Expression of a 74-kDa Nuclear Factor 1 (NF1) Protein Is Induced in Mouse Mammary Gland Involution

INVOLUTION-ENHANCED OCCUPATION OF A TWIN NF1 BINDING ELEMENT IN THE TESTOSTERONE-REPPRESSED PROSTATE MESSAGE-2/CLUSTERIN PROMOTER

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Testosterone repressed prostate message-2 (TRPM-2)/clusterin gene expression is rapidly induced in early involution of the mouse mammary gland, after weaning, and in the rat ventral prostate, after castration. A search for involution-enhanced DNase I footprints in the proximal mouse TRPM-2/clusterin gene promoter led to the identification and characterization (by DNase I footprinting and EMSA) of a twin nuclear factor 1 (NF1) binding element at −356/−309, relative to the proposed transcription start site; nuclear extracts from 2-day in-vitro, in a primary cell culture system, triggered the appearance of the 74-kDa NF1 protein. This protein was not found in lactation where three other NF1 proteins of 114, 68, and 46 kDa were detected. Reiteration of the epithelial cell apoptosis associated with early mammary gland involution, in vitro, in a primary cell culture system, triggered the appearance of the 74-kDa NF1. Overlaying the cells with laminin-rich extracellular matrix suppressed the apoptosis and the expression of the 74-kDa NF1 and, in the presence of lactogenic hormones, initiated milk protein gene expression and the expression of two of the lactation-associated NF1 proteins (68 and 46 kDa). This study, thus, identifies for the first time the occurrence of a switch in expression of different members of the family of NF1 transcription factors as mammary epithelial cells move from the differentiated to the involution/apoptotic state, and it is likely that the involution-specific 74-kDa NF1 accounts for the enhanced NF1 footprint detected on the TRPM-2/clusterin promoter with extracts of mouse mammary gland.

Removal of hormonal stimuli, by weaning, in the case of the mammary gland, and by castration, in the case of the prostate, induces both glands to undergo involution (1, 2). This process has been shown to occur in two overlapping phases (3): there is an initial regression of the epithelial elements by apoptosis, resulting in an 80% loss of the lobuloalveolar epithelial cells that had functioned in lactation and a 90% (approximate) loss of prostatic epithelial cells (1, 2). After this phase has initiated, a phase of glandular remodeling begins. Loss of cell-cell contact and breakdown of cell-extracellular matrix (ECM) interactions may contribute significantly to triggering the epithelial cell apoptosis (4–6). This displays biochemical and morphological characteristics that include DNA fragmentation, chromatin condensation, and cell wall blebbing (1, 7, 8). The remodeling is associated with a dramatic transient increase in extracellular protease activity (9). The initial phases of apoptosis have been shown to be accompanied by a transient triggering of expression of a range of genes that encode early response factors, cell cycle regulators, and apoptosis-associated proteins. These include c-Fos, c-Jun, and JunD (10); p53, transforming growth factor-β, and c-Myc (11); and tissue transglutaminase and testosterone-repressed prostate message-2 (TRPM-2)/clusterin (11, 12). We have been interested in identifying transcriptional regulators that play a central role in the triggering of this new gene expression and that may be key targets for cellular signals that initiate this series of events.

Increased expression of the protein TRPM-2/clusterin (also known as sulfated glycoprotein-2 (13) and SP-40/40 (14)), has been shown to be closely associated with the outset of involution and apoptosis in a range of different cell types (15–17). However, the role played by TRPM-2/clusterin in the apoptotic response or in involution has not been defined. TRPM-2/clusterin mRNA levels increase rapidly and significantly once involution is initiated in both the mouse mammary gland (11) and the rat ventral prostate (12), and run-on transcription studies suggest that this increase arises from an increase in TRPM-2/clusterin gene transcription (10). In our efforts to identify transcription factors that are central to the triggering of the new gene expression that accompanies the onset of mammary gland and prostate involution, we looked for involution-enhanced DNase I footprints on the proximal promoter of the TRPM-2/clusterin gene. Our studies identify a novel twin nuclear factor 1 (NF1) binding element in the proximal TRPM-2/clusterin promoter whose occupation is enhanced during involution (see below).

NF1 and CCAAT-binding transcription factor/NF1 (CTF/NF1) proteins are a heterogeneous family of transcription factors that can range in size from 30–100 kDa (18, 19). NF1s have a highly conserved N-terminal DNA binding and dimerization domain,

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‡ The abbreviations used are: ECM, extracellular matrix; CTF, CCAAT-binding transcription factor; EMSA, electrophoretic mobility shift assay; FACS, fluorescence-activated cell sorting; HIP, hydrocortisone, insulin, and prolactin (lactogenic hormones); kb, kilobase(s); bp, base pair(s); NF1, nuclear factor 1; Stat5/MGF, signal transducer and activator of transcription 5/ mammary gland factor; TRPM-2, testosterone-repressed prostate message-2.

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which enables them to bind to the inverted repeat, 5'-TGGA/CA/N/GCCCA-3' (20). NF1s bind to DNA as a dimer through an unusual DNA binding domain, which contains four cysteines that are essential for binding (21). Some NF1s can bind to “half-sites” where a promoter contains only one copy of the motif: TGGCA (22). The characterized NF1 proteins have carboxyl-terminal proline-rich transactivating domains that are highly divergent in size and sequence. Thus, related products encoded by four different genes have been characterized (NF1-A, B, C, and X), and multiple splice variants that differ in the carboxyl terminus of three of these have been described (23–25). This diversity allows possible heterodimer formation (24) and a high potential degree of selective control through NF1 binding sites. Further control of these transcriptional regulators occurs at a post-transcriptional level by regulation of NF1 mRNA stability (26) or by phosphorylation (19, 27). NF1s have been shown to play a role in cell-specific and function-specific regulation of gene expression (28–31). In the mammary gland NF1s play a critical role in activating milk protein gene promoters during lactation, and the DNA binding activity of one form of NF1 has been shown to be ECM-dependent, in mammary epithelial cells (32–34). In addition, NF1 and the activated glucocorticoid receptor coordinately drive transcription from the murine mammary tumor virus enhancer (29). However, the particular forms of NF1 that are active in these processes have generally not been characterized.

Here we report the characterization of a twin NF1 binding element in the proximal mouse TRPM-2 clustering gene promoter, which contains a proximal high affinity binding element and a distal low affinity binding element. In the involuting mammary gland, significantly enhanced occupation of the proximal site is seen, whereas in the involuting prostate, occupation of both binding sites is induced. The enhanced occupation of the proximal element in the involuting mammary gland was associated with the de novo expression of a single 74-kDa NF1 protein. In contrast three NF1 proteins of 114, 68, and 46 kDa, which are prominent in the lactating gland are lost in involu- tion. When midpregnant mouse mammary epithelial cells in primary culture were maintained on plastic, they undertook apoptosis, as happens in involu- tion; under these conditions, the expression of the 74-kDa NF1 was initiated. When such cells were overlaid with laminin-rich ECM, both apoptosis and the expression of the 74-kDa NF1 was suppressed; in the presence of lactogenic hormones, the ECM-overlaid cells expressed differentiation markers, e.g. β-casein mRNA, and the 68- and 46-kDa lactation-associated NF1s. This represents the first report of an involu- tion/apoptosis-specific NF1 and a switch in expression of NF1 proteins in the lactation/involu- tion transition in the mouse mammary gland.

MATERIALS AND METHODS

Plasmid Constructs and Antibodies—A mouse TRPM-2 clusterin gene promoter fragment was cloned by screening a genomic library with a mouse TRPM-2 3′ cDNA probe (a gift from F. Li, University of Berne) and subsequent Southern blot analysis with an oligonucleotide derived from the 5′-end of the rat cDNA sequence. The 680-bp EcoRI/PvuII mouse TRPM-2 promoter fragment was subcloned into the EcoRI/SmaI restriction sites of pBS KS+ to yield p-629/TRPM-3/KS. One copy of the 46-nucleotide proximal NF1 binding site within the mouse TRPM-2/clusterin promoter (−356–309) and one copy of each of the three mutant oligonucleotides (m1, m2, and m3) (see Fig. 2A) were subcloned into the SmaI site of pBSS KS+ for sequencing and labeling for footprinting analysis.

Gifts of four anti-NF1 rabbit polyclonal antibodies directed against the conserved amino-terminal DNA binding domain were obtained: against a bacterially expressed fusion protein containing human NF1 from Dr. N. Tanese (New York University Medical Center) (35); against rat NF1 (amino acids 1–237) from Dr. J. Dekker (University of Utrecht) (36); against amino acids 1–222 of the human NF1/NF1 expressed by baculovirus-infected SF9 cells from Dr. R. Hay (University of St. Andrews) (37), and a Sepharose G column-purified polyclonal anti-N-terminus antibody from Dr. E.-L. Winnacker (Ludwig-Maximilians University).

Preparation of Tissue Extracts and EMSA: Pure NF1—Baculovirus-expressed and purified NF1 was prepared and characterized as described previously (38). Nuclear extracts from rat liver, rat ventral prostate (normal adult male and 2- and 4-day postcastration) and mouse mammary gland (virgin, 8- and 16-day pregnant, lactating; and 2- and 4-day postweaning) were prepared essentially as described by Andrews et al. (39) in the presence of a mixture of protease inhibitors (40). Cytochalasin was obtained by saving the supernatant fraction after the tissue homogenate had been centrifuged at 30,000 rpm for 10 min in a Beckman ultracentrifuge rotor, TLA 100.3; the nuclear extract was obtained by salt extraction of the resulting pellet (41). Whole cell extracts were prepared from primary mammary epithelial cells as described by Andrews et al. (41) but in the presence of the following mixture of protease inhibitors (40). Electromobility shift assays (EMSA) were performed as described by Frain et al. (42) using 10 μg of protein, with the [32P]phosphate end-labeled (43) double-stranded oligonucleotides (see Fig. 2A).

DNase I Footprinting Analysis—The mouse TRPM-2/clusterin gene promoter was 3′-end-labeled on the coding strand using the EcoRI site (at −629) and on the noncoding strand using the internal DraI site (at −255). The probe was released at the unlabeled 3′-end by DraI digestion. The fragment. The oligonucleotides subcloned into pBS KS+ (see Fig. 2A) were labeled with the Klenow fragment of DNA polymerase I and [α-32P] dCTP following restriction with NotI (coding strand) and XhoI (noncoding strand). Nuclear extract (30–200 μg of protein) was preincubated with 300 ng of poly(dI–dC) in a buffer containing 20 mm Hepes-KOH, pH 7.9, 1.9. 2.5 mm MgCl2, 60 mm NaCl, 5 mm Na2EDTA, 20% (v/v) glycerol on ice for 10 min. Purified labeled fragment (1.5 × 10⁴ cpm) was then added, and the reaction was incubated on ice for 15 min. DNase I digestion was carried out at room temperature for 1 min using a predetermined DNase I dilution of the stock. The stock DNase I (Worthington) was stored at 1 mg/ml and contained approximately 2.6 units/ml. The reactions were terminated by adding twice the reaction volume of 50 mm EDTA, 0.1% SDS, and 200 μg/ml proteinase K and were then incubated at 4°C for 30 min. The products were precipitated with ethanol and separated on 8% polyacrylamide gels containing 8% urea. Maxam and Gilbert G + A reactions for the appropriate fragment were run in adjoining lanes. The procedures used were essentially as described in Ref. 43.

EMSA/Western and Western Blot Analysis—For the EMSA/western analyses a 5′ × 32P EMSA was run on a 6% nondenaturing polyacrylamide gel. The wet gel was autoradiographed for 3 h at room temperature. The bands of interest were then excised and eluted overnight in 100 μl of 2× loading buffer (20% glycerol, 2% SDS, 125 mm Tris-HCl, pH 6.8, 25 μg/ml bromophenol blue, and 50 μl/μl mercaptoethanol) at 37°C. The samples were boiled for 3 min before loading on an 8% SDS-polycrylamide gel electrophoresis for Western blot analysis.

Western blot analysis was carried out essentially as described in Ref. 43; tissue nuclear extracts (30 μg of protein) were boiled in an equal volume of 2× loading buffer and separated on an 8% SDS-polyacrylamide gel (45). The proteins were transferred to reinforced nitrocellulose (Opti- tran, Schleicher & Schuell) using a wet transfer system in 20 mm Tris, pH 8.5, 150 mm glycine, 0.1% SDS, and 20% methanol (v/v). The membrane was blocked for 45 min with 1.5% BSA in TBST (25 mm Tris-HCl, pH 7.6, 150 mm NaCl, and 0.1% Tween 20) followed by three 5-min washes in TBST. The primary antibody was incubated with the filter in 1.5% BSA in TBST overnight followed by three 5-min washes in TBST. The secondary antibody (goat anti-rabbit immunoglobulin G) was horseradish peroxidase; DAKO was incubated with the filter at a 1:2,000 dilution in 1.5% BSA in TBST for 2 h. After three 15-min washes in TBST, the bands were detected by enhanced chemiluminescence (ECL).

Northern Blot Analysis—Total RNA from mouse mammary glands and liver was isolated by a one-step guanidium/phenol method (43), and poly(A)+ mRNA was prepared using the PolyATtract mRNA isolation system (Promega). Four μg of poly(A)+ RNA from each sample were separated on a 1% agarose/formaldehyde gel (43) and transferred to nylon membrane (Hybond-N, Amersham Corp.) in 10× SSC (1.5 mm NaCl, 0.15 mm Na2-HPO4, pH 7.0). For NF1 mRNA analysis, the filter was probed with a random prime-labeled (Prime-a-Gene labeling system, Promega) human CTF/NF1 cDNA (the entire 1.7-kb cDNA fragment was excised from pCT1-1 (25) by BamHI/EcoRI digestion) and hybridized in 50% formamide, 5× SSPE, 10% dextran sulfate, 5× Denhardt’s solution, 1% SDS, and 100 μg/ml salmon sperm DNA at 42°C overnight. The filter was washed three times in 1× SSC and 0.1% SDS at 60°C prior to autoradiography. The filter was stripped by boiling in 0.1% SDS and was reprobed with a coronavirus terminus NF1-X-specific probe (−600 bp EcoRI/XhoI fragment from the human NF1-X cDNA).
Equal loading of the gel was measured by subsequently reprobing the blot with a 1.06-kb HindIII fragment of GAPDH cDNA probe (43).

Northern blot analysis for β-casein mRNA in the cultured primary mammary epithelial cells was carried out essentially as described above; 15-μg aliquots of total RNA were separated on a 1.5% agarose/formaldehyde gel and transferred to a nylon membrane and hybridized with a >500-bp EcoRI mouse β-casein cDNA probe (45).

**Primary Mammary Epithelial Cell Culture—**Mouse mammary epithelial cells were prepared from midpregnant CD-1 mice essentially as described by Barellos-Hoff et al. (46) and Streuli et al. (47, 48, 34). Cells were seeded on tissue culture plastic at a density of 2.4 × 10⁶ cells/ml and grown for 48 h in a proliferation medium containing F12 (Life Technologies, Inc.), 10% heat-inactivated fetovine serum (Life Technologies, Inc.), 5 mg/ml epidermal growth factor (Promega), 5 μg/ml insulin (Sigma), 1 μg/ml hydrocortisone (Sigma), 1 mg/ml fetal bovine serum (Sigma), and 50 μg/ml gentamicin (Sigma). The cells were washed and then cultured for a further 24 h in F12 medium (with gentamicin) in the absence of serum but in the presence of 5 mg/ml epidermal growth factor, 5 μg/ml insulin, and 1 μg/ml hydrocortisone. The cultures were subsequently washed extensively, and the medium was changed to a differentiation medium (F-12; Dulbecco’s modified Eagle’s medium (1.1), 50 μg/ml gentamicin, 5 μg/ml insulin, 1 μg/ml hydrocortisone, and 3 μg/ml prolactin (Sigma)). These cells were then cultured in the absence or presence of an overlaid laminin-rich ECM matrix (Matrigel, 200 μg/ml, Becton Dickinson), which had been diluted in the above medium. After 3.5 days, the cells were harvested. The ECM-overlaid cells were treated with dispase (2.0 mg/ml, Becton Dickinson); the nonoverlaid cells were recovered by scraping and were pelleted by centrifugation. The cell pellets were snap frozen and stored at −80°C prior to protein extract preparation or RNA extraction. Cell nuclei were prepared for FACS analysis as described by Gurley et al. (49).

**Isolation of Genomic DNA—**High and low molecular weight DNA was isolated from cultured primary mammary epithelial cells essentially as described in Ref. 50. The cells were lysed by incubation in 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 25 mM EDTA, pH 8.0, 0.5% SDS, and 0.2 mg/ml proteinase K at 50°C for 12 h. After phenol/chloroform extraction, the DNA was precipitated using ammonium acetate/ethanol at −80°C for 20 min. The DNA was pelleted, washed with 70% ethanol, and resuspended in TE (10 mM Tris-HCl, pH 8.0, 1.0 mM EDTA, pH 8.0). Twenty μg of each sample was run on a 1.5% agarose gel, stained with ethidium bromide, and visualized by UV transillumination and photographed.

**RESULTS**

**An Involution-enhanced DNase I Footprint in the TRPM-2/Clusterin Promoter—**The sequence of the mouse TRPM-2 promoter (−629/−68) fragment that was cloned by screening a mouse genomic library is shown in Fig. 1A. The promoter sequence is identical to that independently lodged in GenBank (accession number X84792). This region of the 5′-flanking sequence shows a strong homology with the rat promoter sequence (51) and with human promoter sequence (52). We hypothesized that as the transcription of this gene is rapidly amplified in the postlactational involuting mouse mammary gland (10, 11) and in the involuting rat ventral prostate (12), we might be able to detect involution-enhanced association of transcription factors with its promoter. This in turn would act as a means of detecting transcription factors that play a central role in triggering the new gene expression that accompanies involution/apoptosis in these epithelia.

A TRPM-2/clusterin promoter fragment (−629 to −276) was end-labeled and subjected to DNase I footprinting analysis in the presence of increasing concentrations of nuclear extracts prepared from lactating and 2-day-involuting mouse mammary gland and normal and 2-day-involuting rat ventral prostate. A weak footprint (FI) detectable with high concentrations of lactating extract (−309 to −355, noncoding strand) (Fig. 1B, lanes 3–5) became significantly more intense with increasing concentrations of involuting extract (Fig. 1B, lanes 6–8). Similarly, in the presence of increasing concentrations of nuclear extract from involuting rat ventral prostate, the same footprint is also present (Fig. 1B, lanes 12–14). There is also some binding to a more 5′ element with 2-day-involuting rat ventral prostate extracts (FII) (lanes 12–14), which is not seen.
using extracts from normal prostate (lanes 9–11) or the involuting mouse mammary gland. (FII) was also associated with DNase I hypersensitivity sites at approximately −356 and −359 (lanes 12–14). Similar observations were made on footprinting the coding strand (not shown). The F1 footprint at −309 to −335 lies over a classical NF1 binding element (TGGN₇CCA) (20) (Fig. 1B, lower part), and the extended F1I footprint, detected with the involuting prostate extract, also covers a second potential NF1 binding element (TGGN₇CCA) (Fig. 1B, lower part). This was the only involvement-enhanced footprint detected over the 651 bp of the TRPM-2/clusterin promoter studied. We proceeded to characterize this potential twin NF1 binding element.

**Binding of Pure Recombinant NF1 and Tissue Extracts to the Twin NF1 Binding Element**—Four 46-mer double-stranded oligonucleotides were synthesized based on the sequence of the twin NF1 binding element (Fig. 2A). The first retained the wild-type sequence (wt); in m1 the TGG and CCA motifs of the proximal (F1) NF1 binding site were mutated; in m2 the TGG and CCA motifs of the distal (FII) NF1 binding site were mutated; and m3 contained both sets of mutations. NF1 binding to the element was characterized by EMSA using a purified recombinant 36-kDa NF1, produced with baculovirus (3B). In the presence of a low concentration of pure NF1, a single shifted species was formed on the wild type oligonucleotide probe (Fig. 2B, lane 1); however, titration with increasing amounts of NF1 led to the appearance of a second more retarded shifted species (lanes 3 and 4). This observation probably reflects the filling of both NF1 binding sites at high factor concentrations. Mutation of the proximal site (m1) led to loss of the slower moving shifted species and a significant reduction in the intensity of the faster moving band (lane 5); mutation of the distal site (m2) also led to loss of the slower moving shifted species, but the intensity of the faster moving band was equivalent to wild type (lane 6). Specific mutation of both NF1 binding elements inhibited formation of both shifted species (lane 7).

It is likely, therefore, that the more slowly migrating complex seen at high NF1 concentrations reflects binding of factor to both elements. Furthermore, comparison of NF1 binding to mutants m1 and m2 would suggest that the more proximal element binds NF1 with a relatively higher affinity than does the distal element. This may reflect the structure of the two elements. The proximal element has the accepted consensus structure TGGN₇CCA, while the distal element is TGGN₇CCA, which may be less ideal (Fig. 2B). Preincubation of 20 ng of pure NF1 with the anti-NF1 antibody inhibited the formation of both retarded complexes (lanes 8 and 9).

As NF1 comprises a family of transcription factors, which can range in size from ∼30 to 100 kDa (18, 19), it would not have been useful to compare the occupancy of the twin binding element with the pure 36-kDa NF1 protein with the range of NF1 proteins present in tissue extracts by EMSA. Therefore, the comparative occupancy of this element was analyzed by DNase I footprinting analysis using the wt and mutant oligonucleotide probes (Fig. 2C). On the coding strand, the proximal NF1 binding element was occupied in 2-day involuting mouse mammary gland extracts (lanes 3 and 4, Fig. 2C); a similar observation was made with lower concentrations of prostate extract (lanes 5 and 6, F1), but with higher concentrations of prostate extract an indication of enhanced occupancy of the distal site could also be observed (lanes 7 and 8, FII). A comparison of occupancy of the F1 and F1I sites with extract of 2-day involuting prostate and pure recombinant NF1 on the wt oligonucleotide showed very similar footprints (lanes 9 and 13), and with the mutant oligonucleotides the occupation of the binding sites was constrained in the same manner with the 2-day involuting prostate extract and pure NF1 (lanes 10–12 and 14–16); mutation of the proximal site (m1) inhibited binding to both sites (lanes 10 and 14), while mutation of the distal binding site (m2) led to occupation of only the proximal site (lanes 11 and 15). Mutation of both putative NF1 binding elements (m3) resulted in no binding (lanes 12 and 16). Similar observations were made on footprinting the noncoding strand (not shown). Therefore, footprinting of the oligonucleotides 1) confirmed the involvement-enhanced occupancy of the proximal (F1) site seen in Fig. 1B, in both extracts of mammary gland and ventral prostate; 2) showed similar footprints on the wild-type element and footprint pattern over the mutant series with tissue extract and pure recombinant NF1 (note also, that the boundaries of the footprint arising from occupation of the high affinity proximal site with involuting mammary gland extract on the wt probe (lane 4) are identical to those on mutant m2 obtained with pure NF1 (lane 15) and involuting prostate extract (lane 11), i.e. under conditions where they can only occupy the proximal site; 3) suggested that there may be cooperativity of binding, with the distal site (FII) being occupied more readily if the proximal site (F1) is occupied.

When the twin site oligonucleotide was used in EMSA with tissue extracts (Fig. 2D) we could detect the presence of three retarded species with nuclear extracts of rat liver (complexes A, B, and C, lane 2). Complexes B and C were present with normal prostate extract but were lost in involuting with which an intense complex A band appeared (lanes 3 and 4). The involution-induced complex A could reflect the occupation of the twin binding element observed in the footprinting analysis (Figs. 1B and 2C). With the lactating mammary gland extracts the larger (complex A) and the smaller complex (C) seen in the liver extracts were apparent (lane 5). The principal change seen in involution was the appearance of the intermediate sized complex B (lane 6) and the eventual loss of complexes A and C by 4-day involution (lane 7). The appearance of the complex B band may reflect the involution-enhanced occupancy of the proximal NF1 site on the TRPM-2/clusterin promoter seen in the footprinting studies (Figs. 1B and 2C). When both NF1 binding elements were mutated (m3) all three complexes failed to form, suggesting their dependence on intact NF1 binding elements as shown using extracts from lactating (Fig. 2D, lanes 8 and 10) and involuting mammary gland (lanes 9 and 11).

This series of experiments satisfied the first goal of the study, namely to identify a transcription factor(s) whose activity changes on the initiation of mammary gland and/or prostate involution by detecting changes in binding site occupancy in an involution-activated promoter. However, because the NF1 binding patterns seen on the twin binding element in EMSA studies could be arising from the occupation of both binding sites by different NF1 proteins, we chose to further study the changing NF1 DNA binding patterns using a single NF1 binding site.
Functional analysis of the twin NF1 binding sites in the TRPM-2/clusterin gene promoter. A, the twin NF1 binding element. Sequences of the wt and the mutation element series (m1–m3) are shown. The sequence of the single NF1 binding site subsequently studied and a consensus NF1 binding element sequence are also shown. B, EMSA analysis of recombinant NF1 binding to the twin NF1 binding element and effect of an anti-NF1 antibody on shifted complex formation. Lanes 1–4, recombinant NF1 (2.5, 5, 10, and 20 ng of protein, respectively) incubated with the wt probe; lanes 5–7, recombinant NF1 (20 ng of protein) incubated with the m1, m2, and m3 probes; lane 8, wt probe with 20 ng of NF1 and 1 µl of normal rabbit serum; lane 9, as lane 8 but with 1 µl of anti-NF1 polyclonal antiserum (36). The putative single and double NF1 site retarded complexes are indicated by filled arrowheads. C, DNase I footprinting analysis of the twin NF1 oligonucleotide (wt) with mammary gland and prostate extracts and pure NF1. A comparison of the binding pattern of tissue extracts and pure NF1 on the mutant series (m1–m3) is shown. An analysis of factor association with the coding strand is shown. Lane 1, Maxam and Gilbert G sequence; lanes 2 and 17, [32P]phosphate-labeled fragment (wt), with BSA (40 µg); lanes 3–8, wt probe with lactating mammary gland nuclear extract, 2-day involuting mammary extract, normal rat prostate extract, 2-day involuting prostate extract (all 100 µg of protein), respectively. Lanes 9–12, 2-day involuting prostate extract (100 µg of protein) and end-labeled DNA fragments containing wt, m1, m2, and m3 sequences, respectively. Lanes 13–16, with pure recombinant NF1 (50 ng) and end-labeled DNA fragments containing wt, m1, m2, and m3 sequences, respectively. The proximal and distal footprints are labeled FI and FII, as in Fig. 1B. The DNA fragments used in this footprinting analysis were generated by cloning the oligonucleotide set (double-stranded 46-mers) described for panel A into the SmaI site of pKS and were radiolabeled with Klenow fragment of DNA polymerase I at the restricted EcoRI site (coding strand) or XhoI site (noncoding strand) of the polycloning cassette before release of the probe fragment. D, EMSA analysis with tissue extracts on the twin NF1 binding element. Lane 1, [32P]phosphate-labeled wt probe; lanes 2–7, with liver nuclear extract, normal rat prostate extract (p n), 2-day (2d) involuting prostate extract (p invol), lactating mammary gland nuclear extract (m lact), 2-day involuting mammary extract (m invol 2d), 4-day involuting mammary extract (m invol 4d), (all 10 µg of protein), respectively. The major shifted complexes are labeled A–C. Lanes 8 and 9, [32P]phosphate-labeled m3 probe with lactating mammary gland nuclear extract and 2-day involuting mammary extract, respectively; lanes 10 and 11, [32P]phosphate-labeled m3 probe with lactating mammary gland nuclear extract and 2-day involuting mammary extract (all 10 µg of protein), respectively.
extract with an anti-NF1 antibody (36) inhibited formation of the lactation-enhanced complexes I and IV, but not II, and partially inhibited the formation of the involution-specific complex III (data not shown). This is the first reported instance of an increase in activity of an NF1 protein-DNA complex in involution; enhanced NF1 activity has previously been associated with the differentiated state of mammary epithelial cells. With rat ventral prostate extracts (results not shown) we did not detect any complex whose activity increased during involution, which suggests that the formation of the involution-enhanced footprint (Fig. 1B) and the enhanced EMSA complex A (Fig. 2D) with prostate extracts involves a critical component that does not recognize the single NF1 binding element with high affinity.

In order to further characterize the NF1 species in complexes III and IV, the proteins were eluted from the shifted bands and subjected to Western analysis using a polyclonal anti-NF1 antibody directed against the CTF/NF1 DNA binding domain (35) (Fig. 3B). In the left part the gel areas excised for elution and further analysis are indicated. After elution, these complexes were analyzed by Western blotting with total nuclear extracts being used for comparison (right part). The involution-specific complex III (EMSA studies (left part, lane 2)) contains a single predominant NF1 of 74 kDa (right part, lane 4) (and a smaller amount of a faster migrating species, which may be a degradation product). The 2-day involution nuclear extract is also dominated by the 74-kDa protein with some of a 114-kDa protein also being detected (right part, lane 3). The lactation-enhanced complex IV (left part, lane 1) contains two anti-NF1 reactive proteins of 114 and 68 kDa (right part, lane 2). Nuclear extracts from lactating mammary gland contain these and smaller NF1 proteins (right part, lane 1). This suggests that the relative change in abundance of the 68-, 114-, and 74-kDa NF1 proteins in mammary epithelial cell nuclei may be responsible for the different pattern of shifted species seen in lactation and involution in the EMSA studies (Fig. 3). We could not detect any proteins by Western analysis on eluting complexes I and II.

A more comprehensive Western analysis (Fig. 4A) confirmed the clear induction of the 74-kDa NF1 protein during involution. It is present at very low levels in lactation, peaks in 2-day involution, and has decreased by 4-day involution (compare lanes 3, 4, and 5). This protein is not present in normal or involuting rat ventral prostate (lanes 6 and 7) and so may be specific to mammary gland involution. NF1s of 114-, 68-, and 46-kDa proteins were most abundant in lactation and were lost in involution (lanes 3 and 4). The 114-kDa NF1 is also abundant in the normal prostate, as is a protein of ~80 kDa and a range of NF1s of 50–60 kDa not seen in the mammary gland (lanes 6 and 7); all are reduced in abundance in involution (lane 7). HeLa cells (lane 9), which are of epithelial origin show a similar spectrum of NF1 proteins to that seen in prostate and mammary gland, but in liver (lane 8) none of the larger species are abundant. The exception is the relative abundance of proteins of about 50–60 kDa that are predominant in liver. HeLa cells, and prostate but not in mammary gland. A 30-kDa NF1 was also detectable in variable amounts in lactating mammary gland (see Fig. 4B, lane 1).

A possible mechanism that could account for the switch in presence of the 114-, 68-, and 46-kDa species in nuclear extracts from lactating mammary gland to the 74-kDa NF1 is that this is unlikely. Comparative Western analysis of pairs of nuclear and cytosolic extracts show that the species that are abundant in lactation are not detectable in cytosol at any stage (compare lane 1 to lanes 4–6). The involution-specific 74-kDa

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**Fig. 3.** EMSA and EMSA/Western analysis of NF1 proteins in lactating and involving mouse mammary gland. A, EMSA analysis of binding of nuclear extracts to the single NF1 binding element. *Lane 1*, NF1 32P-end-labeled probe; *lanes 2–7*, probe with nuclear extract from virgin, 8-day pregnant, 16-day pregnant, lactating, 2-day involuting, and 4-day involuting mouse mammary gland (all 10 μg of protein), respectively. Open arrowheads indicate the major NF1 species seen in the 2-day involuting mammary gland. The major shifted complexes are numbered I–IV. B, Western analysis of EMSA-shifted species. *Left part*, typical EMSA analysis. NF1 32P-end-labeled probe with nuclear extract (10 μg of protein) is shown from lactating (lane 1) and 2-day involuting (lane 2) mouse mammary gland. The areas of each gel lane excised and used for elution of the shifted protein species are indicated by dotted lines. *Right part*, Western analysis of proteins eluted from shifted bands. *Lanes 1 and 3*, nuclear extracts from lactating and 2-day involuting mouse mammary gland, respectively; *lane 2*, proteins eluted from lactating extract shifted complex (see left part); *lane 4*, proteins eluted from 2-day involuting mammary gland extract shifted complex (see left part). Open arrowheads indicate the major species recovered from the shifted complex in lactation, and a closed arrowhead shows the major species recovered from the shifted complex with the 2-day involuting mammary gland extract. The transfected proteins were analyzed using a polyclonal anti-CTF/NF1 antibody (35); a horseradish peroxidase-linked anti-mouse IgG was used as second antibody, and the signals were generated by ECL. The positions to which the molecular weight markers migrated are indicated. NE, nuclear extract; RC, recovered complex.
NF1 is not seen in cytosol at lactation (compare lane 2 with lane 4), but a slightly smaller protein is reasonably abundant in the 2-day involuting cytosol (lane 5).

Analysis of NF1 mRNA in Lactating and Involuting Mouse Mammary Gland—Northern blot analysis was used to determine if there were selectively transcribed NF1 mRNAs detectable during lactation and involution, one of which might encode the 74-kDa NF1. Poly(A)$^+$ mRNA was isolated from lactating mammary glands, epithelial cell organoids recovered from collagenased lactating glands, 2-day involuting glands, and liver. The blot was hybridized with a full-length CTF1 cDNA probe (23), which should be able to detect not only CTF1 but also other NF1 species through their conserved DNA binding domain sequence. From Fig. 5 (left part) it can be seen that five products were detected, which we estimated to be of 6.5, 5.8, 4.5, 3.8, and 1.6 kb. The 6.5- and 5.8-kb bands were present in the lactating mammary gland samples and in liver (lanes 1, 2, and 4). During involution, the 6.5-kb band disappears, but the 5.8-kb band persists (lane 3). A 4.5-kb band was also present in the lactating mammary gland and liver, and its abundance is reduced in involution (compare lanes 1 and 3). A band at 3.8 kb was present in all lanes but appeared to be of highest abundance in liver. Interestingly, high levels of a 1.6-kb product were seen in the 2-day involuting mRNA. This was only present at very low levels (if at all) during lactation.

When the blot was reprobed with an NF1-X-specific probe (24, 26) three of the mRNA transcripts shown in Fig. 5 (left part) were detected, those of 5.8, 4.5, and 1.6 kb (Fig. 5, right part). Nebl et al. (26) previously detected a 5.8-kb NF1-X transcript in immortalized mouse mammary epithelial cells but did not report the presence of smaller transcripts. The 4.5-kb transcript may be the splice variant, NF1-X2, reported by Apt et al. (24). Of the three, only the 1.6-kb transcript varies in expression levels between lactation and involution and, as found with the general probe, hybridizes strongly and only in involution. Such a small transcript could not encode a 74-kDa protein, but the possibility would remain open that the 74-kDa NF1 might be significantly post-translationally modified. It is interesting that a 1.6-kb NF1 mRNA was found to be expressed in a tissue-specific manner in rat testis and to be the most abundant NF1 transcript in that tissue (53). Nebl et al. (26) detected CTF1-specific transcripts in immortalized mouse mammary epithelial cells of 6.5 and 4.8 kb. Thus, the transcripts we see lost in involution could very well be CTF1s. It is noteworthy that the 4.5-kb NF1-X transcript overlaps the −4.5-kb transcript that is lost in involution. The blot was finally reprobed with a GAPDH-specific cDNA fragment (44) to determine equivalence of mRNA loading on the gel (Fig. 5, bottom part).

Western Analysis of NF1 in a Primary Cell Culture Model that Reiterates Mammary Epithelial Cell Differentiation and Programmed Cell Death—Boudreau et al. (5) and Streuli et al. (47, 48) have demonstrated that mouse mammary epithelial cells maintained in culture can reiterate certain functional characteristics of the epithelial cells in situ, such as proliferation and differentiation and apoptosis associated with involution. We harvested mammary epithelial cells from 16-day pregnant mice by collagenase/trypsin digestion and cultured them as monolayers on plastic under proliferating conditions for 48–72 h (see “Materials and Methods”). They were subsequently maintained either by being overlaid with a laminin-rich ECM (Matrigel) or in the absence of an ECM. The cell population maintained in the absence of ECM displayed significant levels of DNA fragmentation (nucleosomal laddering), indicating programmed cell death by apoptosis (11, 54) (Fig. 6A, lane 1), which was not present in the cell population overlaid with ECM. Apoptosis in this cell population was confirmed using FACS (cell cycle) analysis, which showed a substantial "sub-G0/G1 nuclear population" on propidium iodide staining of these cells (data not shown). Overlay with ECM both suppressed the appearance of the DNA fragmentation (Fig. 6A, lane 2) and reduced the sub-G0/G1 population by 78% (mean value) (data not shown).

The cells overlaid with ECM maintained in the presence of HIP expressed β-casein mRNA as judged by Northern analysis (Fig. 6B, lane 5), while the cells maintained in the absence of ECM did not (lane 6). The expression of milk protein genes such as the β-casein gene is an accepted marker of mammary epithelial cell differentiation and shows a requirement for ECM association. Note the expression of β-casein mRNA in lactation (lanes 1 and 2) and at a lower level in the 16-day pregnant gland (lane 3) as well as its loss from the 16-day pregnant mammary epithelial cells after the collagenase/trypsin single cell preparation (lane 4). Thus, the conditions of culture generate a population of differentiated cells protected from cell death by association with ECM and a population of cells displaying the programmed cell death characteristic of involution.
Investigation of the NF1 proteins in these cell populations was carried out by Western analysis; the 74-kDa species was not present in the pregnant gland (Fig. 6C, lane 1), in the epithelial cells immediately following harvest (lane 2), or in the lactating gland (lane 3) but was clearly present in the 2-day involuting gland (as previously, lane 4) and in the apoptosing cell population maintained in the absence of ECM (lane 5). When these cells were overlaid with ECM, the presence of this band was suppressed (lane 6). The differentiated cells selectively expressed the lactation-specific 68-kDa NF1 and the 46-kDa NF1 (lane 6), but we were not able to detect the lactation-specific 114-kDa species. The cells maintained in the absence of ECM did not express the 68-kDa NF1 but did show the presence of the 46-kDa protein. Thus, in primary cultures of mammary epithelial cells deprived of their essential survival association with a laminin-rich ECM and, consequently, undergoing apoptosis, we have been able to detect the unique expression of the involuting-specific 74-kDa NF1. In contrast, when overlaid with ECM the presence of this latter NF1 is suppressed, and the lactation-specific 68-kDa NF1 is selectively detected.

**Discussion**

Our studies on the involuting-enhanced occupation of the TRPM2/clusterin promoter (Figs. 1 and 2) have highlighted a potentially important role for NF1 proteins in this process in both ventral prostate and mammary gland and led to the identification of an apoptosis/involving-specific 74-kDa NF1 protein in mouse mammary gland (Figs. 3, 4, and 6). The appearance of an involuting-specific NF1 during mammary gland involuting is surprising. The NF1 family of transcription factors has been shown to be critical for the tissue-specific transcription of a range of genes selectively expressed in terminal differentiation, e.g., in activating an adipocyte-specific transcriptional enhancer (31), in activating transcription of liver-specific genes such as albumin (28), in brain-specific gene expression (55), and in driving epithelial cell-specific milk protein gene expression in the mammary gland (56–59). In the differentiating lactating mammary gland, NF1 protein(s) have been shown to contribute to casein gene expression (57), α-lactalbumin (58), and whey acidic protein (59) gene expression, in addition to the activation of the murine mammary tumor virus enhancer (56). Some studies suggest the existence of a mammary gland-specific NF1 (56). However, the nature of the NF1 proteins responsible for these latter activities has not been established.

In EMSA studies we have identified three NF1 species, complexes I, II, and IV, in extracts of lactating mammary gland, that show an increase in abundance in lactation versus mid-pregnancy (complexes I and IV) or are lactation-specific (complex II) and all of which are lost during involution. This would be expected because milk protein gene expression is switched off as the secretory cells are channeled into the apoptotic pathway. Sequential EMSA-Western analysis of complex IV revealed that it contained two proteins, of 114 and 68 kDa; this suggests that this complex contains an NF1 heterodimer. Western blot analysis showed the presence of three major NF1 species in extracts of lactating mammary gland, of 114, 68, and 46 kDa; these are lost in involution. These species, thus, would constitute the NF1 proteins that potentially contribute to activation of the milk protein gene promoters (60, 61). One of the three, the 114-kDa NF1, was also present in the 16-day pregnant mammary gland; however, some milk protein gene expression is already evident at this point in pregnancy, and its presence could correlate with this state of partial differentiation. The data, therefore, indicate a switch in expression of NF1 proteins as the mammary epithelial cell changes state from lactation (differentiation) to involuting/apoptosis with the loss of the 114-, 68-, and 46-kDa NF1s and the appearance of the 74-kDa protein. Goyal et al. (62) have also observed a change in NF1-DNA complexes (and presumably NF1 proteins) in cells as they change their functional status; they found different NF1-DNA complexes in exponentially growing, confluent, and serum-starved NIH 3T3 cells.
was associated with the presence of the 68- and 46-kDa lactation-specific NF1 proteins; the cell culture environment must not reiterate the requirements for the expression of the 114-kDa NF1. Streuli et al. (34) have recently demonstrated the ECM dependence of expression of an NF1 DNA binding activity under similar cell culture conditions. The data presented here suggest an ECM-regulated switch in the expression of NF1 proteins.

NF1 proteins are functionally defined by their ability to bind the palindromic DNA consensus sequence (TGGN\textsubscript{7}CCA) as dimers (64); additionally, factors have been partially characterized that show a higher relative binding affinity for the consensus half-site (56). Those NF1 subfamilies whose genes have been cloned (CTF/NF1, NF1-A and -B, NF1-L, and NF1-X) contain a highly conserved amino-terminal DNA binding domain that allows this binding. Four independently produced antibodies, all directed against different NF1 amino-terminal DNA binding domains could detect the involuion-specific 74-kDa NF1, and two or more of the antibodies could detect the 114-, 68-, and 46-kDa lactation-associated NF1s, in Western analysis. This strongly suggests that each of these proteins contains the conserved DNA binding domain. However, it is presently not possible to assign any of these proteins as products of a particular NF1 gene subfamily on the basis of available information. A previous study of NF1 mRNAs in a mouse mammary epithelial cell line identified two CTF/NF1 transcripts of 6.5 and 4.5 kb and an NF1-X transcript of 5.8 kb. Our study also detects these transcripts and suggests that the 6.5-kb (and possibly the 4.5-kb) CTF1 transcript is selectively lost in involution; there seems to be no change in the levels of the 5.8-kb NF1-X transcript. The major expression change seen in involution was the appearance of a strongly hybridizing NF1-X band of 1.65 kb, too small to encode a 74-kDa protein. An NF1-X transcript of this size has only previously been identified in rat testis (53). As its expression is induced to such a high level during mammary gland involution, it would be very interesting to characterize its function. In addition, the expression of this 1.6-kb mRNA may be ECM-suppressed in the differentiated gland. On the basis of this data it might be reasonable to hypothesize that at least some of the lactation-associated NF1s are CTF/NF1 gene products, but the 74-kDa protein cannot yet be assigned. Nebel et al. (26) have shown that a Ha-ras-mediated down-regulation of both CTF/NF1 and NF1-X mRNA levels in mouse mammary cell lines occurs through a decrease in the stability of the NF1 transcripts. Destabilization of mRNA may also account for the reduction in the levels of the 6.5-kb (and 4.5-kb) CTF1 mRNAs during mammary gland involution, and this could be triggered by a loss in ECM-epithelial cell contact.

We considered that the lactation/involution switch in nuclear NF1 proteins and EMSA-detectable NF1 DNA binding might be achieved by a cytoplasmic/nuclear shuttling reminiscent of the NFpB activation; interestingly, Ivanov et al. (85) have reported that a bovine lactation-activated 50-kDa NF1 protein, when isolated from cytosol, is associated with a 20-kDa factor. However, we could find no evidence of a 50-kDa lactation-activated NF1 protein or of cytoplasmic/nuclear shuttling in the mouse mammary gland. There remains the possibility that post-translational modifications such as phosphorylation (19, 27) or O-glycosylation (66) may play a significant role in the changes under discussion, but there is as yet no evidence of this. Cdc2 kinase has been shown to phosphorylate NF1, but its effect on NF1’s DNA binding capacity and transcriptional activity is unknown (27).

Expression of a different set of NF1 proteins in lactation and involution may have significant consequences. First, the differ-

FIG. 6. Western analysis of NF1 proteins in a primary cell culture model that reiterates mammary epithelial cell differentiation and programmed cell death. A, genomic DNA fragmentation in mammary epithelial cells cultured in the presence and absence of ECM. Agarose gel analysis is shown (with ethidium bromide staining) of genomic DNA (20 µg) from mammary epithelial cells after culture without ECM overlay in the presence of HIP (lane 1) and after culture on plastic with ECM overlay and HIP (lane 2). B, effect of ECM overlay on β-casein mRNA levels in cultured primary mammary epithelial cells. Top part, total RNA (20 µg) from lactating, lactating (2 µg of total RNA), and 16-day pregnant mammary gland and from mammary epithelial cells directly after harvesting by collagenase/trypsin digestion of the presence of HIP (lanes 1–6, respectively) were separated by electrophoresis on an agarose gel, transferred to nitrocellulose, and probed with a \textsuperscript{32}P-labeled β-casein cDNA-specific probe. Bottom part, methylene blue-stained nitrocellulose membrane after RNA transfer (28 S mRNA band). C, Western analysis of NF1 proteins from cultured mammary epithelial cells. Western analysis is shown (using conditions described in the legend to Fig. 3B) of nuclear extract from mouse mammary gland (16-day pregnant (lane 1), lactating (lane 3), and 2-day involuting (lane 4)) and whole cell extract from 16-day pregnant mouse mammary epithelial cells (after collagenase/trypsin harvesting (lane 2), after culture on plastic in the absence of ECM + HIP (lane 5), and after overlay with ECM + HIP (lane 6)) (30 µg of protein in all cases). Open arrows indicate the major NF1 species seen in lactation, and a closed arrow shows the major NF1 species seen in the 2-day involving mammary gland.

pressed by ECM association with mammary epithelial cells. Overlay of the epithelial cells with ECM, in the presence of lactogenic hormones, initiated β-casein gene expression and
ent proteins may show qualitative or quantitative promoter selectivity. This may be dictated by DNA binding element selectivity or cooperative association with other transcription factors. Transactivation/cooperativity between NF1 and the glucocorticoid receptor in activating the MMTV enhancer and between NF1 and Stat5/MGP in milk protein promoter activation has previously been described (29, 34), and cooperativity between CTF/NF1 and AP2 in lactation-specific β-1,4-galactosyl-transferase gene expression may occur (67). Second, Apt et al. (30) demonstrated that a high NF1-X:CTF1 ratio restricts the activity of the human papilloma virus enhancer in fibroblasts, while a high CTF1:NF1-X ratio was facilitative in epithelial cells (24, 30). Thus, changes in the relative expression of different NF1 proteins can have a significant qualitative influence on cellular gene expression. Third, there is considerable recent evidence of a silence/repressor role for NF1 proteins in transcriptional regulation. Silencer elements in the growth hormone gene, cartilage matrix protein gene, and peripherin gene promoters have been shown to contain palindromic and NF1-first binding elements, and a particular role for NF1-L in occupying these sites has been proposed (68–70). Last, with the potential presence of different NF1 proteins transcribed from closely related genes (e.g. NF1-A, -B, -C, and -X) and alternatively sliced products (e.g. CTF-B1, -2, and -3 or NF1-X1 and -X2), all with different transactivation activity and the possibility of heterodimer formation on the binding element, there is the possibility for very subtle regulation of transcription by this family of nuclear modulators.

Our immediate future aim must be the identification of the genes that encode the apoptosis/involution-specific and lacta-
tion-associated NF1 proteins in order that their functional significance may be confirmed by modulating their expression in vivo.

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