CHANGES IN THE PATTERN OF POLY(A)-CONTAINING RNA DURING TERMINAL DIFFERENTIATION IN NEUROBLASTOMA CELLS

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1. Introduction

One of the difficulties in analysing the control of genetic expression during the terminal differentiation of somatic cell systems is to decide whether the parameter under study relates to the acquisition of a new phenotype or to more general preparative events, such as the entrance into the post-mitotic stage. In the case of a neuronal system, such as mouse neuroblastoma cells, this situation is complicated by the fact that the developmental stages under comparison often refer to cell populations cultivated in different nutritional or physical conditions. Two stages are often analysed: round immature neuroblasts grown in suspension in the presence of serum or cells exhibiting the properties of mature neurones after being attached to culture dish in the absence of serum.

This remark particularly applies to previous observations from the literature in which the synthesis of various enzymes, or of tubulin, have been compared in the course of neuroblastoma differentiation [1–4]. It also refers to our previous work on the transcriptional activities of immature and differentiated neuroblastoma [5]. Analysis of the hydrodynamic distribution of cytoplasmic poly(A)-containing RNA species from suspension or monolayer grown cells has revealed interesting differences with the virtual disappearance in the latter kind of cells of the 16 S pulse-labelled fraction and a relative variation in the amplitudes of some other fractions. Interestingly the 15 S fraction, observable after pulse-labelling of the suspension grown cells but not after labelling of the monolayer culture, could however be detected in this latter case by hybridization to [^{3}H]poly(U) when using polysomal poly(A)-containing RNA. This was taken as evidence that morphological differentiation does not involve major alteration of the transcription program, but variations at the level of the relative stability of some cytoplasmic messengers.

A question that remained unanswered however was whether changes observed in the pattern of pulse-labelled species did in fact correspond to the morphological events accompanying terminal differentiation or to the modifications in the culture conditions which were experimentally introduced to trigger the developmental program. In particular, the relevance of the transition between the logarithmic growth of suspension cells and the postmitotic stationary phase of monolayer cultures had to be explored.

The present study attempts to answer the question. Cytoplasmic poly(A$^+$) RNA distribution has been reinvestigated in populations of neuroblastoma attached to culture dish, in a stationary phase, under conditions where no neurite extension occurs. This was achieved by changing the culture conditions in the case of the NIE 115 clone or by using a special neuroblastoma variant, clone NIA 103, which does not undergo morphological differentiation after induction by serum withdrawal [6]. Our results are consistent with the conclusion that there is a causality relationship between the changes in the distribution of poly(A)-containing RNAs previously described [5] and the formation of neurites.

2. Materials and methods

2.1. *Suspension cultures*

NIE 115, a clone which has the capacity to morphologically differentiate when placed under per-

missive conditions, and NIA 103, a variant clone kindly provided by Dr Nirenberg, which is unable to develop neurites, were both derived from mouse neuroblastoma C1300. They were grown on Greiner 'non-treated' petri dishes in Dulbecco's modified Eagle's medium containing 7.5% foetal calf serum, supplemented with 100 U/ml penicillin, 50 μg/ml streptomycin and 0.25 μg/ml fungisone in an atmosphere of 5% CO_2.

2.2. *Monolayer cultures*

2.2.1. Clone NIE 115

Confluent monolayer stage

Aggregated suspension cells were centrifuged, washed and dissociated by pipetting up and down in the same medium. The cells were then transferred to a Falcon or Greiner 'treated' tissue culture dish. The cells attached on the less hydrophobic surface of these dishes and continued to divide in the presence of serum. When the cell concentration reached approx. $4 \cdot 10^6$ per 10 cm diameter dish, the cells attained a stationary phase without extending neurites.

Differentiated monolayer cultures

Aggregated suspension cells were centrifuged, washed and dissociated by pipetting up and down in the same medium without serum. The cells were then transferred to a culture dish to which they readily attached. After two days in this serum free medium long neurites, several times the cell diameter in length, could be observed.

2.2.2. Clone NIA 103

The culture conditions achieved were those leading to the differentiation of clone NIE 115 when maintained in monolayers without serum. NIA 103 cells were kept two days under these conditions; they remained attached without dividing, and did not form neurites.

2.3. *Labelling and extraction of total cytoplasmic RNA*

Suspension cultures were sampled at the end of the logarithmic growth phase and resuspended in 3 ml medium (in 10 cm diameter dish) prior to labelling. Monolayer cultures were labelled in 3 ml fresh medium, two days after the beginning of the stationary phase.

Labelling was for 6 h, with 20 μCi [^{3}H]uridine (spec. act. 28 Ci/mmol). In some double labelling experiments, clone NIE 115 was labelled with 20 μCi of [^{3}H]uridine, and clone NIA 103 with 4 μCi of [^{14}C]uridine (spec. act. 53.9 mCi/mmol).

After the incubation, the suspension cells were collected by centrifugation and rinsed in a saline solution. Cells grown on a solid substrate were rinsed and scraped with a rubber policeman. Both suspension and monolayer cells were harvested in a buffer (1 ml/dish) containing 0.14 M NaCl, 10 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$ and 250 μg/ml heparin. Cells were then lysed for 2 min by addition of 0.1% Nonidet NP40. The nuclei were rapidly removed by centrifugation at 12 000 × *g* and the submitochondrial supernatant was taken for RNA extraction. Cytoplasmic RNA was deproteinized at least three times with equal vol. chloroform/phenol (1/1), 1% isoamyl alcohol, 0.2% dodecyl sulfate and 0.1 M EDTA. RNA was precipitated overnight with 2 vol. ethanol at –30°C.

2.4. *Analysis of RNA*

Cytoplasmic RNA was analysed by sucrose gradient sedimentation as described [5], the amount of poly-(A)-containing RNA present in each fraction being determined by hybridization to poly(U)–glass fiber filters [7].

3. Results

Cultures of neuroblastoma cells, either grown in suspension or maintained in monolayers for two days, were submitted to 6 h labelling with [^{3}H]- or [^{14}C]-uridine (see Materials and methods). After appropriate washings, cells were lysed in a buffer containing heparin and NP40, and total RNA extracted from the submitochondrial supernatant. RNA preparations were centrifuged on sucrose gradients and the distribution of pulse-labelled poly(A)-containing RNA was determined.

Figure 1 illustrates the distribution of poly(A)-containing RNA, after pulse-labelling for 6 h, in suspension cultures from NIE 115 (a) and NIA 103 (b) at the end of logarithmic growth. The population of poly(A)-containing RNA is highly heterogeneous in

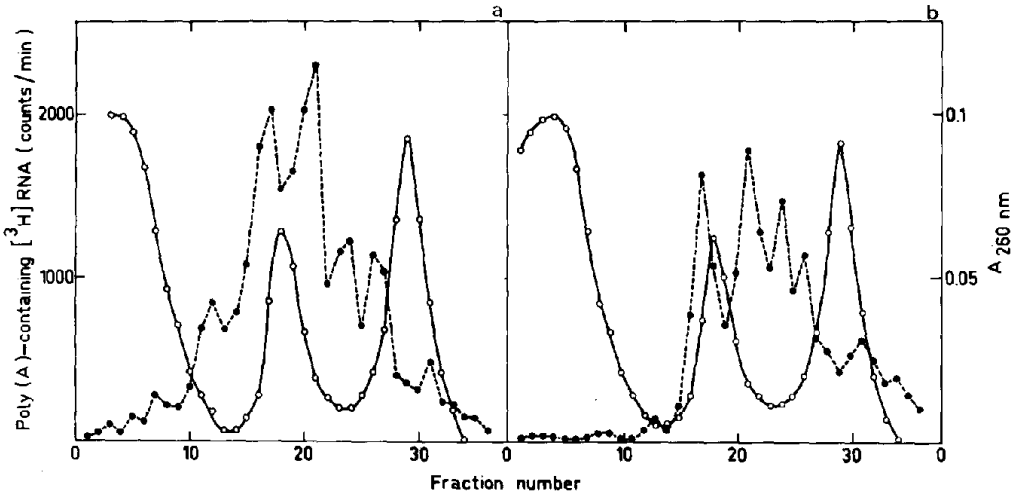

Fig.1. Poly(A)-containing RNA from suspension neuroblastoma cultures of a NIA 103 clone (a) and a NIE 115 clone (b). Cells were labelled with 20 μCi [^{3}H]uridine for 6 h. Total cytoplasmic RNA was extracted and sedimented on a 5–20% sucrose gradient. An aliquot of each fraction was filtered on a poly(U)-containing glass-fiber filter, dried and counted. (•- - -•) Poly(A)-containing [^{3}H]RNA (in 10^6 cells); (o——o) absorption profile of the total RNA (in 10^6 cells).

both cases and displays approximately the same hydrodynamic characteristics comprising material that sediments between 16 S and 30 S with 5 prominent peaks at 16 S, 21 S, 24 S, 26 S, and 30 S, confirming our earlier published results [5]. One can observe a slight difference in the profile corresponding to the NIA 103 cells with the presence of a somewhat pronounced shoulder in the 13 S region.

Thus two independent clones of mouse neuroblastoma exhibit the same distribution profile in their messenger populations when pulse-labelling takes place during exponential growth.

Figure 2 illustrates the profile of poly(A)-containing RNA extracted from monolayer NIE 115 cells in a stationary phase, under conditions where neurite outgrowth is prevented by the continued presence of serum. One can observe the same characteristics as those shown in the profile corresponding to the suspension grown cells. In particular, the 16 S component remains detectable and no appreciable change is observed in the amplitude of the 21 S, 26 S and 30 S fractions.

As shown in fig. 3, the profile corresponding to monolayer cultures of clone NIA 103 reaching the stationary phase in the absence of serum is similar

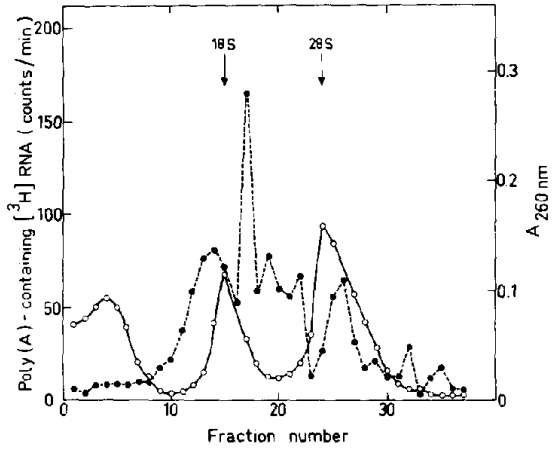

Fig.2. Poly(A)-containing RNA from monolayer NIE 115 neuroblastoma cultures. The cells attached on the dish surface were kept in a stationary phase in the presence of serum. There was no outgrowth of neurites. All technical conditions were the same as described for fig.1. (•- - -•) poly(A)-containing [^{3}H]RNA (in 10^6 cells); (o——o) absorption profile of the total RNA (in 10^6 cells).

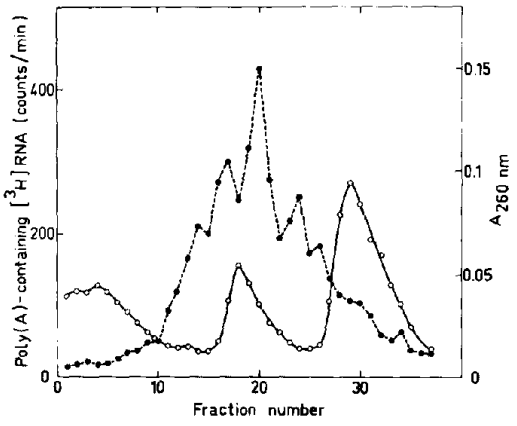

Fig.3. Poly(A)-containing RNA from monolayer NIA 103 neuroblastoma cultures. Monolayer cultures were kept for two days in the absence of serum. These cells cannot extend neurites. (•- - -•) poly(A)-containing [^{3}H]RNA (in 10^6 cells); (o——o) absorption profile of the total RNA in 10^6 cells).

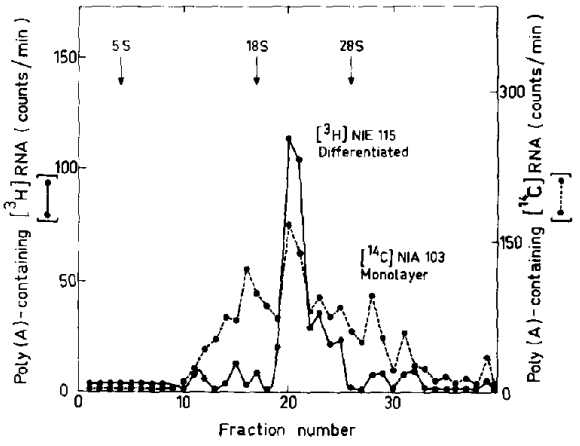

Fig.4. Poly(A)-containing RNA from monolayer NIE 115 and NIA 103 cultures. Cell differentiation was induced by withdrawal of serum from the medium of the NIE 115 cultures. The cells were then pulse-labelled for 6 h with 20 μCi [^{3}H]-uridine. NIA 103 cultures were kept during the same time in the absence of serum, then pulse-labelled for 6 h with 4 μCi of [^{14}C]uridine. Both types of cultures were harvested and pooled for RNA extraction and analysis. (•——•) poly(A)-containing [^{3}H]RNA from NIE 115 cells (in 10^6 cells). (•- - -•) poly(A)-containing [^{14}C]RNA from NIA 103 cells (in 10^6 cells).

to the profile corresponding to the suspension cultures with approximatively the same hydrodynamic characteristics.

Figure 4 illustrates an experiment in which monolayer cultures of NIE 115 and NIA 103, both in the absence of serum, were labelled by a different tracer, mixed and the cells processed for RNA extraction. NIE 115 cells were cultivated in a monolayer form for two days in the absence of serum; neurite outgrowth was evident. The cells were subsequently pulse-labelled with [^{3}H]uridine for 6 h. The NIA 103 cells were cultivated under the same conditions (i.e., attached to the plates, in a stationary phase of growth without serum). No formation of neurite could be detected. These cells were pulse-labelled for 6 h in the presence of [^{14}C]uridine. NIE 115 and NIA 103 cells were then harvested and pooled for RNA extraction and analysis.

The profile corresponding to the poly(A)-containing RNA from 'differentiated' NIE 115 cells resembles those we have described [5]. If one compares figs 2 and 4 the main change appears to be the virtual disappearance of the 16 S component and a relative increase in the 21–22 S peaks. This contrasts with the ^{14}C profile of clone NIA 103 whose characteristics are the same as those observed with suspension grown cells (i.e. presence of a 16 S poly(A)-containing fraction) (Compare figs 3 and 4).

4. Discussion

In a previous paper [5], we have shown that mouse neuroblastoma grown in suspension or in monolayer, conditions where they respectively appear like immature round neuroblasts or neurites bearing cells, exhibit specific differences in their profiles of cytoplasmic poly(A)-containing RNAs. After a relatively short labelling with tritiated uridine, monolayer-grown neuroblastoma were lacking in their distribution profile a rapidly renewable 16 S RNA fraction which was prominent in suspension grown cells. Several differences albeit less drastic could be observed in the relative amplitudes of other subfractions.

The present paper confirms this observation and attempts to specify whether these changes in the pattern of poly(A)-containing RNAs are actually related to terminal differentiation proper or to alterations in growth conditions. The following question has been examined:

Is the alteration in the poly(A)-containing RNA profiles related:

(i) To the formation of neurites?
(ii) To the phase of growth, i.e., the change from logarithmic to the stationary phase?
(iii) To some kind of surface modifications due to the cell attachment on a solid support?

To determine whether the phase of culture growth was critical, two cell models were used:

(i) NIA 103, a clone which is unable to extend neurites under any type of culture conditions assayed [6], but which can be analysed either in the logarithmic or in the stationary phase.
(ii) NIE 115, a clone which is able to differentiate in the absence of serum but in which neurite outgrowth was prevented when the cells reached stationary phase in monolayer culture in the presence of serum.

In none of these monolayer populations, entrance into the stationary phase, either in the absence (NIA 103) or in the presence of serum (NIE 115), did cause any detectable change in the pulse-labelled poly(A)-containing RNA profile with reference to the logarithmic suspension grown cells.

Attachment to a solid support did not appear either as an essential factor in promoting the modification of the poly(A)-containing RNA distribution profile:

(1) When clone NIA 103 was grown in monolayer under conditions where it could attach to a culture dish, no change in the poly(A) RNA pattern was noticeable even in the absence of serum. In particular no relative disappearance of the 16 S peak nor any alteration of other molecular species could be observed.
(2) When the NIE 115 clone was cultivated attached to the substrate in a confluent state, but in the presence of serum (no formation of neurites) a messenger profile typical of logarithmic grown cells was observed.

Since the alteration of the poly(A)-containing RNA profile is related neither to the phase of growth nor to the state of attachment on a solid surface, we conclude that the modifications observed must be directly related to the formation of neurites and thus constitute an interesting marker of neuroblastoma differentiation.

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