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POLYADENYLIC ACID-CONTAINING CYTOPLASMIC RNA INCREASES IN ADENOSINE 3′,5′-CYCLIC MONOPHOSPHATE-INDUCED 'DIFFERENTIATED' NEUROBLASTOMA CELLS IN CULTURE

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SUMMARY

The amount of polyadenylic acid [poly(A)]-containing cytoplasmic RNA in malignant and 'differentiated' mouse neuroblastoma cells in culture was determined. 4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidinone (R020-1724), prostaglandin (PG)E_1, serum-free medium, X-irradiation and sodium butyrate were used to induce 'differentiation'. The cAMP-induced 'differentiated' cells had more poly(A)-containing cytoplasmic RNA than the malignant cells; the increase in poly-(A)-RNA was greatest in R020-1724-treated cells. The amount of poly(A)-containing cytoplasmic RNA did not significantly change in serum-free medium- or sodium butyrate-treated cells; however, it decreased in cells of confluent phase of growth. The percentage of total radioactive RNA in the cytoplasms of cAMP-induced 'differentiated' cells after 1 h of labeling was three-fold greater than that in malignant cells, indicating that transport of RNA from nucleus to cytoplasm occurs at relatively higher rate in 'differentiated' cells. The stability of RNA in cAMP-induced 'differentiated' cells increased in comparison to malignant cells. The binding of actinomycin D with DNA suggests that the proportion of guanine residues that are accessible to actinomycin D are similar in the nuclei of malignant and cAMP-induced 'differentiated' cells. The present and previous studies suggest that cAMP induces many differentiated functions in neuroblastoma cells by increasing both the transcription and translation of genetic information which is probably masked during malignant transformation of nerve cells.

All cell mRNAs except the histone mRNA [1] contain long stretches (100–200 nucleotides) of polyadenylic acid [poly(A)], whereas the ribosomal(r)- and transfer(t)-RNAs do not [4, 6, 10, 32). The rate of synthesis, accumulation and degradation of poly(A)-containing RNA have been measured under a variety of experimental conditions in order to obtain insight on the molecular mechanisms of growth and differentiation in mammalian cells in culture. For example, the amount and rate of synthesis of cytoplasmic poly(A)-containing RNA do not appreciably change with the alteration in growth rate [27]. However, the turnover rate of poly(A)-containing RNA in the non-transformed mouse fibroblasts is proportional to the doubling time of cells [11]. The dividing immature muscle cells have high levels of synthesis of poly(A)-containing cytoplasmic RNA; the half-life of poly(A)-containing RNA increases from 10 h in the dividing muscle cells to 50 h in the differentiated muscle cells [3]. The poly(A)-containing RNA fractions from the liver and the hepatoma polyribosomes are similar

[36]. The fertilization of sea urchin ova elicits a 2.5-fold increase in the synthesis of poly(A)-containing RNA [34].

An elevation of intracellular level of adenosine 3′,5′-cyclic monophosphate (cAMP) in neuroblastoma cells by prostaglandin E_1, inhibitor of cAMP phosphodiesterase or by analogs of cAMP irreversibly induces many differentiated functions which are characteristic of mature neurons. These include formation of neurites [16–18], increase in size of soma and nucleus associated with an increase in total RNA and protein contents [19], elevation of activities of tyrosine hydroxylase [26, 38], choline acetyltransferase [20] and acetylcholinesterase [5, 21], loss of tumorigenicity [22] and increase in sensitivity of adenylate cyclase to catecholamines and acetylcholine [12, 13]. The amount of poly(A)-containing cytoplasmic RNA in 'differentiated' neuroblastoma cells is higher than that in malignant cells [2]. In this paper we have further examined the changes in poly(A)-containing RNA in cells which are induced to express more than one differentiated functions and in cells of different growth phase. We now report that the amount of poly(A)-containing cytoplasmic RNA increases in cAMP- and X-irradiated-induced 'differentiated' cells; however, it does not change in cells treated with serum-free medium or sodium butyrate or in cells of confluent phase of growth.

MATERIALS AND METHODS

Cell culture and treatment with drugs

The procedures for culturing and maintaining the mouse neuroblastoma cells were described [16]. A previously defined clone $NBE^-(A)$ which contains choline acetyltransferase but no tyrosine hydroxylase [14] was used in this study. The doubling time of neuroblastoma cells is about 18 h. Cells produce tumors when injected subcutaneously into male A/J mice. 4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidinone (R020-1724), which inhibits cAMP phosphodiesterase rather selectively when compared with cGMP phosphodiesterase [33], induces many differentiated functions in neuroblastoma cells [12–13, 18–20, 22]; therefore, R020-1724 was primarily used to induce differentiation. The amount of poly(A)-containing cytoplasmic RNA was measured in malignant and cAMP-induced 'differentiated' neuroblastoma cells. Since this clone is relatively insensitive to prostaglandin $(PG)E_1$ in causing morphological differentiation [14], $(PG)E_1$ was also used to induce differentiation in order to estimate its relative efficiency with respect to R020-1724 in producing changes in poly(A)-containing cytoplasmic RNA. Agents such as X-irradiation [15], serum-free medium [31] and sodium butyrate [12, 16, 19–21, 38] cause some of the differentiated functions which are induced by cAMP; therefore, the amount of poly(A)-containing cytoplasmic RNA was also determined after treatment with the above agents.

Cells (0.25×10^6 for control, 0.4×10^6 for R020-1724 and PGE_1, 1×10^6 for X-irradiation, sodium butyrate, serum-free medium and confluency) were plated in Falcon plastic flasks (75 cm^2), and R020-1724 (200 μg/ml), PGE_1 (10 μg/ml), sodium butyrate (0.5 mM), serum-free medium or X-irradiation (600 rads) was given 24 h later. The procedures for irradiating the cells were previously described [15]. The control cultures received an equivalent volume (1 : 100 dilution) of 95 % ethyl alcohol. The drug and medium were changed every day after treatment, and the amount of poly(A)-containing cytoplasmic RNA was determined 3 days after treatment. The time period of 3 days was selected because cAMP-induced differentiated functions became irreversible at this time. For the study of cells of confluent phase of growth, cells were allowed to remain in the medium for 4 days; the medium was changed every day.

Assay of poly(A)-containing RNA

To determine the amount of poly(A)-containing cytoplasmic RNA, cells were incubated in the presence of 2,8-^{3}H-adenosine (2 μCi/ml, spec. act. 32.4 Ci/mM, New England Nuclear, Inc.) for 18 h, and then removed from flask surface using 0.25 % Viokase solution. The cells were washed thrice with growth medium and an aliquot was taken for counting the cell number, and the poly(A)-containing cytoplasmic RNA in the remainder was then determined [2, 35].

Each cell sample was homogenized in 5 ml of 0.32 M sucrose containing 200 mg chick brain as a carrier, and then centrifuged (1 000 g, 10 min) to remove the nuclei and intact cells. The cytoplasmic supernatant was brought to 0.1 M glycine, 0.1 M NaCl, 0.01 M EDTA, 1 % sodium deoxycholate, pH 9.5 and shaken with an equal volume of chloroform : phenol (1 : 1 v/v). After high speed centrifugation, 1/20 vol 3.0 M sodium acetate and 2 vol ethanol were added to the aqueous supernatant and held at −20°C for 1 h [35]. The RNA precipitates were then centrifuged. To purify the RNA from whole cells, DNA was removed by incubating the RNA preparations for 30 min at 37°C with DNase (100 μg/ml, Sigma Chemical Co.) in 0.01 M magnesium acetate, 0.15 M NaCl, 0.025 M Tris-

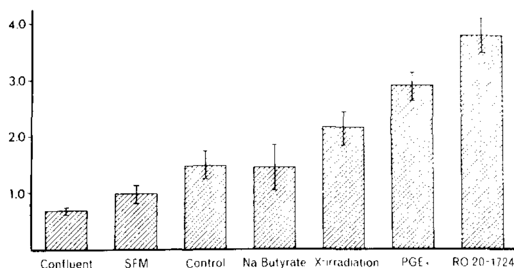

Fig. 1. Ordinate: percentage of radioactivity in cytoplasmic RNA that is within the mRNA fraction. Proportion of labeled cytoplasmic RNA that is messenger, in neuroblastoma cells after various treatment. Neuroblastoma cells were placed in Falcon plastic flasks (75 cm^2) and serum-free medium (SFM), sodium butyrate (Na butyrate), prostaglandin E_1 (PGE_1), 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (R020-1724), or X-irradiation was given 24 h later. The fresh growth medium and drug were changed every day. ^{3}H-Adenosine (2 μCi/ml) and drug were added 54 h after treatment, and the cells were further incubated for 18 h. For the study on cells of confluent phase of growth, cells were allowed to remain in the medium for 4 days before the addition of radioactive adenosine. The polyadenylic acid containing cytoplasmic RNA in cells treated with various agents was determined. Each value represents an average of at least 6 samples ±S.E.M.

HCl, pH 7.2 [30]. This mixture was reextracted with an equal volume of chloroform : phenol.

The RNA fraction in the supernatant was reprecipitated with sodium acetate and ethanol as described above. The DNA content of this RNA fraction was less than 2%. The cytoplasmic RNA precipitates were suspended in 1 ml water and reprecipitated with 0.05 ml 3.0 M sodium acetate and 2.1 ml ethanol. This final RNA precipitate was centrifuged, suspended in 0.5 ml of a high ionic strength buffer (0.1 M Tris-HCl, 0.3 M NaCl, 0.001 M EDTA, pH 7.0) and applied to a cellulose column (0.5×4.0 cm) previously washed with the same high ionic strength buffer. Chromatography was carried out at 37°C and all media used were at this temperature. This was to ensure that only poly-(A)-containing RNA would be retained by the column [35]. The high ionic strength buffer (3.5 ml) was passed through the column to elute non-retained RNA. The column was washed with 4 ml water which removed poly(A)-containing RNA. No residual radioactivity was left on the column.

Aliquots of the fractions were mixed with a compatible scintillation mixture (3a70, Research Products International, Elk Grove, Ill.) and the radioactivity was determined in a liquid scintillation counter at an efficiency of 31–34%. The cpm in samples ranged between 900 and 20 000. We have defined mRNA as that RNA fraction which is retained on the cellulose column at high ionic strength. The RNA with polyadenylate chain of greater than about 40 nucleotides in length is retained under these conditions (Roberts, W. K., personal communication).

Ribonuclease resistance

The messenger and non-messenger RNA fractions were incubated with pancreatic ribonuclease at high ionic strength (37°C, 30 min) in 0.3 M NaCl, 0.1 M Tris-HCl, pH 7.0. The yeast RNA (200 μg) was then added to each sample which was immediately brought to 0.5 N $HClO_4$ with 70% perchloric acid. After centrifugation, the radioactivity in supernatant containing the digested RNA was determined. The precipitate was washed with cold 0.5 N $HClO_4$ and briefly boiled in 1 ml 0.5 N $HClO_4$. The radioactivity in this solution was derived from polynucleotides that had not been degraded by ribonuclease under our incubation condition. This was taken to consist largely of the polyadenylate segment of mRNA. Control experiments using ^{3}H-uridine rather than ^{3}H-adenosine showed that 97.7% of labeled RNA was solubilized by RNase. Thus a background value of 2.3% was deducted from the estimation of non-digestible polynucleotide after ^{3}H-adenosine labeling. This may in part have been due to counts trapped in the precipitate of the non-radioactive yeast RNA that was used as a carrier.

Binding of ^{3}H-actinomycin D to nuclei

The nuclei were prepared from malignant and cAMP-induced 'differentiated' cells by homogenization (0.32 M sucrose, 0.005 M KCl, 0.002 M $MgCl_2$) and centrifugation (1 000 g, 10 min). The resulting pellet was examined by phase contrast microscopy and found to consist largely of nuclei although cytoplasmic tags on nuclei were apparent. Each pellet was suspended in 1 ml 0.32 M sucrose, 0.005 M KCl, 0.002 M $MgCl_2$, and 0.0025 M of ^{3}H-actinomycin D (spec. act. 1.04 Ci/mole, Schwarz–Mann, Inc.) was added in 30 μl. After 20 min incubation at 25°C, samples were brought to 8 ml 0.32 M sucrose and centrifuged (5 000 g, 10 min). The precipitate was washed with cold 5% trichloracetic acid and then with warm chloroform : ethanol (1 : 1 v/v). The final precipitate was taken up in 1 ml 0.5 N $HClO_4$, heated (90°C, 20 min) and centrifuged (5 000 g, 10 min). Absorbance at 265 nm and radioactivity were determined in this final supernatant. DNA concentration was calculated by a method of Santen & Agranoff [28], and the value was corrected for nuclear RNA.

RESULTS

To determine the concentration of poly(A)-containing cytoplasmic RNA, the cells were exposed to ^{3}H-adenosine for 18 h. Fig. 1 shows the effect of various agents which have been shown to induce more than one differentiated functions in mouse neuroblastoma cells in culture. R020-1724-, PGE_1- and X-irradiated-induced 'differentiated' neuroblastoma cells had signifi-

Table 1. *Percentage of total radioactive RNA present within the cytoplasms of neuroblastoma cells after various times of exposure to ^{3}H-adenosine*

Labeling time	Malignant cells	'Differentiated' cells
1 h	20±1[a]	57±3
18 h	77±3	90±2

[a] S.E.M.
4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidinone (R020-1724), a specific inhibitor of cAMP phosphodiesterase, was used to induce differentiation. Each value represents an average of 6 samples.

Table 3. *Percentage of counts in RNA fractions that are RNase-resistant after exposure of cells to ^{3}H-adenosine for 18 h*

Fraction	Malignant cells	'Differentiated' cells
Cytoplasmic messenger RNA	15.8±2.0[a]	13.4±1.2
Cytoplasmic non-messenger RNA	1.9±0.33	0.9±0.25

[a] S.E.M.
4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidinone (R020-1724), a specific inhibitor of cAMP phosphodiesterase, was used to induce differentiation. Each value represents an average of 6 samples.

cantly more radioactivity within poly(A)-containing cytoplasmic RNA than the malignant cells; the increase in poly(A)-containing RNA was greatest in R020-1724-treated cells. The relative amount of labeled poly(A)-containing cytoplasmic RNA did not significantly change in serum-free medium- and sodium butyrate-treated cells; however, it decreased in cells of the confluent phase of growth.

The cytoplasms of malignant and 'differentiated' cells after 1 h of labeling contain 20 and 57% of total radioactive RNA, respectively (table 1). The cytoplasms of malignant cells after 18 h of labeling contain 77% of total radioactive RNA, whereas

Table 2. *Incorporation of ^{3}H-adenosine into whole cell RNA*

Time of labeling	Malignant cells (cpm/1 000 cells)	'Differentiated' cells (cpm/1 000 cells)
18 h	320±20[a]	870±10
18 h plus 30 h in medium without exogenous radioisotope	220±20	960±10

[a] S.E.M.
4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidinone (R020-1724), a specific inhibitor of cAMP phosphodiesterase, was used to induce differentiation. Each value represents an average of 6 samples.

the cytoplasms of 'differentiated' cells contain 90% of total RNA.

The incorporation of ^{3}H-adenosine into whole cell RNA in 'differentiated' cells after 18 h of labeling was twice as high as that in malignant cells (table 2). When the labeled cells (18 h) were further incubated in the medium containing no exogenous radioactive adenosine for 30 h, the radioactivity in the RNA fraction decreased in malignant cells, but it was relatively unchanged in 'differentiated' cells.

The percentage of poly(A)-containing cytoplasmic RNA which was resistant to RNase, was similar in malignant and in 'differentiated' cells (table 3). The very small amount of RNase-resistant polynucleotide in the non-mRNA fraction indicates that the chromatographic separation of poly(A)-containing RNA was relatively complete. If it is assumed that adenosine residues within RNA are randomly labeled after 18 h, about 15% of these residues in mRNA are within the poly(A) segment. Thus this segment constitutes around 4% of the total length of the mRNA.

The binding of actinomycin D with DNA has been used to estimate the amount of DNA within nuclei available for the template activity [8, 37]. Therefore, we

Table 4. *Binding of ^{3}H-actinomycin D with nuclei of malignant and 'differentiated' neuroblastoma cells*

Status of cells	Bound cpm/mg DNA
Malignant	7 200±460[a]
'Differentiated'	7 930±580
Calf thymus DNA	84 000

[a] S.E.M.
4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidinone (R020-1724), a specific inhibitor of cAMP phosphodiesterase, was used to induce differentiation.

have compared the binding of ^{3}H-actinomycin D with the nuclei from malignant and cAMP-induced 'differentiated' neuroblastoma cells. The binding of ^{3}H-actinomycin D with DNA was similar in malignant and in 'differentiated' cells (table 4).

DISCUSSION

The present study shows that the 'differentiated' cells induced by cAMP have the highest increase in poly(A)-containing cytoplasmic RNA. This is consistent with the previous studies in which activities of many enzymes increase in 'differentiated' neuroblastoma cells [5, 12, 13, 20, 21, 26, 38]. There is evidence that enzyme induction in eukaryotic cells is preceded by poly(A) synthesis [29]. X-irradiated-induced 'differentiated' cells also show an increase in poly(A)-containing cytoplasmic RNA, but to a lesser degree. It has been shown that X-irradiation causes the expression of some of the differentiated functions which are induced by cAMP. For example, X-irradiation induces neurites [15], and increases the activities of acetylcholinesterase [21], choline acetyltransferase [20] and catechol-*o*-methyltransferase [23], but it does not increase the activities of tyrosine hydroxylase [38], cAMP phosphodiesterase [25] and adenylate cyclase [13]. Therefore, an increase in the amount of poly(A)-containing cytoplasmic RNA is consistent with the observations that the activities of several enzymes increase in the X-irradiated cells.

Sodium butyrate- and serum-free medium-treated cells do not show any change in the amount of poly(A)-containing cytoplasmic RNA. Sodium butyrate does not induce neurites in neuroblastoma cells [16], but increases the activities of tyrosine hydroxylase [26, 38], choline acetyltransferase [20], acetylcholinesterase [21], catechol-*o*-methyltransferase [23] and adenylate cyclase [12]. Sodium butyrate inhibits the activities of glucokinase and hexokinase in normal liver and hepatoma cells [39]. It is possible that sodium butyrate may inhibit the activities of some enzyme in neuroblastoma cells. Thus, there may be no net increase in the cellular enzyme activities in sodium butyrate-treated neuroblastoma cells in comparison to control, and therefore, there is no increase in the amount of poly(A)-containing cytoplasmic RNA.

Serum-free medium induces neurites [31] and increases acetylcholinesterase activity [7] without changing the activity of tyrosine hydroxylase [7, 38], choline acetyltransferase (Prasad, unpublished observation) or adenylate cyclase [12]. Thus, there may not be a major increase in the cellular enzyme activities in serum-free medium-treated cells; and this may be why we found no increase in the amount of poly(A)-containing cytoplasmic RNA. The lack of increase in poly(A)-containing cytoplasmic RNA in cells of confluent phase of growth is consistent with the observation that these cells do not express any of the differentiated functions which are induced by cAMP [20, 21, 38]. On the contrary, the activities of some enzymes may decrease, because the

cells undergo degenerative changes during the confluent phase of growth.

The cytoplasms of cAMP-induced 'differentiated' cells after 1 h of labeling with ^{3}H-adenosine contained three-fold more radioactivity within RNA than the cytoplasms of malignant cells. This indicates that the transport of mRNA from nucleus to cytoplasm is relatively fast in 'differentiated' cells. However, after incubating the labeled cells for 30 h in the absence of exogenous ^{3}H-adenosine, the total radioactivity in RNA per cell decreased in malignant cells, but it did not change significantly in 'differentiated' cells. This indicates that the stability of total RNA in cAMP-induced 'differentiated' cells is greater than that in malignant cells. Although the malignant cells can undergo division about every 18 h during exponential growth, the rate of proliferation declines markedly after 3 days of plating. This could only partially account for the difference in specific radioactivity of RNA in the malignant and 'differentiated' cells.

The binding of actinomycin D with DNA suggests that the proportion of guanine residues that are accessible to actinomycin D are similar in the nuclei of malignant and cAMP-induced 'differentiated' cells (8.6% and 9.4%, respectively). A previous study [8] has shown that the binding of actinomycin D to chromatin from erythroid cells does not change during maturation. Thus, while the major morphological and biochemical changes are induced by R020-1724, the quantitative amount of genetic material available for the transcription may remain relatively constant. This may be due to the fact that some genetic information is activated, while other is suppressed during differentiation. In addition to the role of cAMP in affecting gene repression, we have previously shown that cAMP can also modify protein synthesis by increasing the stability of cytoplasmic mRNA, thus elevating the translation rate of specific proteins [12, 20, 21, 25, 26]. The fact that cAMP induces many differentiated functions, some of which are independent of actinomycin D, suggests that cAMP mediates its effect by increasing both the transcription and translation of genetic information which is probably masked during malignant transformation of nerve cells [24]. The present study which shows that the concentration of poly(A)-containing RNA and the stability of total RNA increase in 'differentiated' cells supports the above hypothesis. The transcription activating role of cAMP has been observed in other cell system. It has been shown that the effect of cAMP in restoring some of the normal characteristics of rat sarcoma cells is mediated by an increased activity of RNA polymerase II leading to greater mRNA synthesis [9].

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