Mouse and human chromosomal assignments of mortalin, a novel member of the murine hsp70 family of proteins

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Abstract Mortalin has been shown to exhibit differential distributions in cells with mortal and immortal phenotypes. In the present study, we report mot-2 cDNA cloning from RS-4 cells – an immortal clone from CD1-ICR mouse embryonic fibroblasts – and the chromosomal assignments of mortalin related genes to mouse chromosomes 18 and X by fluorescence in situ hybridization. Similar analysis assigned the gene to chromosome 5q31.1 in human.

Key words: Mortalin; Chromosomal assignment; Mouse; Human

1. Introduction

A genetic program implicated in the mechanisms that lead to cellular senescence, the limited division potential of cells in culture [1,2], has led to the quest for genes that limit cellular proliferation potential on the one hand and permit transformed cells to divide indefinitely on the other. Subtraction hybridizations, differential expression screenings and expression studies have implicated the following genes in cell proliferation control: *p53*, *Rb*, BTG, prohibitin, SAG and $p21^{sdi-1}$ [3–9]. Microcell mediated chromosome transfer has identified chromosome 1, 4, 6, 7 and X as carriers of putative senescence genes [9–13].

We have earlier shown that mortal and immortal cells exhibit differential cellular localization of mortalin. All immortal human as well as mouse cells, tested so far, are devoid of the uniformly distributed cytosolic form of the protein which is characterisitic of normal cells [14–16]. Senescent MEF (doubling time >5 days) were transiently induced to divide by the microinjection of anti-mortalin antibody. Expression of the cytosolic form ($p66^{mot-1}$) induced senescence in NIH 3T3 cells that normally harbor the perinuclear form ($p66^{mot-2}$) which differs from $p66^{mot-1}$ by two amino acids [14,17]. This protein has recently been identified by three other independent studies. It was identified and cloned as a peptide-binding protein 74 (PBP-74) from antigen presenting cells [18]. It was also cloned as C3H mouse strain specific antigen [19]. It has also been identified as one of the mitochondrial target proteins, hsp70-like protein, during in vivo nephrotoxicity induced by nephrotoxin S-(1,1,2,2-tetrafluoroethyl)-L-cysteine [20]. Lack of heat inducibility of the protein, the presence of a signal peptide, involvement in the life span determining pathways and the mitochondrial targets of the protein are the observations shared by at least two of these independent studies.

In the present study, we have cloned mortalin cDNA from an immortal cell line, RS-4, established from CD1-ICR mouse fibroblasts. The cDNA exhibits *mot-2* structure similar to the cDNA isolated from NIH 3T3 cells and consistent with the perinuclear distribution of the protein in these cells. The isolated 3.0 kb clone is used for mouse and human chromosomal assignments of mortalin genes. Fluorescence in situ hybridization assigns the mortalin related genes on mouse chromosomes 18 and X. In human cells the gene assigns to 5q31.1, the locus of a putative tumor suppressor gene involved in myeloid malignancies.

2. Materials and methods

2.1. Cell culture, cDNA cloning and sequencing

Details of fibroblast culture from CD1-ICR mouse, establishment of spontaneously immortalized cells from CD1-ICR mouse embryonic fibroblasts (CMEF), synthesis and screening of cDNA library, and sequencing have been described earlier [14–17].

2.2. Chromosome preparation and fluorescence in situ hybridization (FISH)

Mouse and human chromosomes were prepared from lymphocytes and peripheral blood, respectively, as described earlier [21]. For replication R-banding, cells were treated with thymidine (300 μg/ml) for 14 h after 44 h of initial seeding (~0.5×10^6 cells/ml) for mouse and 15.5 h after 48 h of initial seeding for human. Cells were washed twice with serum free medium and then cultured in BrdU (30 μg/ml) supplemented medium for 4 h and 6 h for mouse and human, respectively. Colcemid (0.02 μg/ml) was added 20–30 min before harvesting. The cells were collected by mild centrifugation, treated with 0.075 M KCl, fixed with methanol/glacial acetic acid (3:1), dropped on glass slide and air-dried. The chromosome slides were stained with 1 μg/ml Hoechst 33258 in Sorensen's phosphate buffer (pH 6.8) for 5 min, rinsed with 2 × SSC and mounted. The slides were exposed for 3.5–4 min to a HBO-200 W/A mercury vapor lamp (Osram) at a distance of about 5 cm, and stored at −80°C.

Mortalin cDNA (3 kb, the isolate from RS-4 cells cDNA library) cloned in pBluescript was labeled by nick translation with biotin -16 dUTP (Boehringer Mannheim, Germany) following the manufacturer's directions for FISH studies. The labeled DNA fragments were ethanol precipitated by using sonicated salmon sperm DNA and tRNA as carriers. Precipitated probe was denatured for 10 min at 75°C in 100% formamide and was mixed with an equal volume of hybridization solution to the final concentration of 50% formamide; 2 × SSC, 10% dextranesulfate and μg/ml BSA-1. The chromosome slides were hardened at 65°C for 2 h, denatured in 70% formamide, 2 × SSC, pH 7.0, at 70°C for 2 min and dehydrated in a 4°C ethanol series and were subjected to the probe (20 μl of hybridization mixture containing 200–300 ng labeled DNA was put on the denatured slide, covered with parafilm and

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incubated overnight at 37°C). After 12–15 h, the slides were washed with 50% formamide in 2 × SSC at 37°C for 20 min and with 2 × SSC and 1 × SSC for 20 min each at room temperature. After a rinse in 4 × SSC the slides were incubated with 3 mg/ml fluoresceinated avidin antibody (Vector Labs, Burlingane, CA) in 4 × SSC containing 1% BSA for 1 h at 37°C. Washings were performed with 4 × SSC, 0.1% Nonidet P-40 in 4 × SSC and 4 × SSC for 5 min each and then stained with fluorescein-antigoat IgG (Nordic immunology, Tilburg, The Netherlands) at 1:500 dilution for 1 h at 37°C. After washings with 4 × SSC, 0.1% Nonidet P-40 in 4 × SSC and 4 × SSC for 10 min, the slides were rinsed with 2 × SSC and stained with 1 μg/ml propidium iodide. Excitation at wavelength 450–490 nm (Nikon filter set B-2A) and near 365 nm (UV-2A) were used for observations. Kodak Ektachrome ASA 100 films were used for photomicrography.

2.3. Southern blot analysis

Genomic DNAs from a panel of monochromosomal hybrids with a single human chromosome in chinese hamster background were used for Southern analysis. *Bam*HI digested DNAs were probed with 2.1 kb mortalin cDNA labeled with [α-^{32}P]dCTP to a specific activity of 10^7 cpm/μg DNA according to the standard protocols [22].

3. Results and discussion

We have earlier reported the identification and characterization of mortalin (23–27). cDNA cloning of the cytosolic (*mot-1*) and the perinuclear (*mot-2*) forms from CMEF and NIH 3T3 cells, respectively, has also been reported (17). A 3 kb cDNA clone (harboring the full open reading frame for mortalin) is presently isolated from RS-4 cells, a spontaneously immortalized clone derived from CMEF, that show the perinuclear distribution of mortalin Upon seqencing it revealed the *mot-2* structure, A at 1,941 and G at 1,959 bp cDNA positions corresponding to M618 and G624 amino acid residues. The fact that these cells have the perinuclear distribution of mortalin strongly suggests that the change from *mot-1* (cytosolic protein) to *mot-2* (perinuclear protein) expression is involved in the conversion of the normal cellular phenotype to an immortal one. The isolated clone has one kb extended sequence than our earlier isolated clones from CMEF and NIH 3T3 cells. The extended 3′ sequence, however, is found to be completely identical to the one reported earlier from Balb/c mouse. An additional change (S522F) however, is observed with respect to sequence reported by Domanico et al. [18]

Cytogenetic mapping studies by FISH on mouse chromosomes detected mortalin signals on R positive C band of mouse chromosome 18 and band A6 of chromosome X (Fig. 1). The location of fluorescence signals were determined as described previously [21]. In the case of these cells, strong twin spots on both homologues were observed in about 32% out of 86% signal positive metaphase spreads, the rest showed incomplete signals on either or both homologues or twin spots on either homologue. The signal on X chromosome was weak and was observed in about 30% of spreads. These observations suggest that mortalin related genes are present on chromosome 18 and X in mouse. The FISH signals obtained for human chromosome localized the gene to 5q31.1, the signal though somewhat less intense than that of the mouse chromosome was observed in 22% of metaphase spreads. To support human chromosomal assignment, we also performed mortalin Southern hybridization on monochromosomal hybrid panel containing single mouse chromosomes against the chinese hamster background. The Chromosome 5 containing hybrid cell line showed additional bands of 6 kb and 2 kb (Fig. 2B) and suppport the chromosomal assignment by FISH analysis. The mortalin related gene was not detected on human X chromosome either by FISH or by monochromosomal hybrid panel analysis. The present study adds to the mouse and human genomic maps and extends another locus that assigns to mouse 18 and human 5q chromosome. The weak intensity of the signal on mouse X chromosome, the absence of corresponding signal on human chromosome and the earlier reported indication of the presence of mortalin related pseudo gene in mouse [28] suggest that the signal on mouse X chromosome may correspond to a pseudo gene. Earlier, the hsp70 gene family members have been mapped to chromosome 2, 12 and 17 in mouse and 1, 6 and 14 in human [29]. Therefore, mortalin is the first hsp70 family member assigned to chromosome 18 in mouse and 5q in human. Hukku and Rhim [30] have suggested a role for chromosome 5 in immortalization and tumorigenesis of human

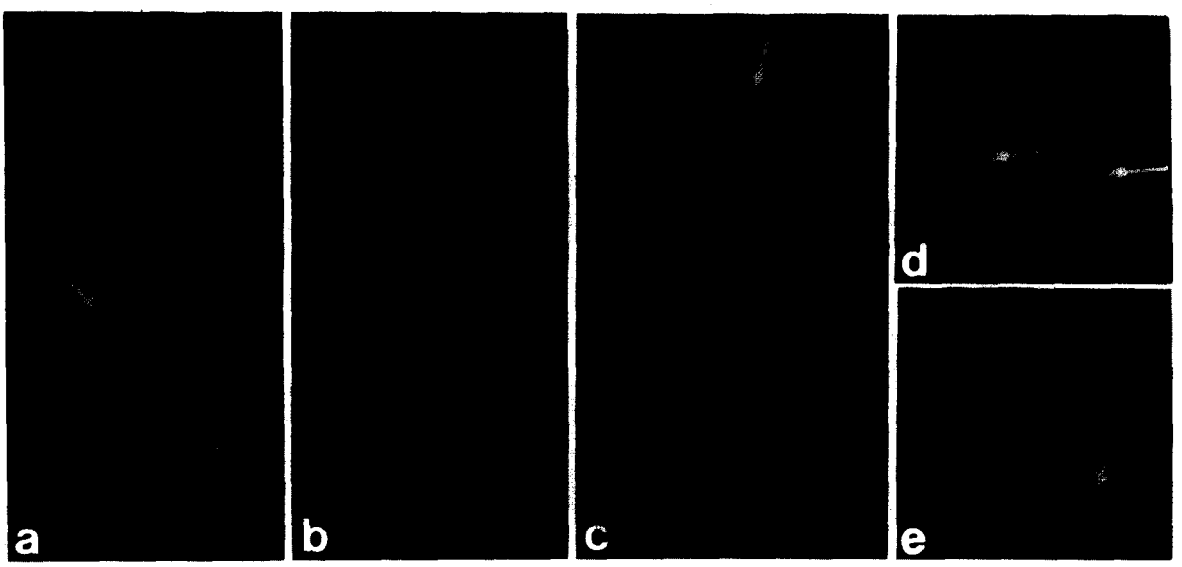

Fig. 1. Cytogenetic mortalin mapping on mouse chromosomes. The metaphase spreads were photographed with B-2A (a,c–e), and UV2A (b) filters, and show R- and G-banding, respectively. The hybridization signals obtained by biotinylated mortalin probe are indicated by long (chromosome 18 C band) and short (chromosome X A6 band) arrows.

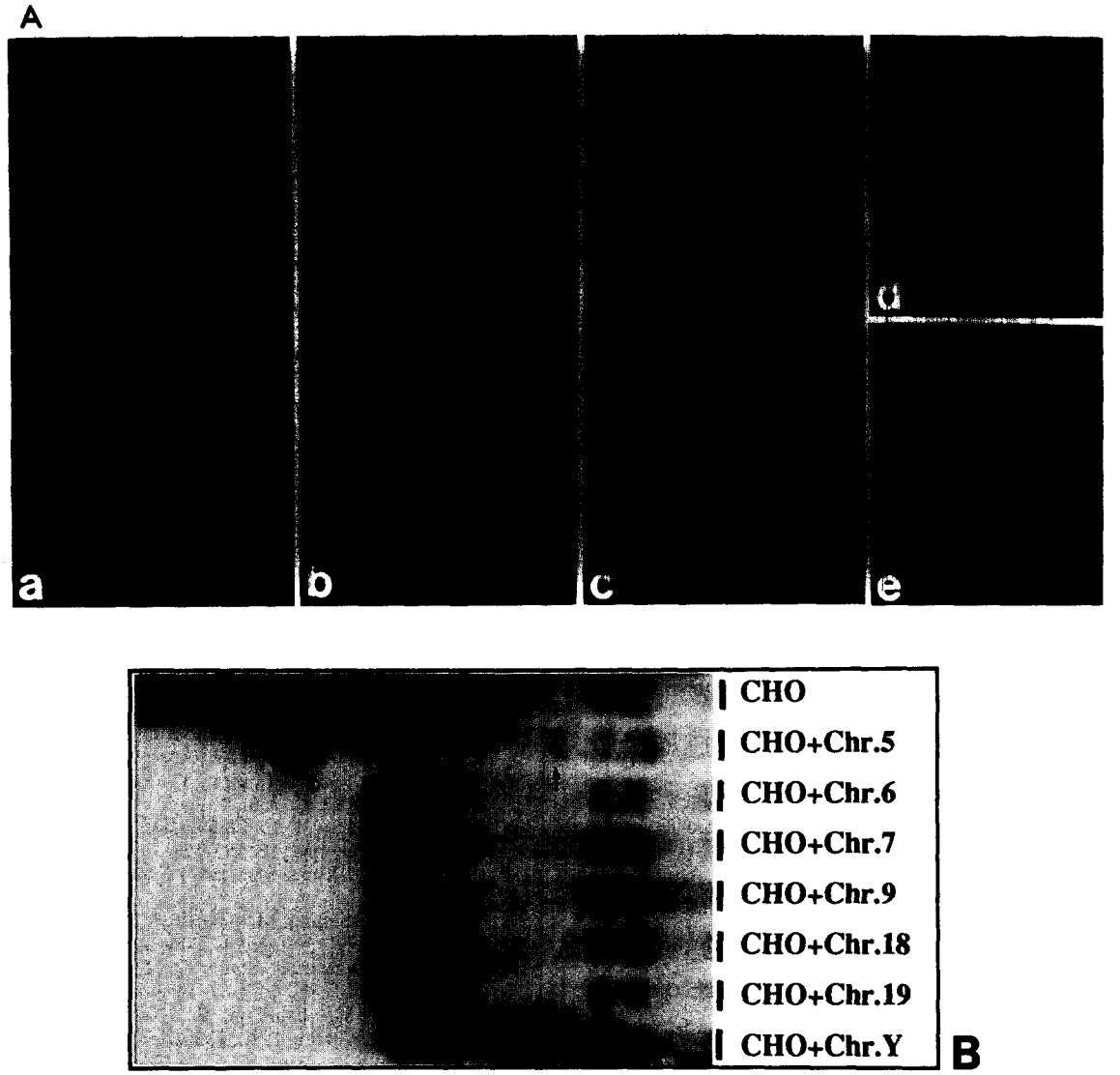

Fig. 2. Assignment of mortalin to human chromosome. (A) FISH signals on metaphase spreads as seen through B-2A (a,c–e) and B2E (b) filters corresponding to R-banding and fluorescein isothiocyanate fluorescence, respectively, are shown. The signal on chromosome 5 is marked with an arrow. (B) Southern hybridization of genomic DNAs from a monochromosomal hybrid panel with a mortalin cDNA probe. The lanes with Chinese hamster ovary (CHO) cell DNA and the ones along with human chromosome 5, 6, 7, 9, 18, 19 and Y are shown. 6.0 kb and 2.0 kb signals in the chromosome 5 lane are indicated with arrows.

keratinocytes. Furthermore, the transfer of chromosome 5 to carcinoma cell lines by microcell fusion has been found to suppress their tumorigenic properties [31, 32]. Recently, 5q31.1 has been identified as a critical common deleted region of chromosome 5 in multiple myeloid malignancies [33] and therefore expected to harbor a putative tumor suppressor gene. One possible candidate has been reported as the interferon regulatory factor 1 (IRF-1) which shows anti-proliferative and tumor suppressive properties [34]. In this regard, the assignment of mortalin to 5q31.1, its unique associations with cellular normal and immortal phenotypes [14–17] attracts greater interest. We have recently found that the immortal human cells assigned to four complementation groups for immortality [35] exhibit four different types of mortalin immunofluorescence patterns corresponding to the differential localization of the protein within the cell [16]. The study demonstrated that an alteration in the mortalin distribution is one common end point of the four different paths to immortalization. The isolation of *mot-1* cDNA and *mot-2* cDNA by immunoscreenings of cDNA libraries from normal and immortal cells, established from the same mouse, support that these are related to mortal and immortal phenotypes, respectively. Michikawa et al. [28] have reported that the two altered base pairs between *mot-1* and *mot-2* reside in the same exon of the gene thus possibility of the differential splicing of the gene to yield different transcripts can be ruled out. Since the two changes have also been found to be always linked in mouse cells and tissues, so far analyzed, by RT-PCR analysis (data not shown), the chance that these can arise by mutational events is unlikely. We have also found that the transfection of *mot-1* cDNA results in the induction of mortal phenotype in NIH 3T3 cells whereas transfection of *mot-2* in these cells is either neutral or induce transformed

phenotype at a low frequency ([17] and unpublished observations). Such differential activity of the transcripts suggests that they are unlikely to represent allelic polymorphisms. The genetic relation and regulation(s) that control the cytosolic expression in normal and the perinuclear expression in immortal mouse cells as well as the genetic routes and relationship between the forms of mortalin with different subcellular localization in immortal human cells will be adressed in the future study. Nevertheless, the present study describing the chromosomal assignments of mortalin gene on mouse and human chromosomes would be helpful in defining its in vivo function(s).

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