridized mRNA for MCGF mRNA in *Xenopus* oocytes. Eight pools of plasmid DNA, each containing 600–1000 random cDNA clones, were examined in the first round of hybrid selections; several contained sequences that could hybridize MCGF mRNA activity. One, containing 672 clones, reproducibly selected mRNA that yielded high levels of MCGF activity after injection into the oocytes. This pool was subdivided further into 14 pools, each containing 48 of the original clones. Only one of these pools was positive in the hybrid selection assay (data not shown).

The 48 clones in the positive subpool were then screened with two enriched ^{32}P-labeled cDNA probes. Fig. 2*A* shows that four positive colonies hybridized with the T-cell-specific

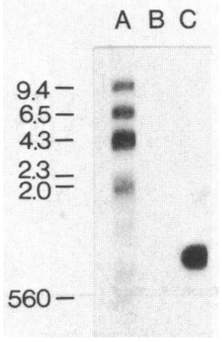

FIG. 3. RNA blot analysis of induced and uninduced Cl.Ly1$^+$2$^-$/9 mRNA. Clone 5G, a partial MCGF cDNA clone, was nick-translated to a specific activity of 1×10^8 cpm/μg (20). Five micrograms of poly(A)$^+$ mRNA isolated from uninduced or Con A-induced Cl.Ly1$^+$2$^-$/9 cells was denatured, treated with formaldehyde, and then separated on a 1% agarose/formaldehyde gel (24). The RNA was transferred from the gel to nitrocellulose (25) and the filter was hybridized with nick-translated clone 5G DNA. Lanes: A, λ DNA restricted with *Hin*dIII; B, uninduced Cl.Ly1$^+$2$^-$/9 mRNA; C, Con A-induced Cl.Ly1$^+$2$^-$/9 mRNA.

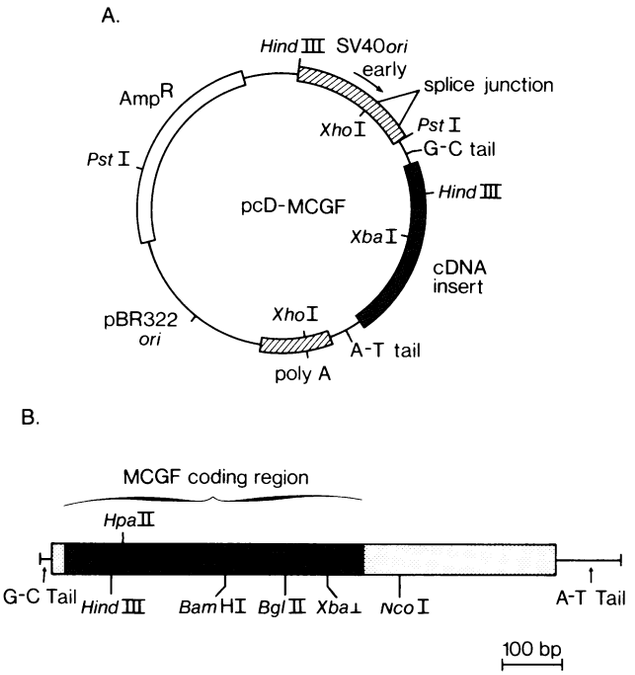

FIG. 4. (*A*) Diagram of pcD–MCGF, the plasmid carrying a nearly full-length MCGF cDNA insert. The direction of transcription from the SV40 early promoter is indicated by the arrow. The locations of the splice donor and acceptor sites are shown. The polyadenylylation signal, also derived from SV40, is located at the 3′ end of the cDNA insert. The cDNA insert (950 bp) is shaded in black. The remainder of the vector sequences are derived from pBR322, including the β-lactamase gene (*Amp^R*) and the origin of replication. (*B*) Restriction endonuclease cleavage map of the cDNA insert of MCGF. The MCGF coding region is shaded in black, and the noncoding regions are lightly shaded.

cDNA probe; only three of these four clones also hybridized with the Con A-induction-specific cDNA probe (Fig. 2*B*). Plasmid DNA prepared from each of these clones was annealed with poly(A)$^+$ RNA containing MCGF mRNA, and the eluted RNA was assayed in the oocyte system. One clone, 5G, reproducibly hybridized functional MCGF mRNA.

Isolation of a Full-Length MCGF cDNA Clone and Expression in Monkey Cells. Clone 5G plasmid DNA was labeled

Table 2. Transient expression of MCGF in monkey cells

Clone	cDNA start point*	MCGF activity, units/ml
Mock	—	20
B4	41	5,228
B5	ND†	7,371
B6	1	3,307
B8	1	6,929
B9	1	3,362
Cl.Ly1$^+$2$^-$/9 supernatant	—	19,769

Five of the six initial positive clones were examined in detail in transient expression experiments. Individual MCGF cDNA plasmids were introduced into COS-7 cells using DEAE-Dextran. Cell growth medium was harvested 72 hr after the transfection and assayed for MCGF activity by measuring stimulation of [^{3}H]thymidine incorporation by the MC/9 cell line. Mock-infected COS cells were treated identically but DNA was omitted. MCGF units are defined in the legend to Table 1.

*The 5′ end of MCGF cDNA inserts are numbered as in Fig. 5.

†Not determined; cDNA start point is located to the 5′ side of position 41.

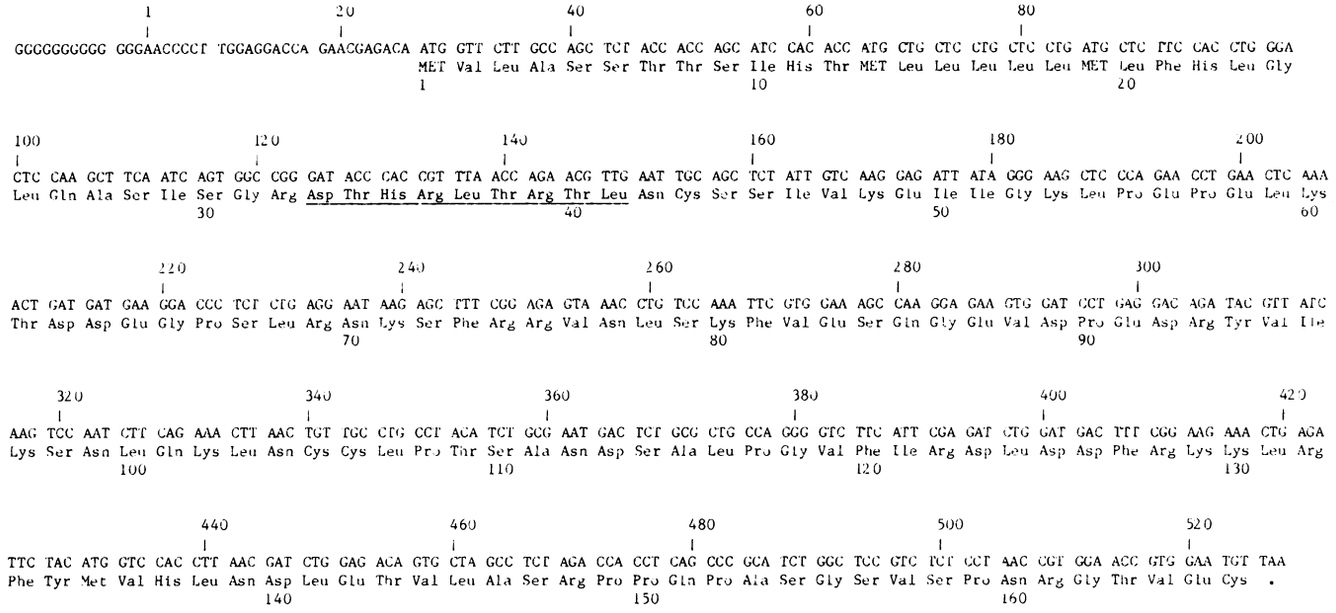

FIG. 5. Nucleotide sequence and predicted amino acid sequence of the MCGF coding region. The nucleotide sequence begins with position 1 at the first nucleotide following the oligo(dG) segment. The amino acid sequence begins with the first in-phase ATG codon for the single long open-reading frame. The underlined amino acids from 33–41 are those that are identical to the sequence reported by Ihle *et al.* for IL-3 (12).

with ^{32}P by nick-translation and used as probe with an RNA blot of induced and uninduced Cl.Ly1$^+$2$^-$/9 mRNA. Fig. 3 shows that the cDNA of clone 5G hybridizes to a single mRNA species, ≈1 kb long, that is strongly inducible by Con A. Restriction nuclease analysis, as well as nucleotide sequence analysis indicated that the clone 5G cDNA was only about 650 base pairs (bp) long and therefore was not a full-length cDNA. Accordingly, the 5G cDNA clone was used to screen a sublibrary that had been enriched for cDNA inserts 1–2 kb long; approximately 2×10^4 colonies were screened and 19 positive clones were identified.

Because the cDNA library had been constructed in a mammalian expression vector (Fig. 4*A*), we examined the 19 positive clones for their ability to produce MCGF. The SV40 origin of replication contained on the pcD vector allows replication of the plasmid in COS-7 monkey cells, thereby amplifying the plasmid copy number and expression of the encoded genes. Each plasmid was transfected into COS-7 cells using DEAE-Dextran, and the supernatants from the transfected cells were assayed for MCGF activity. Six of the cDNA clones produced high levels of MCGF activity (Table 2); no activity was detected with the remaining clones. Thus, it appears that six cDNA inserts contain sufficient information to direct the synthesis of a functional MCGF polypeptide.

Nucleotide Sequence of the MCGF cDNAs. Fig. 4*B* shows the restriction map of one of the longest cDNA inserts (clone B9 in Table 2). The DNA sequences of the cDNA inserts for several of the active cDNA clones were determined and the sequence for clone B9 is shown in Fig. 5. The cDNA insert contains a single long open-reading frame. The first ATG is found 28–30 nucleotides from the 5′ end, and is followed by 166 codons before the termination triplet TAA at nucleotide positions 536–538. The NH_2-terminal segment of the predicted MCGF amino acid sequence is hydrophobic, as would be expected for a signal peptide. The 5′ end of the shorter cDNA insert of clone B4 is at nucleotide position 41. The clone isolated initially (5G) starts at nucleotide position 191.

DISCUSSION

We describe here the isolation of cDNA clones that encode the MCGF produced by the murine T-cell clone Ly1$^+$2$^-$/9. When this study was undertaken, there was no nucleotide or amino acid sequence information available for the synthesis of specific nucleic acid probes, nor were there antibodies against MCGF that could be used for immunological detection of the protein. However, a convenient and reliable proliferation assay for MCGF did exist. We showed that *X. laevis* oocytes injected with mRNA from Cl.Ly1$^+$2$^-$/9 synthesize active MCGF detectable by this proliferation assay. Therefore, in the initial stages of the screening for MCGF cDNA clones, we used hybrid selection in conjunction with oocyte injection to identify groups of clones that could hybridize to MCGF mRNA. After the initial screen, a ^{32}P-labeled cDNA probe, highly enriched for MCGF sequences, was used to further restrict the number of candidate MCGF clones. Finally, one cDNA clone was found that hybridized with MCGF mRNA that was translated into biologically active MCGF in oocytes, but this cDNA segment was smaller than the mRNA that encodes MCGF. The frequency of MCGF cDNA clone was estimated to be ≈0.02% of total clones in a cDNA library.

A clone library in the pcD expression vector provided an opportunity to identify complete cDNA clones by expression in mammlian cells. COS-7 monkey cells were used as the recipient for this plasmid because the SV40 large T antigen they produce drives the replication of the pcD DNA from the SV40 origin of replication (26). The amplified copy number of the plasmid in COS-7 cells increases the transient expression of genes on the plasmid. We assumed that a full-length MCGF cDNA clone in COS cells would direct the synthesis of a polypeptide that would be secreted into the culture medium. Nineteen putative MCGF cDNA clones were isolated from a sublibrary containing cDNA inserts in the 1–2 kb range. The six clones containing the longest cDNA inserts all produced detectable MCGF in the cell supernatants.

The production of biologically active MCGF after transfection of COS-7 monkey cells with pcD–MCGF strongly suggests that these clones contain a nearly full-length MCGF cDNA. We conducted reconstruction experiments to determine the sensitivity of this method for the identification of particular clones in a cDNA library. These experiments showed that a mixture of DNAs containing 1% full-length

MCGF clones transfected into COS-7 cells produces readily detectable levels of MCGF (data not presented). Thus, transfection of randomly picked or pre-enriched pools of cDNA clones into mammalian cells combined with an appropriate sensitive assay, provides an alternative to the more laborious hybrid selection procedure. Direct expression might be particularly useful in cases in which large amounts of mRNA for hybridization are difficult to obtain.

Three of the cDNA inserts that yielded MCGF after transfection of COS cells were analyzed and found to have identical sequences at the 5′ end of the insert. It is therefore likely that these independently isolated cDNA clones contain full-length or nearly full-length copies of MCGF mRNA. These cDNA inserts contain a single open-reading frame consisting of 166 codons beginning with the methionine codon at position 28. In addition to this putative initiation codon, two additional methionine codons occur 12 and 18 codons downstream from the first. A fourth cDNA clone starts 40 bp downstream from the 5′ ends of the other three inserts. This shorter cDNA clone lacks the first methionine codon but still makes active MCGF on introduction into COS cells. This suggests either that the initial methionine codon is not the primary site of translation initiation *in vivo* or that one of the two ATG codons downstream serves as the initiation codon; alternatively, the shortened protein is also biologically active.

Ihle *et al.* have purified IL3 to apparent homogeneity from supernatants for the WEHI-3 cell line and have shown that it has MCGF activity (11). The deduced amino acid sequence that begins 20 codons downstream of the second in-phase ATG contains nine contiguous amino acids, which are identical to the nine NH_2-terminal amino acids reported for IL3 (12). The identity of the IL3 amino acid sequence and our deduced amino acid sequence indicates that the MCGF produced by Cl.Ly1$^+$2$^-$/9 is related to IL3. The coding region between the first ATG and the beginning of the sequence contained in IL3 is rich in hydrophobic amino acids, as would be expected for a leader sequence for a secreted protein. It is likely, therefore, that the mature form of secreted MCGF begins with an aspartic acid residue, as does IL3, and the preceding 20 amino acids constitute the putative leader sequence that is removed by proteolytic processing. If this is true, mature MCGF would consist of 134 amino acid residues with a calculated molecular weight of about 15,000. Analysis of purified MCGF from Cl.Ly1$^+$2$^-$/9 supernatants must be carried out to confirm this predicted structure.

It has been suggested that mouse IL3 is glycosylated (11) and has an apparent molecular weight of 28,000 by $NaDodSO_4$/polyacrylamide gel electrophoresis (11, 12). Judging from the deduced amino acid sequence of mouse MCGF cDNA clone, there are four potential *N*-glycosylation sites (27) (Asn-X-Ser at positions 42–44, 70–72, 77–79, and 112–114, respectively). Therefore, the discrepancy between the reported size of IL3 and the calculated molecular weight of MCGF deduced from our clones may be partly due to glycosylation of the molecule. Previous studies have failed to separate MCGF activity from granulocyte–macrophage colony stimulating factor (GM-CSF) activity in Cl.Ly1$^+$2$^-$/9 supernatants (5–7). The availability of MCGF produced by transfected mammalian cells will help to define the spectrum of biological activities that may be attributed to this intriguing lymphokine.

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