Identification of a Gene That Reverses the Immortal Phenotype of a Subset of Cells and Is a Member of a Novel Family of Transcription Factor-Like Genes†

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Based on the dominance of cellular senescence over immortality, immortal human cell lines have been assigned to four complementation groups for indefinite division. Human chromosomes carrying senescence genes have been identified, including chromosome 4. We report the cloning and identification of a gene, mortality factor 4 (MORF 4), which induces a senescent-like phenotype in immortal cell lines assigned to complementation group B with concomitant changes in two markers for senescence. MORF 4 is a member of a novel family of genes with transcription factor-like motifs. We present here the sequences of the seven family members, their chromosomal locations, and a partial characterization of the three members that are expressed. Elucidation of the mechanism of action of these genes should enhance our understanding of growth regulation and cellular aging.

Cellular senescence, the terminal nondividing state that normal cells enter following completion of proliferative potential, is the dominant phenotype in hybrids of normal and immortal cells (1a, 23, 24). Fusions of immortal human cell lines with each other have led to their assignment to at least one of four complementation groups for indefinite division, indicating a minimum of four genetic pathways to senescence (7, 25, 41). Microcell fusion studies have identified chromosomes 1 (group C), 4 (group B), and 7 (group D) as the loci of three of these cell senescence-related genes. The identification was based on the ability of the chromosome to induce senescence in multiple immortal cell lines assigned to one of the complementation groups, with no effect on the proliferation of cell lines assigned to the other groups (10, 20–22). We here describe the cloning and identification of a gene on chromosome 4, mortality factor 4 (MORF 4), that causes a senescent-like phenotype when introduced into two complementation group B cell lines. This gene is a member of a novel family of genes with transcription factor gene-like motifs. MORF 4 is expressed at low levels in all cell types examined, but its expression is higher in HeLa cell clones that lose proliferation following transfection with the gene. Two MORF-related genes (MRGs) that are more highly expressed demonstrate changes in RNA levels during the cell cycle and at senescence. The results suggest that these genes play a role in growth regulation and replicative senescence.

MATERIALS AND METHODS

Generation of the cell line A9+F4. The cell line A9+F4 was generated as follows. Microcell hybrids obtained following the introduction of an intact chromosome 4 into HT 1080, a fibrosarcoma cell line (group A), were analyzed cytogenetically, since HT 1080 has a pseudodiploid karyotype. One hybrid had chromosomal DNA in addition to the expected HT 1080 karyotype, and fluorescent in situ hybridization (FISH) analysis with a centromeric probe specific for chromosome 4 demonstrated this was a fragment of chromosome 4. This microcell hybrid was used as a donor in microcell fusion with A9 as the recipient cell to generate A9+F4. F4 DNA could not be detected by FISH with the centromeric probe in the A9+F4 cell line, indicating that not all of the original chromosome 4 DNA from the HT 1080 microcell hybrid was transferred. Pulsed-field gel electrophoresis revealed less than 800 kb of human DNA in A9+F4. *Alu* PCR products, generated from A9+F4 genomic DNA and used as a probe with a human monochromosomal hybrid panel, mapped only to chromosome 4 (16, 35). When this cell line was used as a donor in microcell fusion with immortal cell lines assigned to complementation group B, the fragment of human DNA caused loss of proliferation as efficiently as the intact chromosome.

Transfection of genes and analysis of PD achieved. The genomic equivalents of three cDNAs that mapped to the human DNA in A9+F4 were used in transfection studies. A 2.3-kb *NheI* band and a 7.5-kb *HindIII* band corresponding to cDNA 200901 from bacterial artificial chromosome (BAC) 526e7, a 4-kb *EcoRI* band corresponding to cDNA 231653 from BAC 526e7, and a 7.5-kb *HindIII* band corresponding to cDNA 195885 from BAC 316k7 were isolated by using the Qiaquick gel extraction kit (Qiagen) and cloned into the respective enzyme sites of pExSVNEO by standard ligation techniques. pExSVNEO was derived from pCMVex (34) by digestion with *SpeI* and *XbaI*, to remove the cytomegalovirus (CMV) promoter, and religation with a 38-bp oligonucleotide encoding the restriction sites *SpeI*, *ApaI*, *BglII*, *KpnI*, *NheI*, *NruI*, *SacII*, and *XbaI*. The simian virus 40 neo expression cassette was derived from pRC/CMV as a 1.5-kb *BamHI-EcoRI* fragment and cloned into a unique *DraII* site with *AscI* linkers.

Transfections were performed with Lipofectamine (Life Technologies) according to the manufacturer's directions. The cell culture conditions and estimation of population doublings (PD) were described previously (26).

FISH. FISH analysis was performed on normal human cells in the FISH core facility at Baylor College of Medicine. In the case of MORF 4, four separate BACs carrying the gene were used to determine 4q33.34.1 as the locus. These were done with two different normal individuals, and the same locus was identified on all four normal chromosomes.

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† This work is dedicated to the memory of Ruth Sager, a visionary scientist in the fields of senescence and tumor biology, among others.

Senescence-associated β -galactosidase and motalin staining of cells. Senescence-associated β -galactosidase staining and motalin staining were done according to previously published methods (5, 37, 38).

Oligomer probing of MRGs and confirmation of a frameshift mutation in MRG 1. Poly(A)⁺ RNA isolated from immortal cells of different complementation groups (EJ, HeLa, T98G, J82, CMV-MJ-HeL⁺ and SUSM1) and from young and senescent fibroblasts was reverse transcribed with the Ready-To-Go first strand beads (Pharmacia Biotech). cDNAs flanked by the 5' gen (GGAGGTG GCAAATCACTTATA) and 5' Race (GAATGGAATCCACATTCTTCTGG) primers were amplified by PCR, and 200 ng was transferred to a Hybond-N membrane (Amersham). The same primers were used to amplify BAC DNA corresponding to each family gene, and the amplicons were transferred to membranes. Oligonucleotides (50 pmol) were end labelled with [γ -³²P]ATP by using T4 polynucleotide kinase, and unincorporated nucleotides were removed by using NAP-5 columns (Pharmacia Biotech). Prehybridization, hybridization, and washing steps were performed at the same temperature, which had to be determined empirically for each oligonucleotide (2, 3, 9). Membranes were prehybridized for 30 min in 1 ml of rapid hybridization solution and 200 μ g of denatured salmon sperm DNA per ml, hybridized for 2 h in the presence of 10⁶ cpm of labeled oligonucleotide per ml. Membranes were washed twice for 10 min in 5 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.5% sodium dodecyl sulfate (SDS) and for 20 min in 2 $\times$ SSC-0.1% SDS and exposed for 2 h by autoradiography. The oligonucleotides were Mrg11 (5'-TGCAGAAAGA ATAAGAAC-3' [42°C]), Mrg5 (5'-TGCAGAAACAACCTCAAA-3' [45°C]), Mrg1-5'B (AGACCCCTCAGCTCCTTGGAA), and Mrg1-3'B (GACATG GGTGCATCGGGATG). Genomic DNA was amplified with the above primers by using Pfu polymerase. The amplification conditions were 95°C for 3 min, followed by 30 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 2 min and a final incubation at 72°C for 10 min. The amplicon with a size of about 400 bp was resolved in agarose and purified with the Qiaquick gel purification kit (Qiagen). The purified products were sequenced with the Mrg1-5'B primer. BACs 328i14 and 364h20 from the human genomic DNA library (Genome Systems, Inc.) and two monochromosomal hybrid cell lines which carry chromosome 1, A9+1 (from J. Carl Barrett, National Institutes of Environmental Health Sciences, Research Triangle Park, N.C.) and GM 10880 (from the Coriell Institute for Medical Research, Camden, N.J.), were analyzed.

Database analysis for motifs in DNA sequence. BLAST searches were performed to identify related genes. A profile scan revealed the bipartite nuclear localization signals in MORF 4 and MRG 15, and Scan Prosite identified the phosphorylation sites, leucine zipper, and helix-loop-helix (HLH) regions. The chromodomain of MRG 15 was predicted by the BLOCKS program (Expasy) as well as hydrophobic cluster analysis (15, 42).

Tagging of the MORF 4, MRG 15, and MRG X genes. The MORF 4, MRG 15, and MRG X genes were tagged with an enhanced green fluorescent protein at the 3' end of the open reading frame by PCR. Cells were transiently transfected with these constructs by using the reagent Lipofectamine (Life Technologies) according to the manufacturer's directions, and the cells were observed under a fluorescence microscope for green fluorescence.

RT-PCR and in gel hybridization analysis. MORF 4 underwent reverse transcription (RT) from 5 μ g of total RNA with the M4-3'B primer (ATTGTGTG AGTAGCTGGGTG) with the Superscript II preamplification kit (Life Technologies) according to the supplier's protocol. PCR was performed with 2 μ l of the RT mixture in a 50- μ l total volume with the primers M4-L1 2205 (CAGA GGTGCAATCCTAGTC) and 386 5'RACE (GAATGGAATCCACATTCTT CTTGG) in 1 $\times$ PCR buffer (Life Technologies), containing 1.5 mM MgCl₂, 200 μ M deoxynucleoside triphosphates, and 2.5 U of Platinum *Taq* DNA polymerase (Life Technologies). The reaction mixtures were denatured at 94°C for 2 min and subjected to 40 cycles of 94°C for 15 s, 58°C for 30 s, and 72°C for 55 s, followed by 10 min at 72°C. Twenty microliters of the PCR mixture was run out on a 1.25% agarose gel and visualized either with ethidium bromide or with the oligomer probe CAGTGAGACTCCCCAGCCTCCTCGAAAG, which is specific for MORF 4 (4, 11).

The cell lines tested included HCA2; normal human diploid fibroblasts at PD of 23, 67, and 87 (senescent); early-PD normal fibroblasts CSC303, IMR90, GRC 173, and GRC 536; immortal cell lines GM639, EJ (group A), GM 2096, HeLa, J82, Mli019, T98G, UAB Co23 (group B), CMV-MJ-HeL⁺, 143BTK⁻ (group C), A1698, and SUSM1 (group D) (25); and three HeLa+MORF 4 clones which had ceased proliferation and one which was unaffected. Mli019 and UAB Co23 were assigned to group B on the basis of their motalin staining pattern (38).

Northern analysis. For Northern analysis, total RNA was harvested from normal human foreskin fibroblasts (HCA2) at various PD. In the cell cycle analysis, young HCA2 cells (PD 23) were made quiescent by removal of serum growth factors for 1 week. The cells were then stimulated with 10% serum and harvested at various times.

RESULTS

Identification of MORF 4 as a gene reversing the immortal phenotype in a subset of immortal human cells. *Alu* PCR probes generated from A9+F4 genomic DNA were used as a probe on high-density filters of a human genomic library

cloned into a BAC vector (Genome Systems, Inc.). Seventeen positive BAC clones were identified and aligned in a contig spanning the break points of the fragment (manuscript submitted). BACs used as probes for FISH were found to localize to the chromosome 4q33-34.1 border. *Alu* PCR probes from A9+F4 had localized to the same region by FISH. Interestingly, studies of normal and matched bladder and head and neck carcinomas had indicated loss of heterozygosity in this region (27, 28).

cDNAs encoded by the genomic contig were identified by probing 24 I.M.A.G.E. consortium cDNA libraries arrayed on high-density filters (Genome Systems, Inc.) with inserts from representative BACs (1). We also used a subset of BACs in a PCR-based screen of a normal human brain cDNA library and obtained the same cDNAs that were isolated from the filters, confirming the correct identification of the cDNAs (36). cDNAs containing repetitive elements were eliminated from further analysis, and the dbEST and UNIGene EST (National Center for Biotechnology Information) cluster databases allowed identification of cDNAs representing the same gene. The cDNA with the largest insert for each gene was obtained from Genome Systems, Inc., and used as probe in Southern analysis of the BACs encompassing the contig. Six cDNAs were found to have coding sequence within the contig (1). One spanned the junction break point in A9+F4 and was eliminated from further analysis. The other cDNAs were used to probe multitissue Northern blots (Clontech). One was expressed only in placenta and was eliminated as a candidate gene. We arbitrarily chose and used genomic DNA encoding three of the four remaining cDNAs (I.M.A.G.E. clone identification no. 200901, 195885, and 231653) in our initial transfection studies.

We had previously monitored the effect of the complementation group B gene under the control of its own promoter when introducing either the intact chromosome 4 or the chromosome fragment. We therefore used genomic DNA rather than the cDNAs in the transfection experiments. This eliminated potential complications of overexpression of a gene from a strong promoter, such as CMV, resulting in proliferative loss or cell death. Indeed, we subsequently found that expression of a CMV-driven construct encoding the gene that caused loss of proliferation in a subset of immortal human cells also caused nuclear fragmentation within 12 h following transient transfection. Two genomic fragments of BAC 526e7 (encoding a homolog of cDNA 20091) when transfected into immortal cell lines representative of all the complementation groups suppressed the immortal phenotype in two group B cell lines, with no effect on six cell lines assigned to the other groups (Table 1). We refer to the gene as MORF 4. Transfection of the genomic equivalents of cDNAs 231653 and 195885 had no effect on proliferation of any of the cells. The MORF 4-transfected clones achieved between 19 and 35 PD before ceasing proliferation. The cells then morphologically resembled senescent cells and were positive for the senescence-associated β -galactosidase activity (Fig. 1a to d) (5). Furthermore, the motalin staining pattern, which distinguishes normal from immortal cells as well as identifying the complementation group to which immortal cells are assigned, had reverted to that of a normal cell (38). The perinuclear staining usually observed in group B cells (Fig. 1e) was restored to the uniform cytoplasmic staining characteristic of the normal cell (Fig. 1f and g).

Transfection of a mutant with a frameshift in the start codon of the 2.3-kb *NheI* fragment of BAC 526e7, which disrupts the open reading frame and thereby production of a protein, the vector control or other genomic DNAs and cDNAs had minimal to no senescence-inducing activity on any of the cell lines

TABLE 1. Transfection of immortal human cell lines

Cell line	Complementation group	DNA transfected ^a	No. of clones that lost proliferation/ total no. of clones	Range of PD achieved prior to loss of proliferation
EJ	A	MORF 4 Other DNAs	0/10 0/15	
GM639	A	MORF 4 Other DNAs	0/10 0/6	
HeLa	B	MORF 4 Frameshift mutant Other DNAs	29/44 2/12 8/73	18–35 27–30 19–28
T98G	B	MORF 4 Frameshift mutant Other DNAs	25/33 0/10 3/34	19–33 26–33
CMV-MJ-HeL1	C	MORF 4 Other DNAs	0/12 0/22	
143BTK ⁻	C	MORF 4 Other DNAs	0/7 0/9	
A1698	D	MORF 4 Other DNAs	0/10 0/10	
SUSM1	D	MORF 4 Other DNAs	1/10 0/16	19

^a The control DNAs (other DNAs) included empty vectors pCMVexSVneo, pCDNA, cDNA 386h22, cDNAs, and genomic equivalents of I.M.A.G.E. clone identification no. 231653 and 195885 and XON1 and MRG 1. MORF 4 transfections include transfer of both *NheI* and *HindIII* fragments.

tested (Table 1). These controls were included to determine the number of transfectants that would lose proliferation because of positional effects rather than the activity of the gene itself and because some percentage of clones from untreated immortal cell lines exhibit limited division potential (17). The results indicate that the fragment of genomic DNA from BAC 526e7 contains a gene, MORF 4, that is capable of reversing the immortal phenotype of a subset of immortal cells. The fact that some clones did not cease proliferating following the introduction of the gene is not surprising, because it takes a single immortal variant cell in the population to mask the senescence phenotype. Similar results have been obtained in whole-cell and microcell fusion studies (1a, 7, 10, 20–25, 41). The strength of these analyses lies in the fact that the majority of the clones ceased proliferating.

Sequencing of the genomic clones revealed one intronless open reading frame with a 3' untranslated region and a poly(A)⁺ track (GenBank accession no. AF100614). Interestingly, the MORF 4 gene is inserted into a LINE 1 (L1) element very similar to L1.39 (33), and direct repeats corresponding to bases 2262 to 2270 of the L1.39 sequence flank the gene. Within the L1 sequence 5' of the MORF 4 gene, two putative promoters (distinct from the L1 promoter) were identified by using the Neural Network Promoter Prediction program (29–31). Neither of these promoters is the L1 promoter. One is located 476 bases upstream of the MORF 4 gene within the 5' direct repeat with a putative transcription start 420 bases upstream. The other is located 65 bases downstream of the MORF 4 5' repeat.

Identification of MRGs. The MORF 4 sequence differed slightly from that of cDNA 200901 (GenBank accession no. AF100615), indicating they were members of a family of genes, which we refer to as MRG. A major difference between the protein products of these two genes is caused by a single base deletion in MORF 4. This results in a stop codon at nucleotide

233 with the probable use of the second ATG start site and production of a protein lacking 88 amino acids of the N-terminal sequence encoded by cDNA 200901 (Fig. 2). A search of the database by using the cDNA 200901 and MORF 4 sequences revealed an expressed sequence tag (GenBank accession no. AF100620) with sequence very similar to that of these genes, except for the first 150 to 82 bp, respectively. The overall sequence identity of cDNA 200901 and D14812 at the DNA level 72%, and the identity at the protein level was 63% (Fig. 2). Using probes that distinguished MORF 4 and cDNA 200901 from D14812 (Fig. 3a), we screened high-density BAC filters from Human Genome Systems, Inc. Eighteen BACs, in addition to and different from the ones encoding MORF 4, were identified, and these were reduced to four groups. Ten BACs similar to the D14812 probe were reduced to two groups. FISH analysis mapped the various MRG to chromosomes 1q4.1-4.2 (MRG 1; GenBank accession no. AF100616), 5p14-15.1 (MRG 5; GenBank accession no. AF100618), 11ptelomere (MRG 11; GenBank accession no. AF100619), 15q24 (MRG 15; GenBank accession no. AF 100615), 4q1.2 (MRG 4; GenBank accession no. AF 100617), and Xq22 (MRG X; GenBank accession no. AF 100620).

Sequencing of the genomic regions corresponding to chromosomes 1, 4q1.2, 5, and 11 revealed that these were intronless and flanked by short L1 element sequence fragments and most likely processed pseudogenes which were not transcribed since they all lacked promoter sequences. There were also base changes, deletions, and insertions in the sequences of MRG 4, 5, and 11, indicating they would not code for a functional protein. Indeed, analysis with specific 18-mer probes indicated that although the MRG 5 and MRG 11 genes could be amplified from the corresponding BACs by PCR, they were not expressed in young or senescent normal cells and various immortal human cells (data not shown). Probes specific for MRG 1 could not be designed, because MRG 1 is highly homologous

FIG. 1. Analyses of MORF 4-transfected cells. Senescence-associated β -galactosidase staining of a stably transfected clone of HeLa+MORF 4 cells at 20 PD (a), 30 PD (b), and 35 PD (c), when the cells had ceased proliferation. (d) Four weeks after the cells had stopped dividing. (e to g) Mortalin staining of HeLa cells (e), normal human fibroblasts (f), and a stably transfected HeLa+MORF 4 cell that had ceased division (g). (h) Fluorescence 8 h after transfection of EJ cells transiently transfected with the MORF 4 green fluorescent protein-tagged construct. (i) Fluorescence 12 h after transfection.

to cDNA 200901, which is encoded by MRG 15. However, analysis of three independent sources of normal human genomic DNA demonstrated there is a stop codon at nucleotide 627, suggesting that if this processed pseudogene is transcribed, a truncated protein will result from the transcript. Analysis of 2 kb of sequence 5' of MRG 1 revealed no promoter elements and multiple independent *Alu* and L1 fragments, indicating it is not likely to be transcribed. MRG 15 and MRG X contain introns and encompass at least 5 and 4.5 kb of DNA, respectively. MORF 4 and MRG 1, 5, and 11 were most likely derived from MRG 15, and MRG 4 was most likely derived from MRG X.

The motifs common to the predicted proteins MORF 4, MRG 15, and MRG X include an HLH region, within which lie three separate regions of homology to and in the same spatial order of the *Euplotes crassus* telomere binding subunit p51 (39) and a leucine zipper region (Fig. 2). Both MORF 4 and MRG 15 contain a bipartite nuclear localization signal (NLS) flanked by phosphorylation sites, whereas MRG X contains a single NLS. In addition, MRG 15 encodes a chromatin organization

modifier (chromo) domain at its amino terminus, which is absent in MORF 4 and MRG X.

Zoo blot analysis with MRG 15- or MRG X-specific 132-bp probes designed at the 5' end of their open reading frame showed that they are conserved from human to chicken (data not shown). However, when the MRG 15 sequence was compared to those in the BLASTP database, the most homologous sequences included two predicted yeast homologs (Z98977 for *Schizosaccharomyces pombe* and ypr023c for *Saccharomyces cerevisiae*). Additional homologous sequences were a Cu^{2+} -transporting ATPase homolog in *Arabidopsis thaliana*, the *Drosophila* male-specific lethal-3 protein (msl-3), and the human retinoblastoma binding protein 1 isoform I (RBP1) and retinoblastoma-associated protein 2 (RBP2). The yeast and *Arabidopsis* genes had homology to the entire MRG 15 cDNA, although the *Arabidopsis* gene had an additional 2 kb of non-homologous sequence. The similarity to RBP1 and RBP2 was entirely in the chromodomain.

MORF 4, MRG 15, and MRG X proteins localize to the nucleus. The coding sequences of the MORF 4, MRG 15, and MRG X genes were cloned into the pEGFP-N1 vector (Clontech), which results in an enhanced green fluorescent protein tag at the C terminus of the proteins. When the MORF 4 construct was transiently transfected into HeLa cells, the protein was targeted to the nucleus (Fig. 1h), consistent with the NLS motif. However, monitoring of the cells over time following transfection demonstrated that overexpression of this gene ultimately caused abnormal nuclear morphologies (Fig. 1i) and cell death. The MRG 15 and MRG X proteins also localized to the nucleus (data not shown), following transient transfection. Overexpression of MRG 15 also resulted in abnormal nuclear morphologies and cell death (data not shown), but the effect was less severe than that observed with MORF 4. Overexpressed MRG X did not change nuclear morphology or kill cells.

Expression of MORF 4, MRG 15, and MRG X mRNAs. The nucleic acid sequence of the MORF 4 transcript contains L1 sequence upstream of the 5' direct repeat and is approximately 96% identical to that of MRG 15 downstream of the 5' direct repeat. This includes the 3' untranslated region, which is 95% conserved between the two genes, suggesting a possible role in the regulation of these genes. Despite this high level of conservation, we were able to analyze the MORF 4 transcript by the use of RT-PCR utilizing primers specific to MORF 4. The MORF 4 transcript, detected by in gel hybridization of the PCR products with a 24-mer probe that distinguishes MORF 4 from MRG 15, was present at very low levels in multiple normal and immortal human cell lines analyzed. However, HeLa cells which had stopped proliferating after the stable transfection of the genomic fragment of MORF 4 appeared to express higher levels of the transcript, because fewer PCR cycles were needed for detection. Furthermore, a mutant MORF 4 construct with a single base insertion in the putative translation start codon, which should result in the loss of production of a functional protein, did not cause the induction of the senescent-like phenotype seen with the wild-type gene (Table 1). These results together indicate that the production of a functional protein is responsible for the observed effects.

Expression of MRG 15 and MRG X could be detected by Northern analysis with the 5'-specific probes for each (Fig. 3A). The results demonstrate that the MRG 15 gene encodes two transcripts at 1.8 and 1.4 kb, whereas the MRG X gene encodes a single transcript at 1.8 kb (Fig. 3). The less-abundant transcript at 1.4 kb detected by MRG 15 may be an alternative spliced variant or a truncation due to use of an alternate polyadenylation site. Expression of both transcripts of MRG 15 and

FIG. 2. Comparison of the protein sequences and predicted motifs in the expressed MRG and MORF genes. The various predicted motifs are indicated. +, potential cyclic AMP (cAMP) phosphorylation site; ‡, protein kinase C phosphorylation site; ¥, a tyrosine phosphorylation site. The three regions of homology to the *E. crassus* telomere binding subunit p51 within the HLH domain are underlined.

the single MRG X transcript declined slightly in normal senescent fibroblast cell populations and was also decreased in young normal fibroblast cells made quiescent by removal of serum growth factors (Fig. 3B and C). The RNA levels of MRG 15 increased ~2 fold at 4 to 8 h post serum stimulation of the normal quiescent cells and remained at this level up to 28 h poststimulation (Fig. 3C). MRG X expression, in contrast, increased 8- to 10-fold at 4 h after serum stimulation, remained at this level at 8 h, began to decline at 18 h, and was low 28 h

later (Fig. 3C). Expression of the MRGs was not significantly different in various immortal human cell lines analyzed and showed no correlation with complementation group assignment (data not shown). The levels of expression of these genes varied in different tissues (Fig. 3D), the significance of which is currently not clear.

To determine whether MRG 15 might have biological activity similar to MORF 4 (although this seemed unlikely, since RNA levels decrease with senescence), we used microcell fu-

FIG. 3. Analysis of RNA levels of MRG 15 and MRG X by using probes specific for each gene. (A) Dot blot analysis of BAC and cDNA encoding MRG 15 or MRG X probed with 132 bp from the 5' end of each gene demonstrates the specificity of the probes. (B) RNA levels of MRG 15 and MRG X with increasing PD. (C) RNA levels of MRG 15 and MRG X in normal human fibroblast cells (PD 23) made quiescent by removal of serum growth factors for 1 week and then stimulated with 10% serum. The RNA was harvested 0, 4, 8, 18, and 28 h after serum stimulation. (D) RNA levels of MRG 15 and MRG X in various tissues. Poly(A)⁺ RNA blots were obtained from Clontech. Lanes from left to right: heart (h), brain (br), placenta (pl), lung (lu), liver (li), skeletal muscle (sk), pancreas (pa), spleen (s), thymus (th), prostate (pr), testis (te), ovary (ov), small intestine (si), colon (co), and peripheral blood lymphocytes (pbl). In panels B and C, total RNA was used and 28S RNA was the loading control.

sion to introduce a normal human chromosome 15 into HeLa cells. No effect on proliferation of the microcell hybrids was observed (data not shown). We did not test a normal chromosome X in such fusions, because of the negative result with chromosome 15, and the fact that RNA levels of this gene also decline with senescence. Additionally, although chromosome X has been implicated in causing loss of proliferation in a nickel-transformed Chinese hamster cell line (13), no effect on human cells has been reported. The position of MRG X at Xq22 is also not consistent with the proposed location of the hamster senescence gene, which is very close to the centromere and most likely on the p arm (40).

DISCUSSION

MORF 4 is an intronless, functional gene inserted in an L1 element and appears to be transcribed from a promoter that is not the same as the L1 promoter. Although the literature on processed pseudogenes indicates that most are not transcribed because of lack of promoters and will not be translated into a

functional protein due to frame shifts, point mutations, insertions, or deletions, there are examples of such functional genes. These include the testis-specific phosphoglycerate kinase gene, the calmodulin-like gene in human cells, and the ferritin L subunit in the mouse (4, 18, 19, 32, 43). The genomic fragments we used for transfections did not affect cell proliferation as rapidly as the intact chromosome, in that cells ceased dividing after 19 PD when the genomic DNA was transfected, whereas microcell hybrids lost proliferation as rapidly as 4 PD after introduction of the chromosome. This may be due to the fact that the genomic constructs used for transfection lack some enhancer elements that lie further upstream or that another gene in the region encoded by F4 contributes to and causes the induction of senescence. Additionally, since MORF 4 does not have classic tumor suppressor gene characteristics (e.g., RNA is expressed in cell lines that lose proliferation following transfection of the gene), we cannot conclude that it is the complementation group B gene. However, it clearly has negative growth regulatory function in that subset of immortal human cells.

MRG 15 is of particular interest, because it encodes a chromodomain. The chromodomain has been previously identified in several proteins which act as negative or positive regulators of transcription. Many of these chromatin regulators bind to specific loci in chromatin, although they do not seem to bind DNA in a sequence-specific manner, but rather are recruited to these sites in multiprotein complexes. It has been hypothesized that the chromodomain helps deliver positive or negative transcription regulators to the sites of action on chromatin (12, 14). The homology to the *Drosophila* msl-3 protein is interesting because it is in a region preceding the chromo shadow domain of msl-3. This protein has been implicated in the regulation of dosage compensation in *Drosophila* by acting in a multimeric complex which binds to hundreds of specific sites on the male X chromosome and induces hypertranscription (8). Thus MRG 15 has the potential to cause global changes in gene expression in cells.

The fact that both MRG 15 and MRG X are regulated during the cell cycle is also of relevance. Young cells 4 to 8 h poststimulation are in the early to mid-G₁ phase of the cell cycle. Cells begin to enter S phase at 16 h postexposure to serum, and DNA synthesis is maximal at 24 h. Thus, it appears that upregulation of MRG 15 may be needed for cell cycle progression and that MRG X expression initially increases but may have to decrease for cells to enter S phase.

The data taken together indicate that MORF 4 encodes a protein that is involved in causing loss of proliferation in a subgroup of immortal cells that regain limited division potential after the gene is restored. Since the protein is localized in the nucleus and has a leucine zipper motif, it has the potential to bind DNA and/or form transcriptionally active homo- or heterodimers. Thus, MORF 4 could act as a transcription factor that either directly upregulates genes necessary to stop division or downregulates genes required for cell cycle progression. Alternatively, it could interact with other proteins, such as MRG 15 and MRG X, and thereby inhibit or enhance their function, resulting in loss of cell division. The similarity to an N-terminally truncated MRG 15 raises the possibility that it may act in a dominant negative manner in transcriptional complexes involving MRG 15. If MORF 4 prevented the chromatin-modifying activity of the chromodomain of MRG 15, it could cause major changes in gene expression, such as those observed when cells become senescent. Elucidation of the precise mechanism of action of these genes and determining whether they act together, competitively, or independently in transcriptional control and DNA binding will enhance our

understanding of the regulation of cell growth control, cellular senescence, and cancer.

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