FEBS Letters 348 (1994) 273–277

FEBS 14237

# Increased expression of nucleoside diphosphate kinases/nm23 in human diploid fibroblasts transformed by SV40 large T antigen or $^{60}$Co irradiation

Kinuko Ohneda[a,e], Mitsugu Fukuda[a], Nobuko Shimada[a], Naoshi Ishikawa[a,b], Tateo Ichou[c], Kazuhiko Kaji[d], Takayoshi Toyota[e], Narimichi Kimura[a,*]

*Departments of [a]Molecular Biology, [b]Molecular Pathology, [c]Cell Bank and [d]Isotope, Tokyo Metropolitan Institute of Gerontology, 35-2 Sakaecho, Itabashi-ku, Tokyo-173, Japan*

*[e]3rd Department of Internal Medicine, Tohoku University, 2-1 Seiryoucho, Aoba-ku, Sendai-shi, Miyagi-980, Japan*

Received 20 May 1994; revised version received 9 June 1994

**Abstract**

When the expression levels of nucleoside diphosphate (NDP) kinase/nm23 were examined in four human normal diploid fibroblast cell lines in comparison with their corresponding immortalized cells transformed by SV40 large T antigen or $^{60}$Co irradiation, mRNA levels of the two isoforms (NDP kinase A/nm23-H1, NDP kinase B/nm23-H2) were increased in the immortalized cell lines. The increase was found to be associated with increased translation products. Furthermore, the cell extracts prepared from these immortalized cell lines demonstrated slightly higher enzyme activity than those from their normal counterparts. Neither the growth state nor the in vitro aging largely affected their expression in a normal cell line (TIG-3) examined. The results suggest possible involvement of NDP kinases/nm23 in acquiring an infinite growth property of these cells.

*Key words:* NDP kinase; nm23; Gene expression; Enzyme activity; Immortalization; Human diploid cells

## 1. Introduction

Nucleoside diphosphate (NDP) kinase is a ubiquitous enzyme that has been believed to play a housekeeping role through catalyzing transphosphorylation of nucleoside diphosphates to produce corresponding triphosphate nucleotides [1]. The enzyme is now known to be multifunctional [2–4]; it is identical to nm23 (a candidate tumor metastasis suppressor) [5,6], Awd (a morphological regulator in *Drosophila*) [7], I-factor (a differentiation inhibitor of mouse myeloid leukemia cell) [8] and PuF (a transcription factor of human c-*myc* gene) [9]. Among these functions, the latter two do not require the enzyme activity to elicit their actions [10], whereas Awd function seems to be associated with the enzyme activity [7]. Whether the action of nm23 parallels the enzyme activity remains uncertain, but a recent report describes that serine phosphorylation rather than enzyme activity may be associated with the antimetastatic potential of nm23 [11].

Growth associated increase in either the enzyme activity or its transcript levels of NDP kinase has been documented for fission yeast *Schizosaccharomyces pombe* [12], slime mold *Dictyostelium discoideum* [13], mouse [14] and human [15] cells. This leads to the speculation that the enzyme may function in association with growth property of the cell. A recent finding [16] that microinjection of an NDP kinase/nm23 antibody into rat embryo fibroblasts inhibited cell division with no apparent effect on DNA synthesis is an additional support for such notion. However, the relationship between NDP kinase expression and cell growth property is not straightforward. For example, we [17] recently observed that overexpression or disruption of a *Saccharomyces cerevisiae YNK* gene that encodes NDP kinase affected neither growth rate nor spore formation. Further, a recent report [18] describes that increased levels of *nm23*-H1 mRNA in proliferative phase of cancer cell growth were not accompanied by definite changes in the translation product. In this study we investigated whether the expression of NDP kinase/nm23 is related to an acquisition of the infinite cell growth property of normal human diploid cells in terms of the transcription and translation products, and the enzyme activity.

## 2. Materials and Methods

### 2.1. Cells and cell culture

Four human normal diploid fibroblasts (TIG-3 [19], KMS-6 [20], WI-38 [21] and IMR-90 [22]) and their corresponding immortalized cells (TIG-3SVts, KMST-6, WI-38VA13, and IMR-90SV) were used. Population doubling levels (PDL) of normal cell lines, methods of transformation and doubling time of these cell lines are listed in Table 1. All of these transformed cells were unable to form colonies when cultured on soft agar plates. Cells were maintained in Eagle's minimal essential medium (MEM, Nissui Pharmaceutical Co. Ltd., Tokyo) supplemented with kanamysin and 10% fetal bovine serum in a humidified atomosphere of 5% $CO_2$ and 95% air. The cells were grown at 37°C except TIG-3SVts which was cultivated at 34°C.

### 2.2. Preparation of cell extracts and NDP kinase assay

Subconfluent cells in 100 mm culture dishes were detached by 0.25% trypsin, rinsed three times with ice-cold Dulbecco's phosphate buffered saline and lysed with 0.8 ml of 0.5% sucrose monolaurate (Mitsubishi Kasei Kogyo Co. Ltd.) in STME buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 1 mM EDTA). NDP kinase activity

*Corresponding author. Fax: (81) (3) 3579-47761.


*SSDI* 0014-5793(94)00623-4

was determined by pyruvate kinase-lactate dehydrogenase coupled enzyme system as described previously [23].

*2.3. Immunoblotting*

The cell extracts (15 mg of protein) were subjected to SDS-PAGE and then electrophoretically transferred to a PVDF (polyvinylidene difluoride) membrane (Bio-Rad). The membrane was treated with affinity purified anti-rat NDP kinase rabbit polyclonal antibody (NK-2) that reacts specifically with human [24] as well as rat [23,25] NDP kinases, followed by peroxidase conjugated anti-rabbit IgG, and detection was carried out using an ECL assay kit (Amersham).

*2.4. RNA extraction and Northern blotting*

Total cellular RNA was prepared from cells in a 100 mm culture dish using 1.5 ml of Isogen (Wako Pure Chemicals), a modified extraction solution of single-step purification [26]. 15 μg of RNA was electrophoresed on a 1% agarose gel and transferred to a nitrocellulose membrane after separation. Hybridization was performed with either a full-length nm23-H1 (NDP kinase A) or nm23-H2 (NDP kinase B) cDNA probe labeled by the random priming method essentially as previously described [4]. The filters were washed with 2 × SSC/0.2% SDS, followed by two times washing with 0.2 × SSC/0.2% SDS at 65°C for 30 min.

## 3. Results

The immortalized human cells obtained by transformation with SV40 large T antigen or $^{60}Co$ irradiation were all morphologically of transformed phenotype and neither of them showed anchorage independent growth properties. So far, two isoforms of NDP kinase/nm23 have been identified in human [27] as well as rat [4] and mouse [28]. Northern blot analyses (Fig. 1) revealed that mRNA levels of both isoforms were concomitantly elevated in the immortalized cells; especially an increase in the NDP kinase-A/nm23-H1 mRNA was dramatic in the immortalized cells except WI-38VA13 cell. Neither the growth state (log phase or stationary phase) nor serum deprivation (from 10% to 0.5%, and serum refeeding also) largely altered these transcript levels when examined with TIG-3 cell (not shown). It is known that normal human diploid cells cease to grow after a limited number of PDL. In an attempt to know the effect of in vitro aging on the expression of NDP kinase, we compared these transcripts levels of normal and immmortalized TIG-3 cells with different PDL (normal: 17.6 vs. 59.3 PDL; immortalized: 178 vs. 303 PDL) (Fig. 2). The results demonstrated that the transcripts levels were essentially the same between the cell lines with different PDL in both groups. It seems therefore that the levels of NDP kinases/nm23 transcripts fluctuate in relation to infinite growth property (immortalization) of the human fibroblasts rather than their growth state or in vitro aging (PDL) of the cells.

Whether the increased levels of nm23 transcripts are accompanied by those of translation products and enzyme activity was further examined. Western blot analysis with an affinity purified anti-rat NDP kinase antibody (NK-2) identified a doublet with 18 kDa major protein band (Fig. 3). In agreement with the data on the mRNA levels, all these transformed cell lines contained more NDP kinase protein than did their parental normal counterparts although the difference seemed smaller in each case than that of the transcript level. Moreover, small but significant difference was also observed for the NDP kinase enzyme activity (Fig. 4). The enzyme activity present in the normal cell extracts varied between 3.8–5.7 mmol/mg protein/min. Nevertheless, the activities in the immortalized cell extracts were consistently higher than those in their normal counterparts (10–38% increase). It seemed unlikely that NDP kinase activity is correlated with doubling time of these cells (see Table 1).

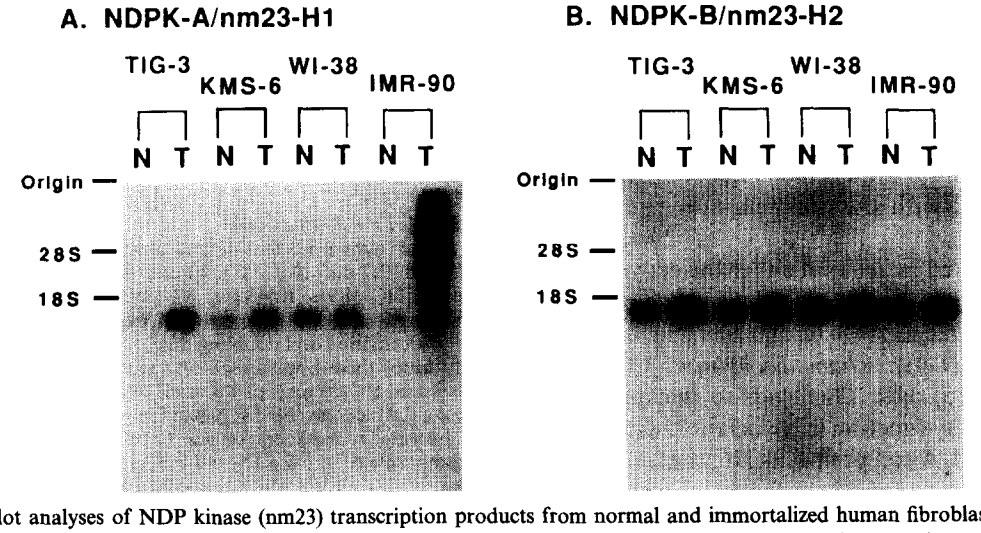


Fig. 1. Northern blot analyses of NDP kinase (nm23) transcription products from normal and immortalized human fibroblasts. Total RNAs (15 μg each) from cultured human cell lines were separated on a 1% agarose gel and transferred onto a nitrocellulose membrane. Hybridization with either nm23-H1/NDP kinase A (A) or nm23-H2/NDP kinase B (B) cDNA probe was carried out as described in the text. N = normal cells; T = corresponding immortalized, transformed cells. Strong ladder bands, which are present in higher position compared with that of nm23-H1 (NDP kinase A) in IMR-90 SV cells but not in other cells, seem unlikely to be physiologically relevant, since the Western blot data provided neither extra protein bands nor extremely strong signal at the expected size (18 kDa).

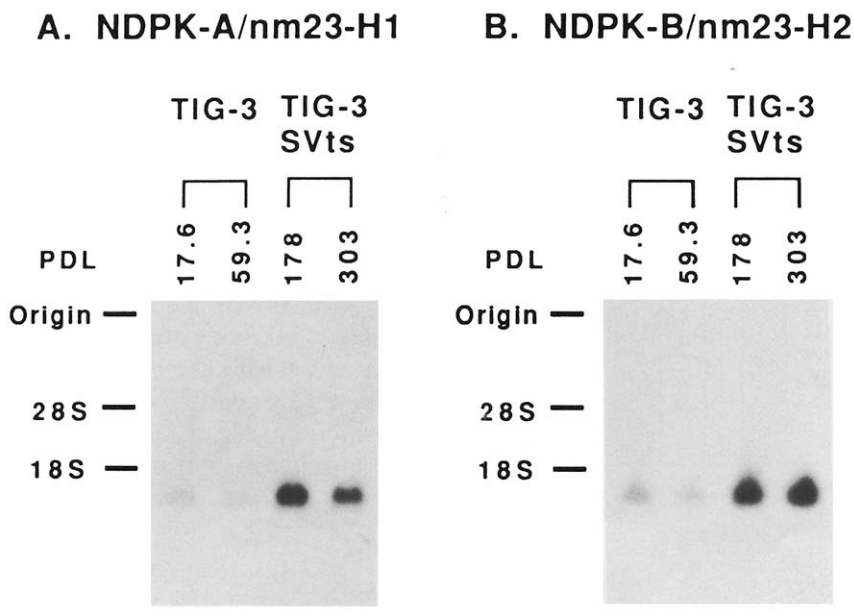


Fig. 2. Expression of NDP kinase (nm23) mRNAs in normal TIG-3 and immortalized TIG-3SVts cells with different PDL. Total RNAs from normal TIG-3 with 17.6 PDL (lanes 1 and 5) and 59.3 PDL (lanes 2 and 6), and those from immortalized TIG-3SVts with 178 PDL (lanes 3 and 7) and 303 PDL (lanes 4 and 8) were used and analyzed as described in Fig. 1.

## 4. Discussion

Some biochemical and immunohistochemical studies from this and other laboratories on the behavior of NDP kinase/nm23 of cancer tissues from human and other origins provided an indication that not all but most cancer tissues including human breast cancers seem to be associated with enhanced expression of NDP kinase/nm23 compared with neighboring normal tissues [29–32]. Such observations led us to speculate that the NDP kinase/nm23 expression may alter in association with an early step of the tumorigenic processes in addition to metastatic phenotype of the cell. The present study demonstrated for the first time that the expression of NDP kinase/nm23 is potentiated in human fibroblasts with infinite cell growth potential. The expression did not change depending on the growth state or in vitro aging of the cells. Although the alteration of the enzyme activity was smaller than that of the transcript levels, the observation seems important because, in most other cases so far examined, detection of such altered enzyme activity (or protein level) has been rare even under the condition where their transcript levels changed drastically [11,18]. It should be noted, however, that WI-38VA13 cell showed significant increased levels of the protein amount and enzyme activity despite insignificant change in the transcripts levels. This observation was unexpected and remains to be investigated further. Considering the fact that neither of the immortalized cell lines used in this study possessed anchorage independent growth property, NDP kinases/nm23 (probably through their enzyme activity) seem to be involved in an early phase of tumorigenic processes.

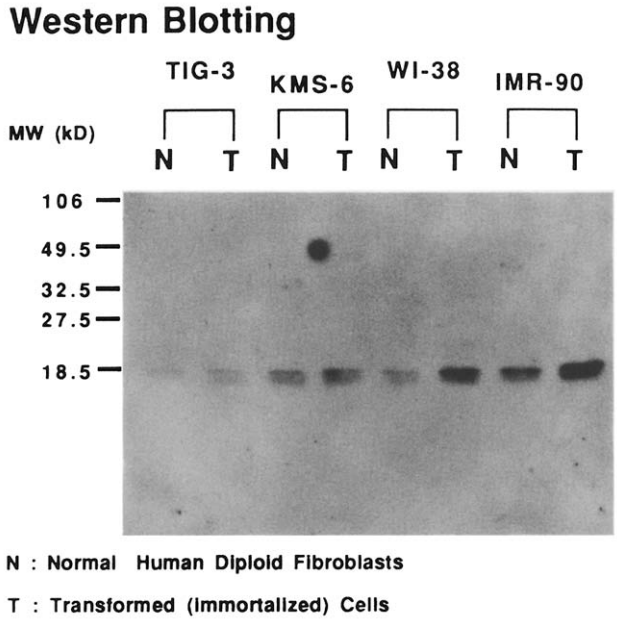


Fig. 3. Western blot analyses of NDP kinases from human normal and immortalaized cultured cells. The cell extracts (15 μg protein each) from normal and immortalized human fibroblasts were electrophoresed on a SDS-PAGE gel, followed by electrophoretic transfer onto a PVDF membrane and immunodetection by an affinity purified anti-rat NDP kinase rabbit antibody. N = normal cells; T = corresponding immortalized, transformed cells.

Normal human diploid cells can proliferate in in vitro culture systems but die after a limited number of PDL. These cells acquire infinite growth property after transformation and then undergo tumorgenic alterations. Re-

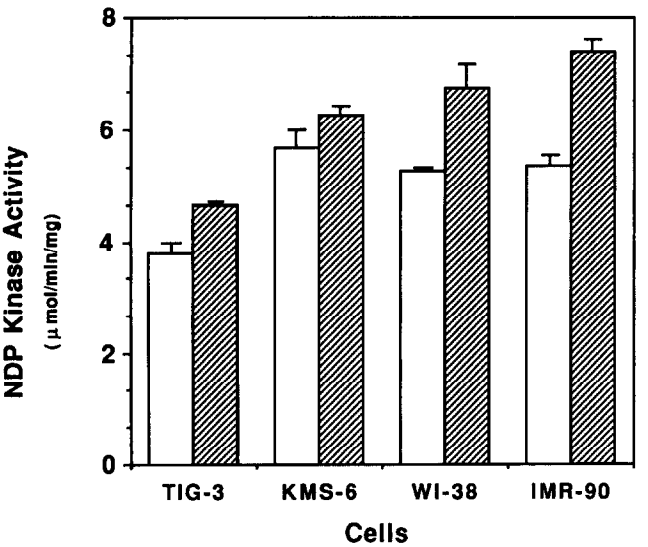


Fig. 4. NDP kinase activity in the extracts from normal and immortalized human fibroblasts. The cell extracts were prepared as described in section 2 and the enzyme activity was determined by the pyruvate kinase-lactate dehydrogenase coupled enzyme assay method. Open columns = normal cells; hatched columns = corresponding immortalized, transformed cells. The values are means ± standard errors (vertical bars). Similar results were obtained from three independent experiments and the representative data are shown.

cent studies revealed that the former process (immortalization) can be achieved by the participation of at least four complementary recessive factors [33]. These factors are suggested to include tumor suppressor gene products, such as, Rb and p53 [34,35]. In view of the fact that the immortalized human cell lines used in this study except KMST-6 cell were transformed by SV-40 large T antigen that is known to bind and inactivate Rb and p53 proteins [34–36], the expression of NDP kinases may be influenced, directly or indirectly, by these tumor suppressor gene products.

Table 1
Profiles of human normal diploid fibroblasts and their immortalized counterparts

| Cells | PDL* | Doubling time (h) |
|---|---|---|
| *Normal cells* | | |
| TIG-3 | 18.3 (21.8%) | 24.0 |
| KMS-6 | 30.3 (79.1%) | 32.2 |
| WI-38 | 25.3 (45.5%) | 26.1 |
| IMR-90 | 33.3 (47.5%) | 26.7 |
| *Immortalized cells* | | |
| TIG-3SVts** | | 27.0 |
| KMST-6*** | | 23.1 |
| WI-38 VA13** | | 21.6 |
| IMR-90 SV** | | 24.3 |

*% lifespan completed.
**Transformed by SV-40 large T antigen.
***Transformed by $^{60}$Co irradiation.

Two isoforms of mammalian NDP kinases are extremely homologous to each other in their amino acid sequences [4]; 89% identical in rat and human, for example. Although their specific roles have not been clearly demonstrated [25], the observations, such as tissue-dependent expression of isoforms [4] and preferencial decreased expression of one isoform compared with the other in highly metastatic cells and tissues [27], may suggest their respective roles and different expression control mechanisms. It should be noted, in this respect, that mRNAs of both isoforms were increased in the immortalized cell lines except WI-38VA13. This may suggest a common, in addition to independent, regulatory mechanism of the gene expression for the two isoforms relating to an acquisition of infinite cell growth property.

*Acknowledgements:* The authors are grateful to Drs. P.S. Steeg and L.A. Liotta (Laboratory of Pathology, NCI, NIH) for providing us with nm23-H1 and nm23-H2 cDNA probes. This work was supported in part by grants from the Ministry of Education, Science, and Culture, Japan and a research grant from Human Frontier Science Program Organization.

## References

[1] Parks Jr., R.E. and Agarwal, R.P. (1973) in: The Enzymes (Boyer, P.D. ed.) vol. 8, pp. 307–333, Academic Press, New York.
[2] Kimura, N. (1993) in: GTPases in Biology, Handbook of Experimental Pharmacology Vol. 108/II (Dickey, B. and Birnbaumer, L. eds.) pp. 485–498, Springer-Verag, Berlin.
[3] Kimura, N., Shimada, N., Nomura, K. and Watanabe, K. (1990) J. Biol. Chem. 265, 15744–15749.
[4] Shimada, N., Ishikawa, N., Munakata, Y., Toda, T., Watanabe, K. and Kimura, N. (1993) J. Biol. Chem. 268, 2583–2589.
[5] Steeg, P.S., Bevilacqua, G., Kopper, L., Thorgeirsson, U.P., Talmadge, J.E., Liotta, L.A. and Sobel, M.E. (1988) J. Natl. Cancer Inst. 80, 200–204.
[6] Leone, A., Flatow, U., King, C.R., Sandeen, M.A., Margulies, M.K., Liotta, L.A. and Steeg, P.S. (1991) Cell 65, 25–35.
[7] Biggs, J., Hersperger, E., Steeg, P.S., Liotta, L.A. and Shearn, A. (1990) Cell 63, 933–940.
[8] Okabe-Kado, J., Kasukabe, T., Honma, Y., Hayashi, M., Henzel, W.J. and Hozumi, M. (1992) Biochem. Biophys. Res. Commun. 182, 987–994.
[9] Postel, E.H., Berberich, S.J., Flint, S.J. and Ferrone, C.A. (1993) Science 261, 478–480.
[10] Postel, E.H. and Ferrone, C.A. (1994) J. Biol. Chem. 269, 8627–8630.
[11] MacDonald, N.J., De La Rosa, A. Benedict, M.A., Freihe, M.P., Krutsch, H. and Steeg, P.S. (1993) J. Biol. Chem. 268, 25780–25789.
[12] Creanor, J. and Mitchison, J.M. (1989) J. Cell Sci. 93, 185–189.
[13] Wallet, V., Mutzel, R., Troll, H., Barzu, O., Wurster, B., Veron, M. and Lacombe, M.-L. (1990) J. Natl. Cancer Inst. 82, 1199–1202.
[14] Shimada, N., Kimura, N., Varga, Z., Utsuyama, M., Kasai, M. and Hirokawa, K. (1990) Biomed. Gerontol. 14, 121–122 (Abstract).
[15] Keim, D., Hailat, N., Melhem, R., Zhu, X.X., Lascu, I., Veron, M., Strahler, J. and Hanash, S.M. (1992) J. Clin. Invest. 89, 919–924.
[16] Sorscher, S.M., Steeg, P., Feramisco, J.R., Buckmaster, C., Boss,

G.R. and Meinkoth, J. (1993) Biochem. Biophys. Res. Commun. 195, 336–345.
[17] Fukuchi, T., Nikawa, J., Kimura, N. and Watanabe, K. (1993) Gene 129, 141–146.
[18] Igawa, M., Rukstalis, D.B., Tanabe, T. and Chodak, G.W. (1994) Cancer Res. 54, 1313–1318.
[19] Matsuo, M., Kaji, K., Utakoji, T. and Hosoda, K. (1982) J. Gerontol. 37, 33–37.
[20] Namba, M., Nishitani, K., Hyodoh, F., Fukushima, F. and Kimoto, T. (1985) Int. J. Cancer 35, 275–280.
[21] Hayflick, L. (1965) Exp. Cell Res. 37, 614–636.
[22] Nichols, W.W., Murphy, D.G., Cristofalo, V.J., Toji, L.H., Greene, A.E. and Dwight, S.A. (1977) Science 196, 60–63.
[23] Kimura, N. and Shimada, N. (1988) J. Biol. Chem. 263, 4647–4653.
[24] Nakamori, S., Ishikawa, O., Ohhigashi, H., Kameyama, M., Furukawa, H., Sasaki, Y., Inaji, H., Higashiyama, M., Imaoka, S., Iwanaga, T., Funai, H., Wada, A. and Kimura, N. (1993) Clin. Exp. Metastasis 11, 151–158
[25] Fukuchi, T., Shimada, N., Hanai, N., Ishikawa, N., Watanabe, K. and Kimura, N. (1994) Biochim. Biophys. Acta 1205, 113–122.
[26] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[27] Stahl, J.A., Leone, A., Rosengard, A.M., Porter, L., King, C.R. and Steeg, P.S. (1991) Cancer Res. 51, 445–449.
[28] Urano, T., Takamiya, K., Furukawa, K. and Shiku, H. (1992) FEBS Lett. 309, 358–362.
[29] Hirayama, R., Sawai, S., Takagi, Y., Mishima, Y., Kimura, N., Shimada, N., Esaki, Y., Kurashima, C., Utsuyama, M. and Hirokawa, K. (1991) J. Natl. Cancer Inst. 83, 1249–1250.
[30] Francis, B., Overmeyer, J., John, W., Marshal, E. and Haley, B. (1989) Mol. Carcinogen. 2, 168–178.
[31] Haut, M., Steeg, P.S., Willson, J.K.V. and Markowitz, S.D. (1991) J. Natl. Cancer Inst. 83, 712–716.
[32] Hailat, N., Keim, D.R., Melhem, R.F., Zhu, X-X. Eckerskorn, C., Brodeur, G.M., Reynolds, C.P., Seeger, R.C., Lottspeich, F., Strahler, J.R. and Hanash, S.M. (1991) J. Clin. Invest. 88, 341–345.
[33] Pereira-Smith, O.M. and Smith, J.R. (1988) Proc. Natl. Acad. Sci. USA 85, 6042–6046.
[34] Shay, J.W., Pereira-Smith, O.M. and Wright, W.E. (1991) Exp. Cell Res. 196, 33–39.
[35] Hara, E., Tsurui, H., Shinozaki, A., Nakada, S. and Oda, K. (1991) Biochem. Biophys. Res. Commun. 179, 528–534.
[36] DeCaprio, J.A., Ludlow, J.W., Figge, J., Shew, J., Huang, C., Lee, W., Marsilio, E., Pancha, E. and Livingston, D.M. (1988) Cell 54, 275–283.