COMPLEXITY OF POLYSOMAL POLY(A) RNA IN DIFFERENT DEVELOPMENTAL STAGES OF A NON-DIFFERENTIATING NEUROBLASTOMA CLONE

Francis BERTHELOT, François GROS and Bernard CROIZAT
Laboratoire de Biochimie Cellulaire, Collège de France, 11, Place Marcelin Berthelot, 75231 Paris Cedex 05, France

Received 1 October 1980
Revised version received 27 October 1980

1. Introduction

In [1], using RNA–DNA molecular hybridizations according to [2–4], we analyzed the sequence complexity and the frequency distribution of the polysomal poly(A) RNA from N1E115 neuroblastoma cells at two developmental stages: either as round immature neuroblasts grown in suspension in the presence of serum, or as neurite-bearing cells attached to a culture dish, in the absence of serum. Both mRNA populations exhibited a total complexity corresponding to ~7200 average-sized sequences of 1750 nucleotides distributed in 3 abundance classes. These data agree well with those in [5]. All the sequences from differentiated cells were present in the polysomes of undifferentiated cells. Conversely, the mRNA from differentiated cells failed to hybridize with ~15% of the cDNA pertaining from cell suspensions. Our interpretation implied that morphological differentiation was accompanied by the disappearance of a small number of messengers corresponding to the intermediate frequency class in the suspension cells. This loss of sequences may be related to the expression of neuronal morphogenesis. However, the possibility that these changes reflected, at least partly, the disappearance of mRNA species involved in cell replication was not ruled out by studies on other systems [4,6].

Here, we analyze the sequence complexities and frequency distributions of polysomal poly(A) RNA from a neuroblastoma variant, clone N1A103, which is unable to undergo neuronal morphogenesis, as expressed by the extension of neurites, under any type of culture conditions assayed. To compare both studies, we used the same technical procedure as for the analysis of N1E115 complexities. Furthermore, N1A103 cells have been maintained in the same conditions as N1E115 cells, i.e., cells growing as a suspension in the presence of serum (S cells) and not dividing cells attached on the substrate in the absence of serum (A cells).

2. Materials and methods

2.1. *Cell culture*

The clone N1A103 derived from mouse neuroblastoma C1300 was obtained from Dr M Nirenberg (NIH, Bethesda, MD). The conditions of culture have been described [7]. The cells were cultured either as round immature neuroblasts grown in suspension in the presence of serum or attached to a culture dish in the absence of serum. In the latter conditions, they do not divide and do not form neurites.

2.2. *Extraction of polysomal RNA and preparation of polysomal poly(A) RNA*

The whole procedure was performed as in [1] except that diethyl pyrocarbonate was omitted during the RNA extraction by CsCl buoyant density centrifugation. CsCl (Merck) was sterilized before use. All poly(A) RNA titrations were performed using a batch of poly([^{3}H]U) synthesized according to [8].

2.3. *cDNA synthesis*

Avian myeloblastosis virus reverse transcriptase was used to copy polysomal poly(A) RNA from both suspension and monolayer cultures. The reaction mixture (0.25 ml) contained: 40 mM Tris–HCl (pH 8 at 37°C), 100 mM KCl, 5 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM EDTA, 0.64 mM dATP, dGTP, dTTP (Calbiochem), 0.08 mM d[^{3}H]CTP (7450 cpm/pmol) (Amersham), 200 μg/ml actinomycin D, 5.6 μg/ml

oligo(dT)$_{12-18}$ (Boehringer), 30 μg/ml poly(A) RNA and 600 units/ml reverse transcriptase. After 15 min incubation at 45°C, the reaction was stopped by the addition of 0.45 ml H_2O, 0.2 ml 10 × standard saline citrate buffer (standard buffer: 0.15 M NaCl and 0.015 M sodium citrate, pH 7), 0.05 ml 0.5 M EDTA and 0.05 ml 10% sodium dodecylsulphate. The cDNA was extracted and purified by Sephadex G-50 chromatography as in [1]. Its specific activity was ~5.6 × 10^6 cpm/μg for a total amount of 2.1 μg (S cells) and 2.5 μg (A cells).

3. Results

3.1. *Characterization of polysomal poly(A) RNA*

Polysomal poly(A) RNA extracted from polysomes sedimenting faster than 100 S and separated from non-poly(A) RNA by two passages on oligo(dT)-cellulose column, was analyzed on a sucrose–formamide gradient as in [1]. For both S cells and A cells, the average length was taken as 1750 nucleotides. The average size of the poly(A) tract was estimated to represent 10% of poly(A) RNA.

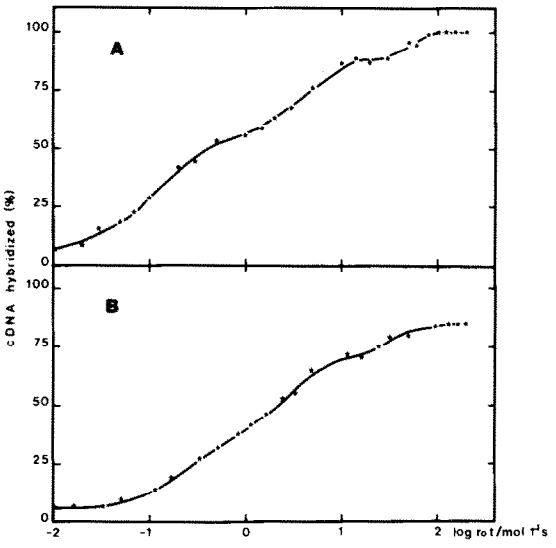

Fig.1. Homologous hybridization kinetics cDNA from S cells (A) and A cells (B) was hybridized with its poly(A) RNA template. For r_0t values up to 0.5 mol . l^{-1} s, the hybridization reaction contained 62.5 μg RNA/ml; for r_0t values of 0.5–15 mol . l^{-1} s, 250 μg RNA/ml; and for greater r_0t values, 750 μg RNA/ml. The extent of hybridization was determined by S_1 nuclease digestion. Each point was corrected for 1.2% background of S_1 nuclease-resistant cDNA (self-annealed fraction), obtained by digestion of cDNA after incubation with *E. coli* ribosomal RNA. r_0t is the product of [RNA] (mol nucleotide/l) and time (s). The difference between the hybridization levels, due to variations in the efficiency of the cDNAs, was corrected as usual by normalisation to 100%.

3.2. *Polysomal poly(A) RNA sequence complexity and comparative distribution in abundance classes*

The conditions for RNA–DNA hybridization and detection of the hybrids were those in [9]. The RNA/DNA ratio ranged from 70–800, with cDNA at 0.9 μg/ml. The kinetic curves of the homologous hybridization between poly(A) RNA from S cells and A cells and their corresponding cDNA (fig.1A,B) show that complete hybridization is obtained at a r_0t value of 100 mol . l^{-1} s in both cases. The curves extend over several r_0t decades, thus indicating that RNA populations are distributed in distinct abundance classes. Numerical analysis of the curves reveals that we are dealing with 3 classes. The $r_0t_{1/2}$ value, the sequence complexity and the number of average sized RNA copies for each abundance class are shown in table 1 for S cells and A cells. Globin mRNA was used as a complexity standard. The total complexity appears to be very similar in both cases, and corresponds to ~6300 sequences, assuming an average RNA size of 1750 nucleotides. However, the frequency distribution within the 3 classes is not identical. The largest discrepancy concerns the abundant classes, which correspond to 44% and 29% of the hybridizable cDNA for S cells and A cells, respectively. The corresponding numbers of copies/cell are 1486 and 407. These differences appear clearly on the heterologous curves.

Fig.2A represents the heterologous hybridization reaction between cDNA from S cells and poly(A) RNA from A cells and, for comparison, the homologous kinetics from the cDNA participating in cross hybridization (solid line). The reciprocal cross hybridization is shown on fig.2B. It can be seen that:

(i) The heterologous RNAs are able to hybridize to cDNAs with the same efficiency as the homologous RNA, thus indicating that both RNA populations comprise the same sequences;

(ii) The homologous and heterologous kinetics are different. This confirms that the RNA sequences are not at the same concentrations in the two populations. The heterologous kinetics between the cDNA of S cells and the RNA of A cells

Table 1
Numerical analysis of homologous hybridization kinetics

Class of abundance	Fraction of hybridizable cDNA (α)	$r_0t_{1/2}$ (mol . l^{-1} s)		Base sequence		Copies/ cell	mRNA molecules/ cell
		Observed	Corrected	Complexity in nucleotides (*NT*)	Different mRNA species		
S cells							
Abundant	0.44	0.089	0.037	6.73×10^4	38	1486	56 468
Intermediate	0.35	2.11	0.712	1.29×10^6	737	62	45 694
Rare	0.21	26.2	5.29	9.64×10^6	5510	5	27 550
Total	1.00				6285		129 712
A cells							
Abundant	0.29	0.165	0.089	1.62×10^5	92	407	37 444
Intermediate	0.40	1.57	0.597	1.09×10^6	621	84	52 164
Rare	0.31	18.14	5.34	9.71×10^6	5551	7.2	39 967
Total	1.00				6264		129 575

The hybridization curve has been analyzed as in [10]; α represents the fraction of hybridizable cDNA reacting in each abundance class. $r_0t_{1/2}$ values are expressed in mol . l^{-1} s. Corrected $r_0t_{1/2}$ is given by $r_0t_{1/2} \times \alpha$. The sequence complexity of each abundance class (*NT*) is calculated according to the formula: $NT = 1200 \times$ (corrected $r_0t_{1/2}$)/(6.6×10^{-4}), where 6.6×10^{-4} corresponds to the $r_0t_{1/2}$ of the hybridization reaction between $\alpha + \beta$ globin mRNA (sequence complexity = 1200 nucleotides) and its total cDNA. The number of messenger RNA species is calculated considering an average size of 1750 nucleotides. The number of copies per cell is given by: [poly(A) RNA/cell (g)]$\alpha.6 \times 10^{23}/330\,NT$, the amount of poly(A) RNA being estimated at 1.25×10^{-13} g/cell

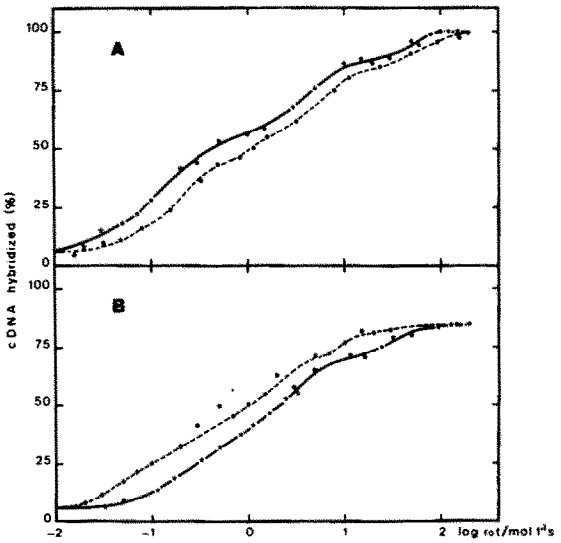

Fig.2. Heterologous hybridization kinetics. (A) cDNA from S cells was hybridized with poly(A) RNA from A cells (•——•). (B) cDNA from A cells was hybridized with poly(A) RNA from S cells (•——•). In both cases, the homologous kinetics corresponding to the cDNA was plotted for comparison (solid line). Experimental procedure was as in fig.1.

starts to diverge from the homologous kinetics at low r_0t values. A proportion of the abundant class cDNA hybridizes at higher r_0t values with monolayer RNA, thus showing that these RNAs are rarer in monolayer cultures. The abundant class in S cells represents 44% of total complexity as compared to 29% for A cells. The divergence decreases at the level of the intermediate class and finally both kinetics reach the same plateau.

The situation is confirmed by the reciprocal cross hybridization kinetics. Conversely, at the beginning of the kinetics, the heterologous hybridization reaches the same percentages as the homologous hybridization at lower r_0t values, thus meaning that the sequences are more frequent in the mRNA population of S cells.

4. Discussion

Transition of cellular states in the N1A103 clone does not imply detectable variations in the number of mRNA species. However, the molecular species

are not equally distributed within the two mRNA populations as shown by:

(i) The numerical analysis of homologous hybridization kinetics;

(ii) The examination of heterologous hybridization curves.

This situation is different from that observed in the N1E115 clone where ~150 sequences, belonging to the intermediate class, disappear in the differentiated state. Both N1A103 and N1E115 cells were maintained in strictly identical culture conditions. They adhere to the substrate and stop dividing when transfered to a falcon culture dish in the absence of serum. However, only N1E115 cells extend neurites. It is thus probable that the loss of sequences in differentiated N1E115 cells corresponds to neither cell adhesion nor to the arrest of replication since these phenomena occur in N1A103 cultures without implying any detectable loss of sequences. It may therefore be correlated with the formation of neurites in the N1E115 cells.

The quantitative modulation of mRNA species observed during the transition of N1A103 cultures might reflect a significant decrease in the concentration of species involved in cell replication. The species, largely represented in S cells, would be decreased in A cells in response to metabolic alterations linked to the stationary phase. This interpretation is not favoured by the analysis of the N1E115 complexities where no such detectable modulations have been observed. However, N1E115 and N1A103 clones are not identical systems; differences have actually been shown concerning membrane excitability and the synthesis of neurotransmitters [11]. Thus it is possible that the change of cellular state in N1A103 cells involves larger metabolic alterations implying detectable changes, within the limits of the technical sensitivity, in the number of sequences.

The major result reported here is that no sequences disappear in N1A103 A cells where no morphogenesis occurs. One is thus tempted to assume that the loss of sequences, found in N1E115, is related to neuronal morphogenesis. Since only polysomal sequences have been analysed it is impossible to ascertain whether these mRNA changes are directly related to transcriptional events. It is possible, however, that the neuroblastoma constitute a convenient model for studying the 'switch off' of genes in the terminal phase of differentiation. A major advantage of the neuroblastoma system is that no abundant marker masks the evolution of minor products within messengers or proteins.

References

[1] Felsani, A., Berthelot, F., Gros, F. and Croizat, B. (1978) Eur. J. Biochem. 92, 569–577.

[2] Bishop, J. O., Morton, J. G., Rosbash, M. and Richardson, M. (1974) Nature 250, 199–204.

[3] Birnie, G. D., MacPhail, E., Young, B. D., Getz, M. J. and Paul, J. (1974) Cell Diff. 3, 221–232.

[4] Getz, M. J., Elder, P. K., Benz, E. W. jr, Stephens, R. E. and Moses, H. L. (1976) Cell 7, 255–265.

[5] Grouse, L. D., Schrier, B. K., Letendre, C. H., Zubairi, M. Y. and Nelson, P. G. (1980) J. Biol. Chem. 9, 3871–3877.

[6] William, J. G. and Penman, S. (1975) Cell 6, 197–206.

[7] Croizat, B., Berthelot, F., Felsani, A. and Gros, F. (1977) Eur. J. Biochem. 74, 405–412.

[8] Bishop, J. O., Rosbash, M. and Evans, D. (1974) J. Mol. Biol. 85, 76–86.

[9] Affara, N. A., Jacquet, M., Jakob, H., Jacob, F. and Gros, F. (1977) Cell 12, 509–520.

[10] Jacquet, M., Affara, N..A., Robert, B., Jakob, H., Jacob, F. and Gros, F. (1978) Biochemistry 17, 69–79.

[11] Amano, T., Richelson, E. and Nirenberg, M. (1972) Proc. Natl. Acad. Sci. USA 69, 258–263.