Metastasis-inducing DNA Regulates the Expression of the Osteopontin Gene by Binding the Transcription Factor Tcf-4

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ABSTRACT

Small 1,000-bp fragments of genomic DNA obtained from human malignant breast cancer cell lines when transfected into a benign rat mammary cell line enhance transcription of the osteopontin gene and thereby cause the cells to metastasize in syngeneic rats. To identify the molecular events underlying this process, transient cotransfections of an osteopontin promoter-reporter construct and fragments of one metastasis-inducing DNA (Met-DNA) have identified the active components in the Met-DNA as the binding sites for the T-cell factor (Tcf) family of transcription factors. Incubation of cell extracts with active DNA fragments containing the sequence CAAAG caused retardation of their mobilities on polyacrylamide gels, and Western blotting identified Tcf-4, β-catenin, and E-cadherin in the relevant DNA complexes in vitro. Transfection of an expression vector for Tcf-4 inhibited the stimulated activity of the osteopontin promoter-reporter construct caused by transiently transfected active fragments of Met-DNA or permanently transfected Met-DNA. This stimulated activity of the osteopontin promoter-reporter construct is accompanied by an increase in endogenous osteopontin mRNA but not in fos or actin mRNAs in the transfected cells. Permanent transfection of the benign rat mammary cell line with a 20-bp fragment from the Met-DNA containing the Tcf recognition sequence CAAAG caused an enhanced permanent production of endogenous osteopontin protein in vitro and induced the cells to metastasize in syngeneic rats in vivo. The corresponding fragment without the CAAAG sequence was without either effect. Therefore, the regulatory effect of the C9-Met-DNA is exerted, at least in part, by a CAAAG sequence that can sequester the endogenous inhibitory Tcf-4 and thereby promote transcription of osteopontin, the direct effector of metastasis in this system.

INTRODUCTION

Most cancers, including those of the breast, are thought to arise in a benign proliferative form and at a later stage in development to acquire multiple genetic alterations that promote dissemination and metastasis (1, 2). To identify those genetic alterations that are important in promoting metastasis in a dominant manner, we have transfected a stably diploid rat mammary epithelial cell line Rama37 that induced the cells to metastasize in syngeneic rats (1). To identify the particular inhibitory transcription factor and located this factor and its partners in the relevant DNA complex in vitro. Therefore, the regulatory effect of the C9-Met-DNA is exerted, in part, by sequestering this particular inhibitory transcription factor, thereby promoting transcription of opn, the direct effector for metastasis in this system.

MATERIALS AND METHODS

Cell Culture and Transient Transfections. The Rama 37 and the C9-Met-DNA permanently transfected Rama 37 cells were obtained and cultured as described previously (3, 11). For the transient transfection assays, the cell lines were cultured in DMEM, 10% (v/v) FCS, 100 μg/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc., Glasgow, United Kingdom), harvested, and seeded in multiwell plates at 2.5 × 104 cells/3.5-cm well in 1 ml of serum-free medium. After 24 h, cells were cotransfected with given amounts of C9-Met-DNA or its DNA fragments, 200 ng of β-galactosidase control expression plasmid and 650 ng of opn promoter-reporter plasmid (see below), as described previously (19). Cells were incubated usually for 48 h and were harvested in 300 μl of Reporter Lysis Buffer (Promega, Madison, WI), and CAT and control β-galactosidase activities were assayed as described previously (20) on 100-μl and 150-μl aliquots, respectively. CAT activity was normalized relative to β-galactosidase activity, and maximum activity was reached by 48 h; results at 72 h were similar.

Cloned DNAs and Oligonucleotides. The C9-Met-DNA (21) and the reverse orientation were obtained by digestion of the relevant recombinant pBluescript vector (11) using XhoI and SalI or EcoRI and XhoI restriction enzymes, respectively, to excise the Met-DNA, and cloned into PSI vector (Promega). C9-Met-DNA was cut further with suitable restriction enzymes to yield pM1 to pM8-DNA and subcloned into pBKCMV vector. pM9-DNA was obtained by amplification of C9-Met-DNA with Taq PCR using forward primer TTA GAG TGC CGT CAG and reverse primer ATG AGA GTT AGC CTT GAA, cloned into PCR 2.1 vector (Invitrogen, Carlsbad, CA), then digested by EcoRI, and subcloned into pBKCMV vector (Stratagene, La Jolla, CA). The synthetic oligonucleotides pM10, 11, 12, 12, AA-DNA, and the CAAAG-deleted pM12/D-DNA correspond to the following sequences: ATG AGC TCA TGG GAA AGG GGA GAA CCA GCC AAA GGT GTG GCG TGT GAC; CTC AGA ATT ATG AGG GCC AAA GGT TCA AGG CTA ACT CTC ATG AYA GAG; AAC CAG GCA; AAG GTG TTG GC; AAC CAA ACA AAG GTG TTG GC; and AAC CAG GTG GGT GCG. They were cloned into the EcoRI sites of the pBKCMV as above. The 2.3-kbp fragment of the 6-kbp opn promoter from Dr. Amy Ridall (University of Texas, Dental
Branch, Houston, Texas; Ref. 22) was amplified with Taq PCR using AAG CTT CCTG TCT GTT GTA GCA AAG CCA AGG ATG as forward and reverse primers, respectively, cloned into PCR 2.1 vector (Invitrogen), released by digestion with HindIII and SalI, and then coupled to a CAT reporter construct to measure its activity, as described previously (19). The amplified product was free of any mutations. Tcf-4 and Tcf-1 cDNAs were obtained from Prof. Hans Clevers (University Hospital, Utrecht, Holland; Ref. 23) and were separately cloned into the pBKCMV expression vector as above. The cDNAs for smooth muscle α-actin, c-fos, and opn were obtained as described previously (11, 12).

**Gel Shift and Supershift Assays.** The gel shift assays were performed essentially as described (24) by incubating 0.5 ng of 32P-labeled Met-DNA fragments with 10 μg of nuclear or whole cell protein extract in 25 μl (100 mM KCl, 10 mM Tris-HCl (pH 7.9), 0.5 mM EDTA, 0.5 mM DTT, 4% (w/v) Ficoll, 2 μg of poly(dexoyinosinic-dexoyctydilic acid) (Pharmacia, Uppsala, Sweden), and 0.01% (w/v) SDS) for 40 min at 0°C. Where indicated, 20 ng of nonradioactive pM12-DNA was added initially or at the end of the incubation period, and in the latter case, the incubations were continued for an additional 40 min at 0°C. For supershift assays, 0.25 μg of MAb to Tcf-4 or Tcf-1 was added at the end of the first incubation period, and then the incubations were continued for an additional 40 min at 0°C. Samples were electrophoresed through nondenaturing 4% (w/v) polyacrylamide gels, which were dried, exposed to Fuji X-Omat film for 18 h with an intensifying screen, and processed for autoradiography. Nuclear and whole cell protein extracts were isolated from Rama 37 cells by standard methods (25). The Met-DNA fragments were radioactively labeled by random-primed synthesis to a specific activity of ~108 dpm/μg. Mouse MAb to Tcf-1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and that to Tcf-4 was a gift from Prof. Hans Clevers (University Hospital, Utrecht, Holland).

For gel shift comparison and competition experiments, Tcf-4 was produced by transcription and translation for 90 min with RNasin in reticulocyte cell-free protein-synthesizing lysates (Promega) directed by pCDNAI expression vector for Tcf-4 obtained directly from Prof. Hans Clevers (23). The programmed lysates contained Tcf-4 as its major product as determined by [35S]methionine-labeled proteins and independently by Western blotting, and this component was absent from unprogrammed lysates without the vector for Tcf-4. Gel shift comparative assays were performed by incubating 100 ng of 32P-end-labeled 20-bp pM12- or pM12AA-DNA (specific activity, ~107 dpm/μg) with 6 μl of programmed or unprogrammed lysates in a total volume of 30 μl of 0.03 mM KCl, 0.1 mM EDTA, 0.01 mM HEPES (pH 7.9), 0.25 mM dithiothreitol, 1 mM Na2HPO4 (pH 7.9), 10% (w/v) glycerol, 1 μg of single-stranded salmon sperm DNA, 0.4 μg of poly(dexoyinosinic-dexoyctydilic acid), and 0.01% (w/v) SDS for 40 min at 0°C. For supershift assays, 0.25 μg of MAb to Tcf-4 was added for 30 min at 0°C to the Tcf-4-containing lysate. Then the reaction buffer containing the 32P-labeled DNA was added, and the mixture was incubated for an additional 40 min at 0°C. For competition experiments, different amounts in the range 0.2 to 10 μg of pM12- or pM12AA-DNA were included with the 100 ng of 32P-labeled pM12AA-DNA. Reaction products were electrophoresed through nondenaturing 4% (w/v) polyacrylamide gels as above.

**Western Blotting for Proteins.** For detection of proteins, 10 μg of whole cell or nuclear extract or their equivalent from the gel mobility assays of Fig. 3 obtained by cutting out the relevant areas were electrophoresed through 10% (w/v) polyacrylamide, 1% (w/v) SDS gels, and a portion of the gel stained with silver-containing solutions (25). The proteins were then transferred from the remainder by blotting onto an Immobilon P membrane (Millipore Corporation, Watford, United Kingdom; Ref. 25). The membranes were incubated with blocking buffer containing 5% (w/v) Marvel, 0.02 Mr Tris-HCl (pH 7.0), 0.9% (w/v) NaCl, 0.1% (v/v) Tween 20, and then with 1:500 Mab to Tcf-4 or 1:50 rabbit polyclonal antibodies to β-catenin or E-cadherin. Bound antibodies were located by a further incubation with 1:5000 horseradish peroxidase-conjugated rabbit antimouse (for anti-Tcf-4) or swine antirabbit IgGs (for anti-β-catenin/ E-cadherin), visualized with Super Signal West Pico Chemiluminescence System (Pierce and Warner, Chester, United Kingdom), and exposed to Kodak XAR5 film (Anachem, Luton, United Kingdom). Prior incubation of primary antibody with its corresponding antigen abolished bands observed on these Western blots. Mouse Mabs to Tcf-1 and Tcf-4 were the gifts of Prof. Hans Clevers. Rabbit antibodies to β-catenin and E-cadherin were purchased from Santa Cruz Biotechnology. Western blots with whole cell or nuclear extracts isolated from transiently transfected Rama 37 cells or from C9-Met-DNA permanently transfected Rama 37 cells were also performed. The products of 90 reactions with 32P-labeled pM9- or pM12-DNAs and whole cell extracts were run in parallel on a thick DNA gel produced with a wide comb, and the excised bands were combined and rerun on a thick protein gel. The combined trypsin in-gel digests from the M, 43,000 band were microsequenced using an Applied Biosystems model 471A automated sequencer, and the first 10 amino acid residues from a major peptide were compared with those of other proteins using the Swiss-Prot database. Western blots for secreted opn protein were conducted on 10% (w/v) polyacrylamide gels and visualized with a polyclonal antisemur to rat opn as described previously (12). The stained bands were quantified by densitometry.

**Northern Blotting for mRNAs.** Northern blots for detection of mRNAs were performed on total RNA isolated from the relevant cell lines by TRIzol reagent (Life Technologies, Inc.), as described previously (20). The blots were hybridized separately in turn to 32P-labeled cDNAs for Tcf-4, α-actin, c-fos, and opn and subjected to autoradiography for 48 h using Fuji X-Omat film and an intensifying screen, as described previously (19). The cDNAs were radioactively labeled by random primed synthesis to a specific activity of 5 × 108 dpm/μg (25).

**Permanent DNA Transfections and Metastasis.** Rama 37 cells growing exponentially were harvested, seeded at a density of 0.5 × 106 cells/10 ml of routine medium (DME, 5% (v/v) FCS, 50 ng/ml insulin, and 50 ng/ml hydrocortisone) in four 90-mm diameter cell culture plates, and incubated for 24 h at 37°C. One plate was required for each transfection experiment, and one extra plate was required to estimate the number of cells at the time of transfection. Calcium phosphate-mediated transfection experiments with 5 μg of the pM12-DNA, pM12/DNA, both cloned in pBKCMV, and with pBKCMV vector alone were then performed exactly as described previously (11, 12), and the transfectants were passed after 24 h at a dilution of 1:10 in selective medium containing 1 mg/ml Geneticin. Some transfectant colonies were isolated; the remaining were pooled and expanded to give stocks for freezing. Southern blotting was undertaken with a 32P-labeled probe of a 420-bp fragment (pVUIpM12-Nhel) of the pM12-DNA-pBKCMV vector radioactively labeled by random primed synthesis (high primer; Boeringer, Mannheim, Germany) to a specific activity of 109 dpm/μg before hybridizing to 5 ng of pVUI- and Nhel-digested cellular DNA run on 0.8% (w/v) agarose gels, as described previously (11). The radioactive bands corresponding to hybridization of the probe were detected by exposure to X-ray film and quantified by scanning the images using a Schimadzu CS9000 scanning densitometer (11).

For the metastasis assay, pooled cells were harvested and 105 cells were injected into the right inguinal mammary fat pads of 6–10-week-old female Futh-Wistar rats (Ludwig-Wistar OLA strain), 20 rats/group (3). Rats were autopsied after 12 weeks, and tumors and relevant tissues were fixed in Methacarn before histological examination of five microscopic fields from two sections of each. The average of five independent experiments is shown. The activity of the opn promoter was performed on histological sections with the polyclonal antisemur to rat opn, detected with a peroxidase-conjugated rabbit antigoat secondary immunoglobulin, and photographed as described previously (11, 12).

**Animals.** Animal experiments were conducted under United Kingdom Coordinating Committee for Cancer Research guidelines with Home Office Project License PPL 40-00151 to Prof. Philip S. Rudland in accordance with the New York Academy of Sciences Committee on animal research.

**RESULTS**

**Identification of Positive Regulatory Regions of Met-DNA for the Opn Promoter.** To identify which region(s) of the most potent Met-DNA, C9-Met-DNA were responsible for up-regulating the expression of the opn gene, cloned C9-Met-DNA and its fragments (Fig. 1) in suitable constructs were transiently cotransfected into the benign Rama 37 cells together with an entirely separate 2.3-kbp fragment of the opn promoter coupled to a CAT reporter gene to measure opn promoter activity. The full-length C9-Met-DNA in either orientation stimulated the activity of the opn promoter in a concentration range of 1–10 ng, with a maximum at 1 ng/reaction (Fig. 2A). These results suggested that the active sequence for stimulation of the promoter is
nondirectional and is not capable of being expressed as mRNA. The restriction enzyme-digested or PCR-amplified fragments of C9-Met-DNA, pM1 to pM9-DNA were individually tested in this transient transfection, promoter-reporter assay. Only pM2-, pM6-, pM7-, and pM9-DNAs were positive, reaching 80% or above of the stimulation achieved by C9-Met-DNA (Fig. 2B) at their optima of 1, 1, and 10 ng/reaction, respectively. These results suggested that the active moiety resided solely in the region of C9-Met-DNA contained within pM9-DNA (339–435 bp; Fig. 1). The pM9-DNA (96 bp) and its two chemically synthesized halves, pM10 and pM11-DNAs (each 48 bp), stimulated the opn promoter at their optimum concentrations of 10, 20, and 20 ng/reaction to 81%, 56%, and 53%, respectively, of that with C9-Met-DNA (Fig. 2C). The region of pM9 contained within pM10, pM12-DNA (362–381 bp), a fragment of just 20 bp, stimulated the opn promoter by 59% of that of the full-length C9-Met-DNA, but pM12/D-DNA (15 bp) with the middle CAAAG sequence deleted was inactive (Fig. 2C).

These results suggested that the important sequence for activity was CAAAG, because it occurred once in all of the active fragments of pM9-DNA and twice in pM9-DNA itself. No other common sequence occurred in all of these active fragments. This sequence conforms to

Fig. 1. Fragments of C9-Met-DNA. The pattern of DNA fragments produced from C9-Met-DNA is shown. Rev. C9-Met-DNA is C9-Met-DNA in the reverse orientation. pM1-pM8 were obtained by restriction enzyme digestion, pM9 was obtained by PCR, and pM10, 11, and 12, including the CAAAG-deleted pM12/D, were obtained by chemical synthesis of DNA (see “Materials and Methods”).

Fig. 2. Effect of C9-Met-DNA fragments on opn promoter activity in Rama 37 cells. Rama 37 cells were transiently cotransfected for 48 h with an opn promoter-CAT reporter gene construct and (A) different amounts (ng) of C9-Met-DNA in PSI vector (C9 DNA) in one (●) or the reverse (○) orientation; (B) the optimum concentrations of 1 ng of C9-Met-DNA in PSI vector (C9), 1 ng of its truncated versions (Fig. 1.) pM1 to pM8-DNA or 10 ng of pM9-DNA in pBKCMV vectors; or (C) different amounts of truncated versions pM9 (■), pM10 (○), pM11 (●), pM12 (○), and pM12/D (▲)-DNA of C9-Met-DNA (Fig. 1.) in pBKCMV vectors. The CAT activity of the promoter-reporter construct alone (Con.) has been set at one in all of the cases. The relative CAT activity is the CAT activity with different DNAs relative to that with the promoter-reporter construct alone, normalized as described in “Materials and Methods.” Results are the mean ± SD of three separate experiments, but for clarity the minus SD bars have been omitted.
one of the core consensus sequences for the Tcf family of transcription factors given by $M_1 + M_2 = C$ or A; Ref. 26) and occurs at positions starting at nucleotides 364 and 404 in C9-Met-DNA. Although there are seven other potential Tcf-binding sites in C9-Met-DNA, none corresponded to CAAGA. The C9-Met-DNA fragments that contained only the other possible Tcf-binding sequences CACAG in pM1 ($\times 3$), pM3 ($\times 3$), pM4, pM5, and pM8-DNA and AAAAG in pM1, pM3, pM4, and pM5 ($\times 2$)-DNA (Fig. 1) were inactive in this assay (Fig. 2B). The fourth potential Tcf recognition sequence AACAG was not present in C9-Met-DNA. The only other transcription factor recognition sequence present in pM9-DNA, and that just once, is ATTCN for HIP1a (27) at position 393 in C9-Met-DNA. However, this sequence was also present once in an inactive fragment, pM5-DNA (Fig. 2A), at position 198 in the whole sequence (Fig. 1). The 14 remaining potential transcription factor recognition sequences that occurred in C9-Met-DNA did not occur in pM9-DNA (data not shown). In controls to check for the stimulatory effect of Met-DNA on the opn promoter, there was no stimulation of activity of a rat S100A4 promoter-CAT reporter construct by C9-Met-DNA, pM9-DNA, or pM12-DNA (data not shown). When Rama 37 cells, permanently transfected with C9-Met-DNA, were tested for opn promoter-reporter activity, they were more active than the untransfected Rama 37 cells. However, the Tcf-4-expressing vector still inhibited, and the Tcf-1-expressing vector still stimulated this elevated promot-er-reporter activity, they were more active than the untransfected Rama 37 cells. However, the Tcf-4-expressing vector still inhibited, and the Tcf-1-expressing vector still stimulated this elevated promot-er-reporter activity in the same concentration range as in the Rama 37 cells with similar results, 85% inhibition and 3.3-fold stimulation, respectively (Fig. 6B).

Effect of C9-Met-DNA Fragments on Endogenous mRNA and Protein Levels. As anticipated, pM9- or pM12-DNA fragments transiently transfected into Rama 37 cells suppressed the level of Tcf-4 protein in the nucleus but also unexpectedly partially suppressed the level of Tcf-4 mRNAs at 48 h. They had no effect on the levels of c-fos and actin mRNAs. These suppressed levels were also observed in Rama 37 cells permanently transfected with C9-Met-DNA (Fig. 7, A

Fig. 3. Gel mobility shift assay for Met-DNA fragments incubated with nuclear extracts from Rama 37 cells. A, gel retardation assay. $^{32}$P-labeled pM9-DNA in Lanes 1–3, $^{32}$P-labeled pM12-DNA in Lanes 4 and 6, and $^{32}$P-labeled pM12-D/deleted-DNA in Lane 5 were incubated with either buffer (Lanes 3 and 6) or nuclear protein extract (Lanes 1, 2, 4, and 5) from Rama 37 cells with Tcf-4, served as a supershift control. $^{32}$P-labeled pM9-DNA in Lanes 1–2 was incubated with nuclear protein extract from Rama 37 cells without (Lane 1) or with (Lane 2) MAb to Tcf-4 electrophoresed through a polyacrylamide gel, and the resultant radioactive bands were identified by autoradiography. The positions of the unbound pM9-DNA (Lane 3) and pM12-DNA (Lane 6) are located by arrows (→). B, supershift assay. $^{32}$P-labeled pM9-DNA in Lanes 1–2 was incubated with nuclear protein extract from Rama 37 cells without (Lane 1) or with (Lane 2) MAb to Tcf-4 electrophoresed through a polyacrylamide gel, and the radioactive bands were identified by autoradiography as above. Nonspecific binding is indicated by the arrowhead (↔).
Fig. 4. Comparison of gel mobility shifts for pM12-DNA and pM12AA-DNA incubated with reticulocyte cell-free protein-synthesizing lysates directed by an expression vector for Tcf-4. A, comparison assay. Reticulocyte lysates containing in vitro synthesized Tcf-4 protein were incubated with 32P-radioactively labeled pM12-DNA (Lanes 1 and 3) or with 32P-labeled pM12AA-DNA (Lanes 2 and 4), and nonradioactive pM12-DNA or 32P-labeled pM12AA-DNA (Lanes 5 and 6, respectively). In addition, MAAb to Tcf-4 was included in some reaction mixtures (Lanes 3 and 4). The reaction products were electrophoresed through polyacrylamide gels, and the resultant radioactive bands were identified by autoradiography. The positions of the unbound pM12-DNAs are located by thin arrows ( ), and the positions of the bound pM12-DNAs are located by thick arrows ( ). B and C, competition assays. Reticulocyte lysates containing in vitro synthesized Tcf-4 protein were incubated with 32P-labeled pM12AA-DNA and increasing amounts of either (B) nonradioactive pM12-DNA or (C) nonradioactive pM12AA-DNA. The reaction products were electrophoresed through polyacrylamide gels, and the resultant radioactive bands were identified by autoradiography. The positions of the unbound 32P-labeled pM12-DNA are located by thin arrows ( ), and the positions of the bound 32P-labeled pM12AA-DNA are located by thick arrows ( ).

Fig. 5. PAGE of proteins from Rama 37 cell extracts bound to Met-DNA fragments. A, silver-stained gels of proteins eluted from the retarded radioactive bands (Fig. 3A) of pM9-DNA (Lane 1), pM12-DNA (Lane 2), and pM12D-DNA (Lane 3). B, C, and D, Western blots of (B and C) nuclear or (D) whole-cell extracts (Lane 1) of proteins eluted from the retarded radioactive bands (e.g., Fig. 3A) of pM9-DNA (Lane 2) and pM12-DNA (Lane 3; B and C only) using antibodies to (B) Tcf-4, (C) β-catenin, and (D) E-cadherin. The molecular weights (in thousands) of standard proteins are also shown, and arrows point to the position of authentic proteins. The proteins eluted from pM12-DNA and Western blotted for E-cadherin are not shown.

and B). The CAAAG-deleted pM12/D-DNA, which failed to stimulate the opn promoter, also failed to suppress protein and mRNA levels (Fig. 7, A and B). The endogenous levels of opn mRNA were also increased in the pM9- and pM12-DNA transiently transfected cells at 72 h to similar levels seen in the C9-Met-DNA permanently transfected Rama 37 cells, but transient transfection with pM12/D-DNA was again without effect (Fig. 7C). The kinetics of these mRNA changes and those attributable to the opn promoter-reporter activity were investigated further. The increase in opn promoter-reporter activity and decrease in Tcf-4 mRNA occurred maximally at 48 h after transfection with pM9- or pM12-DNAs, whereas the increase in opn mRNA occurred maximally at 48–72 h after transfection (Fig. 8, A and B). Rama 37 cells permanently transfected with C9-Met-DNA were also stimulated to produce an enhanced opn promoter-reporter activity and enhanced levels of opn mRNA by transient transfection with pM9-DNA. The maximum for both increases also occurred after 48 h. However, the low levels of Tcf-4 mRNA were relatively unaffected by transient transfection with pM9-DNA (Fig. 8C). Upon quantitation at 48 h after transient transfection, the basal opn promoter-reporter activity in the C9-Met-DNA permanently transfected cells was 7–9-fold higher than in the Rama 37 cells, and the level of endogenous opn mRNA was 9-fold higher in the permanently transfected, compared to the untransfected, Rama 37 cells. At the same time, there was a 3-fold drop in the level of Tcf-4 mRNA. When Rama 37 cells were transiently transfected with pM9-DNA, the promoter-reporter activity rose 3–4-fold, there was a 3-fold increase in opn mRNA, and a fall of 6-fold in Tcf-4 mRNA. In contrast, when the C9-Met-DNA-transfected cells were transiently transfected with pM9-DNA, the promoter-reporter activity rose only 1.6-fold, the opn mRNA only 1.4-fold, and the Tcf-4 mRNA dropped by only 2-fold after 48 h (Table 1). Northern hybridizations and Western blots with cDNA and antibodies to Tcf-1 failed to detect any Tcf-1 mRNA or protein, respectively, in either untransfected or transfected Rama 37 cells (data not shown).

Effect of Permanent Transfection of a Met-DNA Fragment on Production of Opn and Metastasis in Vivo. Transfection of Rama 37 cells with pM12-DNA construct, pM12D-DNA construct, or pBKCMV vector alone yielded 1- to 5-mm diameter colonies of cells that were visible after 10 days. The transfection frequency of 1.7 ± 0.3 × 10^4 was not significantly different for the three constructs. Control experiments with no transfected DNA yielded no colonies even after 3 weeks of incubation in selective medium. Col-
ometastasis-inducing DNA and Tcf-4

Fig. 6. Effect of Tcf overexpression on pM9-DNA stimulation of the opn promoter. A, Rama 37 cells were transiently transfected for 48 h with the opn promoter-CAT reporter gene construct in pBKCMV vector and 10 ng of pM9-DNA in pBKCMV. B, C9-Met-DNA permanently transfected Rama 37 cells were transiently transfected for 48 h with the same promoter-reporter construct as above but without the pM9-DNA vector. Increasing amounts of Tcf-4 (□) or Tcf-1 (○) cDNAs in the pBKCMV expression vector were cotransfected/3-cm dish. The CAT activity of the promoter-reporter construct alone in Rama 37 cells has been set at one in both cases. The relative CAT activity is the CAT activity with different DNAs and/or transfected cells relative to that with the promoter-reporter construct alone in Rama 37 cells, normalized as described in “Materials and Methods.” Results are the mean ± SD of three separate experiments, but for clarity the minus SD bars have been omitted.

Fig. 7. Effect of Met-DNA fragments on endogenous mRNA and protein levels. A, Fos, actin, and Tcf-4 mRNA. Northern hybridizations with cDNAs to fos, smooth muscle α-actin, and Tcf-4 mRNAs were conducted on 20 μg of total RNA isolated from untransfected Rama 37 cells (Lane 1), Rama 37 cells transiently transfected for 48 h with 20 ng of pM12-DNA (Lane 2), Rama 37 cells transiently transfected for 48 h with 10 ng of pM9-DNA (Lane 3), Rama 37 cells transiently transfected for 48 h with 20 ng of pM12-DNA (Lane 4) all in pBKCMV vectors, and Rama 37 cells permanently transfected with C9-Met-DNA (Lane 5). The positions of the relevant mRNAs are indicated. B, Tcf-4 protein. Western blots were conducted on 10 μg of nuclear protein isolated from the same Rama 37 transfected cells as above. The position of the Tcf-4 and marker proteins is shown. C, opn mRNA. Northern hybridizations with cDNAs to opn mRNA were conducted as in (A) above and to smooth muscle α-actin (Actin) mRNA, except that the transient transfections were continued for 72 h. The lanes contain the same transfecting DNAs as in (A) above. The position of the opn and actin mRNAs are shown by the arrows.

Fig. 8. Effect of Met-DNA fragments on endogenous mRNA and protein levels. A, Fos, actin, and Tcf-4 mRNA. Northern hybridizations with cDNAs to fos, smooth muscle α-actin, and Tcf-4 mRNAs were conducted on 20 μg of total RNA isolated from untransfected Rama 37 cells (Lane 1), Rama 37 cells transiently transfected for 48 h with 20 ng of pM12-DNA (Lane 2), Rama 37 cells transiently transfected for 48 h with 10 ng of pM9-DNA (Lane 3), Rama 37 cells transiently transfected for 48 h with 20 ng of pM12-DNA (Lane 4) all in pBKCMV vectors, and Rama 37 cells permanently transfected with C9-Met-DNA (Lane 5). The positions of the relevant mRNAs are indicated. B, Tcf-4 protein. Western blots were conducted on 10 μg of nuclear protein isolated from the same Rama 37 transfected cells as above. The position of the Tcf-4 and marker proteins is shown. C, opn mRNA. Northern hybridizations with cDNAs to opn mRNA were conducted as in (A) above and to smooth muscle α-actin (Actin) mRNA, except that the transient transfections were continued for 72 h. The lanes contain the same transfecting DNAs as in (A) above. The position of the opn and actin mRNAs are shown by the arrows.

DISCUSSION

Fragmentation of C9-Met-DNA has shown that only the smaller pM-DNAs that possess the CAAAG sequence are active in stimulat-
ing the entirely separate opn promoter-reporter CAT construct when transiently transfected into the Rama 37 cells. There are two such sequences in the whole-length C9-Met-DNA, as well as in pM2, pM6, pM7, and pM9-DNAs, and one such sequence in pM10, pM11, and pM12-DNAs (21). This distribution of CAAAG sequences is reflected in the relative maximum activities of the opn promoter-reporter construct when cotransfected with either of the two groups of DNA fragments. The first group produces maximum stimulations of 80% or above of that with C9-Met-DNA, none of which are significantly different from that with the full-length molecule (Student’s t test, \( n = 3, P > 0.05 \)), whereas the second group produces maximum stimulations of 50–60% of that with C9-Met-DNA, all of which are significantly different from that with C9-Met-DNA (\( P < 0.05 \)) and that with the promoter-reporter construct alone (\( P < 0.03 \)). Although three of four combinations of potential core Tcf-binding sequences given by \( M_1 M_2 A G (M_1, M_2 = C \text{ or } A; \text{Ref } 26) \) are present in C9-Met-DNA, only those fragments possessing the CAAAG sequence are active in stimulating the cotransfected opn promoter-reporter construct. The C9-Met-DNA contains seven other potential core Tcf-recognition sites, four of which occur in pM1 and three in pM5, but neither of these fragments is active (Fig. 2B). These results eliminate the CACAG and AAAAG combinations of potential core Tcf-recognition sequences (21) from possessing any activity for stimulating the opn promoter-reporter construct in this system. The fact that pM9-DNA possesses two CAAAG sequences and pM12-DNA possesses one such sequence that are active in stimulating the cotransfected opn promoter-reporter construct is consistent with results obtained in the gel retardation/supershift assays. Here, pM9-DNA produces two retarded bands after incubation with cell nuclear extracts, whereas pM12-DNA produces only one retarded band in these assays (Fig. 3A). Both retarded bands of pM9-DNA (Fig. 3B) and the one retarded

Table 1

Comparison of opn promoter-report activity, opn, and Tcf-4 mRNAs in different transiently transfected cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>pM9-DNAa</th>
<th>Cpm</th>
<th>Opn mRNAb</th>
<th>Tcf-4 mRNAc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rama 37</td>
<td>-</td>
<td>1.00 ± 0.2</td>
<td>1.00 ± 0.2</td>
<td>1.00 ± 0.2</td>
</tr>
<tr>
<td>Rama 37</td>
<td>+</td>
<td>3.6 ± 0.4</td>
<td>3.1 ± 0.2</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>Met-DNA R37</td>
<td>-</td>
<td>2.5 ± 0.7</td>
<td>8.9 ± 0.6</td>
<td>0.31 ± 0.02</td>
</tr>
<tr>
<td>Met-DNA R37</td>
<td>+</td>
<td>12.1 ± 0.6</td>
<td>12.7 ± 0.9</td>
<td>0.15 ± 0.05</td>
</tr>
</tbody>
</table>

a Cell lines used, Rama 37, benign rat mammary cells or Met-DNA R37, Rama 37 cells permanently transfected with C9-Met-DNA, were transiently transfected for 48 h with the opn promoter-reporter construct either without (+) or with (+) 10 ng of pM9-DNA/reaction (Fig. 1).b CAT activity of the transiently transfected opn promoter-reporter construct, normalized as described in “Materials and Methods,” expressed relative to that for the Rama 37 cells transfected with only the promoter-reporter construct (1 = 2801 cpm). Results are the mean ± SD of three separate experiments. c Hybridizable levels of opn mRNA, normalized with respect to actin mRNA, and expressed relative to that for the Rama 37 cells transiently transfected with only the promoter-reporter construct (1 = 500 cpm). Results are the mean ± SD of three separate experiments. d Hybridizable levels of Tcf-4 mRNA, normalized with respect to actin mRNA, and expressed relative to that for Rama 37 cells transiently transfected with only the promoter-reporter construct (1 = 560 cpm). Results are the mean ± SD of three separate experiments.

Fig. 8. Kinetics of the changes in Tcf-4 mRNA, opn promoter-reporter, and opn mRNA after introduction of Met-DNA fragments. A and B, parental Rama 37 cells. Rama 37 cells were transiently transfected with the opn promoter-reporter construct in pBKCMV vector and (A) 10 ng of pM9-DNA or (B) 20 ng of pM12-DNA in pBKCMV vector. C, permanently transfected cells. C9-Met-DNA permanently transfected cells were transiently transfected with the opn promoter-reporter construct in pBKCMV vector and 10 ng of pM9-DNA in pBKCMV vector. Cells were isolated at different times in h after transient transfections and assayed for opn promoter activity using the CAT assay (○), for endogenous opn mRNA (●), and for endogenous Tcf-4 mRNA (□); see “Materials and Methods”). The CAT activity of the promoter-reporter construct alone in (A and B) Rama 37 or in (C) C9-Met-DNA-transfected Rama 37 cells has been set at one at 0 h, and all of the other CAT activities are expressed relative to these. All of them have been normalized as described in “Materials and Methods.” The relative levels of mRNA have been normalized with respect to the level of actin mRNA, and the results for time zero for opn or the results for the 72-h point for Tcf-4 mRNA have been set at one. Results are the mean ± SD of three separate experiments, but for clarity the minus SD bars have been omitted.

Fig. 9. Effect of permanent transfection of a Met-DNA fragment on production of opn. Western blots with antibody to rat opn are shown for the transfectants R37-AC1 (Lane 1), AC2 (Lane 2), AC3 (Lane 3), AC4 (Lane 4), high-passage pool R37-A (Lane 5), R37-AE1 (Lane 6), AE2 (Lane 7), AE1 again (Lane 8), low-passage pool R37-A (Lane 9), all from the pM12-DNA construct, blots of the transfectants R37-BC1 (Lane 10), BC2 (Lane 11), BE1 (Lane 12), high-passage pool R37-B (Lane 13), low-passage pool R37-B (Lane 14), all from the pM12/D-DNA construct, and a blot of the pool R37-C from the pBKCMV vector construct alone (Lane 15). The position of authentic rat opn protein is shown by the arrow (○). Equal amounts of total cellular protein were loaded in each lane of the polyacrylamide gel.
band of pM12-DNA (Fig. 4A) contain Tcf-4, because they are all subject to supershift with MAb to Tcf-4. The two gel shifted bands of pM9-DNA probably correspond to pM9-DNA with one Tcf-binding site occupied and to pM9-DNA with two Tcf-binding sites occupied. The reason for the relative efficiencies of C9-Met-DNA and its fragments in stimulating the cotransfected opn promoter-reporter with the smaller fragments requiring 10–20 times greater concentration by weight than the whole-length and larger fragments of C9-Met-DNA for maximum stimulation is unknown. It is not related to the number of CAAAG sequences but may be attributable to their secondary and tertiary structures in either protecting the fragments from degradation and/or stabilizing any resultant complexes still further. In the latter respect, the fact that incubation of the complexes with excess nonradioactive pM12-DNA or with pancreatic DNase fails to dislodge or digest any DNA suggests that these complexes are extremely stable and involve more than the five nucleotides in the Tcf-recognition sequence. The results obtained for stimulation of the opn promoter-reporter construct are not unique to the rat mammary cell line used for its assay, because identical results have been obtained when the C9-Met-DNA fragments and the opn promoter-reporter construct have been cotransfected in human HeLa cells and in monkey COS-1 cells (data not shown).

When pM9- or pM12-CAAAG-containing DNA fragments of C9-Met-DNA are incubated in vitro with Rama 37 cell extracts, the Tcf family factor that is found to be preferentially bound is Tcf-4 and not Tcf-1 in both gel shift and supershift assays. That β-catenin is also found in gel-shifted bands containing pM9- or pM12-DNAs and that this finding is dependent on an intact CAAAG sequence suggest that β-catenin binds directly to Tcf-4 on the DNA, as reported in other systems (30, 31). Although E-cadherin is not found binding to the Tcf-4/Met-DNA complex in experiments with nuclear extracts, consistent with the idea that E-cadherin and Tcf-4 bind to overlapping sites on β-catenin and therefore are mutually exclusive (31), it is found in association with Met-DNA fragments containing the CAAAG sequence, Tcf-4, β-catenin, and actin in experiments with whole-cell extracts. Thus, it is possible that Tcf-4 and β-catenin form complexes with Met-DNA fragments in the nucleus and that E-cadherin in the remainder of the cell can interact indirectly through actin-associated proteins (e.g., α- and γ-catenins) with β-catenin (32, 33), thereby permitting Tcf-4 to bind to β-catenin and anchor the entire complex at the correct site on the DNA.

There are three CAAAG sequences in the rat opn promoter (22), all of which are capable of binding Tcf-4 and then are transcriptionally activatable by β-catenin or inhibitable by excess Tcf-4.4 However, these three CAAAG sequences are preceded by the dinucleotide TA, AA, or TT rather than GG, which is present in both instances in C9-Met-DNA. The moderately degenerate heptamer motif A/TA/TCAAAG is considered to be a complete binding sequence for Tcf-1 in T lymphocytes (28, 29), but there was no difference in gel mobility shifts for either the AA-containing or the GG-containing pM12-DNAs. In the systems used here, both oligonucleotides competed for binding to reticulocyte lysates containing Tcf-4 in the same concentration range. Both complexes contained Tcf-4, and the complexes with the active Met-DNA fragments pM9 and pM12-DNA contained Tcf-4 in the Rama 37 system. Thus, it is possible that further degeneracy in the first two positions of the original heptamer motif for Tcf-1 (26) may occur in the binding of extracts containing Tcf-4. Thus, Tcf-4 presumably acts as a negative transcription factor, as reported previously (31) for matrilysin in intestinal cells, and the GGCAAAG-containing Met-DNA fragments compete for Tcf-4 with the A/TA/TCAAAG-containing opn promoter reporter construct in this Rama

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**Table 2 Comparison of levels of opn, incidence of tumor formation, and incidence of metastasis produced by permanent transfection of a Met-DNA fragment**

<table>
<thead>
<tr>
<th>Transfectants</th>
<th>Transflecting DNA</th>
<th>Opn protein</th>
<th>Tumor incidence</th>
<th>Incidence of metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>R37-A</td>
<td>pM12-DNA construct</td>
<td>9.4 ± 1.8</td>
<td>14/20</td>
<td>8/14*</td>
</tr>
<tr>
<td>R37-B</td>
<td>pM12/D-DNA construct</td>
<td>1.2 ± 0.4</td>
<td>10/20</td>
<td>1/10</td>
</tr>
<tr>
<td>R37-C</td>
<td>Vector construct alone</td>
<td>1.0 ± 0.0</td>
<td>18/20</td>
<td>0/18</td>
</tr>
</tbody>
</table>

* Transfecting DNA was incorporated into the expression vector pBKCMV.

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4 M. El-Tanani, R. Barraclough, and P. S. Rudland, unpublished data.

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**Fig. 10.** Immunocytochemical staining for opn of tumors and metastases produced by Rama 37 cells permanently transfected with a Met-DNA fragment. *A*, tumor from pooled transfectants R37-B produced with the pM12/D-DNA construct showing no staining. *B*, tumor from pooled transfectants R37-A produced with the pM12-DNA construct showing cellular cytoplasmic staining. *C*, lung micrometastasis from pooled transfectants R37-A above showing cytoplasmic staining of the tumor cells (*i*) but no staining of the parenchymal tissue (*f*). Magnification, ×220. Bar, 50 μm.
The transfectected GGCAAAG-containing DNAs in large excess can then sequester Tcf-4 in the Rama 37 cells and thereby stimulate the activity of the transiently cotransfected, but entirely separate, opn promoter-reporter construct. This stimulation is achieved by relieving the inhibition caused by Tcf-4 binding at the A/TATA/GCAAG sequences in the opn promoter.

The above interpretation is supported by the fact that transiently transfected vectors expressing Tcf-4 but not Tcf-1 inhibit the increased activity of the opn promoter-reporter construct produced by transiently cotransfected GGCAAAG-containing fragments of C9-Met-DNA or by permanently transfected C9-Met-DNA in Rama 37 cells. However, the identification of proteins complexed with GGCAAAG-containing DNA fragments in vitro does not necessarily prove that such complexes occur in vivo, and direct recourse to other types of experiments that assay for complex formation in vivo, such as the yeast one hybrid system (34, 35), will be necessary to establish this fact unequivocally. The reason for the contrasting effects of Tcf-4 and Tcf-1 in either inhibiting or enhancing the opn promoter-reporter activity is unknown. Both transcription factors are reported to recognize the same regulatory sequences (26), and only one of them, CAAAG, occurs in pM9-DNA (Fig. 1). Thus, higher order interactions involving additional proteins may be responsible for the differential effects of the expression vectors for Tcf-4 and Tcf-1 (Fig. 6).

However, because only Tcf-4 and not Tcf-1 protein and mRNA can be detected in Rama 37 and its transfected cells, the stimulatory effects of Tcf-1 do not occur normally in these cells in vivo.

The similar time courses for induction of endogenous opn mRNA and for opn promoter-reporter activity of the exogenously added construct suggest that C9-Met-DNA and its active fragments are capable of inducing endogenous opn mRNA by activation of its endogenous promoter in the same way as that of the construct. This effect is relatively specific, because there is no effect on the exogenously transfected promoter-reporter construct (36) for the metastasis-inducing protein S100A4 (p9Ka; Ref. 37) or on the enhancement of levels of mRNA of endogenous genes for c-fos and actin. The relative increase in activity of the transiently transfected opn promoter-reporter construct, both in pM9-DNA transiently transfected Rama 37 cells and in C9-Met-DNA permanently transfected Rama 37 cells, is also reflected in a similar relative increase in the levels of opn mRNA (Table 1). That the much higher basal levels of opn promoter-reporter activity and endogenous opn mRNA in the C9-Met-DNA permanent transfecants (7–9-fold) are less readily stimulatable by transient transfection of pM9-DNA (1.4–1.6-fold) than the parental Rama 37 cells (3–4-fold) is also consistent with the idea that the multiply integrated copies of C9-Met-DNA (11) are responsible for sequestration of Tcf-4 protein. However, sequestration of Tcf-4 cannot be the entire explanation for this phenomenon. Although Tcf-4 protein is dramatically depleted in nuclear extracts presumably, in part, by complexing with transfected DNA in the Rama 37 cells (Fig. 7), the levels of mRNA (Fig. 7) and total cellular Tcf-4 protein (data not shown) are also specifically reduced in the same time period by GGCAAAG-containing transfected DNAs, although not to the same extent (Fig. 7). Thus, GGCAAAG-containing transfected DNAs can sequester Tcf-4 and also cause reduction in the level of Tcf-4 mRNA by hitherto unknown mechanisms, thereby also reducing the level of Tcf-4 protein. The balance between sequestration and loss of Tcf-4 remains to be determined. Although the relative levels of activity of the opn promoter-reporter construct, opn mRNA, and Tcf-4 mRNA are consistent in a single series of experiments (e.g., Table 1), there are small differences in relative basal levels for Rama 37 and C9-Met-DNA-transfected Rama 37 cells when experiments are well separated in time (e.g., Fig. 6 and Table 1). These small differences probably arise from differences in the passage number and/or culture conditions of the cells and are not sufficiently large to alter the conclusions.

The fact that permanent DNA transfection of Rama 37 cells with the 20-bp fragment of C9-Met-DNA, pM12-DNA causes elevated levels of opn and confers the ability to metastasize in syngeneic rats, whereas transfection with the equivalent CAAAG-deleted fragment pM12/D-DNA fails on both accounts, further substantiates the claim that the CAAAG sequences in C9-Met-DNA are responsible both for the elevated levels of opn and for the induction of metastasis in the Rama 37 cell system. The possibility that our results are attributable to inadvertent selection of a particular clone of permanent pM12-DNA transfecants, which, by chance, overproduces opn and which is metastatic, is unlikely for the following reason. The pooled pM12-DNA transfecants, as well as the six single-cell clones of pM12-DNA transfecants tested, all produce similarly elevated levels of opn. These levels of opn are also similar to those produced by direct transfection of the same cells with an expression vector for opn and cause them to metastasize in vivo (12).

In addition to C9-Met-DNA, there are five other Met-DNAs termed C2, C5, C6, C12, and C20-Met-DNA (11), and they also possess potential core Tcf-CAAAG recognition sequences. The only other transcription factor recognition sequence besides that of Tcf (26) that is common to all of the six Met-DNAs is that for HIP1b (27) at positions 744 and 944 in C9-Met-DNA. The next most commonly occurring transcription factor recognition sequences are for CTFC (38) at position 230 and for HIP1a (27) at positions 198 and 393, which occur in five of the Met-DNAs, including C9-Met-DNA (11). However, fragments of the C9-Met-DNA that contain these other potential transcription factor recognition sequences (pM1, pM3, pM4, pM5, and pM8-DNAs) are all unable to stimulate the opn promoter-reporter construct in the rat mammary Rama 37 cell system used here (Fig. 28) or in human HeLa or monkey COS-1 cells (data not shown). All of the Met-DNAs have been obtained from one malignant metastatic breast cell line, Ca2-83. However, DNAs with similar metastasis-inducing abilities occur in MCF-7 (39) and ZR-75 (40) breast carcinoma cell lines isolated from metastatic pleural effusions (5) and in metastatic rat mammary cell lines (4). Moreover, in pilot studies, C9-Met-DNA has been shown to occur preferentially in genomic DNA from some but not all human breast carcinomas, but not in genomic DNA from normal tissue, when analyzed by Southern blotting techniques (11). How the Met-DNAs arise preferentially in the original malignant metastatic cells is unknown, but it is possibly the result of deletions or rearrangements bringing together the active CAAAG sequences. Our previous subtractive hybridization experiments between the benign and the original human metastatic cell DNA-transfected rat cell lines have shown that opn mRNA is by far and away the largest single change detected in any mRNA species using this technique (12), and all of the six Met-DNA permanently transfected cell lines exhibit elevated levels of opn mRNA that correlate with their metastasis-inducing ability (11). Direct permanent transfection of Rama 37 cells with opn cDNA in a cytomegalovirus expression vector that produces similarly elevated levels of opn mRNA also causes the parental cells to metastasize, establishing a causal link between permanent opn overexpression and metastasis in this system (11, 12). The high number (~100) of integrated copies/cell of all of the CAAAG-containing Met-DNAs (11) as well as of pM12-DNA in the permanently transfected Rama 37 cells must be enough to sequester a sufficient number of Tcf-4 molecules in the nucleus so that the majority of those bound to the endogenous opn promoter are removed, thereby stimulating its transcription. This idea is supported by the fact that transient transfection of Rama 37 cells with pM9-DNA yields an appreciable 3–4-fold stimulation of the opn promoter and opn mRNA, whereas transient transfection of C9-Met-
DNA-transfected Rama 37 cells with pM9-DNA yields only a 1.4–1.6-fold stimulation, albeit over much higher basal levels of 7–9-fold compared with the basal levels in Rama 37 cells (Table 1). Tcf-4 is a member of the high mobility group of architectural transcription factors that control developmental steps in diverse species (23). Recently, Tcf gene products have been found to constitute a downstream component of the Wnt signal transduction pathway (23) that, inter alia, is responsible for the maintenance of the small intestinal crypts (41) and may contribute to morphological organization of the mammalian gland (42, 43). Tcf-related factors introduce sharp bends in the DNA and facilitate interactions with other transcription factors by helping to assemble a large nucleoprotein complex (44) that can then trigger both positive and negative regulatory signals depending on the identity of the other factors present (23). Little is known about the role of the Tcf family in breast cancer, but the Tcf-signaling pathway is thought to be involved in the generation of some colon cancers. Normally, the product of the APC gene binds to β-catenin and prevents β-catenin from forming a complex with Tcf-4. However, in colorectal cancer, mutations in 80% of cases for APC or 10% of the remainder for β-catenin cause the inhibitory effect of APC to be lost and allow β-catenin to bind to Tcf-4 nuclear complex (24, 30). These changes cause β-catenin to be lost from its membrane/cytoskeletal location (where it binds to E-cadherin and/or α-catenin) in 26% of primary tumors and in 60% of liver metastases (45). However, the precise target genes then regulated by β-catenin/Tcf-4 are virtually unknown, although candidate genes have included c-myc (46) and matrilysin (31). Overexpression of Tcf-1, but not Tcf-4, is reported to occur in human colon cancer-derived cell lines, and this overexpression has been correlated with properties associated with metastasis (47), whereas Tcf-4 has been shown recently (42) to be expressed in mammary epithelium. Now we have demonstrated in a rat mammary model that the metastasis-inducing DNAs sequester the Tcf-4 transcription factor in vitro, together with its associated proteins β-catenin and E-cadherin, as well as down-regulating Tcf-4 in vivo. Thereby, they allow activation of the promoter for the direct effector of tumor progression and metastasis in this system, opn, recently (11, 12). Therefore, this report also identifies a novel downstream effector, opn, for the Tcf-signaling pathway that is directly involved in promoting metastasis in a model system.

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Metastasis-inducing DNA Regulates the Expression of the Osteopontin Gene by Binding the Transcription Factor Tcf-4

Mohamed El-Tanani, Roger Barraclough, Mark C. Wilkinson, et al.


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