Journal of Cellular Biochemistry 48:190–202 (1992)

Characterization of the Human Myeloid Cell Nuclear Differentiation Antigen: Relationship to Interferon-Inducible Proteins

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Abstract The human myeloid cell nuclear differentiation antigen (MNDA) is expressed specifically in cells of the granulocyte/monocyte lineage. The MNDA has been isolated by using a monoclonal antibody affinity matrix and reversed-phase high performance liquid chromatography. Its NH_2-terminal sequence has been obtained, as well as additional sequence information derived from peptides produced by cyanogen bromide and SV_8 protease cleavages. Meaningful similarities were observed in extended regions between the MNDA and the reported β interferon-inducible proteins, 202 and 204, from Ehrlich ascites mouse tumor cells. An amphipathic, basic α-helical region, showing no similarity to the 202 and 204 proteins, exhibited close similarity to a region in the interferon response factor-2, a protein which binds the interferon stimulated response element. The relatively high number of S(T)PXX motifs present in the partial amino acid sequence of the MNDA, described herein, suggests that the MNDA binds DNA and is a transcription factor.

Key words: interferon, myeloid differentiation, immunoaffinity chromatography, reversed-phase HPLC, peptides, nuclear protein

Numerous human leukemic cell lines have been blocked at different stages along the myeloid lineage, providing models for investigating the mechanisms underlying cellular differentiation (Metcalf, 1990; Lubbert et al., 1991). Goldberger et al. (1984) reported the use of antisera against chromatin proteins of various myeloid cells as probes to investigate qualitative and quantitative changes in the composition of nuclear proteins isolated from leukemic cells exhibiting a range of differentiation phenotypes. In general, the results were consistent with the observation that cells along a differentiation pathway contain very few unique nuclear proteins (Rabilloud et al., 1991). Notably, a basic protein of M_r 55,000 (MNDA) was found only in the more differentiated myeloid leukemia cell lines and the normal end-effector cells, monocytes and granulocytes. The unique qualitative difference in the MNDA expression during blood cell differentiation was used to target this nuclear protein for further characterization (Goldberger et al., 1984, 1986).

Exposure of HL-60 promyelocytic leukemia cells to cisplatin resulted in cross-linking between MNDA and DNA, an association which was quantitatively modulated during vitamin D_3- and TPA-induced monocyte differentiation of HL-60 cells but not during retinoic acid-induced granulocyte differentiation of HL-60 cells (Gaczynski et al., 1990). As well, Duhl et al. (1989) implicated the MNDA in DNA binding during nuclear fractionation studies of HL-60 cells using endonuclease digestion and differential solubility in 0.35 M NaCl.

A series of rat monoclonal antibodies were produced against the MNDA allowing for its purification and further characterization (Hudson et al., 1988). The rat monoclonal antibody, 3C1, which is reactive with native MNDA, has been purified and coupled to agarose for use as an affinity matrix for the isolation of the MNDA (Hudson et al., 1988). Using the monoclonal antibody affinity matrix and additional purification by reversed-phase HPLC (Regnier, 1983), the MNDA has been purified to a homogeneous band on an SDS polyacrylamide gel as assessed

Received August 21, 1991; accepted September 23, 1991.

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by silver staining. Amino acid sequence was obtained from the NH_2-terminus and also through cleaving the MNDA chemically and enzymically with subsequent purification of the resultant peptides by reversed-phase HPLC. One-half of the MNDA sequence was determined which reveals extensive regions of sequence similarity to interferon-inducible genes as well as motifs which suggest a role in the regulation of gene transcription.

MATERIALS AND METHODS

Cell Culture

U937 human monoblastic leukemic cells and KG1a human myeloblastic cells were obtained from the American Type Culture Collection (Rockville, Md.) and were cultured in a humidified incubator at 37°C with 5% CO_2/95% air atmosphere. The cells were seeded at 1×10^5/ml in RPMI 1640 (Gibco, Gaithersburg, Md.) supplemented with 10% defined bovine calf serum (Hyclone Laboratories, Logan, UT) and antibiotics [penicillin G (50 units/ml) and streptomycin sulfate (50 units/ml)] and harvested at 1×10^6/ml.

Preparation of Nuclear Protein Extract

All procedures were performed at 4°C in the presence of 1 mM PMSF. U937 cells (6×10^9) were harvested by centrifugation at 700*g* for 10 min and washed in 600 ml of PBS, 3 mM $MgCl_2$ (pH 7.2). Cells were lysed (Hallick and Namba, 1974), and the nuclei were then sedimented through 2.2 M sucrose, 10 mM Tris-HCl, 5 mM $MgCl_2$ (pH 7.5) at 100,000*g* for 60 min. Nuclei were resuspended in 0.25 M sucrose, 10 mM Tris-HCl, 5 mM $MgCl_2$ (pH 7.5) (0.25 M STM) and dispersed by homogenization. The purified nuclei were quantitated by absorbance at 260 nm. After centrifugation at 1,000*g*, nuclei were stored at −70°C or further processed. Isolated nuclei (5.4×10^8) were suspended and homogenized in 20 ml of 0.5% NP-40, 0.25 M STM. After centrifugation at 1,000*g*, a nuclear fraction soluble in 5 ml of 0.35 M NaCl, 10 mM Tris-HCl, 1 mM EDTA (pH 7.5) was released by homogenization followed by incubation for 30 min. The insoluble nuclear material was then pelleted by centrifugation at 1,800*g* and the supernatant fraction was saved. The insoluble nuclear pellet was re-extracted and the supernatant fractions were combined. Remaining insoluble material was removed by pelleting at 100,000*g* prior to affinity purification of the 0.35 M NaCl-soluble MNDA.

SDS Polyacrylamide Gel Electrophoresis and Immunodetection

SDS polyacrylamide gel electrophoresis methods described by Laemmli (1970) and by Schagger and Von Jagow (1987) were utilized. In the Laemmli system, a 4% stacking gel overlayed resolving gels of varying polyacrylamide concentration and pH. In the Schagger and Von Jagow system, a 4% stacking gel, a 10% spacer gel, and a 16.5% resolving gel were employed with a Tricine-based buffer system. Separated proteins were visualized by silver stain according to Oakley et al. (1980) or transferred to nitrocellulose as described by Towbin et al. (1979) with some modifications (Glass et al., 1981). Antigens were visualized on the immunoblot using affinity-purified alkaline phosphatase-linked rabbit anti-rat antibodies (Sigma Co., St. Louis, MO) (Turner, 1986).

Monoclonal Antibody Affinity Chromatography

The rat monoclonal antibody to MNDA, designated 3C1, was isolated from the ascites fluid of nude mice and coupled to CDI-activated agarose (Pierce Co., Rockford, IL), as previously described (Hudson et al., 1988). The monoclonal antibody affinity matrix was added to 0.35 M NaCl-extracted nuclear proteins from U937 cells and allowed to incubate with continuous end-over-end mixing for 20 h at 4°C. Beads were washed sequentially with 0.35 M NaCl, 10 mM Tris-HCl, 1 mM EDTA (pH 7.5); 0.5% NP-40, 6.25 mM Tris-HCl (pH 6.8); and 6.25 mM Tris-HCl (pH 6.8). All washes were in volumes of solutions equal to the original extract volume and were performed twice. MNDA was released from the beads by a 10-min incubation with 10% isopropanol, 0.1% TFA, 12 mM HCl (pH 1.9) (AEB 1).

HPLC Procedures

HPLC grade acetonitrile and isopropanol (Baxter Healthcare Corp., Stone Mountain, GA) and sequence grade trifluoroacetic acid (TFA) (Sigma Co.) were used. All solutions were sparged with helium prior to separations. HPLC separations were performed on a Spectra Physics SP8100 solvent delivery system (San Jose, CA) coupled to a Spectra Physics SP4270 integrator. Spectrophotometric detection of protein peaks was accomplished on line using a UV Monitor D

(Milton Roy, Riviera Beach, FL) at a fixed wavelength of 214 nm.

The affinity-purified MNDA in AEB 1 was applied to a 4.6-mm × 50-mm HPLC C_4 reversed-phase column (J.T. Baker Co., Marietta, GA) equilibrated in 0.1% TFA (pH 2.1) at a flow rate of 0.5 ml/min. The column was developed initially with a linear gradient to 39% of 0.1% TFA, 100% acetonitrile over 19.5 min. MNDA was eluted by increasing the concentration of 0.1% TFA, 100% acetonitrile to 42% over 60 min.

CNBr-derived peptides in 15% isopropanol, 0.1% TFA (pH 2.2) were applied to a 2-mm × 15-cm HPLC C_{18} reversed-phase column (Phenomenex, Torrance, CA) equilibrated in 15% isopropanol, 0.1% TFA (pH 2.2) at a flow rate of 0.25 ml/min. The column was developed with a linear gradient to 100% of 0.1% TFA, 45% isopropanol/H_2O over 60 min.

SV_8-derived peptides were applied to the HPLC C_{18} column equilibrated in 0.1% TFA (pH 2.1) at a flow rate of 0.25 ml/min. After 10 min of isocratic elution, the column was developed with increasing percentages of 0.1% TFA, acetonitrile: 20% at 30 min, 40% at 90 min, and 100% at 150 min.

Aliquots of fractions were dried by vacuum centrifugation (Savant Instruments, Farmingdale, NY) and solubilized according to Laemmli (1970) or Feick and Shiozawa (1990). Using the protocol of Feick and Shiozawa (1990), dried pellets were solubilized with sequential additions of 0.6% SDS, 60 mM Tris-HCl (pH 8.0); 5% NaOH; 4% SDS, 10% glycerol, 0.125 M Tris-HCl (pH 6.8), 10% 2-mercaptoethanol; (4:1:5, v/v); and then heated for 30 min at 60°C.

Two-Dimensional Isoelectric Focusing (IEF) and Nonequilibrium pH Gradient Electrofocusing (NEPHGE)

Samples were solubilized according to Peters and Comings (1980) using 2% 3.5–10 ampholines (w/v) (Pharmacia, Piscataway, NJ). Isoelectric focusing was performed using a mini-2D gel apparatus (Bio-Rad Labs, Melville, NY) by the method of O'Farrell (1975). The same apparatus was used for nonequilibrium pH gradient electrofocusing (O'Farrell et al., 1977). The second dimension separation of the MNDA was in a 9% SDS polyacrylamide gel. The pH gradient was determined by using standard proteins of defined isoelectric points (Sigma Co.) separated by a 15% SDS polyacrylamide gel in the second dimension.

Chemical and Enzymic Cleavages

Purified MNDA was vacuum centrifuged to dryness, resuspended in distilled water, and redried. For cyanogen bromide cleavage, purified MNDA was solubilized in 70% formic acid (approximately 200 ng/ml) (Fisher, Springfield, N.J.) and cyanogen bromide (> 100-fold excess by weight, Sigma Co.) was added. After a 24-h incubation in the dark, the same amount of CNBr was re-added, and the incubation continued for 8 h. The cleavage products were diluted tenfold with distilled water, and vacuum centrifuged to dryness. This step was repeated. The resultant peptide mixture was solubilized in 15% isopropanol, 0.1% TFA (pH 2.2), incubated at 37°C for 30 min, and microcentrifuged for 2 min to remove insoluble material.

For SV_8 cleavage, purified MNDA was suspended in 2 M urea, 50 mM NH_4HCO_3 (pH 8.0) (approximately 50 ng/ml). SV_8 protease (Worthington Biochemical Corp., Freehold, NJ) from *Staphylococcus aureus* (Drapeau, 1976) was added at an enzyme-to-substrate ratio of 1:30 (w/w). After 9 h of incubation at 37°C, the peptide mixture was microcentrifuged for 2 min to remove the insoluble fraction.

Amino Acid Sequencing of NH_2-Terminus of MNDA and Peptides

Two protein sequencing facilities were utilized in this study. At Vanderbilt University in the Center in Molecular Toxicology (VUCMT), the samples were applied to polybrene-coated glass fiber discs [Applied Biosystems, Inc. (ABI), Foster City, CA] and sequenced on an ABI 470A Gas Phase Protein Sequencer coupled to a 120A PTH Analyzer (ABI). PTH amino acids were separated on a 2.1-mm × 220-mm C_{18} column (ABI). At the Core Facility for Protein Chemistry in the Department of Medicine/Division of Rheumatology at the University of Alabama (UAB), samples were applied and processed (Bhown and Bennet, 1985) on an enhanced Beckman 890M Protein Sequencer (Beckman Instruments, Inc., Palo Alto, CA). The resultant PTH-amino acids were separated in a Beckman System 334 gradient HPLC using an Altex ultrasphere C_{18} 5-mm (0.46-cm × 15-cm) reversed-phase column (Beckman Instruments, Inc.). Peaks were detected at 254 nm, collected by a HP 3396 Series II integrator (Hewlett-Packard Co., Avondale, PA) and routed to an 80386-based microcomputer. Data were analyzed and compiled

within the Chrom Perfect V 4.0 software system (Justice Innovations, Palo Alto, CA).

Amino Acid Sequence Similarity Searches

The Swiss Protein (University of Geneva, Switzerland) and PIR (Protein Identification Resource of National Biomedical Research Foundation) data banks were searched for sequence homologies to MNDA amino acid sequences using the Intelligenetics Suite (release 5.4) (Intelligenetics, Inc., Mountain View, CA) (Wilbur and Lipman, 1983). Secondary protein structures were evaluated in the Pep module in the Intelligenetics Suite using the Chou and Fasman algorithm (Chou and Fasman, 1974, 1978).

RESULTS

The purification of the MNDA was followed by immunoblot analysis of samples separated on SDS polyacrylamide gels, transferred electrophoretically to nitrocellulose, and probed with a rat monoclonal antibody against MNDA (Fig. 1). The immunoadsorption of the MNDA from a 0.35 M NaCl nuclear protein extract of U937 cells was nearly quantitative (compare lanes 2 and 3, Fig. 1). Antigen-antibody interaction was reversible in AEB 1 and yielded a preparation enriched for MNDA (lanes 4–6, Fig. 1). Elution of the MNDA from the monoclonal antibody affinity matrix was found to be nearly quantitative (compare lanes 6 and 8, Fig. 1). A control experiment in which the monoclonal antibody was omitted during the first incubation of a duplicate immunoblot revealed no contribution from the second antibody to the generation of immunoreactive bands (data not shown).

In order to eliminate possible contaminants which would interfere with NH_2-terminal sequencing and subsequent peptide generation, the affinity-purified MNDA was applied to a HPLC reversed-phase C_4 column and eluted with increasing composition of acetonitrile (Fig. 2, peak 1), yielding a preparation containing one homogeneous band as assessed by silver staining (side lane in Fig. 3B). This preparation is termed purified MNDA.

Control experiments were performed to determine the purity of the MNDA preparation. The monoclonal antibody affinity matrix incubated with 0.35 M NaCl alone and subsequently eluted with AEB 1 released no detectable proteinaceous material (data not shown). As assessed by silver staining SDS polyacrylamide gels, neither

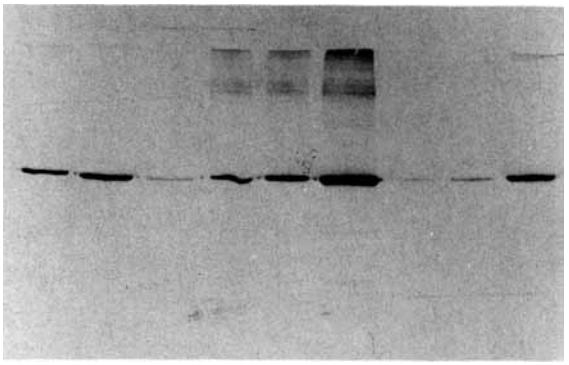

Fig. 1. Immunodetection of MNDA during purification. Protein samples taken during the isolation of MNDA were separated on 9% SDS polyacrylamide gels, transferred electrophoretically to nitrocellulose, and probed with rat monoclonal antibody to MNDA (12G11). **Lane 1,** 0.35 M NaCl nuclear protein extract from 1.35×10^5 U937 nuclei; **lane 2,** same material as in lane 1 from 2.7×10^5 U937 nuclei; **lane 3,** 0.35 M NaCl nuclear protein extract from 2.7×10^5 U937 nuclei showing the amount of the MNDA not immunoadsorbed during incubation with monoclonal antibody affinity matrix; **lane 4,** MNDA immunoadsorbed from 0.35 M NaCl nuclear protein extract from 2.7×10^5 U937 nuclei; **lane 5,** same material as in lane 4, obtained from 4.05×10^5 U937 nuclei; **lane 6,** same material as in lane 4, obtained from 5.4×10^5 U937 nuclei; **lane 7,** MNDA which was not released from the monoclonal antibody affinity matrix in AEB 1 when the 0.35 M NaCl nuclear protein extract from 2.7×10^5 U937 nuclei was used for the initial immunoadsorption; **lane 8,** same material as in lane 7, but twice the amount analyzed; **lane 9,** affinity-purified MNDA fractionated on C_4 reversed-phase column (termed purified MNDA). Amount obtained from 5.4×10^5 U937 nuclei.

the monoclonal antibody affinity matrix incubated with nuclear protein extracts from a human leukemia cell line not expressing MNDA (KG1a) nor a bovine serum albumin coupled matrix incubated with 0.35 M NaCl nuclear protein extract from U937 cells adsorbed proteinaceous material (data not shown), thus demonstrating the specificity of the monoclonal antibody affinity matrix.

Analyses were performed also to control for the possibility that another M_r 55,000 protein was adsorbed with the MNDA out of the U937 nuclear protein extract and co-isolated with MNDA during C_4 chromatography. Purified MNDA and a mixture of proteins of defined isoelectric points were separated by two-dimensional nonequilibrium pH gradient electrofocusing (2D-NEPHGE) and two-dimensional isoelectric focusing (2D-IEF). The separations of proteins of defined isoelectric points by 2D-NEPHGE (Fig. 3A) and 2D-IEF (Fig. 4A) estab-

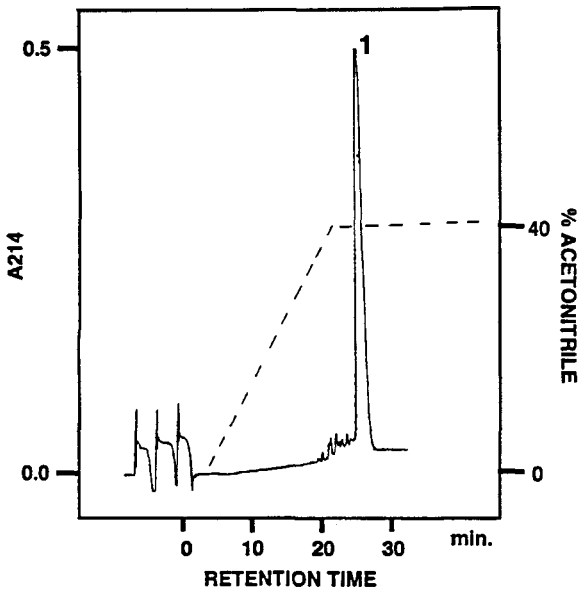

Fig. 2. Chromatograph of the separation of affinity-purified MNDA on a HPLC C_4 reversed-phase column. MNDA in AEB 1 was applied to a reversed-phase C_4 column equilibrated in 0.1% TFA (pH 2.1) at 0.5 ml/min. The column was developed with a complex linear gradient into 0.1% TFA, acetonitrile, as shown by the dashed line. MNDA was released at 26 min (peak 1).

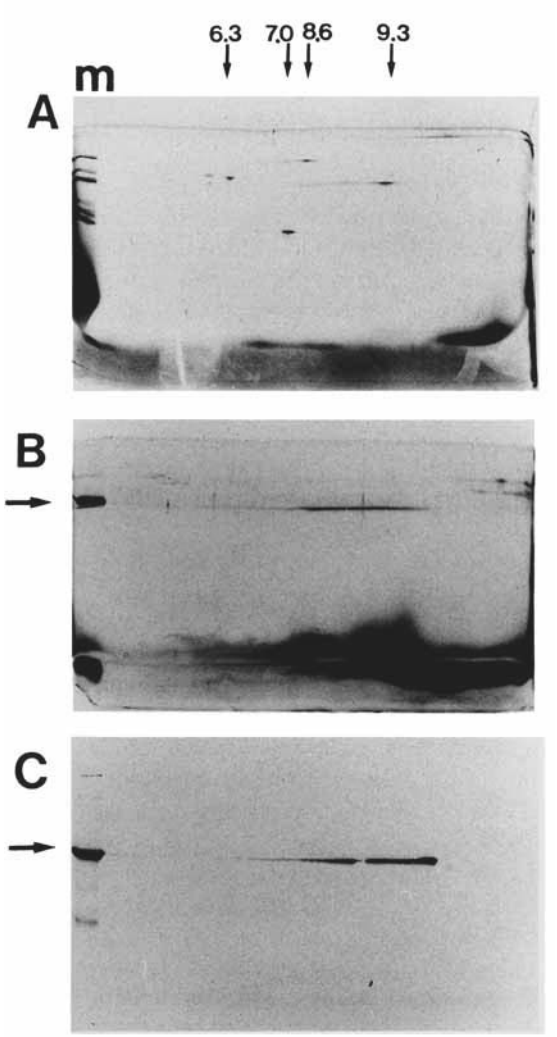

Fig. 3. Two-dimensional nonequilibrium pH gradient electrofocusing of purified MNDA. Protein markers of defined isoelectric point (Sigma Co.) or purified MNDA were separated in 4% polyacrylamide gels under nonequilibrium conditions and then separated electrophoretically in a second dimension. **A:** Protein markers separated in the second dimension on a 15% SDS polyacrylamide gel according to Laemmli. **Lane m,** markers applied to a second-dimension side lane. Arrows on top of figure denote isoelectric points of separable markers. Gel was stained with silver. **B:** Purified MNDA (material isolated from 2.7×10^6 U937 nuclei) separated in second dimension on a 9% SDS polyacrylamide gel. Arrow denotes equal amount of purified MNDA separated only in the second dimension in a side lane. Gel was stained with silver. **C:** Duplicate gel as in B transferred electrophoretically to nitrocellulose and stained with 12G11 rat monoclonal antibody. (Break in immunostaining of smear in Panel C is due to bubble artifact during electrophoretic transfer.)

lished a pH separation range from pH 9.3–4.6. Purified MNDA separated by 2D-NEPHGE (Fig. 3B) revealed only one silver stained streak at M_r 55,000. This streak was confirmed to be the MNDA by electrophoretically transferring a duplicate gel as in Figure 3B to nitrocellulose and probing with a rat monoclonal antibody to MNDA (Fig. 3C). No silver stained proteins were detected at M_r 55,000 in the 2D-IEF separation (Fig. 4B) where proteins of neutral and acidic pIs would be resolved. Thus, the purified MNDA is free of obvious protein contaminants. Three similarly prepared samples of purified MNDA were submitted for NH_2-terminal sequence analysis, all of which gave the same NH_2-terminal sequence. The longest sequence obtained is shown in Table I.

Purified MNDA was cleaved with CNBr, and the resultant peptides were separated on a HPLC reversed-phase C_{18} column using an isopropanol gradient (Fig. 5). Aliquots from fractions of the separation in Figure 5 were analyzed on a 16.5% SDS polyacrylamide gel with a 10% spacer gel according to Schagger and Von Jagow (Fig. 6). Four fractions were submitted for automated Edman degradation sequencing (Table II). CNBr peptides (fractions 5 and 10, Fig. 6) were separated electrophoretically and transferred to nitrocellulose and shown to be immunologically reactive with three different Mabs to MNDA (Fig. 7).

Purified MNDA was cleaved with SV_8 protease and resultant peptides were separated by

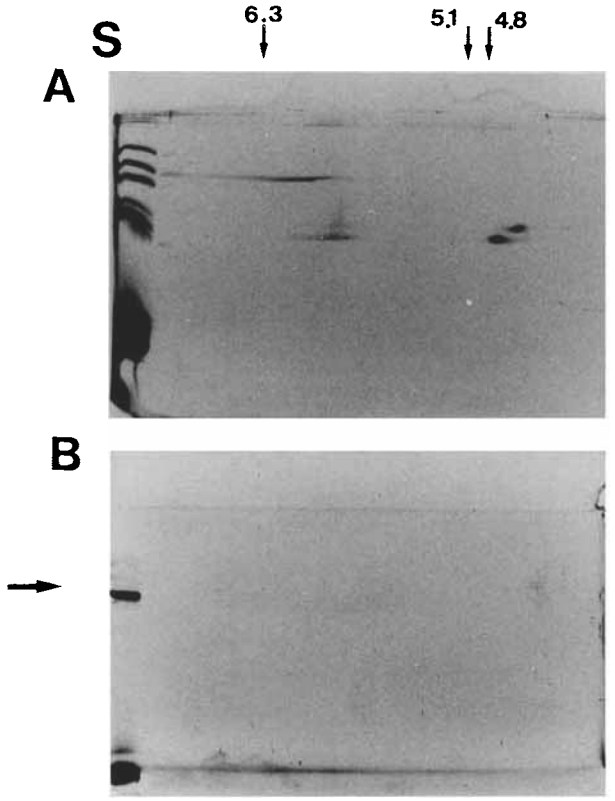

Fig. 4. Two-dimensional isoelectric focusing of purified MNDA. Protein markers of defined isoelectric points or purified MNDA were separated in 4% polyacrylamide isoelectric focusing gels and then separated electrophoretically in a second dimension. **A:** Protein markers separated in second dimension on a 15% SDS polyacrylamide gel. **Lane S,** markers applied to a second dimension side lane. Arrows on top of gel denote isoelectric points of separable markers. Gel was stained with silver. **B:** Purified MNDA (material derived from 2.7×10^6 nuclei) separated in second dimension on a 9% SDS polyacrylamide gel. Arrow denotes equal amount of purified MNDA applied to a second dimension side lane. Gel was stained with silver.

HPLC (Fig. 8). Aliquots of fractions from the separation were analyzed electrophoretically on a 20% SDS polyacrylamide gel according to Laemmli (data not shown). Selected fractions were submitted for automated Edman degradation sequencing (Table III). Both fractions 2 and 4 were found to contain two peptides (designated 2A, 2B, 4A, 4B).

Alignment of all peptide sequences (Tables I–III) revealed overlapping sequences of 70 amino acids (Fig. 9A) and 41 amino acids (Fig. 9B). The overlapping sequences in Figure 9A indicated that the SV_8 protease digestion did not cleave MNDA to completion. As well, an anomalous cleavage between two alanine residues occurred to produce the SV_8-derived peptide from fraction 4B.

TABLE I. Automated Edman Degradation of Purified MNDA*

Cycle no.	Amino acid	PTH (pmol)
1	V	233
2	N	190
3	E	168
4	Y	203
5	K	203
6	K	246
7	I	141
8	L	172
9	L	202
10	L	203
11	K	188
12	G	134
13	F	143
14	E	122
15	L	134
16	M	122
17	D	96
18	D	108
19	Y	96
20	H	48
21	F	99
22	T	44
23	S	20
24	I	55
25	K	100
26	S	16
27	L	54
28	L	63
29	A	60
30	Y	36
31	D	39
32	L	51
33	G	34
34	L	50
35	T	20
36	T	20
37	K	60
38	M	19
39	Q	35

*Purified MNDA was vacuum centrifuged to a small volume (100 μl). HPLC grade acetonitrile was re-added to 40% (v/v). Three samples were submitted to the VUCMT protein sequencing core lab. All three samples (sequences of 16, 25, and 39 aa) were confirmatory for the same N-terminal sequence. The sample giving the longest read is shown with recoveries per cycle (pmol).

Data bank searches showed similarities between three regions of the MNDA and the β interferon-inducible proteins, 202 and 204, from Ehrlich ascites mouse tumor cells. The NH_2-terminal regions of MNDA (39 aa) and 204 are identical in 51% of the sequence (Fig. 10A). The

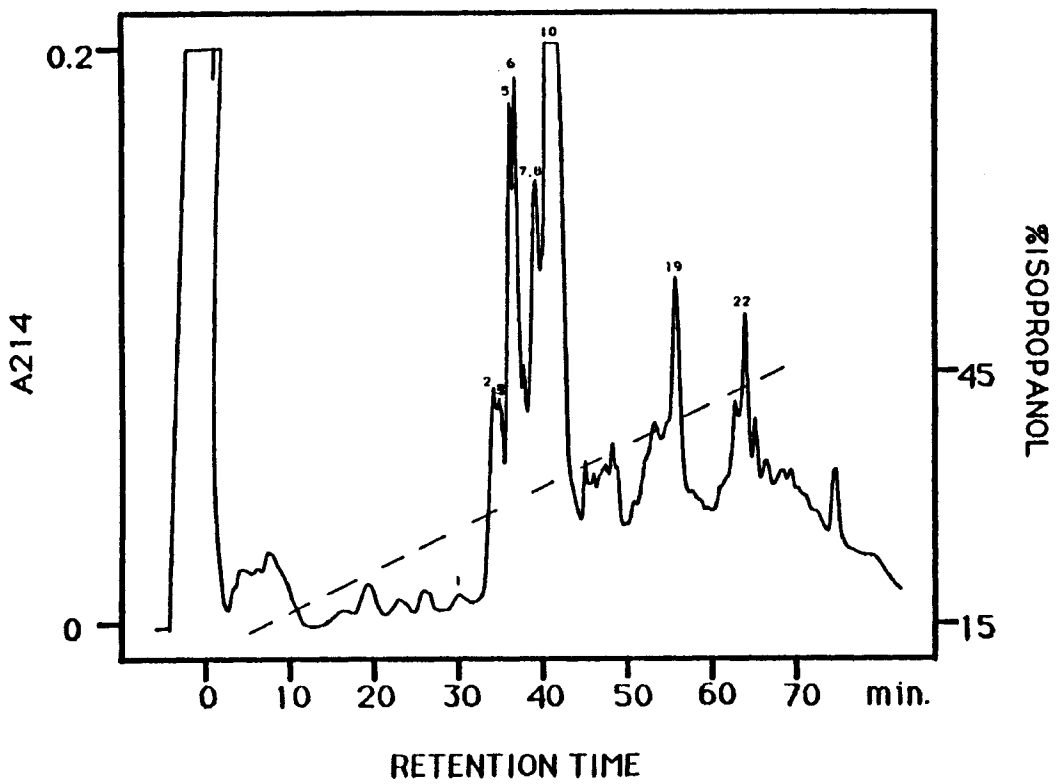

Fig. 5. Chromatograph of HPLC separation of CNBr-derived peptide fragments. The CNBr digest of purified MNDA was applied to a HPLC reversed-phase C_{18} column equilibrated in 15% isopropanol, 0.1% TFA (pH 2.2) at a flow rate of 0.25 ml/min. The column was developed with a linear gradient into 45% isopropanol, 0.1% TFA (pH 2.4) over 60 min, as shown by the dashed line. Peptides were detected by absorbance at 214 nm.

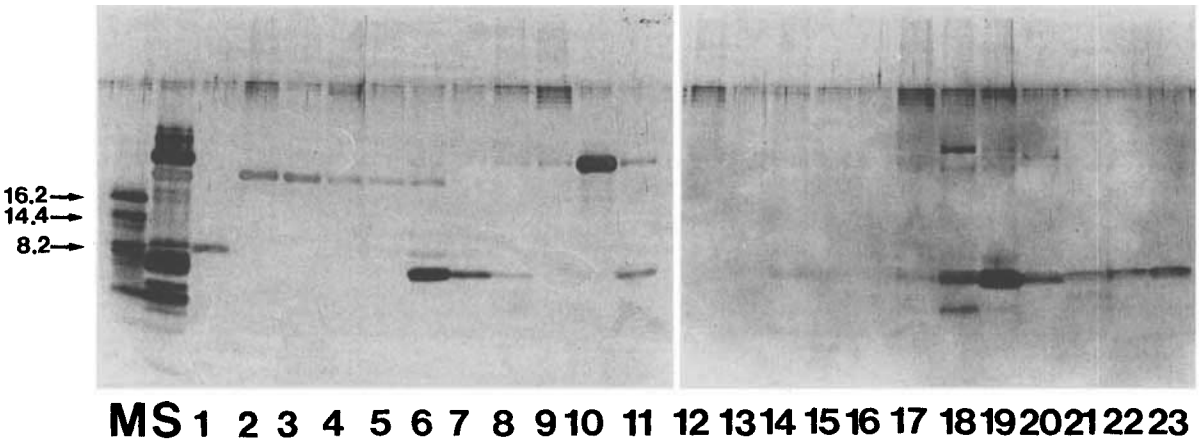

Fig. 6. Electrophoretic analysis of CNBr-derived peptide fragments. Differing quantities of aliquots taken from fractions of a HPLC separation of CNBr-digested purified MNDA were analyzed on a Tricine-buffered SDS polyacrylamide gel composed of a 16.5% resolving gel overlayed with a 10% spacer gel according to Schagger and Von Jagow. **Lane M,** Pharmacia (Piscataway, N.J.) low molecular weight markers (M, × 1,000); **lane S,** CNBr-digested MNDA; **lanes 1–23,** aliquots (differing in amount) of fractions from C_{18} reversed-phase column corresponding in number to fractions on Figure 5. Note only selected fractions in Figure 5 are denoted. Gel was stained with silver.

TABLE II. Automated Edman Degradation of CNBr-Derived Peptides*

Cycle no.	CNBr Fraction 10		CNBr Fraction 7 and 8		CNBr Fraction 19		CNBr Fraction 22	
	Amino acid	PTH (pmol)	Amino acid	PTH (pmol)	Amino acid	PTH (pmol)	Amino acid	PTH (pmol)
1	P	152	E	405	D	92	E	451
2	S	112	I	576	V	85	K	525
3	L	257	K	422	V	214	K	545
4	K	373	E	431	G	119	F	607
5	N	98	A	536	S	16	Q	303
6	L	219	S	47	G	97	G	392
7	V	276	S	51	K	131	V	618
8	N	100	V	488	W	29	A	471
9	N	111	S	47	H	14	ND	ND
10	L	229	D	213	N	52	L	372
11	R	53	F	358	I	80	D	186
12	K	367	N	183	K	51	K	117
13	E	143	Q	194	ND	ND	L	523
14	K	349	N	162	E	49	I	297
15	S	150	F	311	K	76	E	218
16	K	298	E	227	G	46	L	292
17	V	229	V	378	D	28	A	257
18	A	182	P	172	K	35	K	195
19	K	252	N	135	L	55	D	43
20	K	286	R	75	R	12		
21	I	12	I	248	L	61		
22	K	257	I	308	F	39		
23	T	26	E	167	ND	ND		
24	Q	63	I	260	L	46		
25	E	102	A	202	Q	17		
26	K	191	N	93	L	50		
27	A	125	K	95	R	8		
28	P	66	T	26	T	4		
29	V	142	P	77	V	34		
30	K	171	K	53	D	11		
31	K	177						
32	I	29						
33	N	37						
34	Q	35						
35	E	58						
36	E	66						
37	V	98						
38	G	42						
39	L	68						
40	A	70						
41	A	85						
42	P	45						
43	A	74						
44	P	43						
45	T	9						
46	A	59						
47	R	11						
48	N	22						
49	K	67						
50	L	44						

*Fractions from the HPLC reversed-phase C_{18} separation of CNBr-digested MNDA were vacuum centrifuged to small volumes (10 μl). HPLC grade isopropanol was re-added to the fractions to approximate the percentage of organic solvent at which the fractions eluted from the column. Fractions were submitted to the UAB Core Facility for Protein Chemistry.
ND denotes no PTH-amino acid detected.

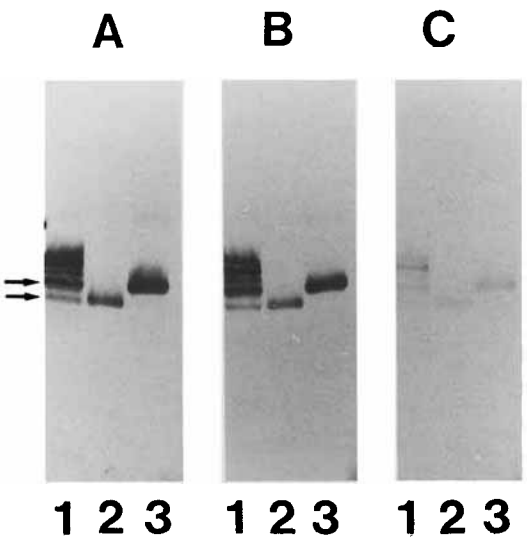

Fig. 7. Immunodetection of CNBr-derived peptide fragments. Aliquots of CNBr-digested MNDA and purified CNBr-derived peptides were separated on a SDS polyacrylamide gel as in Figure 6. After electrophoretic transfer to nitrocellulose, duplicated regions of the immunoblot were separated and probed with different rat monoclonal antibodies to MNDA. **Lane 1,** CNBr digest; **lane 2,** CNBr fraction 5; **lane 3,** CNBr fraction 10. Immunoblots were probed with 12G11 Mab **(A)**; 2F10 Mab **(B)**; 3C1 Mab **(C)**. Arrows denote position in CNBr digest of peptide isolated in fraction 10 (top) and in fraction 5 (bottom).

CNBr-derived peptide from fraction 19 (30 aa) exhibited a 63% identity to a region in the 204 protein (Fig. 10B) and a 50% identity to a region in the 202 protein (Fig. 10C). The overlapping sequence in Figure 9B exhibited 51% identical residues to a region in the 202 protein (Fig. 10D) and a 49% identity to a region in the 204 protein (Fig. 10E). Consensus sequences conserved in the duplicated regions of the 202 and 204 proteins (202 a and b, 204 a and b) were matched within MNDA sequences at nine of ten locations.

Another notable similarity was observed between the MNDA and the interferon regulatory factor 2 (IRF-2) protein in a region that showed no similarity to the interferon-inducible proteins 202 and 204. Residues 10 to 37 of the CNBr-derived peptide from the immunochemically active fraction 10 showed 46% identity to a region in the IRF-2 protein between residues 114 and 141 (Itoh et al., 1989). Though limited by the short stretches of sequence available, a study of potential secondary structure revealed a basic, amphipathic, α-helical region in the sequence related to IRF-2 (residues 10–27). When a helical wheel representation was performed using this region, six of eight basic residues were positioned on one side of the helix, while the other side showed a hydrophobic character.

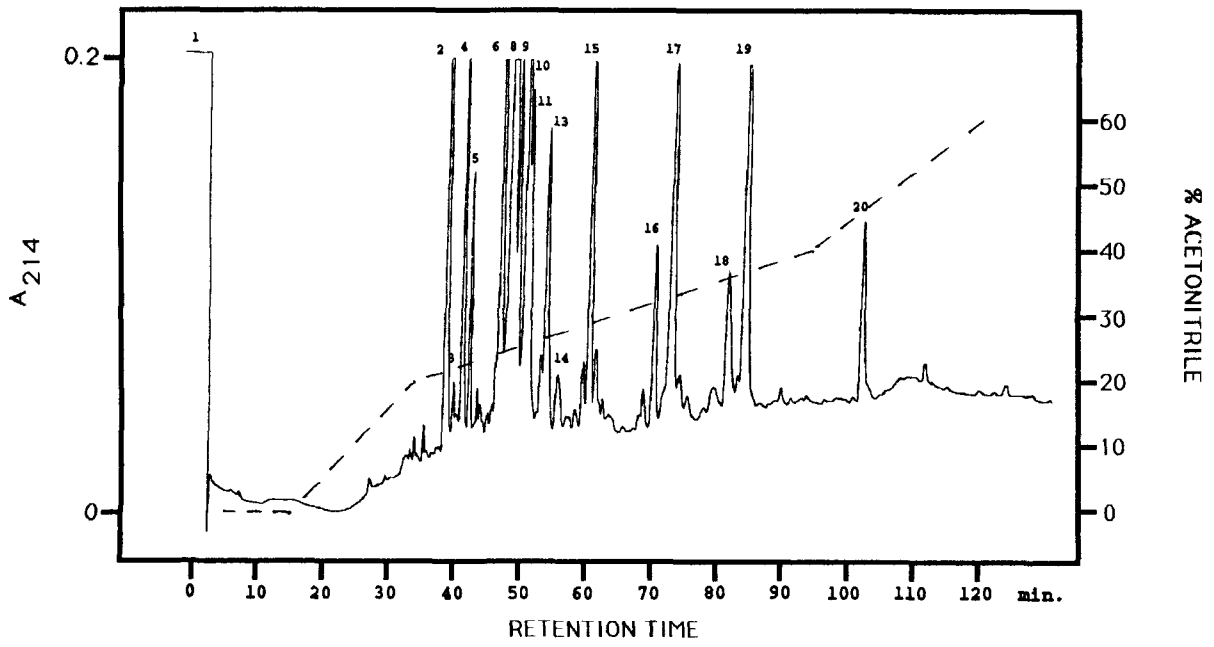

Fig. 8. *Chromatograph of HPLC separation of SV_8-derived peptide fragments. The soluble fraction of a 9-h SV_8* protease digestion of purified MNDA was applied to a HPLC reversed-phase C_{18} column equilibrated in 0.1% TFA (pH 2.1) at a flow rate of 0.25 ml/min. The column was developed with a complex linear gradient into 0.1% TFA, acetonitrile, as shown by the dashed line. Peptides were detected by absorbance at 214 nm.

TABLE III. Automated Edman Degradation of SV_8-Derived Peptides*

Cycle no.	Fraction 2A		Fraction 2B		Fraction 4A		Fraction 4B		Fraction 8		Fraction 9		Fraction 13		Fraction 19	
	Amino acid	PTH (pmol)	Amino acid	PTH (pmol)	Amino acid	PTH (pmol)	Amino acid	PTH (pmol)	Amino acid	PTH (pmol)	Amino acid	PTH (pmol)	Amino acid	PTH (pmol)	Amino acid	PTH (pmol)
1	K	2219[a]	K	2219[a]	A	460[b]	A	460[b]	K	238	A	60	V	108	I	84
2	S	485	A	1149	R	138	P	92	T	408	R	28	G	118	A	78
3	K	701	P	932	G	189	A	160	E	266	G	36	L	115	N	59
4	V	1406[a]	V	1406[a]	R	152	P	66	A	393	R	39	A	121	K	42
5	A	858	K	644	I	221	T	64	K	206	I	25	A	129	T	36
6	K	1232[a]	K	1232[a]	P	132	A	79	R	233	P	41	P	119	P	39
7	K	602	I	545	V	194	R	47	N	210	V	20	A	134	K	23
8	I	676	N	681	A	232	N	53	K	72	A	32	P	91	I	38
9	K	325	Q	586	Q	140	K	49	V	144	Q	28	T	65	S	22
10	T	443	E	476	K	118	L	51	S	87	K	11	A	70	Q	30
11	Q	320	E	367	R	127	T	42	Q	149	R	34	R	69	L	40
12					K	96			E	105	K	9	N	71	Y	21
13					T	103			Q	119	T	24	K	40	K	15
14					P	72			S	54	P	17	L	47	Q	25
15					N	60			K	18	N	11	T	42	A	26
16					K	45			P	112			S	23	S	ND
17					E	37			P	97			E	26	G	18
18									G	107			A	27	T	24
19									P	80			R	32		
20													G	30		
21													R	40		
22													I	26		
23													P	26		
24													V	10		
25													A	20		
26													Q	20		
27													K	4		
28													R	18		

*Fractions from the HPLC reversed-phase C_{18} separation of SV_8-digested MNDA were vacuum centrifuged to small volumes (100 μl). HPLC grade acetonitrile was re-added to the fractions to approximate the percentage of organic solvent at which the fractions eluted from the column. Fractions were submitted to VUCMT protein chemistry core. Fractions 2, 9, and 13 were submitted twice and were confirmatory for the same sequence.

[a]Denotes total amount released for both peptides from fraction 2.

[b]Denotes total amount released for both peptides from fraction 4.

ND denotes value below integrator's detection threshold.

A.

CNBr10	PSLKNLVNNLRKEKSKVAKKIKTQEKAPVKKINQEEVGLAAPAPTARNKL .....
SV_8 2A	KSKVAKKIKTQ
SV_8 2B	KAPVKKINQEE
SV_8 13	VGLAAPAPTARNKLTSEARGRIPVAQKR.....
SV_8 4B	APAPTARNKLT
SV_8 4A	ARGRIPVAQKRKTPNKE
SV_8 9	ARGRIPVAQKRKTPN.....

B.

CNBr 7&8	EIKEASSVSDFNQNFEVPNRIIEIANKTPK.....
SV_8 19	IANKTPKISQLYKQASGT.....

Fig. 9. Overlapping sequences of MNDA peptides. **A:** Overlaps of CNBr and SV_8-derived peptides forming a continuous sequence of 70 amino acids. **B:** Overlap of CNBr and SV_8-derived peptides forming a continuous sequence of 41 amino acids.

DISCUSSION

The MNDA exhibits a very specific pattern of expression in blood cells. Expression begins at the stage where hematopoietic cells exhibit a unique phenotype associated with monocyte/granulocyte differentiation (Cousar and Briggs, 1990; Goldberger et al., 1986). HL-60 cells induced to differentiate toward mature monocytes or granulocytes show no change in overall level of MNDA. However, the association between MNDA and DNA, as assessed through cisplatin cross-linking, is changed during induced monocyte differentiation (Gaczynski et al., 1990). The strict nuclear localization of the MNDA in interphase cells is also consistent with a role in nuclear functions required for monocyte and/or granulocyte differentiation. Our early screening experiments were designed to detect nuclear proteins/antigens expressed in a stage-specific manner in human myeloid cell differentiation. Those results revealed ten nuclear antigens which exhibited progressively a decreased level in association with myeloid cell maturation (Goldberger et al., 1984). These antigens may reflect the pattern of expression of the transcription factor NFE-1, the erythroid cell transcription factor, which was recently evaluated in a series of growth factor-dependent cell lines (Crotta et al., 1990). The results indicate that NFE-1 is activated in an early multipotential progenitor cell and that restriction of NFE-1 expression in mature erythroid and mast cells occurs by gradual extinction of NFE-1 gene expression in progressively differentiated granulomonocytic cells. The results of our immunochemical screening suggests that extinction of expression may account for changes in a number of nuclear proteins in cells progressing through granulocyte/monocyte differentiation.

Our earlier screening experiments showed that only the MNDA was expressed in a stage-specific fashion in association with granulocyte/monocyte differentiation (Goldberger et al., 1984). However, there may be other nuclear proteins expressed specifically in the granulocyte/monocyte lineage in a stage-specific manner which are not antigenic. Overall, the great majority of nuclear antigens detected were at the same levels in all cell lines representing various stages of differentiation (Goldberger et al., 1984). These results are consistent with the observation that many transcription factors are ubiquitously ex-

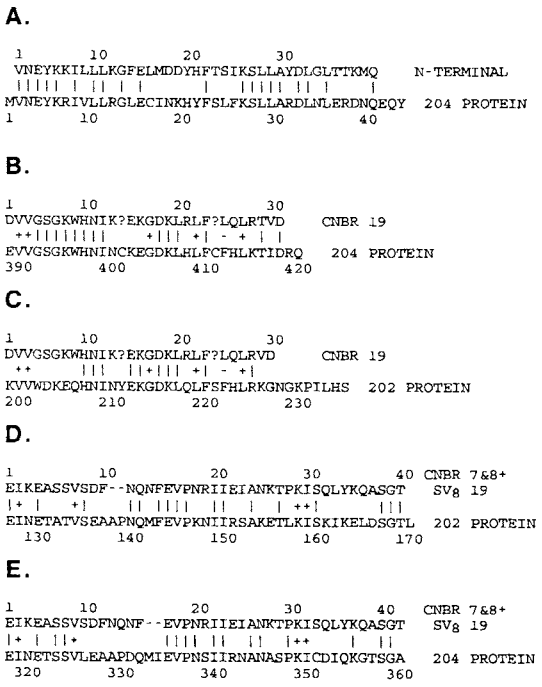

Fig. 10. Amino acid sequence similarities between MNDA and the mouse 202 and 204 proteins. **A:** Sequence similarity between the NH_2-termini of MNDA and the mouse 204 protein. **B,C:** Sequence similarities between the CNBr peptide from fraction 19 and the mouse proteins 204 (B) and 202 (C). **D,E:** Sequence similarities between the sequence, formed by the overlap of the CNBr-derived peptide from fractions 7 and 8 and the SV_8-derived peptide from fraction 19, and the mouse protein 202 (D) and 204 (E). | denotes identical match; + denotes match of consensus site in mouse proteins; - denotes consensus sites in mouse proteins not matched. Note: all consensus sites in mouse 204 and 202 protein family are matched by peptides except one site in CNBr 19 vs. 204 and the same site in CNBr 19 vs. 202.

pressed and only a few show tissue-specific expression consistent with the regulation of tissue-specific gene regulation (Mendel and Crabtree, 1991). Similarly, a comprehensive two-dimensional electrophoretic analysis of nuclear proteins recently revealed few changes over the course of B lymphocyte differentiation (Rabilloud, 1991). It is important to note that the electrophoretic and immunochemical analyses do not differentiate transcription factors from other nuclear proteins.

While the sequence data reported here for the MNDA do not clearly establish MNDA's precise functional role, they do support our previous findings that MNDA is a DNA-binding protein and additionally reveal homologies to interferon-inducible genes. The high degree of similarity between the MNDA and the mouse 202 and 204 proteins suggests a common ancestry. However, the strict lineage- and stage-specific pattern of expression of the MNDA is not observed in the mouse proteins (Choubey et al., 1989). The MNDA sequence also contains a higher-than-normal number of S(T)PXX motifs which are currently thought to perform a nonspecific DNA binding function and are enriched in those proteins involved in gene regulation (Suzuki, 1989). Also, the highly basic α-helical region in the CNBr-derived peptide from the immunochemically active fraction 10 contains a predominance of basic residues on one face of the helix, typical of many DNA binding helices (Churchill and Travers, 1991). This basic region of MNDA in turn shows homology to the interferon regulatory factor 2 (IRF-2) which binds the regulatory elements of interferon and interferon-inducible genes and functions as a repressor (Harada et al., 1989). Interestingly this region in the MNDA is a unique region showing no similarity to the interferon-inducible mouse 202 and 204 proteins.

The involvement of this unique protein, MNDA, in the mechanisms of action of the interferons has not been demonstrated, but the observation that interferons induce cell-specific responses in myeloid cells has been documented (Fan et al., 1989; Radford et al., 1991). The investigation of the possible role of the MNDA in these events is warranted.

ACKNOWLEDGMENTS

We would like to thank Dr. Michael Skinner (Department of Pharmacology, Vanderbilt University) for his suggestions on the composition of AEB 1, Dr. Michael Haralson (Department of Pathology, Vanderbilt University) for his assistance in arranging sequence analysis, and Dr. David Duhl (Department of Psychiatry, Vanderbilt University) for a helpful reading of the manuscript. Supported by NIH grants CA 37097 and ES 00267 and the Vanderbilt University Research Council.

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