Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 5644–5648, August 1986
Immunology

A partial genomic DNA clone for the α subunit of the mouse complement receptor type 3 and cellular adhesion molecule Mac-1

(oligonucleotide probes/macrophage/γ interferon-stimulated premyelocyte maturation/mRNA/cell adhesion molecule LFA-1)

LEANDRO SASTRE*, JANET M. ROMAN†, DAVID B. TEPLOW†, WILLIAM J. DREYER†, CONNIE E. GEE*, RICHARD S. LARSON*, THOMAS M. ROBERTS*, AND TIMOTHY A. SPRINGER*

*Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA 02115; and †Department of Biology, California Instiute of Technology, Pasadena, CA 91125

Communicated by Baruj Benacerraf, April 17, 1986

ABSTRACT **A genomic clone coding for the α subunit of the mouse complement receptor type 3 and the cellular adhesion molecule Mac-1 has been isolated directly from a genomic library using synthetic oligonucleotide probes based on the amino-terminal amino acid sequence of the protein. The identity of the clone has been established by DNA sequencing and *in vitro* translation of hybrid-selected mRNA. The gene is present in a single copy in the murine genome. The region containing the amino-terminal exon has been sequenced. RNA gel blotting shows that the Mac-1 α-subunit mRNA is 6 kilobases in length. Mac-1 α-subunit mRNA is present in macrophages but not T lymphoma or L cells. During γ interferon-stimulated maturation of the mouse premyelocytic cell line M1, Mac-1 α-subunit mRNA is induced. This corresponds with the tissue distribution of the Mac-1 α subunit, showing expression is regulated at least partially at the message level.**

The cellular adhesion molecule Mac-1 is a surface glycoprotein that is expressed on macrophages, monocytes, granulocytes, and large granular lymphocytes in both mouse and human (1, 4). Its expression is also induced during *in vitro* differentiation of premyelocytic cell lines such as the mouse cell line M1 (5) and the human cell lines HL60 and U937 (6). Mac-1 is the complement receptor type 3 for the inactivated complement component iC3b (8), suggesting that Mac-1 plays an active role in the elimination of particles bearing immune complexes. Mac-1 is also involved in monocyte and granulocyte adhesion to nonspecific surfaces and endothelial cells (9). The physiological importance of this molecule has been dramatically demonstrated by the discovery of a human immunodeficiency disease in which the absence of Mac-1 correlates with severe impairment in macrophage and granulocyte complement receptor type 3- and adhesion-dependent functions (10).

Mac-1 is composed of two subunits, α (M_r 170,000) and β (M_r 95,000), noncovalently associated in an $\alpha_1\beta_1$ dimer (11). The nonglycosylated α and β polypeptides are M_r 130,000 and 73,000 (12). High mannose oligosaccharides are added in the rough endoplasmic reticulum to yield precursors of M_r 164,000 and 87,000, respectively (12). The α and β subunits become noncovalently associated before further processing in the Golgi to give rise to the mature molecule (13). Mac-1 has been purified to homogeneity (14), and its α-subunit amino-terminal amino acid sequence has been established (15). Mac-1 has been found to be structurally related to two other leukocyte function-associated antigens, LFA-1 and p150,95 (4). All three proteins share the same β subunit associated with different α subunits (4). Although they differ in cell distribution and function, the LFA-1 and Mac-1 α subunits have 33% amino acid homology in their amino-terminal region (15), suggesting that they could have evolved from an ancestral gene by duplication. All three known members of this family of cell surface glycoproteins are mediators of highly specific cellular adhesion processes.

A better understanding of the relationship between the structure and the function of this molecule, the relationship between Mac-1 and the other related leukocyte adhesion molecules, and the basis of cell lineage-specific expression of each could be obtained by the study of their gene structure. We have taken advantage of the amino-terminal amino acid sequence of the mouse Mac-1 α subunit (15) to isolate directly from a genomic library a DNA clone coding for this protein. Using this clone we have identified the mRNA coding for Mac-1 and studied its expression in different cell lines and during *in vitro* differentiation of the mouse premyelocytic cell line M1.

METHODS

Cells. P388D1, EL-4, M1, and L cells (14) were cultured in complete RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum. M1 cells were induced to differentiate by incubation with γ interferon (IFN-γ) at 10 units/ml (Genentech, South San Francisco, CA) at 5×10^5 cells per ml for 6 days in the same medium. The media was changed at days 2 and 4 of induction and fresh IFN-γ was added to the same concentration.

Synthetic Oligonucleotide Probes. Oligonucleotides were synthesized in the solid phase using phosphoramidite chemistry (17). The correct oligonucleotide was separated on a 20% polyacrylamide gel (40 cm × 4 mm) from shorter fragments arising from incomplete couplings. The desired oligonucleotide band was eluted in 0.5 M ammonium acetate/0.01 M magnesium acetate/0.001 M EDTA/0.1% $NaDodSO_4$, purified on Sephadex G-25, labeled with ^{32}P, separated from free ^{32}P by homochromatography (18), eluted in 20× NET, and used directly in hybridization solution. (1× NET = 150 mM NaCl/1 mM EDTA/15 mM Tris·HCl, pH 7.5.)

Library Screening. A Charon 4A λ library constructed from a partial *Eco*RI digest of BALB/c spleen DNA was the kind gift of R. Perlmutter (California Institute of Technology). Duplicate nitrocellulose filters from each plate (19) were hybridized with synthetic [^{32}P]oligonucleotides as described by Conner *et al.* (20) with minor variations. Filters were prehybridized in 6× NET, 5× Denhardt's (0.1% each of Ficoll, polyvinylpyrrolidone, and bovine serum albumin), 0.1% $NaDodSO_4$, 10% dextran sulfate, 0.5% Nonidet P-40, and salmon testes DNA at 100 μg/ml for 1 hr at 50°C. Hybridizations were in the same solution (minus salmon testes

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: IFN-γ, γ interferon; kb, kilobase(s); bp, base pair(s).

DNA) plus synthetic [^{32}P]oligonucleotide at 10^7 cpm/ml for 12–16 hr at 50°C. Filters were washed at 0°C in 6× SSC, 0.01% sodium pyrophosphate four to six times, 15 min per wash, and then at 50°C in the same buffer two times, 1 min per wash. (1× SSC = 150 mM NaCl/15 mM sodium citrate, pH 7.0.) These conditions of hybridization were also used for plaque purification and initial restriction analyses prior to subcloning. Subsequent mapping and analyses were done at higher stringencies (see Southern and RNA gel blots below).

RNA Preparation. Cells were cultured to a density of 1×10^6 cells per ml and washed twice in phosphate-buffered saline (PBS) at 4°C. (PBS = 140 mM NaCl/10 mM NaH_2PO_4, pH 7.5.) RNA was extracted by homogenization in guanidinium isothiocyanate followed by centrifugation over a cesium chloride cushion (21). Poly(A)-containing RNA was enriched by oligo(dT)-cellulose chromatography (22).

mRNA Translation *in Vitro*. Poly(A)$^+$ RNA was translated *in vitro* using a rabbit reticulocyte lysate system (23). Mac-1 from products translated *in vitro* (24) was immunoprecipitated with a rabbit polyclonal serum raised against homogeneous preparations of mouse Mac-1 (12, 14).

RNA Hybrid-Selection. Plasmid DNAs (20 μg) were linearized by digestion with *Eco*RI (pMM1.8, pUC8) or *Xho* I (human actin plasmid, L. Geddes, Stanford Univ.) restriction enzymes, denatured with NaOH, neutralized, and coupled to 0.5 × 0.5 cm nitrocellulose filters (25). P388D1 poly(A)$^+$ RNA (20 μg) was hybridized to each filter in 65% (vol/vol) formamide/10 mM Pipes, pH 6.4/0.4 M NaCl/0.2% $NaDodSO_4$ (100 μl) at 50°C for 3 hr. The filters were washed 10 times in 150 mM NaCl/15 mM sodium citrate/0.5% $NaDodSO_4$ at 60°C and 3 more times in 10 mM Tris·HCl, pH 7.9/2 mM EDTA at the same temperature (26). The RNA was eluted from the filters by boiling in H_2O for 60 sec, extracted with phenol/chloroform, and precipitated with ethanol.

Sequencing. Sequence analysis of the DNA was accomplished using the dideoxynucleotide method (27) after subcloning of different restriction fragments in M13mp18 (28) or by the partial chemical degradation method (29).

RNA Gel Blots. Poly(A)$^+$ RNA (10 μg per sample) was electrophoresed on 1% agarose gels containing 1 M formaldehyde (30) and transferred to nylon membranes (Zeta-Probe, Bio-Rad) (31). Blots were prehybridized in 50% (vol/vol) formamide/0.9 M NaCl/20 mM NaH_2PO_4, pH 7.4/4 mM EDTA/1× Denhardt's solution/salmon sperm DNA at 100 μg/ml/0.5% $NaDodSO_4$ for 4 hr at 42°C and hybridized with ^{32}P-labeled probes in the same solution with 10% (wt/vol) dextran sulfate for 20 hr at 42°C. ^{32}P-labeled probes were generated by nick-translation (32) of the appropriate DNA fragments to a specific activity of 10^8–10^9 cpm/μg. The blots were washed twice in 2× SSC/0.5% $NaDodSO_4$ at room temperature for 15 min and four times with 0.1× SSC/0.5% $NaDodSO_4$ at 65°C for 30 min. They were exposed to Kodak XAR film at −70°C with intensifying screens.

Southern Blots. DNA was extracted from cells by treatment with proteinase K and $NaDodSO_4$ (33). DNA (10 μg) was digested with the indicated restriction enzyme, fractionated on a 0.5% agarose gel, and transferred to nylon membranes (2). The blots were prehybridized in 6× SSC/5× Denhardt's/0.5% $NaDodSO_4$ salmon sperm DNA at 100 μg/ml for 2 hr at 65°C and then hybridized in the same buffer containing 10 mM EDTA with 10^6 cpm/ml of nick-translated probes. Filters were washed and autoradiographed as described for RNA gel blots.

RESULTS

Isolation of a Mac-1 α-Subunit Genomic Clone. Oligonucleotide probes were synthesized corresponding to the Mac-1 α-subunit amino-terminal amino acid residues 4–10 (probes I and II) and residues 9–15 (probes III and IV) (15). The probes were eicosamers that were 32- or 64-fold redundant, respectively. Probe I was 5′ GC$\frac{A}{G}$TT$\frac{C}{T}$TC$\frac{C}{T}$TG$\frac{A}{G}$AA$\frac{A}{G}$GTCAT 3′; probe II only differed at position 15 $\left[\frac{C}{T}\right]$. Probe III was 5′ GTCATXGG$\frac{A}{G}$TG$\frac{T}{C}$TC$\frac{A}{G}$GT$\frac{G}{A}$TC 3′; probe IV only differed from probe III at position 15 $\left[\frac{C}{T}\right]$. Using hybridization conditions selected to optimize specific hybridization of an eicosamer (20), a library of BALB/c spleen DNA, constructed in the λ vector Charon 4A, was screened. Approximately 1.3×10^6 clones were screened with probe I or probe II, of which 60 clones were selected that hybridized with one or both of the probes. These clones were purified and then independently hybridized with probes III and IV. Two of the 60 purified clones hybridized with both probes III and IV under the conditions of stringency used. These two clones were identical by restriction digest analysis, and one clone designated λMM-1 was selected for further study.

As shown in Fig. 1, this clone contains an 11.9-kilobase (kb) insert with two internal *Eco*RI sites that delineate three fragments of 6, 4.7, and 1.2 kb (A, B, and C, respectively). A *Bam*HI–*Eco*RI fragment containing the sequence hybridizing to the Mac-1 oligonucleotide probe was subcloned into pUC8 (plasmid pMM1.8). The *Eco*RI fragments, designated A, B, and C, were also subcloned into pUC8. These clones were subjected to restriction mapping (Fig. 1). Southern blots of restriction fragments from the λMM-1 and pMM1.8 genomic clones localize the sequences hybridizing to the oligonucleotide probe on a 260-base-pair (bp) *Dde* I fragment, a 550-bp *Hin*fI fragment, and their common 155-bp *Hin*fI–*Dde* I fragment (asterisk, Fig. 1). The *Dde* I fragment that hybridizes to the probe was subjected to Maxam–Gilbert and Sanger nucleic acid sequencing. It contains an open-reading-frame sequence corresponding to the Mac-1 α-subunit amino-terminal amino acid sequence (Fig. 2). The internal *Hin*fI site (marked with a vertical arrow in Figs. 2 and 3) is on the 5′ side of the amino-terminal sequence. Thus the 5′ to 3′ orientation of the gene and the direction of transcription in relation to the restriction map in Fig. 1 is left to right.

The nucleic acid sequence is in perfect agreement with the previously published 18 amino-terminal amino acids of the Mac-1 α subunit, and also confirms 4 additional amino acid determinations at positions 19 and 21–23 (Fig. 2). On the 5′ side of the amino-terminal amino acid of the Mac-1 α subunit, the open reading frame extends for 24 amino acids before reaching a stop codon at position −72 to −70, suggesting that an intron-exon boundary is present. In agreement with this hypothesis, a sequence with homology to consensus splicing

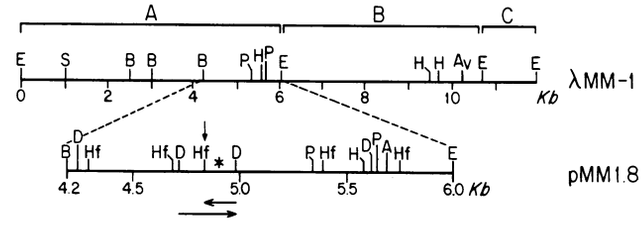

FIG. 1. Restriction map of Mac-1 α-subunit genomic clone. The restriction map of the λMM-1.8 genomic clone was determined by double digestion of λMM-1 and of *Eco*RI fragments A, B, and C inserted into pUC8 (3). The 1.8-kb *Bam*HI–*Eco*RI fragment was inserted into pUC8 (pMM1.8) and a more detailed restriction map obtained by the end-labeling/partial digest procedure (7). The *Hin*fI–*Dde* I fragment hybridizing to the Mac-1 oligonucleotide probe is indicated by an asterisk. The horizontal arrows show the regions and orientation of DNA sequencing. The vertical arrow indicates the internal *Hin*fI site in the sequenced *Dde* I fragment. The 5′ to 3′ orientation of the gene and the direction of transcription in relation to the restriction map is left to right. (A, *Acc* I; Av, *Ava* I; B, *Bam*HI; D, *Dde* I; E, *Eco*RI; H, *Hin*dIII; Hf, *Hin*fI; P, *Pst* I; S, *Sol* I).

```
   -140       -130       -120       -110       -100
5'-GCCCAGGGCCATCAGAGCTTCAATGAATGTGGACAGGAATGGTCA

       -90        -80        -70        -60
CGGGTGGAAATGTGTGTTGTTCTCTAA*CCATATTGGTTCCTTTGC

-50  ↓  -40        -30        -20        -10
TTTGACTCTTACTTCTCCGTTATCCTTCCAG|CC CTG GCC TTG TGT
                                  Leu Ala Leu Cys

          1          10         20         30
CAT GGC TTC AAT CTG GAC ACT GAA CAT CCC ATG ACC
His Gly Phe Asn Leu Asp Thr Glu His Pro Met Thr
      (Phe)Asn Leu Asp Thr Glu His(Pro)Met Thr

             40         50         60
TTC CAG GAG AAT GCA AAA GGC TTT GGA CAG AAT GTG
Phe Gln Glu Asn Ala Lys Gly Phe Gly Gln Asn Val
Phe Glx Glu Asn Ala Lys(Gly)Phe(Gly) X (Asn)Val

   70         80         90
GTC CAG CTT GGC GGA ACC AG|GTAAAGTACCTCA-3'
Val Gln Leu Gly Gly Thr
(Val)
```

FIG. 2. Sequence of the *Dde* I fragment hybridizing to Mac-1 α-amino-terminal oligonucleotide probes. The nucleotide sequence and below it the predicted amino acid sequence and the amino acid sequence determined by protein sequencing are shown. Predicted RNA splice sites are indicated by underlining and a bar. An in-frame termination codon, suggesting that the first splice site is used, is indicated by an asterisk. The internal *Hin*fI site is shown by an arrow. The four most 3' nucleotides are part of the 3' *Dde* I site.

acceptor sequences (16) has been found between nucleotides -26 and -20. The amino acid sequence between the putative splicing site and the amino-terminus contains hydrophobic amino acids, as expected for the carboxyl-terminal portion of a leader peptide. The predicted amino acid sequence extends 28 amino acids from the amino terminus of the protein to a nucleotide sequence that has the potential to be a splice site donor (16) (Fig. 2).

Mac-1 α-Subunit mRNA Hybrid-Selection and *in Vitro* Translation. As a second independent method to authenticate the Mac-1 α-subunit genomic clone, we tested its ability to hybrid-select Mac-1 α-subunit mRNA (25). mRNA from the murine macrophage-like cell line P388D1, which expresses Mac-1, was hybridized to the Mac-1 pMM1.8 DNA or to control plasmid DNA bound to nitrocellulose. The mRNA was then eluted and translated *in vitro*. [^{35}S]Methionine-labeled translation products were immunoprecipitated with rabbit anti-Mac-1 serum and subjected to NaDodSO$_4$/PAGE (Fig. 3). The Mac-1 α-subunit precursor of M_r 130,000 was specifically immunoprecipitated from the translation products of total P388D1 poly(A)$^+$ RNA (Fig. 3, lane A) or poly(A)$^+$ RNA selected by the Mac-1 pMM1.8 DNA (Fig. 3, lane B) but not by poly(A)$^+$ RNA selected by control plasmids containing no insert (Fig. 3, lane C) or a human actin cDNA (Fig. 3, lane D). The same total incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable products was obtained using mRNA selected by the control DNA as by the Mac-1 pMM1.8 DNA; thus the positive selection seen here is not a trivial result of greater protein synthesis.

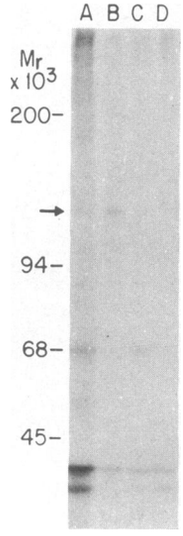

FIG. 3. *In vitro* translation of hybrid-selected Mac-1 α-subunit mRNA. P388D1 mRNA (20 μg) was hybridized to nitrocellulose filters containing 20 μg of linearized plasmid DNA. The hybridized mRNA was eluted and translated *in vitro*. Products were immunoprecipitated with anti-Mac-1 rabbit serum and subjected to 7% NaDodSO$_4$/PAGE and fluorography. Mac-1 translated products from total P388D1 mRNA (lane A) or mRNA selected by the Mac-1 plasmid pMM1.8 (lane B), pUC8 (lane C), or a human actin cDNA clone (lane D) are shown.

Tissue-Specific Expresson of Mac-1 α-Subunit mRNA. Poly(A)$^+$ RNA was prepared from cell lines that are positive (P388D1) or negative (EL-4 and L cells) for Mac-1 α-subunit expression. The mRNA was subjected to formaldehyde/agarose gel electrophoresis. Ethidium bromide staining showed equivalent amounts of mRNA and residual intact 28S and 18S rRNA in all three poly(A)$^+$ RNA preparations. After transfer onto nylon membranes, RNA was hybridized to the ^{32}P-labeled Mac-1 pMM1.8 DNA insert. A 6-kb mRNA from the macrophage-like p388D1 cells hybridized to the Mac-1 DNA (Fig. 4, lane 2), whereas no hybridization was detected with mRNA obtained from the T-cell lymphoma EL-4 or L-cell fibroblasts (Fig. 4, lanes 1 and 3). The lack of hybridization of Mac-1 α-subunit DNA with EL-4 T lymphoma mRNA is of interest because EL-4 cells express LFA-1, a cell surface molecule with an α-subunit amino terminus that is 33% homologous to the α-subunit amino terminus of Mac-1 (as well as a β subunit identical to the β subunit of Mac-1). We verified the expression of LFA-1 α-subunit mRNA in the same EL-4 poly(A)$^+$ RNA preparation by *in vitro* translation and immunoprecipitation (12). Thus, under the conditions of stringency employed, the Mac-1 α-subunit DNA probe does not cross-hybridize with LFA-1 α-subunit mRNA, despite the 33% homology of the amino-terminal amino acids of the Mac-1 and LFA-1 antigens.

Mac-1 Receptor Expression is Transcriptionally Regulated During *in Vitro* Differentiation of the Mouse Myelomonocytic Cell Line M1. The mouse cell line M1 can be induced by IFN-γ to differentiate *in vitro* through the monocytic pathway. Immunofluorescence cytometry showed that uninduced M1 cells expressed little or no Mac-1; while 6 days after IFN-γ induction, they expressed about 50% as much Mac-1 as the mature macrophage-like line P388D1. To test the hypothesis that Mac-1 expression in differentiating M1 cells is regulated at the level of the mRNA, the presence of Mac-1 α-subunit mRNA in M1 cells before differentiation and after 6 days of induction with IFN-γ was tested both by mRNA translation *in vitro* and by RNA gel blot analysis. Translation *in vitro* showed that no M_r 130,000 Mac-1 α-subunit precursor can be synthesized from noninduced M1 mRNA (Fig. 5*A*, lane 2) but that a polypeptide is synthesized

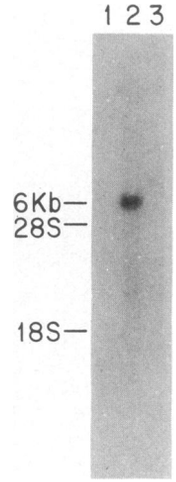

FIG. 4. Tissue-specific expression of Mac-1 α-subunit mRNA. Ten micrograms of poly(A)$^+$ mRNA, isolated from the mouse T-cell lymphoma EL-4 (lane 1), the mouse macrophage-like cell line P388D1 (lane 2), or the mouse L fibroblast cell line (lane 3), was subjected to 1% agarose/formaldehyde gel electrophoresis, blotted to nylon membranes, and hybridized to a ^{32}P-labeled Mac-1 pMM1.8 DNA insert. The relative mobility of the 28S and 18S rRNA and the estimated size of the Mac-1 α mRNA are indicated.

from IFN-γ-induced M1 mRNA (Fig. 5*A*, lane 3) with the same apparent molecular weight as the Mac-1 α-subunit precursor from P388D1 mRNA (Fig. 5*A*, lane 1). The same result was obtained by RNA gel blotting with ^{32}P-labeled pMM1.8 Mac-1 DNA. No Mac-1 α-subunit mRNA could be detected before induction (Fig. 5*B*, lane 1), while a 6-kb mRNA was detectable after induction (Fig. 5*B*, lane 2). A 10-kb mRNA from differentiated M1 cells also hybridized with Mac-1 α-subunit DNA in these experiments. This species may represent a nuclear precursor of Mac-1 mRNA.

Southern Blot Analysis of Mouse Genomic DNA with Mac-1 α-Subunit DNA. The number of different genes hybridizing to the Mac-1 α-subunit genomic clone was analyzed by Southern blotting at high stringency (0.1× SSC at 68° for 2 hr). The three different *Eco*RI fragments of the λ genomic clone of 6, 4.7, and 1.2 kb (fragments A, B, and C, respectively, in Fig. 1) were subcloned into pUC8, and their DNA inserts were ^{32}P-labeled by nick-translation and used as probes on Southern blots of murine P388D1 DNA digested with *Eco*RI or *Hin*dIII. Identical results were obtained with EL-4 and L-cell DNA. Each of the three *Eco*RI fragments of the Mac-1 clone hybridizes to a single band of the corresponding length in total *Eco*RI-digested mouse DNA as expected (Fig. 6, lanes AE, BE, and CE). In addition, the 6- and 4.7-kb *Eco*RI probes hybridize to a common *Hin*dIII fragment of 4 kb (Fig. 6, lanes AH and BH) as predicted by the restriction map (Fig. 1). The 6-kb probe additionally hybridizes to a 10-kb *Hin*dIII fragment resulting from cleavage at position 5.4 kb in Fig. 1 and a flanking site on the 5′ side of the clone. The 4.7- and 1.2-kb probes hybridize to a common 17-kb *Hin*dIII fragment (Fig. 6, lanes BH and CH) corresponding to cleavage at 9.7 kb in the genomic clone (Fig. 1) and a flanking 3′ site. At lower stringency (2× SSC) both the 6- and the 4.7-kb probes (but not the 1.2-kb probe) gave strong background hybridization, suggesting the presence of repetitive sequences in the 4.7- and 6-kb fragments.

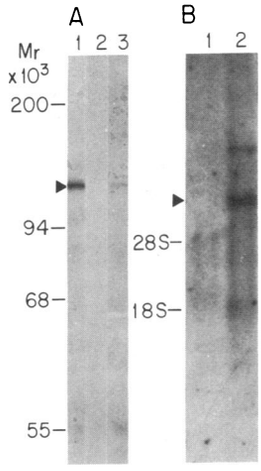

FIG. 5. IFN-γ induction of Mac-1 mRNA as shown by (*A*) *in vitro* translation and (*B*) RNA gel blotting. M1 cells were induced to differentiate *in vitro* by incubation with IFN-γ at 10 units/ml for 6 days. Poly(A)$^+$ RNA was prepared from P388D1, M1 cells, or IFN-γ-induced M1 cells and tested for the presence of Mac-1-specific mRNA. (*A*) [^{35}S]Methionine-labeled protein products translated *in vitro* from mRNA from the macrophage-like cell line P388D1 (lane 1), M1 (lane 2), or induced M1 cells (lane 3) were immunoprecipitated and subjected to 7% NaDodSO$_4$/PAGE and fluorography. (*B*) RNA gel blot of M1 (lane 1) and IFN-γ-induced M1 (lane 2) poly(A)$^+$ RNA using a ^{32}P-labeled Mac-1 pMM1.8 DNA insert. The positions of the M_r 130,000 Mac-1 α-subunit polypeptide and the Mac-1 6-kb mRNA are indicated by arrow heads.

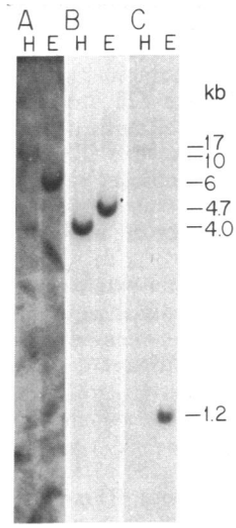

FIG. 6. Southern blot analysis of mouse genomic DNA with Mac-1 α-subunit DNA. P388D1 mouse DNA (10 μg per lane) was digested with either *Eco*RI (E) or *Hin*dIII (H) restriction enzymes, electrophoresed on a 0.5% agarose gel, and transferred to nylon membranes. Triplicate filters were hybridized to ^{32}P-labeled DNA from the three λMM-1 *Eco*RI fragments of 6 (*A*), 4.7 (*B*), or 1.2 kb (*C*), which had been inserted into pUC8. The position and size of the hybridizing bands are indicated.

DISCUSSION

We have isolated, directly from a genomic library, a clone coding for the α subunit of the mouse Mac-1 cell surface receptor using synthetic oligonucleotide probes specified by the amino-terminal protein sequence. The isolated λ clone has an 11.9-kb insert of mouse genomic DNA with three *Eco*RI fragments. Three different lines of evidence demonstrate the authenticity of the clone. (*i*) When segments of this clone were sequenced, we found a sequence that agrees perfectly with the 22 amino-terminal amino acids of the Mac-1 α subunit. (*ii*) The genomic clone specifically hybrid-selects the Mac-1 α-subunit mRNA as demonstrated by *in vitro* translation experiments. (*iii*) The mRNA that hybridizes to the genomic clone is present in Mac-1$^+$ macrophage-like cell lines such as P388D1 or IFN-γ-induced M1 cells but not in Mac-1$^-$ T cells, fibroblasts, or uninduced M1 cells.

The genomic DNA sequence contains intron sequences preceding the amino terminus-coding exon and may also contain intron sequences following it. An intron-exon boundary 20 nucleotides before the amino-terminal phenylalanine codon is predicted by a consensus splicing-acceptor sequence preceded by an in-frame stop codon. At minimum, the exon encodes the last 6 amino acids from a leader peptide and 29 amino acids from the amino terminus of the protein. Nucleotide sequencing and restriction mapping of fragments hybridizing to the oligonucleotide probe showed that the Mac-1 gene is oriented with the 6-, 4.7-, and 1.2-kb *Eco*RI fragments in the 5′ to 3′ direction. The amino terminus is transcribed from the 3′ portion of the 6-kb fragment. The orientation of the gene and size of the mRNA predict that transcription would continue through and beyond the 4.7- and 1.2-kb fragments. Both the 6- and 4.7-kb *Eco*RI fragments hybridize to Mac-1 mRNA. No hybridization was detected with the 1.2-kb fragment (data not shown). Southern blot analysis of total mouse DNA using all three *Eco*RI fragments as probes has shown that the neighboring *Eco*RI fragments hybridize to common *Hin*dIII fragments, proving that they are contiguous in the genome. The most likely explanation for the lack of hybridization of the 1.2-kb fragment to Mac-1 mRNA would be that the 1.2-kb *Eco*RI fragment contains only intron sequences.

The Mac-1 genomic clone specifically hybrid-selects the Mac-1 α-subunit mRNA as shown by translation *in vitro* of a M_r 130,000 polypeptide that was precipitated by Mac-1 antiserum. Mac-1 α chain translated *in vitro* in the presence of dog pancreatic microsomes and the *in vivo* precursor are both M_r 164,000, and the deglycosylated *in vivo* precursor is

M_r 130,000, showing that the M_r 130,000 polypeptide is the *bona fide in vitro* Mac-1 α-chain translation product (12).

RNA gel blots showed the Mac-1 α-subunit messenger is specifically expressed in macrophage-like cell lines. This corresponds with previous studies on the cell surface expression, biosynthesis (14), and translation *in vitro* (12) of the Mac-1 α chain, and shows that tissue-specific expression is regulated at the mRNA level. The Mac-1 α-subunit mRNA is 6 kb. The coding region is about 3 kb, since the unglycosylated polypeptide chain translated *in vitro* is M_r 130,000, corresponding to ≈1000 amino acids. Primer extension with oligonucleotide probes from the amino-terminal sequence of the protein has shown that the distance between this region and the 5′ end of the mRNA is ≈110 bp (data not shown), suggesting the existence of an ≈3 kb 3′-untranslated region.

At the amino terminus the murine Mac-1 α subunit shows 33% amino acid homology with the murine LFA-1 α subunit (15) and 50% homology with the human p150,95 α subunit (L. J. Miller and T.A.S., unpublished data). This level of homology is not necessarily detectable at the level of nucleic acid cross-hybridization. The pMM1.8 Mac-1 insert and the three *Eco*RI fragments from the Mac-1 genomic clone did not hybridize, even under low stringency conditions (Fig. 4 and data not shown), with mRNA preparations from EL-4 T-lymphoma cells, which had been verified by translation *in vitro* to contain LFA-1 mRNA (12). Since the Mac-1 clone isolated here may represent only a portion of the gene, it remains possible that other segments would be more highly conserved and cross-hybridize with LFA-1 mRNA. These findings do, however, rule out the possibility that the 11.9-kb Mac-1 α-genomic clone isolated here contains mRNA-encoding segments of the LFA-1 α gene. Cosgrove *et al.* (34) reported immunological evidence that a single genomic clone in λ could transfect the α chains of the human Mac-1, LFA-1, and platelet glycoprotein IIb-IIIa molecules. Cosgrove *et al.* (34) did not characterize their insert DNA or test for hybridization to message. However, considering the large size of the murine Mac-1 α-subunit mRNA reported here, the presence of intron sequences, and the likelihood that the other two proteins would have similarly large α-subunit genes, it seems unlikely that a single λ clone could transfect them unless their DNA sequences overlapped. Since we found no evidence for hybridization of any part of our 11.9-kb Mac-1 insert with LFA-1 α-chain mRNA, it seems difficult to reconcile our results with those of Cosgrove *et al.* (34) unless the gene organizations in mouse and human DNAs differ radically.

Southern analysis at high stringency showed only one copy of the Mac-1 α-subunit gene is present in the mouse genome. Low-stringency hybridization analysis showed either the presence of repetitive sequences or a large number of related genes, except for the 1.2-kb fragment, which did not hybridize with mRNA and thus would not be expected to be conserved in related genes.

The isolation of a Mac-1 α-subunit genomic clone and the characterization of Mac-1 α-subunit translation products allowed us to study the regulation of Mac-1 expression during differentiation *in vitro* of the mouse premyelocytic cell line M1. Both approaches have shown that the expression of Mac-1 α subunit is regulated by the level of mRNA, since no Mac-1 α-subunit mRNA can be detected by RNA gel blot analysis or translation *in vitro* in undifferentiated M1 cells. The presence of Mac-1 α-subunit mRNA after 6 days of IFN-γ-induced M1 differentiation can be clearly detected by both methods. Whether mRNA levels are controlled by the level of transcription or mRNA stability or both remains to be seen. In any case, the regulation of Mac-1 α-subunit mRNA expression in M1 cells constitutes a very interesting model system for gene expression studies on macrophage differentiation.

To the best of our knowledge, Mac-1 is one of the first macrophage surface molecules to have its gene cloned and characterized, and one of the first examples of cloning a gene directly from a vertebrate genomic library using synthetic oligonucleotide probes. The genomic Mac-1 α-subunit clone isolated here will facilitate a better understanding of the structure of the Mac-1 molecule, its functions as a complement receptor and a nonspecific adhesion molecule, and its relationship with the LFA-1 and p150,95 leukocyte adhesion molecules. This clone also provides a means to investigate the mechanisms of tissue-specific regulation of expression of the Mac-1 antigen and to identify other members of this functionally important family of cell surface proteins.

The authors thank Dr. S. Horvath for the synthesis of oligonucleotide probes, Fred King and Carolyn Anderson Gaines for expert technical assistance, and Janet Casaubon for excellent manuscript preparation. This work was supported by Grants AI20316, CA31799, and AI22304 from the National Institutes of Health.

1. Springer, T., Galfre, G., Secher, D. S. & Milstein, C. (1979) *Eur. J. Immunol.* **9**, 301–306.
2. Southern, E. (1975) *J. Mol. Biol.* **98**, 503–517.
3. Lawn, R. M., Fritsch, E. F., Parker, R. C., Blake, G. & Maniatis, T. (1978) *Cell* **15**, 1157–1174.
4. Sanchez-Madrid, F., Nagy, J., Robbins, E., Simon, P. & Springer, T. A. (1983) *J. Exp. Med.* **158**, 1785–1803.
5. Ralph, P., Ho, M. K., Litcofsky, P. B. & Springer, T. A. (1983) *J. Immunol.* **130**, 108–114.
6. Ralph, P., Harris, P., Punjabi, C. J., Welte, K., Litcofsky, P. B., Ho, M. K., Rubin, B., Moore, M. A. S. & Springer, T. A. (1983) *Blood* **62**, 1169–1175.
7. Smith, H. O. & Bernstiel, M. L. (1976) *Nucleic Acids Res.* **3**, 2387–2398.
8. Beller, D. I., Springer, T. A. & Schreiber, R. D. (1983) *J. Exp. Med.* **156**, 1000–1009.
9. Springer, T. A. & Anderson, D. C. (1986) *Biochemistry of Macrophages*, Ciba Symposium 118 (Pitman, London), pp. 102–126.
10. Anderson, D. C., Schmalstieg, F. C., Arnaout, M. A., Kohl, S., Tosi, M. F., Dana, N., Buffone, G. J., Hughes, B. J., Brinkley, B. R., Dickey, W. D., Abramson, J. S., Springer, T., Boxer, L. A., Hollers, J. M. & Smith, C. W. (1984) *J. Clin. Invest.* **74**, 536–551.
11. Kürzinger, K., Reynolds, T., Germain, R. N., Davignon, D., Maetz, E. & Springer, T. A. (1981) *J. Immunol.* **127**, 596–602.
12. Sastre, L., Kishimoto, T. K., Gee, C., Roberts, T. & Springer, T. A. (1986) *J. Immunol.*, in press.
13. Ho, M. K. & Springer, T. A. (1983) *J. Biol. Chem.* **258**, 2766–2769.
14. Kürzinger, K. & Springer, T. A. (1982) *J. Biol. Chem.* **257**, 12412–12418.
15. Springer, T. A., Teplow, D. B. & Dreyer, W. J. (1985) *Nature (London)* **314**, 540–542.
16. Mount, S. M. (1982) *Nucleic Acids Res.* **10**, 459–472.
17. Hunkapiller, M., Kent, S., Caruthers, M., Dreyer, W., Firca, J., Giffin, C., Horvath, S., Hunkapiller, T., Tempst, P. & Hood, L. (1984) *Nature (London)* **310**, 105–111.
18. Jay, E., Bambara, R., Raumanabhan, R. & Wu, R. (1974) *Nucleic Acids Res.* **1**, 331–353.
19. Benton, W. D. & Davis, R. W. (1977) *Science* **196**, 180–182.
20. Conner, B. J., Reyes, A. A., Morin, C., Itakura, K., Teplitz, R. L. & Wallace, R. B. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 278–282.
21. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18**, 5294–5299.
22. Aviv, H. & Leder, P. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 1408–1412.
23. Pelham, H. R. B. & Jackson, R. J. (1976) *Eur. J. Biochem.* **67**, 247–256.
24. Anderson, D. J. & Blobel, G. (1983) *Methods Enzymol.* **96**, 111–120.
25. Parnes, J. R., Velan, B., Felsenfeld, A., Ramanathan, L., Ferrini, U., Appella, E. & Seidman, J. G. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 2253–2257.
26. Ricciardi, R. P., Miller, J. S. & Roberts, B. E. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4927–4931.
27. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
28. Messing, J., Crea, R. & Seeburg, P. H. (1981) *Nucleic Acids Res.* **9**, 309–321.
29. Maxam, A. M. & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 560–564.
30. Rave, N., Crkvenjakov, R. & Boedtker, H. (1979) *Nucleic Acids Res.* **6**, 3559–3567.
31. Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 5201–5205.
32. Rigby, P. W. H., Dieckmann, M., Rhodes, C. & Berg, P. (1977) *J. Mol. Biol.* **113**, 237–251.
33. Blin, N. & Stafford, D. W. (1976) *Nucleic Acids Res.* **3**, 2303–2308.
34. Cosgrove, L. J., Sandrin, M. S., Rajasekariah, P. & McKenzie, I. F. C. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 752–756.