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Transcription Effect of nm23-M2/NDP Kinase on *c-myc* Oncogene

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Transactivating factor PuF which interacts with a nuclease hypersensitive element locates upstream from the c-myc gene. PuF was recently identified as being encoded by nm23-H2/NDP kinase gene [Postel, E. H., Berberich, S. J., Flint, S. J., and Ferrone, C. A. (1993) Science 261, 428-429]. Here we have studied the correlation of expression between *c-myc* and nm23 genes *in vitro*. By a comparative study of the expression of 2 genes in cell lines, no direct correlation of kinetics was found. A plasmid containing the human *c-myc* fragment (*c-myc* CAT) was cloned upstream from the bacterial chloramphenicol acetyltransferase (CAT) gene. When the murine melanoma cell line was cotransfected with a nm23-M2/NDP kinase expression vector and *c-myc* CAT, CAT activity was elevated. In addition, cell cycle phases in the murine cell lines transfected with nm23/NDP kinase were estimated; an alteration of the cell cycle, prolonged S-phase was found in the cell lines transfected with nm23-M2/NDP kinase, whereas human nm23-H2/NDP kinase did not play a role in transactivating the *c-myc* gene or in S-phase prolongation in murine cell lines. From these results we conclude that the murine nm23-M2 gene transactivates the *c-myc* gene and controls the cell cycle, S-phase, indirectly via a cellular cofactor in the murine cell line, which does not work with the human nm23-H2 gene.

Since the nm23 gene was reported as a potential antimetastasis gene which was initially isolated by differential colony hybridization of cDNAs from low and high metastatic murine K-1735 melanoma cells (Steeg *et al.*, 1988), many investigators have reported several additional functions, including a role in proliferation, differentiation, organogenesis, immortalization (carcinogenesis), signal transduction, and transcription effect, and as a cytokine (Keim *et al.*, 1992; Lakso *et al.*, 1992; Leone *et al.*, 1991; Okabe-Kado *et al.*, 1995; Yamashiro *et al.*, 1994).

The two murine nm23/NDP kinase genes, nm23-M 1 and nm23-M2, and the two human nm23/NDP kinase genes, nm23-H1 and nm23-H2, encode 17 kDa proteins that are 82-89% identical in predicted amino acid and nucleotide sequences (Okada *et al.*, 1996; Stahl *et al.*, 1991; Urano *et al.*, 1992). The nm23/NDP kinase expression correlates inversely with the metastatic potential of several rodent tumors (Steeg *et al.*, 1989) and human breast carcinoma and human hepatocellular carcinoma (Bevilacqua *et al.*, 1989; Tokunaga *et al.*, 1993). Transfection of nm23 cDNA into highly metastatic murine K-1735-TK melanoma cells results in significant reduction of metastasis. However, in colorectal carcinoma, no significant association was found between nm23 expression and metastatic potential, whereas in childhood neuroblastoma, overexpression and mutation of nm23 were associated with advanced tumors.

The nm23 protein was proposed to be a nucleoside diphosphate kinase (NDP kinase) based on cDNA sequence homology with the NDP kinase of *Dictystelium discoideum* (Wallet *et al.*, 1990). It also showed high homology with the *Drosophila* abnormal wing disc (*awd*) gene, mutations of which induce wide-spread developmental abnormalities. The *awd* gene has also been demonstrated to have NDP kinase activity associated with microtubule polymerization (Biggs *et al.*, 1990). Lakso *et al.* (1992) reported that one of the roles of nm23 during mouse organogenesis is normal functional differentiation and/or maintenance of stably differentiated epithelial tissues. In the induced differentiation of human leukemia cell lines, nm23 gene expression was altered (Yamashiro *et al.*, 1994).

Recently, the PuF, human *c-myc* transcription factor was identified as a nm23-H2/NDP kinase, a candidate

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The abbreviations used are: BSK, Bluescript; CAT, chloramphenicol acetyltransferase.

suppressor of tumor metastasis (Postel *et al.*, 1993). With or without a nuclease hypersensitive element (NHE), nm23-H2 transactivation capability to *c-myc* was altered. Namely, PuF/nm23-H2 transactivates the *c-myc* gene via the nuclease hypersensitive element.

In this study, we describe nm23-H2 and nm23-M2 transcription effect on *c-myc* via estimation of the relative acetylation of *c-myc* CAT in transfectant murine cell lines with nm23-H1 and nm23-H2 or nm23-M2. In addition, the alteration of phase proportions in the cell cycle, especially prolonged S phase, was also investigated in nm23/NDP kinase transfectants.

Materials and Methods

Cell culture

Cell lines with low and high metastatic capability, G6 and FE7, were cloned using the murine melanoma cell line, B16. All cell lines were cultured in Dulbeco's minimal essential medium containing 10% fetal bovine serum.

Transfectants

FE7 cells were transfected with the pBSnm23-H2 or pBSnm23-M2 plasmid DNA and control neo gene using Lipofectin (GIBCO-BRL). The supplied Lipofectin protocol was modified to include 10 μg of plasmid and 40 μg of Lipofectin, and the incubation proceeded in 60-mm tissue culture dishes of 80-90% confluent cells for 6 h. These cells were divided into 24 wells and selected for 3-4 weeks in medium containing 1 ng/ml G418 (GIBCO).

Contruction of plasmids for the CAT assay

The 1.6 kb pst-CAT fragment of the *myc* gene was inserted before a promoterless CAT reporter gene in pUC/19 (Takara). The 0.5 kb *XhoI-NotI* fragment of nm23-H2 and 0.5 kb *XhoI-XhoI* fragment of nm23-M2 genes were inserted into pBSK.

DNA transfections and chloramphenicol acetyltransferase assay

Cell lines were seeded at 5×10^5 cells per 60 mm Petri dish, and 24 h later, the cells were transfected with 10 μg of clones by means of the DEAE dextran protocol, with 1 μg of the luciferase expression plasmid pCEV/luc to quantify transfection efficiency. After washing the cells with serum-free medium twice, 3 ml of 10% Nu serum was added. DNA mixture (10 μg of plasmid DNA, 400 μg of DEAE dextran, 50 μl of MEM) was dripped on the cells. Three μl of chloroquine was added and the cells were incubated for 40-45 min. The cells were washed with PBS and incubated for 2 min with 10% Me_2SO. The cells were then washed with PBS and supplied with 7.5% DMEM and incubated for 2 days. The cells were trypsinized for harvest, and sonicated on ice for cell disruption, at a 50% duty cycle for 15 s twice. Cell extract deacetylase was inactivated in a 65 ℃ water bath for 10 min. Fourty μl of cell extract was reacted for 3 h at 37 ℃ with 5 μl of ^{14}C-chloramphenicol (0.25 Ci), 10 μl of 8 mM acetyl-CoA, 25 μl of 1 M Tris-HCl (pH 7.8). After stopping with 250 μl of cold 0.25 M Tris-HCl (pH 7.8), chloramphenicol was extracted by 500 μl of ethyl acetate. Upper organic phase was dried down and redissolved in 20 μl of ethyl acetate and spotted on silica gel (TLC plate). The plate was exposed to a BAS2000 (Fuji) imaging plate for 3 h. The relative intensity of the spots were analyzed by BAS2000.

Northern blot analysis

Total RNA was extracted using acid guanidium thiocyanate-phenol-chloroform. Twenty μg of isolated RNA was electrophoretically separated on a 1% agarose gel containing formaldehyde (Sambrook *et al.*, 1989) and transferred onto a nylon membrane. Hybridization and stringent washing were performed according to the manufacturer's direction. The *Bam*HI-*Eco*RI fragment of pBSK-nm23-H2 and pBSK-nm23-M2, *Eco*RI-*Hin*dIII fragment of pBSK-myc were used as hybridization probes. The probes were labeled with [α-^{32}P]dCTP by a megapriming labeling kit (Amersham, Arlington Heights, IL). The hybridized membranes were exposed to a BAS2000 (Fuji) imaging plate for 3 h. The relative intensity of the bands were analyzed by BAS2000.

Cell cycle

Trypsinized cells were washed with PBS 3 times and centrifuged. Pellets were resuspended in 0.5 ml of PBS with 5 mM $MgCl_2$ and 0.1% Triton X-100 and 2 μl of 10 mg/ml RNase was added. After incubation for 15 min at room temperature, the suspensions were added with 1 ml of propionium iodide solution (50 μg/ml in PBS) and incubated for 15 min in a dark area. The cells were examined with a CellFit soft program in FACS.

Results

Transfectants

FE7-*neo*-1(Neo-1) is a cloned tranfectant of the control neo gene (pBSMGSNeo). FE7-M2-3(M2-3) and FE7-M2-6(M2-6) are two cloned FE7 transfectants with the nm23-M2 gene (pBSMGSN-M2). FE7-H1/2-2(H1/2-2) and FE7-H1/2-4(H1/2-4) are two cloned FE7 transfectants with nm23-H1 and nm23-H2 genes.

Expression of nm23/NDP kinase and c-myc *in Northern blotting*

As shown in Figure 1, the correlation of expression between nm23-H2 and *c-myc,* nm23-M2 and *c-myc* was investigated. HL60 was used as a positive control of *c-myc* expression. More expression of nm23-H 2, nm23-M2, and *c-myc* were shown in G6 cells (low metastatic) than in FE7 (high metastatic). But in

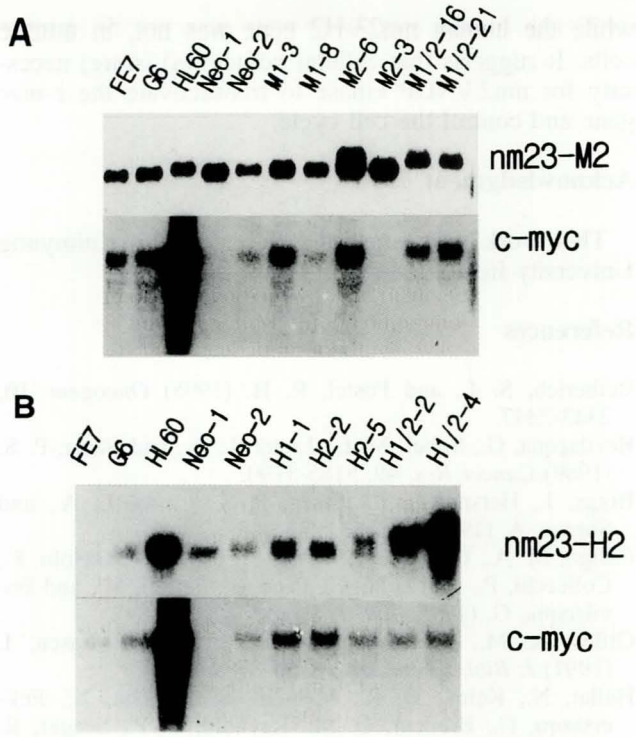

Figure 1. The expression of nm23-H2, nm23-M2, and *c-myc* in nm23 gene transfectants. Panel A, Northern blot analysis of nm23-H2 and *c-myc* in FE7 transfectants with murine nm23 gene and neo gene; panel B, Northern blot analysis of nm23-M2 and *c-myc* in FE7 transfectants with human nm23 gene and neo gene. HL 60 was human promyelocytic leukemia cell line for positive control of *c-myc* expression; G6 and FE7 were described in Materials and Methods; Neo-1 and Neo-2 were FE7 cells transfected with neo gene; M1-3 and M1-8, transfected with nm23-M1; M2-6 and M2-3, transfected with nm23-M2; M1/2-16 and M1/2-21, transfected with nm23-M1 and nm23-M2: H-1, transfected with nm23-H1; H2-2 and H2-5, transfected with nm23-H2; H1/2-2 and H1/2-4, transfected with nm23-H1 and nm23-H2.

transfectants including the neo gene, no similar expression kinetics were found between nm23-H2 and *c-myc* expression pattern, or nm23-M2 and *c-myc* expression pattern.

Myc *transcription activity of nm23/NDP kinase*

CAT assay was done to clarify the *c-myc* transcription activity by nm23/NDP kinase. For this experiment, transfectants M2-3 and M2-6 cells were picked up because of the high expression of nm23-M2/NDP kinase (Fig. 1) as well as H1/2-2 and H1/2-4 because of the high expression of nm23-H2/NDP kinase. To the transfectants, as described previously, *c-myc*-pstCAT and nm23/NDP kinase gene constructs were cotransfected. As shown Figure 2, in nm23-M2/NDP kinase transfectants, M2-2 and M2-6, cotransfection of *c-myc*-pstCAT and nm23-M2/NDP kinase induced a 2-4 fold acetylation of chloramphenicol, comparing control, FE7 and G6 cells, while

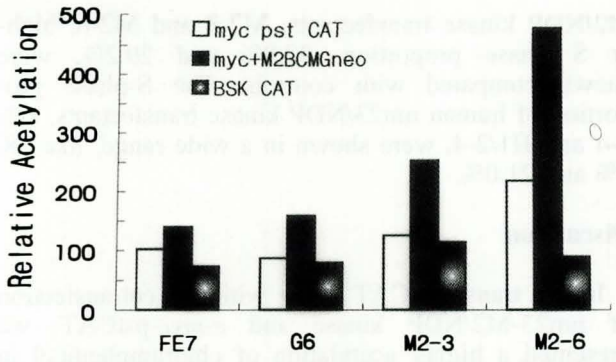

Figure 2. Acetylation of *c-myc* CAT in nm23-M2 transfectants, M2-3, M2-6 in which myc-pstCAT alone, myc-pstCAT and M2BCMGneo combined, and BSK CAT alone for negative control were transfected.

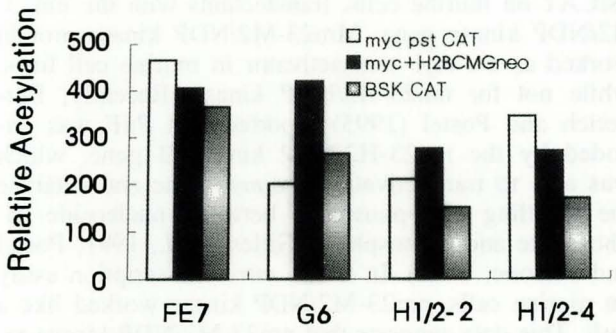

Figure 3. Acetylation of *c-myc* CAT in nm23-H1/H2 transfectants, H1/2-2, H1/2-4, in which *myc*-pstCAT alone, *myc*-pstCAT and H2BCMGneo combined and BSK CAT alone for negative control were transfected.

no correlated induction of acetylation was shown in the cotransfection of *c-myc*-pstCAT and nm23-H2, even in nm23-H1/NDP kinase and nm23-H2/NDP kinase cotransfectants, H1/2-2 and H1/2-4 cells (Fig. 3).

Cell cycle in nm23/NDP kinase transfectant murine cell lines

By the CellFit program of FACS the phase proportion of the cell cycle was estimated and S phase proportions were compared. As shown in Table 1, the proportions of S phase in controls, FE7, Neo-1, and G6, were 21.0%, 21.1%, and 22.6%. In murine nm23-

Table 1. The proportions (%) of cell cycle phases[a]

	G0/G1	S	G2+M	G2+M/G1
FE7	67.9	22.6	9.5	1.92
Neo-1	67.1	21.1	11.8	2.0
G6	71.9	21.0	7.1	1.96
M2-3	57.3	32.9	9.8	1.89
M2-6	64.4	29.2	6.3	1.95
H1/2-2	65.0	28.5	6.4	1.97
H1/2-4	64.6	21.0	14.4	1.92

[a]As described in transfectant in Results previously, FE7, Neo-1, and G6 are controls. M2-3 and M2-6 are nm23-M2 gene transfectants and H1/2-2 and H1/2-4 are nm23-H1 and nm23-H2 genes transfectants.

M2/NDP kinase transfectants, M2-3 and M2-6, higher S phase proportion, 32.9% and 29.2%, were shown compared with controls. The S-phase proportion of human nm23/NDP kinase transfectants, H1/2-4 and H1/2-4, were shown in a wide range, like 28.5% and 21.0%.

Discussion

In the transient CAT assay with the cotransfection of nm23-M2/NDP kinase and *c-myc*-pstCAT, we presented a higher acetylation of chloramphenicol in the murine melanoma cells, FE7, cloned transfectants with nm23-M2/NDP kinase gene. No constant acetylation of chloramphenicol was shown in cotransfection of nm23-H2/NDP kinase and *c-myc*-pstCAT on murine cells, transfectants with the nm23-H2/NDP kinase gene. Mm23-M2/NDP kinase protein worked as a *c-myc* transactivator in murine cell lines, while not for nm23-H2/NDP kinase. Recently, Berberich and Postel (1995) reported that PuF was encoded by the nm23-H2/NDP kinase B gene, which was able to transactivate the *c-myc* gene and catalyze the shuttling of γ-phosphate between nucleoside triphosphate and diphosphate (Gilles *et al.*, 1991; Postel and Ferrone, 1994). In the *in vitro* transcription assay on murine cells, nm23-M2/NDP kinase worked like a PuF. This data suggests that nm23-M2/NDP kinase requires a cofactor which is limiting or transiently present in murine cells. This cofactor does not work with human nm23-H2/NDP kinase to transactivate the *c-myc* gene in murine cells.

Nm23 gene expression correlates with cell growth, especially hepatocyte regeneration (Lee *et al.*, 1997), and S phase (Caligo *et al.*, 1995). In synchronously cycling cells, nm23-H1 mRNA reaches a maximum abundance in the S phase and is present at very low levels during the G0/G1 phase, whereas nm23-H2 is present in growth-arrested cells but is upregulated following growth stimulation. On the estimation of the cell cycle of nm23/NDP kinase transfectants, a prolonged S phase was shown in the murine nm23 (nm 23-M2) transfectants, whereas S phase prolongation was not clear in the human nm23/NDP kinase transfectants.

Other observations, excluding PuF and *awd* gene homology, described previously stated that nm23 genes linked to cell growth as follows: 1) anti-nm23 antibodies recognize mitotic spindle microtubules (Lakshmi *et al.*, 1993); 2) nm23 is highly homologous to the p19 protein, a neuroblastoma cell proliferation-related protein (Hailat *et al.*, 1991); 3) microinjection of an nm23-specific antibody inhibits cell division in rat embryo fibroblasts (Sorscher *et al.*, 1993). However, a direct proof of nm23 involvement in the cell cycle is still missing.

By two different experimental findings, the murine nm23-M2 gene was involved with *c-myc* transactivation and cell cycle control in murine cells, while the human nm23-H2 gene was not, in murine cells. It suggests that cellular cofactor(s) is(are) necessary for nm23/NDP kinase to transactivate the *c-myc* gene and control the cell cycle.

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