Biochimica et Biophysica Acta 1799 (2010) 630–641

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagrm

Massive translational repression of gene expression during mouse erythroid differentiation

Bérengère Pradet-Balade [a,1], Cornelia Leberbauer [b], Norbert Schweifer [c], Florence Boulmé [b,*]

[a] Department of Immunology and Oncology, Centro Nacional de Biotecnologia CNB-CSIC, Campus de Cantoblanco, UAM, 28049 Madrid, Spain
[b] Max F. Perutz Laboratories, University Departments at the Vienna Biocenter, Department of Medical Biochemistry, Division of Molecular Biology, Medical University of Vienna, Dr. Bohr-Gasse 9, 1030 Vienna, Austria
[c] Boehringer Ingelheim Austria RCV GmbH Co & KG, Dr. Boehringer Gasse 5-11, 1121 Vienna, Austria

ARTICLE INFO

Article history:
Received 19 March 2010
Received in revised form 6 August 2010
Accepted 18 August 2010
Available online 8 September 2010

Keywords:
Mouse erythroid differentiation
Translation regulation
Erythropoiesis
Microarray

ABSTRACT

We took advantage of a mouse erythroid differentiation system to determine the relative contribution of transcriptional and translational control during this process. Comparison of expression data obtained with total cytoplasmic mRNAs or polysome-bound mRNAs (actively translated mRNAs) on Affymetrix high-density oligonucleotide microarrays revealed different characteristics of the two regulatory mechanisms. Indeed, mRNA expression from a vast majority of genes was affected, albeit most changes were relatively small and occurred at a low pace. Translational control, however, affected a smaller fraction of genes but was effective at earlier time-points. This analysis unravels six clusters of genes showing no significant variation in mRNA expression levels whereas they are submitted to translational regulation. Their involvement in terminal mouse erythropoiesis may prove to be highly relevant. Furthermore, the data from specific and functional categories of genes emphasize that translational control, not only reinforces the transcriptional effect, but allows the cell to increase the complexity in gene expression regulation patterns.

1. Introduction

A murine erythropoiesis system was set up, under serum-free culture conditions. In this system, proliferation of progenitors as well as differentiation into enucleated erythrocytes was achieved [1,2]. Gene profiling using cDNA macroarrays led to the identification of new candidates, subsequently shown to be implicated in erythroblast differentiation. However this study was restricted to mRNA expression profiling only.

Despite variations in mRNA expression levels, others mechanisms may be involved to explain variations in protein amounts. Many publications demonstrated that it is not possible to extrapolate protein expression levels from corresponding mRNA transcript abundance [3–5]. Tian et al. [6] have shown that differential expression of mRNA accounts for only up to 40% of the variation in protein abundance. Indeed, the importance of translational control has been demonstrated in many different biological processes: T cell activation [5], stress and apoptosis [7], hypoxia [8,9], cell cycle [10,11], erythropoiesis [12] and oncogene transformation [13–15].

Translation involves initiation, elongation and termination of the polypeptidic chain, the first one being considered the rate-limiting step of the process [16]. As a consequence, ribosome loading of a given mRNA species is a robust indicator of its translational status. Separation of transcripts devoid of ribosome from those engaged in polysomes allows the characterization of translationally controlled genes [5,17,18]. To characterize more precisely the mechanisms involved in protein synthesis during erythropoiesis, we simultaneously assayed variations in total mRNA expression levels, as well as in their translational status. Affymetrix high-density oligonucleotide microarrays were hybridised with labelled cRNAs generated from total and polysome-bound RNAs, at different stages of erythroblast differentiation. This enabled us to assess simultaneously the variations in mRNA expression and translation efficiency. Taken together, our data show that regulations of either mRNA expression or translation efficiency differ by kinetics, amplitude of response and the categories of genes affected. As a result, the combination of both regulatory mechanisms would create a higher level of complexity. Furthermore, our data identified genes translationally regulated, which may play a key role in terminal murine erythropoiesis.

* Corresponding author. Present address: Austrian Federal Ministry of Science and Research, Rosengasse 4, 1014 Vienna, Austria. Tel.: +43 1 53120 6723; fax: +43 1 53120 81 6715.
E-mail addresses: berengere.pradet-balade@igmm.cnrs.fr (B. Pradet-Balade), florence.boulme@bmwf.gv.at (F. Boulmé).
[1] Present address: Institut de Génétique Moléculaire de Montpellier, CNRS UMR-5535, 1919 route de Mende, 34293 Montpellier Cedex 5, France.

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doi:10.1016/j.bbagrm.2010.08.006

2. Materials and methods

2.1. Cells and culture conditions

The mouse erythroblast cell line (clone I/11) and the culture conditions for proliferation and differentiation into erythrocytes were previously described [1]. Characteristic variations in cell morphology and haemoglobin content during differentiation were monitored by histological staining and photometric determination, respectively, as described [1].

2.2. RNA isolation, sucrose gradient fractionation, polyA$^+$ purification and Northern blotting

Cytoplasmic RNA extraction, separation of ribosome-free and polysome-bound RNAs on linear sucrose gradients, as well as polyA$^+$ purification from proliferating and differentiating erythroblasts were performed as previously described [5]. The integrity of the RNAs was determined by Northern blotting [5].

2.3. Double-strand cDNA synthesis, cRNA labelling and microarrays hybridization

RNA samples to be used for microarray experiments were spiked using the controls provided by Affymetrix (from either *Escherichia coli*, *Bacillus subtilis* and P1 bacteriophage genes). Polysome-bound RNA (as obtained following sucrose gradient fractionation) to be used for cRNA labelling was cleaned with the RNeasy kit (Qiagen, Valencia, CA.). All the following steps for cRNA generation were performed following rigorously Affymetrix instructions. Total cytoplasmic or polysome-bound RNAs were reverse transcribed into double-stranded cDNAs using the Superscript Choice system *II* (Life Technologics, Rockville, Md.) with a $(dT)_{24}$-T7 promoter primer (5′GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG$[dT]_{24}$) as described by the manufacturer. cDNAs were then transcribed into cRNAs labelled with biotinylated CTP and UTP, using the Bioarray RNA transcript labelling kit (Enzo, Farmingdale, N.Y.). Following fragmentation, 10 μg of cRNAs were hybridised to mouse Mu6500 arrays (Affymetrix, Santa Clara, CA.) overnight at 45 °C. Washes and signal amplification were performed on an Affymetrix GeneChip Fluidics Station 400, according to the manufacturer's instructions.

2.4. Calculation of the translational regulation efficiency

The efficiency with which a given mRNA is translated is characterized by the ratio between the signals corresponding to its polysome-bound fractions and its total RNA level (signal for polysome-bound RNAt/signal for total RNAt) [5,19]. Accordingly, alterations in the translation efficiency of a gene between t_0 (0 h) and a time t was calculated by the following ratio:

$$[(\text{signal for polysome} - \text{bound RNAt}) \times (\text{signal for total RNAt}_0)] \div [(\text{signal for polysome} - \text{bound RNAt}_0) \times (\text{signal for total RNAt})].$$

A gene was considered to be translationally regulated when this ratio changed by a factor of at least two (either inferior to ½ or superior to 2).

3. Results

3.1. Massive diminution of polysome-bound polyA$^+$ RNAs during mouse erythropoiesis

Murine immature erythroblasts were first induced to self-renew in serum-free medium (proliferation state or 0 h) and subsequently induced to differentiate for up to 48 h by addition of appropriate factors [1,20,21]. At 48 h, most of the genes involved in the differentiation process were still active; at longer differentiation times (i.e. 72 h), most of the erythrocytes were already enucleated [1]. As a consequence, samples were processed after 0, 6, 20 or 48 h of differentiation for either total cytoplasmic RNA isolation or sucrose gradient fractionation to separate the RNAs bound to ribosomes from the ribosome-free. Indeed, only the mRNAs bound to ribosomes, (i.e. the polysomes) are actively translated, while the ribosome-free mRNAs are not being translated. PolyA$^+$ RNAs were purified from each population and its quality, including the absence of rRNA contamination, was monitored by Northern blotting. Since there is a large change in cell volume during erythroid differentiation (shrinkage), polyA$^+$ content was normalised to cell volume (Fig. 1A). A small decrease (15%) in total polyA$^+$ RNA density was detected during the first 48 h of the differentiation process. Concomitantly, there was an even stronger decrease in the concentration of polysome-bound polyA$^+$ RNAs (60% of the initial quantity) which could not result solely from the diminution in polyA$^+$ RNAs. This unravels that a strong translational shut-down accompanied the well-characterized repression of mRNA expression, during erythroblast differentiation.

3.2. Reproducibility assays

To demonstrate the robustness of the biological system, we defined a control group of 48 genes plus 15 ESTs, amounting to 63 transcriptional units. We verified the reproducibility of the expression level of those controls, which had been previously described in the same cellular system [1]. Indeed, for all of those 63 elements, we obtained very similar data to that previously published. In addition, reproducibility of the profiling assays was addressed using total cytoplasmic RNAs of the proliferating cells (0 h). This allowed us to estimate the confidence interval associated to each factor of variation, as described [22]. Hence, for a regulation factor of two, the confidence level was 96.9% and for a factor of three, 98.9% (supplementary Fig. 1), so that any change of a factor superior to two very likely reflected variations in transcript levels.

3.3. Quantification of transcriptionally and translationally regulated genes

Among the 6346 genes analysed, 4607 (72%) were expressed at least once during the differentiation kinetics, constituting the global working set of genes. The processed data for the signal obtained using the total cytoplasmic RNAs reflect the mRNA expression levels (as a result from transcription, export and degradation of the mRNA), while the data obtained using polysome-bound mRNAs estimate the molecules engaged in translation. Variations in the amount of translated RNAs, for a given gene, might result from variations in either (i) the mRNA expression level, (ii) its translation efficiency, (iii) or even both. Translational control was thus assessed by determining changes in the ratio polysome-bound RNA/total cytoplasmic RNA, between the successive time-points (see Section 2.4). Three different categories of regulated genes were distinguished: genes showing alterations in (i) only their mRNA level (mRNA expression), (ii) only their translational status (translation), (iii) simultaneously their mRNA expression and translational status (both). The number of genes relevant to each category is presented according to the factor of regulation (Fig. 1B). 51% of the 4607 expressed genes were controlled with a factor superior or equal to two, largely because of variations in mRNA expression level (around 65% of the regulated genes). This proportion drops to 27% of genes regulated with a factor of at least three. Whereas genes regulated simultaneously at the level of transcription and translation (both) represent only 18% of all the regulated genes, this proportion reaches nearly 51% of the 10-fold regulated genes. Indeed, strongly regulated genes were less likely controlled only at the expression level (from 65% of regulated genes by a factor of two to 42% considering a 10-fold