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cDNA CLONING OF MURINE Nrf 2 GENE, CODING FOR A p45 NF-E2 RELATED TRANSCRIPTION FACTOR

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A λgt11 cDNA library was constructed from poly-A rich RNA prepared from circulating murine yolk sac derived nucleated erythroblasts, on day 9 of gestation. In addition to the embryonic globin genes, this library was found to contain clones for the hematopoietic specific p45 NF-E2, as well as p18 NF-E2 and p45 NF-E2 related factor 1 (Nrf 1) genes. Using a degenerate oligonucleotide 17mer probe coding for a part of the highly conserved DNA binding domain for p45 NF-E2, we have isolated murine Nrf 2, a second murine homologue related to p45 NF-E2. The murine Nrf 2 gene is expressed not only in erythroid cells, but also in the 3T3 murine fibroblast cell line.

In the β-globin gene cluster, the locus control region (LCR) situated far upstream of the embryonic ε-globin gene is indispensable for the high level, tissue specific expression of the β-like globin genes (1). The LCR encompasses four erythroid DNAase I hypersensitive sites (HS). Within the LCR, most notably within HS-2, AP-1-like motifs have been found to confer enhancer activity for globin gene expression. These motifs are bound by an erythroid DNA-binding activity, known as NF-E2, as well as AP-1 family members. NF-E2 is comprised of two basic leucine zipper polypeptides, p45 and p18 (2, 3). p45 NF-E2 contains a domain which is highly homologous to that in a transcription factor CNC (cap and collar) found in the Drosophila. The expression of the p45 NF-E2 gene is largely limited to the hematopoietic cells, while that of p18 NF-E2 is widespread. The heterodimer of p45 and p18 binds to the extended AP-1-like sequences (T/C) GCTGA(C/G)TCA(C/T) present in the LCR. Consistent with the proposed role of NF-E2 in globin gene regulation, mouse erythroleukemia cells with the p45 NF-E2 alleles inactivated by Friend virus integration fail to express β-globin at an appreciable level (4).

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Recently, mice lacking p45 NF-E2 have been generated. Unexpectedly, erythropoiesis in such mice is only mildly affected (5). One explanation to account for this finding might be the existence of homologues which might compensate for the lack of p45 NF-E2 during erythropoiesis. In an effort to identify additional p45 NF-E2 related proteins, we constructed a λgt11 cDNA library from poly-A rich RNA obtained from murine yolk sac derived circulating erythroblasts on day 9 of gestation, and screened with a degenerate oligonucleotide 17mer probe within the highly conserved p45 NF-E2 DNA- binding domain. In this manner, we isolated a second murine p45 NF-E2 related factor, mNrf 2.

MATERIALS AND METHODS

Mouse and embryonic erythroblasts

Normal adult C57BL/6J mice were mated, and the morning when the vaginal plug was found was designated as day 0 of gestation. On day 9 of gestation, the pregnant mice were killed by cervical dislocation. The conceptuses with their yolk sacs were carefully dissected and washed free of maternal tissues and blood cells in phosphate-buffered saline. The embryos were decapitated and their circulating peripheral blood was allowed to bleed into Iscove's modified Dulbecco medium. The embryonic erythroblasts were harvested by centrifugation (6).

RNA extraction and cDNA library construction

Total cellular RNA was extracted by RNAzol B (Tel-Test, Inc., Friendswood, TX.). Poly-A rich RNA was obtained with the Poly (A) Quik mRNA isolation kit (Strategene). Complementary DNA synthesis was carried out with oligo (dT) primer, using the Timesaver cDNA synthesis kit (Pharmacia). The double stranded cDNAs were ligated to EcoRI adaptors, which were subsequently ligated and packaged into an EcoRI cleaved λgt11 phage vector (Promega).

Screening for the p45 NF-E2 homologue

A degenerate oligonucleotide 17mer probe, GG(A/C/G/T)AA(A/G)AA(C/T)AA(A/G)GT (A/C/G/T)GC coding for the peptide GKNKVA present in the highly conserved DNA binding domain of the p45 NF-E2 was used to screen the cDNA library. The hybridization was carried out in 3M tetramethylammonium chloride (Sigma) at 48°C, and washing at 50°C. In addition, probes specific for p45 NF-E2 and Nrf 1 genes were used to screen the same filters at high stringency.

The insert in the isolated phage clone was amplified by PCR using λgt11 primers, and sub-cloned into EcoRI-cleaved pUC 18 plasmid vector (7). Sequence analysis was carried out by the dideoxynucleotide chain-termination method using a Sequenase Version 2.0 kit (United States Biochemical). The 5'-rapid amplification of cDNA ends (RACE) reaction was carried out using a kit (GIBCO/BRL) according to the manufacturer's protocol, except that two nested PCR reactions were carried out. Poly-A rich RNA obtained from either mouse erythroleukemia (MEL) cells induced with DMSO or 3T3 cells was used as the templates for the synthesis of the first strand cDNA. Northern blotting analysis was done on total cellular RNA extracted with RNAzol B (Tel-test).

RESULTS

The day 9 yolk sac derived erythroid cell cDNA library

A total of 22 x 10^6 circulating yolk sac blood island derived erythroblasts were obtained from 335 mouse embryos on day 9 of gestation. Cytocentrifuge smears of some of these embryonic peripheral blood preparations confirmed that almost all the cells present were embryonic erythroblasts. These embryonic erythroid cells yielded approximately 200 μg total cellular RNA. After passage through oligo (dT) column and ethanol precipitation, 1 μg of poly-A rich RNA was obtained, which was used for the construction of the λgt11 cDNA library.

The plating of the cDNA library with β-galactosidase revealed that 93% of the phage plaques were β-galactosidase negative and therefore contained recombinant inserts. Of 38 recombinant phage clones isolated randomly and the inserts amplified by PCR, the estimated sizes of the inserts vary between 300 bp to 3.6 kb. Eighteen had an insert size between 300-900 bp. The other twenty inserts measured between 1.0 - 3.6 kb.

Screening of the library with murine embryonic globin gene probes revealed innumerable positive clones (data not shown). In one screening using p45 NF-E2 probe, there were 49 positive clones in a total of 2 x 10^5 plaques plated. Eleven positive clones were randomly isolated. The estimated sizes of ten inserts varied between 1.0 kb to 1.8 kb. Sequence and PCR analyses revealed that all of them were genuine p45 NF-E2 clones.

The p45 NF-E2 homologue

Screening of the library with the oligonucleotide 17mer probe resulted in the isolation of one positive clone, which hybridized with neither the p45 NF-E2 nor Nrf 1 at high stringency. The insert in this clone measured approximately 2.3 kb, and was found to have two internal EcoRI restriction enzyme recognition sites. The three EcoRI digested fragments of the insert were subcloned into pUC 18 plasmid vectors and sequenced. The proper orientation of the three fragments was aligned based on PCR amplification results of the original phage clone with appropriate oligonucleotide primers. The insert in the isolated phage clone is 2295 bp long.

5'-RACE reactions were carried out with poly-A rich RNA derived from MEL cells induced with DMSO and also from 3T3 cells. The final assembled clone is 2484 bp long, and designated as mNrf2. The complete nucleotide sequence has been submitted to the GenBank (Accession no. U20532). The amino acid sequence and its comparison with that of p45 NF-E2 and Nrf 1 are illustrated in Figure 1.

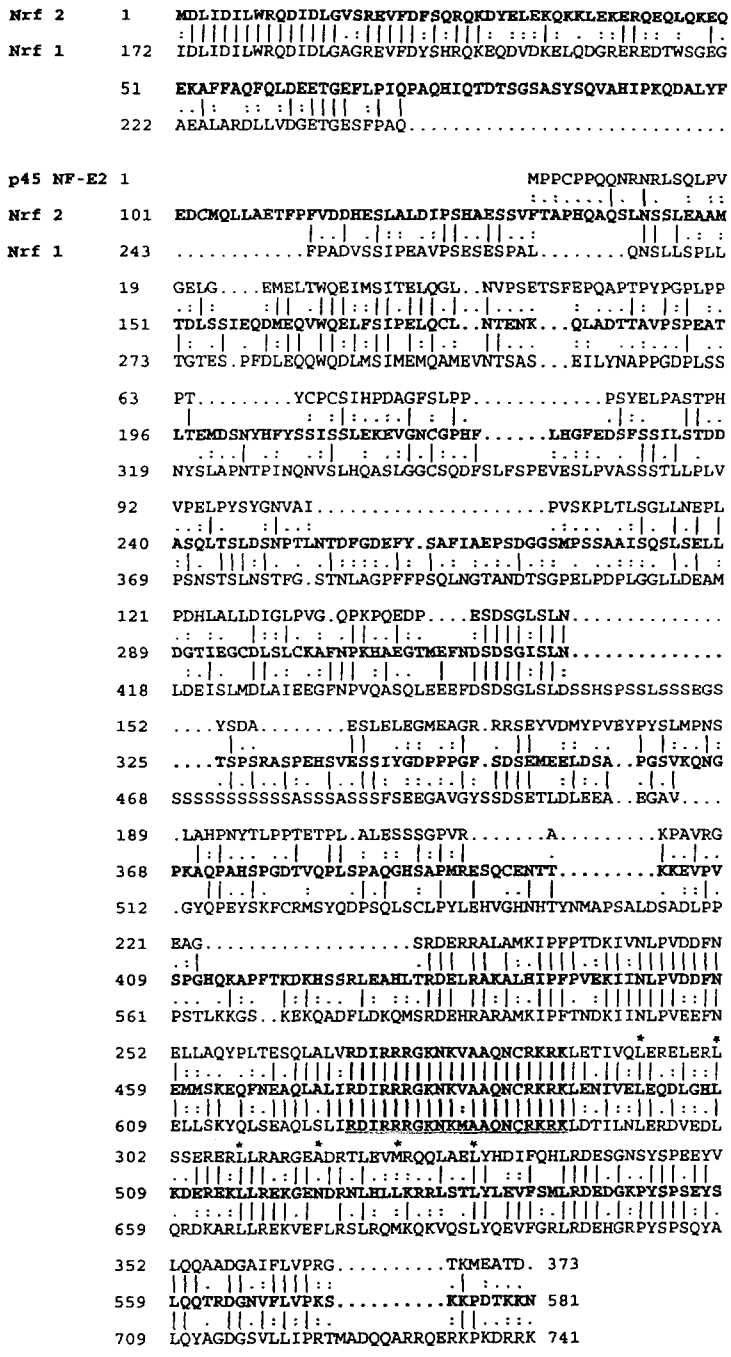

Fig. 1. Amino-acid sequences from murine p45 NF-E2, Nrf 2 and Nrf 1 are aligned for comparison. Amino-acid identity is indicated by connecting lines, while conserved changes are indicated by dots. The DNA binding basic domain is underlined. In the leucine repeats region, the hydrophobic heptad-repeat amino-acids are indicated by asterisks.

Expression of the p45 NF-E2 homologue

A radioactive probe spanning bp 1315 to bp 2383 encompassing the CNC domain and leucine repeats of mNrf 2 was used for Northern blotting analysis of total cellular RNA extracted from adult and fetal tissues as well as from MEL cell lines. Murine Nrf 2 is expressed in normal day 13 fetal hepatic erythroblasts as well as in adult spleen, liver, marrow, and kidney (Data not shown). As shown in Fig. 2, mNrf 2 is also expressed in MEL cells and CB3 cells (4). The level of expression in both cell lines is enhanced after DMSO induction. Also in murine fibroblast 3T3 cells, mNrf 2 is expressed at a level similar to that of DMSO induced MEL cells (Fig. 2).

DISCUSSION

During murine embryonic erythropoiesis, the first site of red blood cell production occurs in the yolk sac blood islands, beginning on day 7 of gestation. By day 9 of gestation, the vasculature is formed and the embryonic heart begins to contract. The yolk sac blood islands derived erythroblasts are then released into the circulation, where they proliferate and differentiate in a relatively synchronous manner. At that stage of development, there are no myeloid or lymphoid cells. Thus, the circulating blood cells on day 9 of gestation consist of only erythroblasts

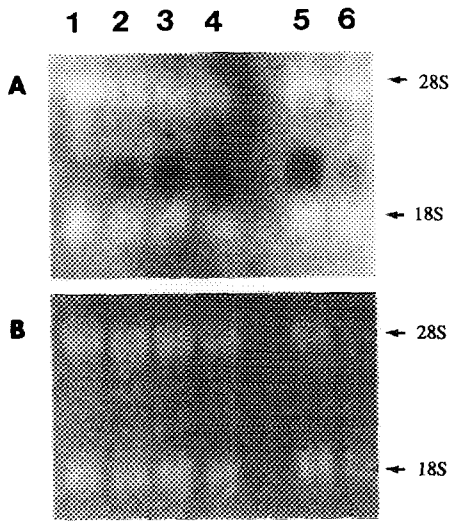

Fig. 2. mNrf 2 expression in cell lines.

A. Northern blot analysis. Lanes: 1, MEL cells; 2, DMSO induced MEL cells; 3, CB3 cells; 4, DMSO induced CB3 cells; 5, 3T3 cells; 6, MEL cells.

B. Agarose gel electrophoresis of above, prior to transfer, stained with ethidium bromide. Twenty micrograms of cellular RNA was loaded to each lane. Arrows indicate the positions of 28S and 18S ribosomal RNAs.

already active in embryonic hemoglobin synthesis (6). In the circulating blood on day 9 of gestation, there are also hematopoietic stem cells which are capable of colonizing the fetal hepatic tissues at later stages (8).

We have harvested this population of "pure" murine embryonic erythroblasts and obtained poly-A rich RNA to construct a λgt11 cDNA library. In addition to numerous cDNA clones for the globin genes, this library also contains clones for the hematopoietic cell specific transcription factors. For example, p45 NF-E2 is found to be present in this library at a frequency of 24 positive clones in 100,000 plaques plated, considerably higher than present in a MEL cell cDNA library (2). The murine p18 NF-E2, Nrf 1 and Nrf 2 genes are also present in the library at a lower frequency, estimated to be 2-4 positive clones per 100,000 plaques plated. This murine embryonic erythroid cell cDNA library should be a useful source for the isolation of novel genes which are important for erythroid and hematopoietic cell differentiation.

Recently, the human Nrf 2 gene was isolated and cloned (9). Comparison of the nucleotide sequences between the human and murine Nrf 2 reveal 84% identity (data not shown). At the level of amino acid sequences, the two genes show 82% identity and 90% similarity. In the C-terminal 151 amino acid residues encompassing the CNC domain including the DNA binding basic region, and the leucine repeats, the two genes are identical except for eight conservative changes and one non-conservative change. The comparisons between the mNrf 2 with the murine NF-E2 p45 and Nrf 1 are shown in Figure 1. In both instances, murine Nrf 2 shares approximately 40% identity and 60% similarity with the other two genes. There is total homology among the three factors in the DNA binding basic domain with only one conservative change in the Nrf 1 factor.

The role of mNrf 2 in erythroid cell differentiation and globin gene expression is presently unknown. In contrast to p45 NF-E2, Nrf 2 as well as Nrf 1, is expressed in non-hematopoietic cells, such as the fibroblast 3T3 cell line. CB3 cells are murine erythroleukemia cells in which both p45 NF-E2 genes were inactivated by Friend leukemia virus integration. These cells fail to express significant β-globin RNA after DMSO induction (4). Forced expression of p45 NF-E2 cDNA, however, partially restores globin expression (4). Whether p45 NF-E2 related factors, such as Nrf 1 or Nrf 2, can replace p45 NF-E2 functions can be tested in this cell system. The present study shows that Nrf 2 is expressed in CB3 cells, and the level of expression is elevated after DMSO induction (Fig. 2). These data suggest that Nrf 2 is unlikely to play a significant role in globin gene expression in that it probably cannot compensate for the

lack of p45 NF-E2 in these cells. Whether Nrf 2 serves any demonstrable functions in erythroid cells in vivo will require additional investigations.

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