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Structure, expression and regulation of the murine 4F2 heavy chain

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ABSTRACT

The murine 4F2 molecule is a 125 kilodalton disulfide-linked heterodimeric cell-surface glycoprotein which has been shown to be involved in the processes of cellular activation and proliferation (1). To elucidate the structure, expression, and regulation of the 4F2 molecule, a murine 4F2 heavy chain (4F2HC) cDNA has been isolated and structurally characterized. The murine 4F2HC is a 526 amino acid (aa) type II membrane glycoprotein which is composed of a 75 aa N-terminal intracytoplasmic region, a single hydrophobic putative transmembrane domain, and a 428 aa C-terminal extracellular domain. Comparison with the human 4F2HC cDNA reveals the highest degree of sequence identity within the transmembrane and intracytoplasmic domains. Northern blot analyses have demonstrated that the 4F2HC gene is expressed at relatively high levels in adult testis, lung, brain, kidney, and spleen, and at significantly lower levels in adult liver and cardiac and skeletal muscle. Studies designed to elucidate the pattern of regulation of the murine 4F2HC gene have demonstrated that it is induced during the process of cell activation, but is subsequently expressed at constant levels throughout the cell cycle in exponentially growing cells.

INTRODUCTION

The 4F2 cell-surface antigen is a 125 kilodalton (kd) heterodimeric glycoprotein which is composed of a 90 kd glycosylated heavy chain (4F2HC) and a 35 kd non-glycosylated light chain (4F2LC). The 4F2 molecule belongs to the set of inducible cell-surface proteins which are involved in the processes of cell growth and proliferation. For example, 4F2 is expressed at relatively low levels on the majority of quiescent cells *in vivo*. However, it is expressed at high levels on all established tissue culture cell lines, and most if not all malignant human cells (2-8). Similarly, human 4F2 is an early B and T cell activation antigen which is expressed at very low levels on resting peripheral blood B and T lymphocytes. However, 4F2 expression is rapidly induced following lectin or antigen-mediated activation of these cells (3,7,9,10). The importance of 4F2 in the process of cell proliferation has been underscored by the finding that monoclonal antibodies directed against

human 4F2 heavy chain epitopes are able to inhibit lectin-induced T-cell proliferation (2) as well as the proliferation of a variety of tumor cell lines in vitro (8). While 4F2 expression has been correlated with the state of cell activation, the cell cycle dependence of 4F2 expression remains controversial. Several studies have reported that cell-surface expression of 4F2 is induced predominantly during the transition from G_0 to G_1 and does not require entry in to the S phase of the cell cycle (10,11). In contrast, other studies have suggested that 4F2 is preferentially expressed during the S phase of the cell cycle (12).

Several reports have suggested that 4F2 may mediate its effects on cellular activation by modulating the concentration of intracellular ionized calcium. Posillico et al. (13) have shown that incubation of human parathyroid adenoma cells with a murine anti-4F2 monoclonal antibody that is directed against a human 4F2HC epitope (2) results in an increase in intracytoplasmic ionized calcium, and an associated decrease in parathyroid hormone secretion by these cells. Michalak and coworkers (14) have reported that incubation of bovine skeletal muscle or sarcolemmal vesicles with anti-4F2 monoclonal antibodies inhibits sodium-dependent calcium exchange by these vesicles. Thus, these investigators postulated that 4F2 may be the sodium-calcium exchange structure or, alternatively, may regulate the activity of this exchanger.

We and others have recently reported the cloning of the human 4F2HC cDNA (12,15-16). The human 4F2HC is a 529 aa type II membrane glycoprotein which is composed of an 81 aa N-terminal intracytoplasmic region, a single hydrophobic transmembrane domain, and a 429 aa C-terminal extracellular domain. No homology has been detected between the human 4F2HC gene and other known genes or proteins including soluble ligand receptors, or ion channels. Thus, while these studies have provided valuable information concerning the structure of the 4F2HC, little has been learned about the function of the molecule. In order to further define important structural and regulatory features of the 4F2 molecule, we have isolated and characterized a murine 4F2HC cDNA clone. Structural analyses of this 1.8 kilobase (kb) cDNA have demonstrated that the murine 4F2HC is a 526 amino acid (aa) type II membrane glycoprotein which has been highly conserved during mammalian evolution. Comparison of nucleic acid and protein sequences of the human and mouse 4F2HC cDNAs has identified regions which display a high level of sequence identity which may be important in the function of the 4F2 molecule. In addition, we have utilized the murine 4F2HC cDNA as a molecular probe in studies of the tissue distribution of this

gene in vivo. Finally, the expression of the murine 4F2HC gene during cell cycle progression of 3T3 fibroblasts has been examined both during serum induced cell activation, and during exponential growth.

MATERIALS AND METHODS

Isolation of Murine 4F2HC cDNA clones

The previously described full length human 4F2HC cDNA JIFB (15), was radiolabelled by random hexanucleotide priming (17) and hybridized to 3.0 x 10^5 recombinant plaques from a λgt11 ICR strain murine spleen cDNA library (Clontech Labs., Palo Alto, CA). Low stringency hybridization was performed in 6X SSC (1X SSC = 150 mM NaCl, 15 mM Na-citrate)/5X Denhardt's solution/0.1% SDS/100ug/ml denatured salmon sperm DNA at 50°C. Final washing conditions were 0.5X SSC/0.1%SDS at 50°C for 60 minutes. One positively hybridizing clone (M4F2HC-1A) containing a 700 base pair (bp) insert was subsequently used to screen approximately 3 x 10^5 recombinant plaques from a λgt10 cDNA library prepared from the murine 70Z/3 (C57BL/6 x DBA/2) pre-B cell line (generously provided by Dr. T. Bender) (18) and a λgt11 ICR strain murine macrophage cDNA library (Clontech Labs., Palo Alto, CA). Six positively hybridizing plaques were isolated from the 70Z/3 cDNA library, and forty positively hybridizing plaques were identified in the macrophage cDNA library. These plaques were purified to homogeneity by sequential hybridization with the same probe and characterized by restriction enzyme analysis as previously described (19). Two of these clones (M4F2HC-27 and M4F2HC-50) were shown to contain 1.6-1.8 kilobase (kb) inserts and were used for all subsequent experiments.

DNA Sequence Analysis

Three methods were used to sequence the murine 4F2HC cDNAs: (i) Appropriate restriction fragments were subcloned directly from low melting point agarose (20) into M13mp18 or M13mp19 for single stranded DNA sequencing by the dideoxy chain termination method of Sanger (21). (ii) Double stranded DNA sequencing using synthetic oligonucleotides complementary to both strands of the 4F2HC cDNA as determined in (i) above was performed following subcloning of the murine 4F2HC cDNAs into the EcoRI site of pUC13 (22). (iii) Finally, the 3' end of the 4F2HC cDNA was sequenced in λgt11 using synthetic oligonucleotide primers. To sequence in the 5'-3' direction a primer complementary to base pairs 1703-1719 of M4F2HC-50 (5'-dTTTGTTCTCCCCAGGCC-3') as determined in (i) and (ii) above was synthesized. To sequence in the 3'-5' direction a primer immediately 3' to the EcoRI cloning site of λgt11 (5'-dCAGACATGGCCTGCCC

GGTTATTA-3') was synthesized. 25 pmol of [^{32}P] end-labelled synthetic oligonucleotide primer was added to 2-3 ug of the recombinant phage and denatured for 10 minutes at 100°C. The reaction mixture was allowed to anneal for 20 minutes and DNA sequencing was performed by the method of Mehra et. al. (23). All restriction enzyme sites were crossed, and all clones were sequenced on both strands.

Northern Blot Analyses

One day old and eight to nine week old BALB/cJ mice (Jackson Labs, Bar Harbor, ME) were sacrificed by cervical dislocation, and the appropriate organs were excised, rinsed in ice cold phosphate buffered saline (PBS) and frozen in liquid nitrogen. RNA from brain, kidney, liver, lung, heart, skeletal muscle, testis and 155.16 cells (C57BL/6 x AKR/J) (24) was prepared by the lithium chloride/urea method of Auffray and Roujeon (25). RNA from spleen and thymus was isolated by the guanidine-HCl method of Strohman et. al (26) and RNA from pancreas was isolated using the guanidinium isothiocyanate method of Chirgwin et al (27). RNA was initially quantitated by spectrophotometry at $E_{260/280}$ with the final quantitation of RNA documented by ethidium bromide staining of 1.2% non-denaturing agarose gels. RNA was fractionated on formaldehyde-containing 1.5% agarose gels and transferred to Hybond N (Amersham, Arlington Heights, IL) nylon membranes as previously described (15). Hybridizations using random hexanucleotide primed [^{32}P]-labelled probes (17) (10^9 cpm/ug) were performed in 3X SSC/50% formamide/5X Denhardt's solution/0.1% SDS/100ug/ml denatured salmon sperm DNA at 42°C. Final washing conditions were 0.1X SSC/0.1% SDS, 55°C for 60 minutes. Probes included the murine 4F2HC cDNAs M4F2HC-27 and M4F2HC-50, the 1.6 kb EcoRI/BamHI 28S rRNA probe (28), the 700 bp murine 4F2HC cDNA M4F2HC-1A, the 1.0 kb ClaI/EcoRI fragment containing the third exon and 3' untranslated region of the human c-myc proto-oncogene (the generous gift of Dr. David Bentley) and a 2.3 kb HindIII/BamHI fragment containing the histone 2b (h2b) gene (29) . Autoradiographs were quantitated using an LKB Model 2200 XL densitometer in conjunction with Gel Scan software (LKB Inc., Bromma, Sweden).

Southern Blot Analyses

High molecular weight DNA was prepared from the murine T-cell hybridoma 155.16 (24), BALB/c, CBA, C57BL/6, and DBA/2 murine livers as previously described (15). 10 ug of high molecular weight DNA was digested overnight with a five-fold excess of the appropriate restriction endonucleases according to the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN), fractionated on 0.8% agarose gels and transferred to nitrocellulose filters as

previously described (15). [^{32}P]-labelled (10^9 cpm/ug) M4F2HC-50 murine 4F2HC cDNA probe was synthesized by random hexanucleotide priming (17). Hybridizations were carried out in 5X SSC/50% formamide/5X Denhardt's solution/5% dextran sulfate (Oncor, Gaithersburg, MO)/100ug/ml denatured salmon sperm DNA at 42°C for 16-20 hours.

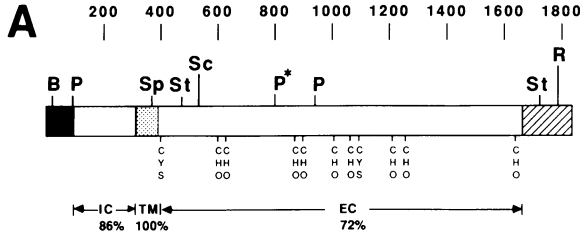
Cell cycle experiments

NIH 3T3 cells were grown to confluence in αMEM medium (Gibco Laboratories, Grand Island, NY) containing 10% fetal bovine serum (FCS). Three days after reaching confluence, cells were deprived of serum for 12 hrs. Following serum deprivation, 15% FCS was added back to the culture medium and aliquots of cells were harvested for RNA at 0, 1, 3, 6, 12, 24, and 36 hrs following serum-induced activation. In addition, RNA was prepared from a culture of exponentially growing 3T3 cells. These RNA samples were subjected to Northern blot analyses as described above. In a second series of experiments, exponentially growing cultures of BALB/c 3T3 cells were separated into subpopulations representing the different stages of the cell cycle by counterflow centrifugation as previously described (30). To confirm that cells were separated into distinct subpopulations representing progressive stages of the cell cycle, cells were stained with propidium iodide and the DNA-staining profile analyzed by fluorescence activated cell sorting (data not shown). RNA isolated from each fraction was analyzed by Northern blot analysis as described above.

RESULTS

Isolation and Structural Characterization of murine 4F2HC cDNAs

Murine 4F2HC cDNAs were isolated by low stringency hybridization with the full-length human 4F2HC cDNA clone J1FB (see Experimental Procedures). A consensus 1835 base pair (bp) murine 4F2HC cDNA sequence was deduced from the detailed sequence analysis of several cDNA clones (Fig. 1). M4F2HC-50 is an 1835 bp clone isolated from the 70Z/3 murine pre-B cell cDNA library, while, M4F2HC-27 is a 1620 bp clone which was purified from a murine macrophage cDNA library. The consensus murine 4F2HC cDNA sequence contains a 1578 bp open reading frame beginning with the 5′ most ATG at bp 93. While the N-terminus of the murine 4F2HC protein has not been determined, we favor the hypothesis that the ATG at bp 93 is the initiation codon, both because it is the 5′ most ATG, and because it conforms to the consensus initiation codon as described by Kozak (31). The cDNA contains 92 bp of putative 5′ untranslated and 165 bp of 3′ untranslated sequence. There is a consensus polyadenylation signal


A
200
400
600
800
1000
1200
1400
1600
1800
Sc
R
B P
Sp St
P*
P
St
IC
TM
EC
86%
100%
72%

B

(AATAAA) at bp 1803 which is followed 20 base pairs downstream by a poly(A) tail. Of note, one 4F2HC cDNA (M4F2HC-59) was found to contain a poly(A) tail at base pair 1780 (vertical arrow Fig. 1B). The frequency of use of this alternative polyadenylation site is unknown. However, it was only observed in one of the six cDNA clones analyzed. A homology search of gene (Genbank) and protein data bases (NBRF) failed to reveal significant homology between the murine 4F2HC and other known nucleic acid and protein sequences. Low level homology was observed between the 4F2HC and members of the Drosophila hdl gene family (32). The significance of this finding remains unclear.

Restriction endonuclease mapping of 4F2HC cDNAs M4F2HC-27 and M4F2HC-50 (Fig. 1A) revealed that M4F2HC-27 isolated from a λgt11 ICR strain murine spleen cDNA library contains a PstI site at bp 791 which is not present in M4F2HC-50 which was isolated from a λgt10 70Z/3 (C57BL/6 x DBA/2) murine pre-B cell cDNA library. DNA sequence analysis of this region of both clones revealed that base pair 786 of M4F2HC-27 is a thymidine, while M4F2HC-50 contains a deoxycytidine at this position. This nucleotide substitution does not alter the amino acid sequence of the 4F2HC proteins encoded by these two cDNAs. In order to determine whether this substitution represents a true sequence polymorphism as opposed to a cloning artifact, a Southern blot

Figure 1. The primary structure of the murine 4F2HC cDNA. A. A partial restriction endonuclease map and schematic representation of the 4F2HC cDNA. The size of the cDNA in base pairs is shown above the map. BamHI (B), PstI (P), SphI (Sp), StuI (St), ScaI (Sc), and EcoRI (R) restriction enzyme sites are shown above the map. The PstI site, (P^*), at base pair 791 is a strain specific sequence polymorphism which is present in 4F2HC cDNA clone M4F2HC-27 and absent in clone M4F2HC-50 (see discussion in text). The 5′ untranslated region is shaded. The putative transmembrane domain is highlighted with dots. The 3′ untranslated region is hatched. Cysteine residues (CYS) and potential N-linked glycosylation sites (CHO) are shown below the cDNA sequence. Comparison of the amino acid sequences (% identity) within the intracytoplasmic (IC), transmembrane (TM), and extracellular (EC) domains of the murine and human 4F2HC cDNAs is shown below the schematic drawing. B. Comparison of the nucleotide and predicted amino acid sequences of the murine and human 4F2HC cDNAs. The nucleotide sequence of the murine 4F2HC cDNA is shown in the top row of each group. Differences between the human and murine nucleotide sequences are shown in the second row of each group. Identical nucleotides are shown as blank spaces. The predicted amino acid sequence of the murine 4F2HC protein is displayed in the third row of each group. Differences between the human and murine amino acid sequences are shown in the fourth row of each group. Identical amino acids are displayed as blank spaces. The putative transmembrane (TM) region and potential N-linked glycosylation sites (CHO) are shown above the murine 4F2HC cDNA nucleotide sequence. The consensus polyadenylation signal (AATAAA) is boxed. The alternate polyadenylation site identified in cDNA clone M4F2HC-59 is denoted with a vertical arrow.

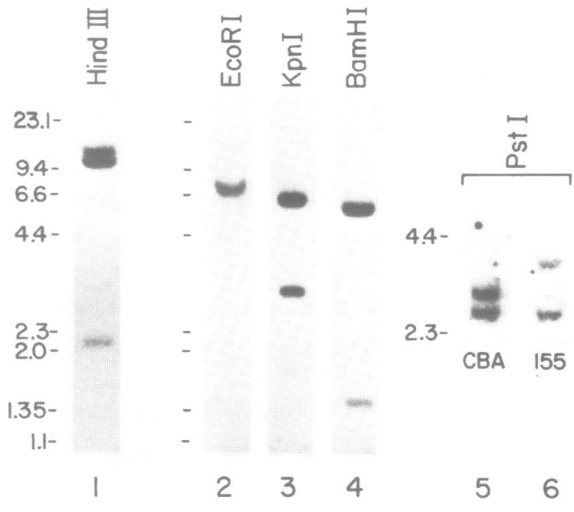


Figure 2. Southern blot analysis of the murine 4F2HC gene. High molecular weight DNA isolated from CBA murine liver (lanes 1-5) was digested with the restriction endonucleases HindIII, EcoRI, KpnI, BamHI and PstI and hybridized to the radiolabelled 1.8 kb murine 4F2HC cDNA probe M4F2HC-50. In order to demonstrate the strain specific sequence polymorphism, high molecular weight DNA obtained from the 155.16 murine T-cell hybridoma cell line was digested with PstI and hybridized to the M4F2HC-50 cDNA probe (lane 6). Molecular size markers are shown in kb to the left of each blot.

analysis of DNA from a number of different in-bred mouse strains was performed. Digestion of 155.16 (C57BL/6 x AKR/J)(Fig. 2, lane 5)(24), C57BL/6, and BALB/c DNAs (data not shown) with PstI followed by hybridization to the 1.8 kb M4F2HC-50 4F2HC cDNA probe produced 2.5 and 4.0 kb bands. A similar Southern blot analysis of CBA (Fig. 2, lane 6) and DBA/2 (data not shown) DNAs digested with PstI produced an identical 2.5 kb band. However, a 3.0 kb band was observed in place of the 4.0 kb band observed following PstI digestion of BALB/c, and C57BL/6 DNAs. Thus, the PstI restriction enzyme site present in DBA/2, CBA, and ICR DNAs but absent from the C57BL/6 and Balb/c DNAS reflects a strain specific DNA sequence polymorphism rather than a cloning artifact. Southern blot analyses using additional restriction endonucleases performed under both high (Fig. 2 lanes 1-4) and low (data not shown) stringency conditions, revealed that the 4F2HC cDNA is encoded by a single copy murine gene because the M4F2HC-50 cDNA probe only hybridized to between one and three EcoRI, KpnI, HindIII and BamHI genomic bands.

A comparison of the human and murine 4F2HC cDNAs and predicted proteins (Fig. 1) revealed a striking level of homology both in terms of the general

structure of the molecules, and in terms of their nucleotide and amino acid sequences. Hydropathicity analyses (data not shown) revealed that, like the human 4F2HC, the murine protein lacks a 5' hydrophobic signal sequence, and contains a single hydrophobic putative transmembrane domain (amino acids 76-98, Fig. 1B). As in the human protein, there are two cysteine residues, but in contrast to the human 4F2HC which contains four potential N-linked glycosylation sites, the murine protein contains nine such sites. All of the potential N-linked glycosylation sites, and both cysteine residues lie on the C-terminal side of the putative transmembrane domain. Taken together, these data suggest that the murine 4F2HC is a type II membrane glycoprotein which is composed of a 75 amino acid N-terminal intracytoplasmic domain, a 23 amino acid transmembrane region, and a 428 amino acid extracellular C-terminal domain.

The human and murine 4F2HC cDNAs are 77% identical at the nucleotide level and 75% identical at the amino acid level. However, marked differences in the levels of identity were observed in the different domains of the two proteins. As summarized in Fig. 1A, the highest levels of identity between the human and mouse polypeptides were observed within the intracytoplasmic (86% identity at the amino acid level) and transmembrane (100% identity at the amino acid level) regions. In contrast, several regions within the extracellular domains of the human and murine 4F2 proteins (i.e. aa 377-396 and 493-515) display only 30-50% identity at the amino acid level reflecting significant evolutionary divergence of these portions of the protein. Other regions within the extracellular domains display a high level of identity (eg. aa 343-366 and 416-436).

Tissue expression of the murine 4F2 heavy chain gene

Previous studies have suggested that the 4F2 molecule is intimately involved in the processes of cell activation and proliferation. In addition, one report has suggested that 4F2 is the sodium-calcium exchange structure (14). These hypotheses lead to certain predictions about the tissue distribution and developmental regulation of the genes encoding the 4F2 heavy and light chains. For example, one might expect to observe high level expression of the 4F2HC gene in cardiac and skeletal muscle if 4F2 is in fact the sodium-calcium exchange molecule. Similarly, if 4F2 is necessary for normal cellular proliferation, growing neonatal organs might be expected to express relatively high levels of 4F2HC mRNA as opposed to their more quiescent adult counterparts. In order to test these predictions, Northern blots containing RNA from a variety of neonatal and adult murine tissues were hybridized with a radio-

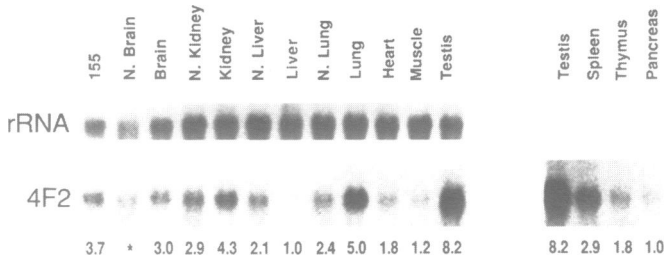


Figure 3. The tissue distribution and developmental regulation of murine 4F2HC gene expression. The top panel (rRNA) shows a Northern blot analysis of RNA samples isolated from adult and neonatal (N. brain, N. kidney, N. liver, N. lung) tissues and the 155.16 T cell hybridoma cell line hybridized to the 1.6 kb 28S rRNA probe (28). The bottom panel shows a Northern blot analysis of the same filters hybridized to a murine 4F2HC cDNA probe. The relative intensity of each autoradiographic band was quantitated by scanning densitometry. The relative levels of 4F2HC mRNA expression normalized against liver 4F2HC mRNA expression were calculated by the equation:

$$\frac{\text{Tissue 4F2HC mRNA intensity}}{\text{Liver 4F2HC mRNA intensity}} \times \frac{\text{Liver 28S rRNA intensity}}{\text{Tissue 28S rRNA intensity}}$$

Of note, the neonatal brain RNA (*) was partially degraded and was therefore, not quantitated by this method. The two panels represent separate experiments in which equal amounts of RNA were loaded in each lane.

labelled murine 4F2HC cDNA probe (Fig. 3). This probe hybridized to a single 1.8 kb band in all tissues tested. The 4F2HC gene is expressed at relatively high levels in the adult testis, lung, kidney, brain, and spleen (Fig. 3). Tissues expressing lower levels of 4F2HC mRNA include heart, skeletal muscle, thymus, pancreas, and liver. A comparison of the levels of 4F2HC gene expression in neonatal and adult (8-9 week old) murine tissues failed to demonstrate a consistent pattern of developmental regulation of 4F2HC gene expression. Specifically, neonatal and adult kidney expressed relatively constant levels of 4F2HC mRNA. Adult liver displayed decreased expression of the 4F2HC gene as compared to it neonatal counterpart. Conversely, comparison of neonatal and adult lung demonstrated an increase in 4F2HC gene expression in the adult tissue. Thus, in the majority of tissues examined the 4F2HC gene is not preferentially expressed during neonatal development.

Expression of the 4F2HC gene during progression through the cell cycle

Previous studies have demonstrated that 4F2 cell-surface expression is induced following cellular activation in a number of different human and murine systems (3,7,9,10). Moreover, recent studies have demonstrated that the 4F2HC gene is transcriptionally induced during the activation of normal

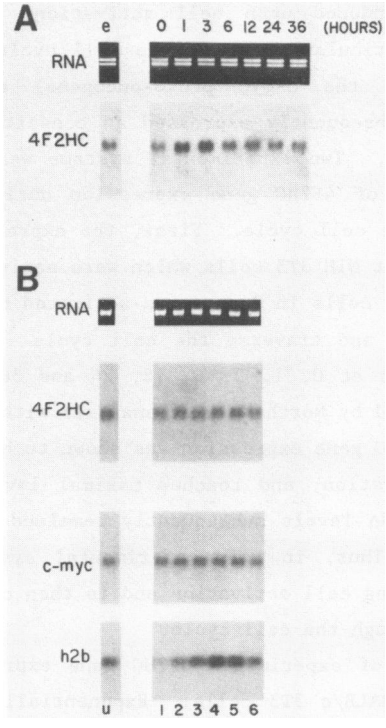


Figure 4. The cell-cycle regulation of 4F2HC gene expression in 3T3 cells. A. A Northern blot analysis of RNA prepared from cultures of serum-activated NIH 3T3 cells. The top panel shows an ethidium bromide stained agarose gel containing equal amounts of RNA isolated from exponentially growing cells (e) as well as cells harvested 0, 1, 3, 6, 12, 24, and 36 hours after serum-activation. The bottom panel shows a Northern blot analysis of these same samples hybridized to the radiolabelled 4F2HC cDNA probe M4F2HC-27. B. A Northern blot analysis of the cell cycle dependent expression of the 4F2HC, c-myc and h2b genes in exponentially growing BALB/c cells. The top panel shows an ethidium bromide stained agarose gel containing equal amounts of RNA isolated from exponentially growing BALB/c 3T3 cells which had been separated by counterflow centrifugation into cell cycle dependent subpopulations. Total RNA was prepared from the unfractionated (u) and fractionated subpopulations (Fractions 1-6). Fraction 1 contains exclusively cells from the G1 phase of the cell cycle, Fraction 4 is the fraction most enriched in S phase cells and Fraction 6 contains mostly cells in G2-M (39). Northern blots were hybridized to the radiolabelled M4F2HC-27 murine 4F2HC cDNA probe (4F2) . The blots were then stripped and rehybridized to the human c-myc (c-myc) and h2b (h2b) gene probes.

human peripheral blood T cells (33). Activation related genes can be divided into two subsets based upon their patterns of expression during progression through the cell cycle. Some genes (eg. the dihydrofolate reductase (DHFR)

and h2b genes) are induced upon cell activation, and are preferentially expressed during a particular phase of the cell cycle (30,34-36). A second subset of genes (eg. the c-myc proto-oncogene) are induced upon cell activation, but are subsequently expressed at constitutive levels throughout the cell cycle (37-39). Two experimental systems were employed in order to determine the pattern of 4F2HC gene expression during cell activation and progression through the cell cycle. First, the expression of the 4F2HC gene was studied in quiescent NIH 3T3 cells which were activated by the addition of serum. The majority of cells in such serum-activated cultures have been shown to synchronously enter and traverse the cell cycle (40). RNA was prepared from duplicate cultures at 0, 1, 3, 6, 12, 24 and 36 hours following serum stimulation and analyzed by Northern blot analysis with a radiolabelled murine 4F2HC cDNA probe. 4F2HC gene expression was shown to be induced 2-fold within one hour of cell activation, and reached maximal levels at 3-6 hours after stimulation. 4F2HC mRNA levels subsequently remained stable during the next 30 hours (Fig. 4A). Thus, in this experimental system the 4F2HC gene is rapidly induced following cell activation and is then constitutively expressed during progression through the cell cycle.

In a second set of experiments 4F2HC gene expression was examined in exponentially growing BALB/c 3T3 cells. Exponentially growing cells can be separated into subpopulations representing the different stages of the cell cycle by counterflow centrifugation (elutriation) (41). This technique is based on the finding that as cells progress through the cell cycle there is a linear increase in cell volume. Exponentially growing cells were separated into size-dependent subpopulation by elutriation and RNA was prepared from each fraction as well as from unfractionated cells and analyzed by Northern blot analyses with a murine 4F2HC cDNA probe. As controls the same blots were stripped and rehybridized to human c-myc and h2b probes. As seen in Figure 4B, the pattern of expression of the 4F2HC gene is almost identical to that observed for the c-myc proto-oncogene, ie. the 4F2HC gene is expressed at constant levels throughout the cell cycle in exponentially growing cells. In contrast, the histone 2b gene is preferentially expressed in the cell fractions most enriched in S phase cells (Fraction 4). These results are in accord with previous findings which have demonstrated that expression of the h2b gene is cell cycle dependent. In summary, the results of both cell cycle experiments demonstrate that, like the c-myc proto-oncogene, 4F2HC gene expression is induced during cell activation, but is subsequently maintained at constant levels throughout the cell cycle in exponentially growing cells.

DISCUSSION

In order to further elucidate the structure and pattern of expression of the 4F2 cell-surface molecule a murine 4F2HC cDNA has been isolated and characterized. Like the human 4F2HC, the murine polypeptide is a type II membrane glycoprotein which is composed of a 75 aa N-terminal intra-cytoplasmic domain, a single hydrophobic putative transmembrane region, and a 428 aa C-terminal extra-cellular domain. The relatively high degree of structural identity observed between the murine and human 4F2HC proteins suggests that 4F2 is a functionally important molecule which has been highly conserved during mammalian evolution. The highest level of amino acid sequence identity between the human and murine 4F2HC homologs was observed in the intra-cytoplasmic and transmembrane domains. In addition, several regions of the extracellular domain, e.g. amino acids 343-366 and 416-436, are 90-95% conserved between the murine and the human 4F2HC homologs. One or more of these highly conserved regions of the 4F2HC could be necessary for association with the highly conserved 4F2LC. In this regard it should be noted that we have demonstrated that retrovirus-mediated expression of the human 4F2HC cDNA in mouse cells results in the cell-surface expression of human 4F2HC/mouse 4F2LC disulfide-linked heterodimers. (J. Leiden, unpublished results). Alternatively, the highly conserved transmembrane and intracytoplasmic domains of the 4F2HC could be involved in transmembrane signalling while the conserved extracellular domains might be involved in the binding of an, as yet unidentified, soluble or cell-associated ligand. Thus, *in vitro* mutagenesis of these highly conserved domains may provide information concerning both the assembly and function of the 4F2 molecule.

Although its precise function is unknown, previous studies have demonstrated that 4F2 is involved in the processes of cellular activation and proliferation (3,7,9,10). Our studies of the tissue distribution of 4F2HC gene expression suggest that the level of 4F2 expression is not strictly correlated with the proliferative state *in vivo*. Thus, 4F2 is expressed at relatively high levels both in adult tissues containing large numbers of proliferating cells (eg. testis), and in tissues containing predominantly quiescent cells (eg. brain). Moreover, the 4F2HC gene is not preferentially expressed in neonatal as compared to adult tissues. These findings suggest that while 4F2 expression may be necessary for cell proliferation, the molecule may serve additional functions in non-proliferating cells.

It has been postulated that 4F2 may mediate its effects on cell growth by modulating intracellular calcium levels. In fact, 4F2 has been suggested as a

potential sodium-calcium exchange structure (13,14). Two experimental findings presented in this report argue against the hypothesis that 4F2 is the sodium-calcium exchange structure. First, 4F2HC mRNA is expressed at low levels in heart and skeletal muscle, two tissues active in sodium-calcium exchange (42). Moreover, in contrast to other previously described ion channels which contain multiple membrane spanning domains (43,44), the 4F2HC protein contains a single transmembrane region. It is noteworthy, that one region of the murine 4F2HC (aa 128-140) does display a sequence which is similar to that of a consensus calcium binding loop sequence which is present in a variety of calcium-binding proteins (45). However, several highly conserved amino acids of this consensus binding loop are not present in the murine 4F2HC. In addition, this sequence is not highly conserved between the murine and the human polypeptides.

Previous studies have provided conflicting results concerning the cell cycle-dependent regulation of the 4F2HC gene. Cotner et al. (9), Yagita and coworkers (11), and Kehrl et. al. (10) quantitated expression of cell-surface 4F2 protein during progression through the cell cycle in activated normal peripheral blood T cells and tonsillar B cells and concluded that the predominant increase in the level of cell-surface 4F2 following cell activation occurs during the transition from G_0 to G_1. Of note, these studies clearly demonstrated that 4F2 induction was not significantly inhibited by hydroxyurea which blocks entry into the S phase of the cell cycle. However, 4F2 expression was significantly reduced by treatment with sodium butyrate which prevents the transition from G_0 to G_1. In contrast to these results, Lumadue et al. (12) examined the expression of 4F2HC mRNA in serum-activated Swiss 3T3 fibroblasts, and reported a biphasic induction of 4F2HC gene expression with an initial increase in expression at 1-2 hours followed by an eight-fold increase in 4F2HC gene expression 24-36 hours after stimulation. Thus, they concluded that the 4F2HC gene is preferentially expressed during the S phase of the cell-cycle.

We have utilized two independent approaches to study the regulation of the 4F2HC gene during progression through the cell cycle in murine fibroblasts. The results of these studies have demonstrated that the 4F2HC gene is rapidly induced following cellular activation, but is subsequently expressed at relatively constant levels throughout the cell cycle. These results are in agreement with the previous findings of Cotner et al. (9), Yagita et. al. (11), and Kehrl et. al. (10), and in addition demonstrate that the pattern of cell-surface 4F2 expression observed by these investigators is mirrored by a

similar pattern of 4F2HC gene expression. Moreover, we have also recently demonstrated that the 4F2HC gene is maximally expressed 6-12 hours following activation of normal human peripheral blood T cells well before the onset of DNA synthesis (32). These findings conflict with the previously reported results of Lumadue and coworkers (12). This apparent disparity may be explained by the fact that these investigators utilized a human 4F2HC cDNA probe in their Northern blot analyses of murine 3T3 RNA. Hybridization of a human 4F2HC cDNA to murine RNA produces a relatively low signal to noise ratio which might have obscured the pattern of 4F2HC gene expression in these experiments.

In summary, the studies presented in this report have demonstrated that the murine and human 4F2HC genes display a high degree of sequence identity. Specific highly conserved domains within the 4F2HC have been identified which may be important in the assembly and/or function of the 4F2 molecule. Studies of the tissue distribution of the murine 4F2HC gene have revealed that 4F2HC mRNA is expressed at relatively high levels in tissues with large numbers of proliferating cells, but is also expressed in some tissues containing relatively few proliferating cells. This finding suggests that 4F2 may be involved in processes other than cellular proliferation. Finally, we have shown that the 4F2HC gene is induced upon cell activation, but is subsequently expressed at relatively constant levels throughout the cell cycle. The availability of a murine 4F2HC cDNA clone should facilitate future studies of 4F2HC structure-function relationships.

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