12-O-Tetradecanoylphorbol-13-acetate and UV Radiation-induced Nucleoside Diphosphate Protein Kinase B Mediates Neoplastic Transformation of Epidermal Cells*

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The molecular changes associated with early skin carcinogenesis are largely unknown. We have previously identified 11 genes whose expression was up- or down-regulated by 12-O-tetradecanoylphorbol-13-acetate (TPA) in mouse skin keratinocyte progenitor cells (Wei, S.-J., Trempus, C. S., Cannon, R. B., Eortner, C. D., and Tennant, R. W. (2003) J. Biol. Chem. 278, 1758–1768). Here, we show an induction of a nucleoside diphosphate protein kinase B (NDPK-B) gene in response to TPA or UV radiation (UVR). TPA or UVR significantly induced the expression of NDPK-B in both in vivo hyperplastic mouse skin and in vitro mouse JB6 Cl 41-5a epidermal cells. Indeed, this gene was also up-regulated in TPA or UVR-mediated skin tumors including papillomas, spindle cell tumors, and squamous cell carcinomas, relative to adjacent normal skins. Functional studies by constitutive expression of nm23-M2/NDPK-B in TPA susceptible JB6 Cl 41-5a and TPA-resistant JB6 Cl 30-7bpreneoplastic epidermal cell lines showed a remarkable gene dosage-dependent increase in foci-forming activity, as well as an enhancement in the efficiency of neoplastic transformation of these cells in soft agar but no effect on proliferation in monolayer cultures. Interestingly, stable transfection of the nm23-M2/NDPK-B delRGD or G106A mutant gene in JB6 Cl 41-5a cells selectively abrogated NDPK-B-induced cellular transformation, implicating a possible Arg105,Gly106,Asp107 regulatory role in early skin carcinogenesis.

Mouse skin carcinogenesis is a complex multistage process that progresses through distinct stages of initiation, promotion, and progression to malignancy (1, 2). The molecular changes associated with the early stages of skin tumor formation have yet to be determined. Tg-AC mouse, which carry the coding region of the v-Ha-ras oncogene fused to a fetal z-globin gene promoter (3), are considered to be genetically initiated and have a higher sensitivity to promotional stimuli including TPA4 (5) and full thickness wounding (4), or carcinogens such as UV radiation (UVR) (5) and 7,12-dimethylbenz[a]anthracene (6). These features establish the in vivo Tg-AC mouse model as a valuable tool to study the early stages of skin carcinogenesis.

In an earlier study with a combination of fluorescence-activated cell sorting, switching mechanism at the 5′-end of RNA templates cDNA amplification, and mouse cDNA array technology, we identified 11 genes whose expression changed significantly in o6 CD34+ keratinocytes harvested from TPA-treated mice relative to cells from untreated mice. Nine genes, including galectin-7, nm23-M2/NDPK-B, cytokines, epidermal keratin 14, deleted in split hand/split foot gene 1 (Dss1), DNA double strand break repair RAD21 homolog, transcription termination factor 1, thymosin β4, calpactin I light chain, and 40 S ribosomal protein SA, were up-regulated, and two genes, apolipoprotein E precursor and acidic keratin complex 1 gene 15, were down-regulated by TPA (7). Dss1, a gene associated with a heterogeneous limb developmental disorder called split hand/split foot malformation (8), has recently been identified as a novel TPA-inducible gene expressed in keratinocyte progenitor cells, with possible involvement in early skin tumorigenesis (7). This novel approach was highly effective in the in vivo identification of TPA-inducible effector genes that might lead to neoplastic transformation. The protein kinase nm23-M2/NDPK-B was another one of nine TPA-up-regulated genes and was selected for further characterization.

Nm23 is a large family of structurally and functionally conserved proteins consisting of 4–6 identical subunits of 17–20 kDa each, known also as nucleoside diphosphate kinases (NDPKs; EC 2.7.4.6) (9). NDPKs were originally identified as essential housekeeping enzymes required for the synthesis of nucleoside triphosphates by catalyzing the transfer of the γ-phosphoryl groups from nucleoside triphosphates to nucleoside diphosphates via a phosphohistidine 118 enzyme intermediate, and they play a role in maintaining intracellular nucleotide concentrations (10). Altered expression of NDPK is also reportedly involved in many cellular processes, including oncogenesis (11, 12), cellular proliferation (13), differentiation (14, 15), motility (16), development (17), DNA repair (18), and apoptosis (19). In Escherichia coli, NDPK functions as a mutator gene and is not essential for viability (20). In Dictyostelium discoideum, the membrane possesses cAMP surface receptor-stimulated NDPK that leads to the activation of G-proteins and phosphorylase C (21). Recent data also indicate that NDPK-B can form complexes with Gβγ dimers and contributes to G protein activation (22, 23). In yeast, an NDPK knockout strain

pAb, polyclonal antibody; RGD, Arg-Gly-Asp; RIP, radioligand precipitation assay; RT, reverse transcription; UVR, UV radiation; WT, wild type; NS, normal skin.
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The full-length mouse nm23-M2/NDPK-B cDNA was generated by reverse transcription (RT) and PCR amplification using total RNA isolated from untreated (normal) skin harvested from TgAC mice. Because the nucleotide similarity (83%) and amino acid identity (88%) between nm23-M2/NDPK-A and nm23-M2/NDPK-B are relatively high (37), mouse nm23-M2/NDPK-B-specific primers were designed to exclude the possibility of cross-reactivity with mouse nm23-M2/NDPK-A. The specific primers used for PCR were as follows: forward (5′-CAC CCT GGA CCA CAA CCT CGG TAC-3′) and reverse (5′-5′-CTG TAC ATG GCC TGG TTT TGA ATC AGC-3′) for full-length mouse nm23-M2/NDPK-B cDNA cloning; forward (512-516) and reverse (321-325) for cDNA amplification; and forward (105-110) and reverse (321-325) for in situ hybridization assay. The amplified cDNA was cloned directly into pcDNA3.1Dvs-His-TOPO mammalian expression vector (Invitrogen). For the in situ hybridization assay, a construct expressing 217 bp of mouse nm23-M2/NDPK-B sense or antisense RNA probe was generated as follows. pcDNA3.1Dvs-His-TOPO

Cloning and Mutagenesis

EXPERIMENTAL PROCEDURES

Reagents

Nmd2-M2/NDPK-B rat monoclonal antibodies (mAb) were obtained from United States Biological (Swampscott, MA). V5 tag mouse mAb was from Invitrogen; α-tubulin mouse mAb was from Zymed Laboratories Inc. (San Francisco, CA); actin rabbit polyclonal antibody (pAb) was from Sigma. Horseradish peroxidase-conjugated secondary antibodies were from Amersham Biosciences. Nmd2-M2/NDPK-B mouse mAb (231.3) was a kind gift from Dr. Michel Véron (Institut Pasteur, Paris, France). Uridine 5′-α,β,γ-[32P]tritiated triphosphate triethylammonium salt (SP6/T7 grade) (~800 Ci/mmol) was purchased from Amersham Biosciences. Restriction enzymes, including BamHI, XbaI, EcoRI, and HindIII, were obtained from New England Biolabs (Beverly, MA). The primers used in this study were purchased from Proligo Corp. (La Jolla, CA). Noble agar was purchased from Difco. The tumor promoter TPA was from Sigma.

Cell Cultures

TPA-susceptible JB6 Cl 41-5a and TPA-resistant JB6 Cl 30-7b mouse epidermal cell clonal variants were from the American Type Culture Collection (Manassas, VA) and grown at 37 °C and 95% air plus 5% CO2 atmosphere in Eagle’s minimal essential medium (MEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS) containing 2 mm glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate (Invitrogen). Mouse fibroblast Rat-1, human keratinocyte HaCaT (generously provided by Dr. Horbet Fusenig, German Cancer Research Center, Heidelberg, Germany), and human epidermoid carcinoma cell A431 were cultured in Dulbecco’s modified Eagle’s medium containing 10% FBS. TgAC43 (a TPA-induced TgAC squamous cell carcinoma cell line) and FVB/N217 (an FVB/N carcinoma cell line) (43) were cultured in RPMI1640/Dulbecco’s modified Eagle’s medium (1:1) with 20% FBS. NIH/3T3 cells were maintained as described previously (44) (Cell lines used in this study were free of mycoplasma infection).

Animals and Treatments

8–10-week-old female homozygous TgAC mice were obtained from the Taconic Laboratory of Animals and Services (Germantown, NY). Mouse studies were carried out in compliance with the National Institutes of Health Guidelines for Human Care and Use of Laboratory Animals. The dorsal skin surface of groups of four homozygous female TgAC mice were dosed twice weekly for 2 weeks with 5 μl TPA in 200 μl of acetone. Untreated control mice were sacrificed on day 1 (designated as NS). Four dosing protocols were used as follows. Mice were dosed on day 1 and sacrificed on day 5 (designated as TPA); mice were dosed on days 1 and 5 and sacrificed on day 8 (designated as 2TPA); mice were dosed on days 1, 5, and 8 and sacrificed on day 12 (designated as 3TPA) and once or twice weekly for 2 weeks with 5 μl TPA in 200 μl of acetone. Untreated control mice were sacrificed on day 1 (designated as NS). Four dosing protocols were used as follows. Mice were dosed on day 1 and sacrificed on day 5 (designated as TPA); mice were dosed on days 1 and 5 and sacrificed on day 8 (designated as 2TPA); mice were dosed on days 1, 5, and 8 and sacrificed on day 12 (designated as 3TPA) and once or twice weekly for 2 weeks with 5 μl TPA in 200 μl of acetone. Untreated control mice were sacrificed on day 1 (designated as NS). Four dosing protocols were used as follows. Mice were dosed on day 1 and sacrificed on day 5 (designated as TPA); mice were dosed on days 1 and 5 and sacrificed on day 8 (designated as 2TPA); mice were dosed on days 1, 5, and 8 and sacrificed on day 12 (designated as 3TPA) and once or twice weekly for 2 weeks with 5 μl TPA in 200 μl of acetone. Untreated control mice were sacrificed on day 1 (designated as NS). Four dosing protocols were used as follows. Mice were dosed on day 1 and sacrificed on day 5 (designated as TPA); mice were dosed on days 1 and 5 and sacrificed on day 8 (designated as 2TPA); mice were dosed on days 1, 5, and 8 and sacrificed on day 12 (designated as 3TPA) and once or twice weekly for 2 weeks with 5 μl TPA in 200 μl of acetone. Untreated control mice were sacrificed on day 1 (designated as NS). Four dosing protocols were used as follows. Mice were dosed on day 1 and sacrificed on day 5 (designated as TPA); mice were dosed on days 1 and 5 and sacrificed on day 8 (designated as 2TPA); mice were dosed on days 1, 5, and 8 and sacrificed on day 12 (designated as 3TPA) and once or twice weekly for 2 weeks with 5 μl TPA in 200 μl of acetone. Untreated control mice were sacrificed on day 1 (designated as NS).
Cells were transfected with empty vector pCDNA3.1, wild type nm23-M2/NDPK-B (WT) or mutant types nm23-M2/NDPK-B including del-RT (5 µm), S122P, and H118F plasmid DNAs using LipofectAMINE PLUS™ reagents (Invitrogen). Transfected cells were cultured for at least 2 weeks in medium containing 400 µg/ml of Geneticin (G418) (Invitrogen). Cells were analyzed by immunoblot to confirm the expression of mouse NDPK-B.

**RT-PCR**

Single-stranded cDNA was prepared from total RNA using the Moloney murine leukemia virus reverse transcriptase SuperScript II (Invitrogen) with oligo(dT) primer and used as a template for PCR. PCR primers for mouse nm23-M2/NDPK-B were as described above. The forward and reverse primers of β2-microglobulin gene (217 bp in size), were used as an internal control, are 5′-GAC TGG TCT TTC TAT ATC CTG G-3′ and 5′-CTT TCT TGC TGG ATC AAT TG-3′, respectively. PCR cycling was as follows: denaturation (94 °C, 45 s), annealing (55 °C, 45 s), and extension (72 °C, 2 min) for 30 cycles. The reaction was carried out in a PerkinElmer-9600 thermal cycler, and PCR products were analyzed using 2% agarose gels. DNA was quantified using Quantity One software version 4.0 (Bio-Rad).

**In Situ Hybridization**

An in situ hybridization assay was performed as previously described (45). Briefly, skin tissues were removed from TgAC mice treated or untreated with multiple doses of TPA and fixed overnight in 10% neutral buffered formalin. Tissues were paraffin-embedded, and sections (6 µm) were cut onto SuperFrost Plus microscope slides (Daigiegbert, Vernon Hills, IL). The sections were deparaffinized and rehydrated by successive washes in xylene and graded alcohols to 2× SSC, and then 2× 10⁶ cpm of [α-32P]UTP-labeled mouse nm23-M2/NDPK-B sense or antisense riboprobes was applied to slides. Riboprobes were prepared from T7/T3-U19/mn23-M2/NDPK-B (105–121) plasmid linearized with EcoRI (antisense) or HindIII (sense) using an in vitro T7 or T3 Riboprobe kit (Promega, Madison, WI). Following 40 °C overnight hybridization, tissues were washed in 2× SSC plus 50% formamide at 40 °C and then in 2× SSC, 1× SSC, 0.5× SSC, and 0.5× SSC for 30 min each wash at room temperature. To remove unbound probe, tissues were incubated with 20 µl of RNase (10 mg/ml). After several washes, the slides were dehydrated in graded alcohols and completely air-dried. The slides were then dipped into NTB-3 autoradiographic emulsion (Eastman Kodak Co.), exposed for 10 days at room temperature in the dark, dried in a light-tight container, and developed in Kodak D19 developer slides were then dipped into NTB-3 autoradiographic emulsion (Eastman Kodak Co.), exposed for 10 days at room temperature in the dark, dried in a light-tight container, and developed in Kodak D19 developer and fixer. The sections were counterstained with hematoxylin, covered with coverslips, and photographed under dark-field illumination (model BX51, Olympus Optical Co., Tokyo, Japan).

**Immunoblot Analysis**

Cells were washed with ice-cold phosphate-buffered saline and lysed in ice-cold modified radioimmunoprecipitation (RIPA) buffer consisting of 50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonfyl fluoride, 10 µg/ml each aprotinin, leupeptin, and pepstatin, 1 mM Na3VO4, and 1 mM NaF. Cell suspensions were gently rocked on an orbital shaker in a cold room for 15 min to lyse cells. Lysates were centrifuged at 14,000 g for 1 h at 4 °C. Protein concentration was determined by Bradford assay (Bio-Rad). Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene difluoride membranes (Amersham Biosciences). Membranes were stained with primary antibodies, detected using horseradish peroxidase-conjugated secondary antibodies (1:3000) (Amersham Biosciences) and enhanced chemiluminescence (Amersham Biosciences). Membranes were stripped and rehybridized with anti-β-actin mouse mAb (1:1000) or anti-actin rabbit pAb (1:1000) as a control to confirm equal loading. Protein quantification was determined by ImageQuant software version 5.1 (Amersham Biosciences), and relative quantity is shown below the panels of Figs. 3B, 4–6, and 8.

**Transformation Assays**

Foci-forming Activity—Cells were seeded overnight at a density of 1 × 10⁴ cells in 10-cm plates. Cells were transfected with 2 or 4 µg of pCDNA3.1/Nm23-M2/NDPK-B or empty vector and selected in medium containing G418 for 14–21 days. Foci were fixed with methanol/acetic acid (v/v = 3:1), stained with 0.4% crystal violet (Sigma) methanolic/acetic acid, and counted as described previously (46).

**Characterization of Cell Growth**—Growth curves were generated as described previously (46). In brief, 2 × 10⁴ cells were grown as described above. The medium was changed every 3–4 days. Cell number was counted triticipate on a hemocytometer every 1–2 days for 14 days. Anchorage-independent Growth Assay—Colonies formation in soft agar was assayed as described previously (46). In a 60-mm tissue culture dish, 1 × 10⁴ cells were resuspended in 0.33% Noble agar in Eagle’s MEM with 10% FBS and layered over 5 ml of 0.5% agar in Eagle’s MEM with 10% FBS. Cells were grown at 37 °C in 5% air plus 5% CO₂, and colonies with more than eight cells were counted and photographed 18 days postseeding.

**RESULTS**

**nm23-M2/NDPK-B Expression Is Induced Following TPA Treatment**—Our previous microarray data indicated that mouse nm23-M2/NDPK-B was increased 5.4-fold in integrin α₆⁺ CD34⁺ keratinocytes isolated from TPA-treated TgAC mice skins (7). We have reported previously that keratinocytes expressing α₆⁺ and CD34⁺ surface markers represent a subpopulation of follicle-derived cells exhibiting properties of progenitor cells (47). This up-regulation was confirmed by relative RT-PCR analysis that revealed a 3.5-fold increase in integrin α₆⁺ CD34⁺ keratinocytes isolated from TPA-treated TgAC mice, relative to integrin α₆⁻ CD34⁺ keratinocytes from untreated mice (Fig. 1). The trend of nm23-M2/NDPK-B overexpression was in agreement with our previous microarray experiment (7). To define the specificity of tissue distribution of nm23-M2/
Fig. 2. Nm23-M2/NDPK-B was expressed in a variety of normal Tg AC mouse tissues. Tissue samples of the heart (H), brain (B), kidney (K), liver (Li), lung (Lu), ovary (O), skin (Sk), stomach (St), spleen (Sp), and small intestine (SI) were homogenized and extracted for preparation of total protein lysates in modified RIPA buffer, as described under “Experimental Procedures.” Sixty micrograms of total protein lysates were separated on a 15% SDS-PAGE, transferred to the polyvinylidene difluoride membrane, and then probed with a specific anti-mouse Nm23-M2/NDPK-B rat mAb (1:200). The primary antibody was detected using horseradish peroxidase-conjugated anti-rat IgG secondary antibody at 1:3000 and enhanced chemiluminescence reagent kit. The specific signal was developed with Amersham Biosciences Hyperfilm ECL. Membranes were stripped and rehybridized with anti-actin rabbit pAb (1:1000). The arrow indicates the molecular mass of mouse Nm23-M2/NDPK-B in size as 17.5 kDa (top panel). Actin (42 kDa) served as an internal control and attested that equivalent amounts of protein were loaded in each lane (bottom panel).

VnNDPK-B, a panel of tissues from normal Tg AC mouse mice was examined by immunoblot analysis using a rat monoclonal antibody specific to mouse Nm23-M2/NDPK-B, with no cross-reactivity with mouse Nm23-M2/NDPK-A molecule (27). As shown in Fig. 2, the specific signal was detected under reducing conditions as a band of 17.5-kDa in size, which was seen in varying intensities in heart, brain, kidney, liver, skin, stomach, spleen, and small intestine. Nm23-M2/NDPK-B was expressed at higher levels in heart and kidney. In liver, spleen, stomach, small intestine, brain, and skin the expression levels of Nm23-M2/NDPK-B were very low, whereas Nm23-M2/NDPK-B was barely detectable in lung and ovary. To determine the localization of nm23-M2/NDPK-B message in mouse skin tissues, in situ hybridization was employed. Although some nonspecific background was apparent in the outermost cornified cell envelope, the signal primarily localized to the stratified squamous epithelial regions (indicated by the arrows in Fig. 3) with antisense but not sense [35S]UTP-labeled mouse nm23-M2/NDPK-B mRNA. Some expression was evident in hair follicles, especially in the third dose of TPA-treated skins in Fig. 3A (Xf). Signal was not detectable in the dermis, adipose, and muscle tissues (Fig. 3A). However, there was no detectable nm23-M2/NDPK-B mRNA message in normal skins (Fig. 3A, II), relative to the sense probe control (Fig. 3A, III). Total protein lysates of skins harvested from Tg AC mice treated with various doses of TPA were used for immunoblot analysis. These doses induce extensive hyperplasia (see Fig. 3A; IV, VII, and X). Fig. 3B revealed an induction of Nm23-M2/NDPK-B in mouse skin homogenates following TPA treatment. Untreated mouse skin was used as a negative control and expressed very low levels of Nm23-M2/NDPK-B (Fig. 3B, lane 1). Mouse nm23-M2/NDPK-B expression was induced 2.5-fold following one dose of TPA (5 µg), with a maximal induction of about 8–10-fold between the second and the fourth dose of 5-µg TPA treatment (Fig. 3D). Nm23-M2/NDPK-B increased with hyperplasia and was maintained at high levels (3.8-fold) even 21 days after the last of four doses of TPA (Fig. 3B). In contrast, Nm23-M1/NDPK-A was not significantly up-regulated in TPA-induced hyperplastic skin tissues (data not shown).

TPA-induced Expression of nm23-M2/NDPK-B in Cultured TPA-susceptible Epidermal Cells—To investigate whether TPA is able to induce mouse nm23-M2/NDPK-B expression, TPA-susceptible JB6 Cl 41-5a and TPA-resistant JB6 Cl 30-7b preneoplastic epidermal cell lines were employed. JB6 Cl 41-5a cells were grown in 5% FBS/Eagle’s MEM with 0, 0.1, 1, 10, or 100 ng/ml TPA. The cells were harvested 8 h post-treatment, and whole-cell lysates were prepared for immunoblot analysis. Fig. 4A showed a 1.8-fold induction of mouse Nm23-M2/NDPK-B, beginning at 0.1 ng/ml TPA, and maximal induction of 2.5–3.2-fold with 1–100 ng/ml TPA. A kinetic analysis of Nm23-M2/NDPK-B expression in JB6 Cl 41-5a cells was conducted at 0, 10, and 30 min and 1, 2, 4, 8, 12, and 18 h following treatment with 10 ng/ml TPA (Fig. 4B). This showed that mouse Nm23-M2/NDPK-B was induced 2.2-fold 10 min after TPA treatment and reached a maximal 3.0-fold induction 8 h post-treatment. Nm23-M2/NDPK-B expression then appeared to decrease from 12 to 18 h post-treatment (Fig. 4B). Interestingly, we did not find that mouse Nm23-M2/NDPK-B was induced in TPA-resistant JB6 Cl 30-7b cell line but rather showed a remarkable decrease 10 min after treatment with 10 ng/ml TPA (Fig. 4C).

Overexpression of nm23-M2/NDPK-B in Skin Tumor Cell Lines and Skin Tumors—In vitro and in vitro studies showed that TPA was able to induce an increase in nm23-M2/NDPK-B gene expression in Tg AC mouse keratinocytes, in hyperplastic Tg AC mouse skins, and in TPA-susceptible mouse JB6 Cl 41-5a epidermal cells. The gene expression of nm23-M2/NDPK-B was also examined by immunoblot analysis in the mouse skin tumor cell lines as well as in TPA-induced skin tumors. The protein level of Nm23-M2/NDPK-B was found to respectively increase 10- and 2.8-fold in two mouse skin tumor cell lines, Tg AC 43 and FVB/N217 (relative to TPA-untreated normal keratinocytes isolated from Tg AC mouse) (TPA−/KCs) (Fig. 5A). In addition, NDK-PK-B expression was also increased in one human epidermoid carcinoma cell line A431 (5.0-fold), when compared with human HaCaT keratinocytes (Fig. 5A). The keratinocytes isolated from the skins of Tg AC mice treated with four doses of TPA (TPA+/KCs) were used as a positive control and showed a 3.5-fold induction (Fig. 5A, lane 2). Nm23-M2/NDPK-B protein was higher in TPA-mediated Tg AC mouse skin tumors, including 15 papillomas (3.1 ± 1.0-fold) and three malignant tumors (3.7 ± 1.8-fold) (one spindle cell tumor (5.8-fold) and two squamous cell carcinomas (2.7- and 2.6-fold)), than in adjacent normal skins (1.0 ± 0.3-fold) (Fig. 5B).

The nm23-M2/NDPK-B Gene Is Induced by UVR—To examine whether nm23-M2/NDPK-B is induced in response to UVR in vivo, we irradiated Tg AC mice with a combination of UVA and UVB (30–40% UVA and 60–70% UVB). After three UVA/UVB exposures, the skin was found to be extensively hyperplastic, keratinized, and inflamed (data not shown). The UV-exposed tissues were collected 24 h after the last exposure and snap frozen in liquid nitrogen for preparation of total protein lysates. Immunoblot analysis revealed that Nm23-M2/NDPK-B was induced by 7.2–10-fold in these four mouse skin tissues following UV exposure (designated as UV1–UV4), relative to the low level expression found in nonexposed skin (designated as NS) (Fig. 6A). To further investigate if this induction persistently occurred in UVR-mediated skin tumors, six papillomas were examined. Our result indicated that Nm23-M2/NDPK-B was increased 7–10-fold in UVR-mediated skin papillomas (designated as UVP1–UVP6), relative to untreated normal Tg AC mice skins (NS) (Fig. 6B).

Overexpression of Mouse nm23-M2/NDPK-B Enhances Neoplastic Transformation in Preneplastic Mouse Epidermal Cells—Mouse nm23-M2/NDPK-B was constitutively expressed in preneoplastic epidermal cells to determine whether increased expression of mouse nm23-M2/NDPK-B potentially plays a role in skin tumorigenesis. One plasmid construct, pcDNA3.1/dnm23-M2/NDPK-B-V5-His, was prepared to constitutively express mouse nm23-M2/NDPK-B. Immunoblot
Fig. 3. Overexpression of nm23-M2/NDPK-B in early TPA-induced hyperplastic mouse skin tissues. A, in situ hybridization. To detect the expression of nm23-M2/NDPK-B, in situ hybridization assay was performed on paraffin-embedded sections of TPA-untreated (Normal Skin; I, II, and III) or TPA-treated TgAC mouse skin tissues including one dose of 5 μg of TPA-treated skins (1TPA; IV, V, and VI), two doses of 5 μg of TPA-treated skins (2TPA; VII, VIII, and IX), and three doses of 5 μg TPA-treated skins (3TPA; X, XI, and XII), using a specific nm23-M2/NDPK-B (105-321) sense (S; III, VI, IX, and XII) or antisense (AS; II, V, VIII, and XI) riboprobe. The silver grains indicate the signals in probe hybridization, and slides were counterstained with hematoxylin and eosin (I, IV, VII, and X) and dark field (II, III, V, VI, VIII, IX, XI, and XII) conditions. The white line (basal layer) indicates the border between epidermis and dermis. E, epidermis; D, dermis; A, adipose tissue; M, muscle tissue; BL, basal layer; HF, hair follicle. Original magnification was ×100. B, immunoblot analysis. The multiple doses of 5 μg of TPA-treated TgAC mouse skin tissues were homogenized and extracted for preparation of total protein lysates in modified RIPA buffer. Mouse tissue total protein lysates (60 μg) were subjected to immunoblotting and probed with anti-mouse Nm23-M2/NDPK-B rat mAb (1:200) (top panel) or anti-actin rabbit pAb (1:1000) (bottom panel). Protein quantitation was determined by ImageQuant software version 5.1 (Amersham Biosciences), and relative quantity is shown under the top panel.
analysis confirmed that mouse Nm23-M2/NDPK-B protein was expressed successfully and efficiently in NIH/3T3, Rat-1, JB6 Cl 30-7b, and JB6 Cl 41-5a cells (Fig. 7A).

Constitutive expression of Ras family proteins and other oncogenic proteins increase focus-forming capability and decrease growth contact inhibition of normal untransformed cells (46). Our results showed that constitutive expression of mouse Nm23-M2/NDPK-B increases foci formation 3.8- (2 μg of DNA) to 4.5-fold (4 μg of DNA) and 4.0- (2 μg of DNA) to 5.3-fold (4 μg of DNA) in mouse JB6 Cl 30-7b and JB6 Cl 41-5a epidermal cell lines, respectively (Fig. 7B). In addition, the increase in foci-forming activity appears to have gene dosage-dependent effects (Fig. 7B). However, expression of mouse nm23-M2/NDPK-B did not change the foci-forming properties in fibroblasts such as NIH/3T3 and Rat-1 cells. These results demonstrate that

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Fig. 4. In vitro dose response and kinetics of TPA-induced expression of nm23-M2/NDPK-B in epidermal cells. JB6 Cl 41-5a (A and B) or JB6 Cl 30-7b epidermal cells (C) were treated with the indicated TPA concentrations or exposed to 10 ng/ml TPA at indicated time points. Cells were harvested, and whole cell lysates were extracted using modified RIPA buffer. Sixty micrograms of whole-cell lysates were analyzed by immunoblotting for the detection of mouse Nm23-M2/NDPK-B (top panel). α-Tubulin (50 kDa) (lower panel) served as an internal control.

Fig. 5. Elevated NDPK-B expression in skin tumor cell lines and TgAC neoplasms. A, skin tumor cell lines. Two mouse skin tumor cell lines, FVB/N217 and TgAC43, and one human epidermoid carcinoma cell line A431 were examined for NDPK-B gene expression by immunoblotting. HaCaT keratinocytes and TPA-untrated normal keratinocytes (TPA−/KCs) (isolated from TgAC mice) were used as negative controls for humans and mice, respectively. TPA-treated TgAC mouse keratinocytes (TPA+/KCs) served as a positive control to indicate the position of NDPK-B. B, TPA-mediated skin tumors in TgAC mice. Total protein lysates were prepared from seven normal skin tissues of TgAC mice (NS1–NS7) and tumors, including 15 papillomas (P1–P15) and three malignancies (M1–M3). All total protein lysates (60 μg) were probed with anti-mouse Nm23-M2/NDPK-B rat mAb (1:200) or anti-human Nm23-H2/NDPK-B mouse mAb (1:1000), anti-actin rabbit pAb (1:1000), or anti-α-tubulin mouse mAb (1:1000).

nm23-M2/NDPK-B alters normal contact inhibition in mouse epidermal cell lines, suggesting that nm23-M2/NDPK-B may have some oncogenic properties.

Transformed cells have a growth advantage in monolayer culture and acquire capacity for anchorage-independent growth (46). The effects of mouse nm23-M2/NDPK-B expression on these growth characteristics were measured in NIH/3T3, Rat-1, JB6 Cl 30-7b, and JB6 Cl 41-5a cells (Fig. 7C). The cells transfected with vector only or mouse nm23-M2/NDPK-B were grown for 48 h and then selected for at least 10 days with medium containing 400 μg/ml of antibiotic neomycin analog G418. Stable cell clones expressing vector only or mouse nm23-M2/NDPK-B were seeded at a cell density of 2 × 10⁶ in a 6-well tissue culture plate to assay for cell growth. Constitutive expression of mouse nm23-M2/NDPK-B was not correlated with increased growth rate in epidermal cells such as JB6 Cl 30-7b and JB6 Cl 41-5a. On the contrary, our results showed that clones overexpressing mouse nm23-M2/NDPK-B in NIH/3T3 and Rat-1 fibroblasts grew at a significantly slower rate than control vector-only cells (Fig. 7C). Furthermore, stable clones
expressing vector only or mouse nm23-M2/NDPK-B were seeded at 1 x 10^4 in a 60-mm soft agar tissue culture plate to assay for anchorage-independent growth. Colony formation efficiency increased ~3.3- and 4.0-fold when mouse nm23-M2/NDPK-B was expressed in JB6 Cl 41-5a and JB6 Cl 30-Tb cells, respectively (Fig. 7D). However, colony forming efficiency did not increase in mouse nm23-M2/NDPK-B-transfected fibroblasts such as NIH-3T3 and Rat-1 cells (Fig. 7D). Background colony formation was higher in JB6 Cl 41-5a cells than in JB6 Cl 30-Tb cells. 

An Increased Release of nm23-M2/NDPK-B from Cells following Exposure to TPA or UVR—Next we examined whether Nm23-M2/NDPK-B is released from the cells and is affected by stresses such as TPA and UVR. For these studies, TPA-susceptible mouse epidermal cell line JB6 Cl 41-5a and human keratinocyte cell line HaCaT, which is routinely used in UVR studies, were employed. Prior to TPA or UV exposure, subconfluent JB6 Cl 41-5a and HaCaT cell monolayers were starved in serum-free medium for at least 12 h. As seen in Fig. 8, Nm23-M2/NDPK-B was detected in serum-free conditioned media collected from untreated 18-h cultures of JB6 Cl 41-5a and HaCaT cells. At this time, cells were observed to still be viable, as ascertained by examination of trypan blue dye exclusion using an inverted microscope (data not shown). The result indicates that Nm23-M2/NDPK-B is released from the cells. More interestingly, the release of Nm23-M2/NDPK-B into the medium was enhanced about 2.5-3-fold in JB6 Cl 41-5a (Fig. 8A) and HaCaT cells (Fig. 8B) 18 h following exposure to TPA (10 ng/ml), UVA (10 J/cm^2) or UVB (10 mJ/cm^2). The increased release of Nm23-M2/NDPK-B may explain why the expression of NDPK-B in JB6 Cl 41-5a cells was significantly down-regulated 12-18 h after treatment with 10 ng/ml of TPA (Fig. 4B).

Deletion of Arg^105>Gly^106-Asp^107 Consensus Sequence Domain or Point Substitution Gly^106 with Ala in Mouse Nm23-M2/NDPK-B Selectively Abrogates Cellular Transformation—To dissect and determine which amino acid residues of Nm23-M2/NDPK-B might be involved in cellular transformation, we employed site-directed mutagenesis to generate mutants for deletion and point substitution and examined anchorage-independent growth using a standard in vitro soft agar assay. The mutants include Arg^105>Gly^106-Asp^107 deletion (delRGD), Gly^106 substitution with Ala (G106A), Ser^122 substitution with Pro (S122P), and His^118 instead of Phe (H118F). We stably transfected the TPA-susceptible mouse preneoplastic epidermal cell line JB6 Cl 41-5a with vector only, wild type (WT), delRGD, G106A, H118F, or S122P nm23-M2/NDPK-B gene and assayed their activity for cellular transformation. Immunoblot analysis showed that the nm23-M2/NDPK-B gene constructs were efficiently expressed and released into the serum-free conditioned media collected from the different JB6 Cl 41-5a stable cell clones, when probed with anti-V5 tag mouse monoclonal antibody (Fig. 9A). The Nm23-M2/NDPK-B (delRGD) mutant protein had a significant shift in electrophoretic gel mobility with a higher rate than the other proteins, whereas the mobility of mutant proteins such as Nm23-M2/NDPK-B (G106A), Nm23-M2/NDPK-B (S122P), and Nm23-M2/NDPK-B (H118F) were not changed (Fig. 9A). The stable clones expressing vector only, WT, delRGD, G106A, H118F, and S122P nm23-M2/NDPK-B genes were seeded separately at 1 x 10^4 in a 60-mm soft agar tissue culture plate to assay for anchorage-independent growth. The colony with more than eight cells was counted 18 days postseeding. As shown in Fig. 9, B and C, colony formation efficiency was reduced ~7.2- and 3.5-fold relative to nm23-M2/NDPK-B (WT), when nm23-M2/NDPK-B (delRGD) and nm23-M2/NDPK-B (G106A) were expressed in JB6 Cl 41-5a cells, respectively. Overexpression of nm23-M2/NDPK-B (S122P) and nm23-M2/NDPK-B (H118F) did not significantly reduce the efficiency of cellular transformation.

**DISCUSSION**

In this study, we further characterize the biological functions of the NDPK-B gene product using the in vitro JB6 mouse epidermal clonal genetic variant cell system that has proven to be valuable in studying tumor promoter-dependent biological events occurring during preneoplastic progression (48). In this way, we first demonstrate that NDPK-B is a potentially important TPA- or UVR-responsive gene required for neoplastic transformation in epidermal cells.

Our RT-PCR data showed a significant up-regulation in mouse nm23-M2/NDPK-B gene expression in integrin α6^+CD34^+ keratinocytes following TPA treatment (Fig. 1). This induction is closely associated with the TPA- and UVR-induced promotion stage of skin carcinogenesis in mice (Figs. 3 and 6A). Immunoblot analysis also showed an in vitro TPA-induced increase in Nm23-M2/NDPK-B protein level in JB6 Cl 41-5a preneoplastic epidermal cells (Fig. 4, A and B). In addition, a dramatic increase in gene expression of NDPK-B occurs consistently in skin tumor cell lines (Fig. 5A) and in TPA- or UVR-mediated mouse skin tumors as well (Figs. 5B and 6B). These results clearly suggest that nm23-M2/NDPK-B is a novel TPA- or UVR-responsive gene that may be a useful marker for early skin tumorigenesis.

Although our results indicate a close correlation between nm23-M2/NDPK-B gene expression and the tumor promotion
FIG. 7. Constitutive expression of mouse nm23-M2/NDPK-B remarkably enhances neoplastic transformation in JB6 Cl 41-5a epidermal cells. A, immunoblot analysis. Sixty micrograms of total protein lysates from parental (P), pcDNA3.1 vector only (V), or nm23-M2/NDPK-B-transfected (N) cell lines including NIH/3T3, Rat-1, JB6 Cl 30-7b, and JB6 Cl 41-5a were separated on 15% SDS-PAGE. The membranes...
stage (induced by TPA or UVR), the specific functions of nm23-M2/NDPK-B in this process, such as proliferation, differentiation, or neoplastic transformation, remains unknown. Earlier reports identified NDPK-B as a differentiation-inhibitory factor, which allows to inhibit the differentiation of several hematopoietic cell lines in vitro, where the inhibition was independent of the phosphotransferase activity, as demonstrated with NDPK-B lacking the enzymatic activity (14, 49). Moreover, several hematopoietic tumor cell lines stained positive for NDPK-B in flow cytometric analysis (50), and up-regulation of the human NDPK-B gene was also observed in normal lymphocytes induced to proliferate with phytohemagglutinin (51). Down-regulation of the NDPK-B and c-myc genes was also reported in 1,25-dihydroxyvitamin D_{3}-induced differentiation (52). These results suggest that NDPK-B may play a critical role in the inhibition of differentiation or the promotion of proliferation of these cells. As seen in Fig. 7, we found that nm23-M2/NDPK-B was efficiently expressed in NIH/3T3, Rat-1, JB6 Cl 30-7b, and JB6 Cl 41-5a (Fig. 7A). The elevated expression of mouse nm23-M2/NDPK-B in genetically modified NIH/3T3, Rat-1, JB6 Cl 30-7b, and JB6 Cl 41-5a stable clones, which were transfected with pDNA3.1/nm23-M2/NDPK-B mammalian expression vector and selected with G418 for at least 10 days, does not significantly promote cell proliferation under standard in vitro tissue culture conditions (Fig. 7C) but appears to markedly increase foci-forming activity in epithelial cell lines (e.g. JB6 Cl 30-7b and JB6 Cl 41-5a) in a gene dosage-dependent manner but not in fibroblast cell lines (e.g. NIH/3T3 and Rat-1) (Fig. 7B). Notably, we found the rate of cell growth to be selectively inhibited in nm23-M2/NDPK-B-overexpressing mouse fibroblasts, NIH/3T3 and Rat-1. Our data consistently showed a decrease of proliferation rate from 100% down to about 1% (NIH/3T3) or 50% (Rat-1) and implicated that more than 50% of the growth inhibition observed in fibroblasts was mediated by activation of Nm23-M2/NDPK-B-initiated signaling pathways (Fig. 7C), presumably suggesting that NDPK-B plays an important role in the maintenance of integrity of skin tissues. More importantly, overexpression of mouse nm23-M2/NDPK-B increased colony-forming efficiency in JB6 Cl 41-5a and JB6 Cl 30-7b cells but not in NIH/3T3 and Rat-1 cells using anchorage-independent growth assay (Fig. 7D).

Thus, the ability of nm23-M2/NDPK-B to regulate cellular transformation may be specific for epithelial cells.

NDPKs have previously been reported to localize at different subcellular localizations, such as nucleus (53), cytoplasm (54), and mitochondria (37), with low levels in the plasma membrane (50). Previous studies have already revealed that inhibitory factor was purified from the conditioned medium of the mouse myeloid cell line M1, although NDPKs have no signal peptide for their secretion (49, 55). Anzinger et al. (56) recently found secretion of NDPK-B by cells isolated from human breast, colon, pancreas, and lung tumors. In addition, Willems et al. (57, 58) also demonstrated that extracellular NDPK modulated normal hematopoietic cell differentiation. Our data have shown NDPK-B release into the serum-free media of mouse JB6 Cl 41-5a epidermal cells and an about 2.5–3.0-fold increase in release levels 18 h following TPA treatment (Fig. 8). All previously identified NDPKs, except that in bacteria, share a specific tripeptide Arg-Gly-Asp (RGD) domain (27). To further determine whether the RGD domain of NDPK-B protein is required for the enhancement of neoplastic transformation in epithelial cells, site-directed mutagenesis was employed to create mutants for deletion or point substitution. Immunoblot analysis showed that Nm23-M2/NDPK-B proteins were released into the serum-free conditioned media collected from JB6 Cl 41-5a individual stable clones, which were transfected with vector only, wild type (WT), delRGD, G106A, S122P, or H118F nm23-M2/NDPK-B gene (Fig. 9A). The elevated expression of mouse nm23-M2/NDPK-B (WT) into JB6 Cl 41-5a cells acquired the susceptibility to transformation in an in vitro soft agar assay. Indeed, constitutive expression of mouse nm23-M2/NDPK-B (delRGD) or nm23-M2/NDPK-B (G106A) mutant gene in JB6 Cl 41-5a cells selectively abolished NDPK-B-induced anchorage-independent growth (Fig. 9, B and C). Thus, the enhancement of cellular transformation requires the presence of the RGD consensus sequence domain. Previous reports indicated that the point substitutions of nm23-H2/NDPK-B at His\textsuperscript{118} with Phe (H118F) and at Ser\textsuperscript{122} instead of Pro (S122P) result in a defective kinase activity and phosphoryl transfer activity, respectively (59, 60). Interestingly, overexpression of mouse nm23-M2/NDPK-B (S122P) (lack of phosphoryl transfer activity) and nm23-M2/NDPK-B (H118F) (lack of kinase activity) did not significantly reduce the efficiency of cellular transformation and suggests that NDPK-B kinase activity is not...
required for transformation (Fig. 9, B and C). Taken together, these results point to a crucial regulatory role of the RGD consensus sequence domain, but not the catalytic domain, in NDPK-B-mediated cellular transformation.

Although NDPK-B protein can be released into the medium in our cell model systems, we cannot exclude the possible effects of NDPK-B in the nucleus. Nm23-H2/NDPK-B has recently been identified as the human PuF factor (40) and can transactivate a human c-myc proto-oncogene via a functional nucleosome hypersensitive element (39). Activation of the c-myc proto-oncogene contributes to cellular transformation, mitogenesis, differentiation, and apoptosis (61–63). Recent reports also indicated that c-Myc promotes differentiation of human epidermal stem cells (64), and c-myc activation in transgenic mouse epidermis results in mobilization of stem cells and differentiation of their progeny (65). Moreover, the constitutive expression of human c-myc has been demonstrated to deplete epidermal stem cells by reducing β1 integrin expression (66).

Mutational analysis of human Nm23-H2/NDPK-B has identified amino acid residues and structural domains that are involved in DNA binding, implicating a multifunctional role in the regulation of genes (e.g., c-myc) important to cell proliferation, differentiation, and cancer development (41, 67). Research is in progress to further identify how NDPK-B, integrin, and c-Myc coordinate to regulate cell growth, differentiation, and transformation in epidermal stem cells following treatment with TPA.

Due to inefficiency of the antibody for immunohistochemical staining in mouse skins, we used in situ hybridization to localize the nm23-M2/NDPK-B mRNA message. Our findings on the localization of nm23-M2/NDPK-B mRNA in homozygous TgAC mice skin tissues following treatment with variable doses of TPA showed a dramatically increased expression in the stratified squamous epithelial regions, in particular in basal layer and spinousum, and with some expression in hair follicles (Fig. 3A). Indeed, the induction of nm23-M2/NDPK-B mRNA seems to occur in a TPA dose-dependent manner, consistent with immunoblot analysis (Fig. 3B). Unlike immunoblot analysis, in situ hybridization did not detect the nm23-M2/NDPK-B signal in normal mouse skin tissues, probably in part due to the relative insensitivity of in situ hybridization. Interestingly, our previous study indicates that the expression patterns of Dss1 and Nm23-M2/NDPK-B were similar in respect to tissue distribution and localization (7). Moreover, recently reported data indicate that human Nm23-H2/NDPK-B binds to single-stranded oligonucleotides in a non-sequence-specific manner but that it exhibits strong specificity for single-stranded DNA (68). One Dss1 functional model, as shown by its 37% acidic residue (aspartic and glutamic acid-rich domains, ~21 charge at pH 7.0) and its 13% aromatic residue content, suggests that it mimics oligonucleotides, possibly regulating the accessibility of a subset of the putative DNA binding sites on the helical and OB1 domains of BRCA2 (69). Recently, a Dss1/BRCA2 interaction was shown to be required for proficiency in DNA repair, recombination, and genome stability in the fungi Ustilago maydis (70).

Fig. 9. Deletion of Arg105–Gly106–Asp107 consensus sequence domain or point substitution Gly106 with Ala in mouse Nm23-M2/NDPK-B selectively abrogates cellular transformation. A, immunoblot analysis. The deletion or point substitution was introduced into mouse nm23-M2/NDPK-B cDNA by the ExSite PCR-Based site-directed mutagenesis kit, as described under “Experimental Procedures.” These mutants include delRGD, G106A, S122P, and H118F. JB6 Cl 41-5a cells were transfected with pcDNA3.1 vector only (Vector), wild type nm23-M2/NDPK-B (WT), or mutant types nm23-M2/NDPK-B including delRGD, G106A, S122P, and H118F plasmid DNAs. To obtain stable clones, transfected cells were selected under 400 μg/ml G418 for at least 10 days. Serum-free conditioned media were collected and concentrated from stable clones and analyzed by immunoblotting to confirm the release of mouse NDPK-B proteins. B, anchorage-indepen-
Nmm23-H2/NDPK-B share an active site, thus implicating a DNA repair function for the NDPK-B molecule. It raises a possibility that Dss1, in addition to binding the DNA repair protein BRC2A2 (71), could interact with the DNA repair protein Nmm23-M2/NDPK-B in mouse skins following exposure with TPA. Although we have found that Dss1 binds directly with Nmm23-M2/NDPK-B in in vitro studies using TNT Quick Coupled Transcription/Translation Systems, further studies will be necessary before the significance of this unique protein-protein interaction can be fully understood.

In summary, our studies provide a new insight into the biological function of mouse Nmm23-M2/NDPK-B and demonstrate that it is likely to play a unique role in mediating TPA- or UVR-induced skin carcinogenesis. NDPK-B was capable of being released outside of cells in culture, and an increase in DNA sequences; Jian-Li Huang (Laboratory of Sig-

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