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Novel DNA Microarray System for Analysis of Nascent mRNAs

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Abstract

Transcriptional activation and repression are a key step in the regulation of all cellular activities. The development of comprehensive analysis methods such as DNA microarray has advanced our understanding of the correlation between the regulation of transcription and that of cellular mechanisms. However, DNA microarray analysis based on steady-state mRNA (total mRNA) does not always correspond to transcriptional activation or repression. To comprehend these transcriptional regulations, the detection of nascent RNAs is more informative. Although the nuclear run-on assay can detect nascent RNAs, it has not been fully applied to DNA microarray analysis. In this study, we have developed a highly efficient method for isolating bromouridine-labeled nascent RNAs that can be successfully applied to DNA microarray analysis. This method can linearly amplify small amounts of mRNAs with little bias. Furthermore, we have applied this method to DNA microarray analysis from mouse G_2-arrested cells and have identified several genes that exhibit novel expression profiles. This method will provide important information in the field of transcriptome analysis of various cellular processes.

1. Introduction

DNA microarray is an important tool for understanding regulatory networks. In fact, this technique was used to analyze changes in the amounts of mRNA in cellular phenomena such as cell differentiation, cellular senescence, and cell cycle progression in a comprehensive manner. Moreover, we have successfully improved the sensitivity and reproducibility of DNA microarray analysis.[1] Although many of the expression profile data obtained through DNA microarray analysis are available at various websites, such data are based on steady-state mRNA levels. It is obvious that both mRNA synthesis and degradation influence steady-state mRNA levels, but usually only the total mRNA is quantified. If we can quantify nascent mRNA in a real-time manner, it will become possible to estimate mRNA synthesis and degradation rates by comparing the nascent amount with the total amount of RNA measured by an ordinary DNA microarray system. One method of detecting nascent mRNA is the nuclear run-on assay.[2] Recently, the transcriptional profiling of radio-labeled RNAs using the nylon-membrane DNA microarray was reported.[3–5] The current standard platforms are GeneChip by Affymetrix, the Stanford-type DNA microarray, and the oligo-DNA microarray. In general, it is very difficult to compare data from such standard DNA microarray systems with data obtained by the nylon membrane DNA microarray. To comprehensively analyze nascent RNAs using these current standard platforms, improved methods for the isolation and labeling of nascent mRNAs were reported. Cleary et al. used uracil phosphoribosyltransferase gene-transformed human cells

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in *Toxoplasma gondii*. Nascent RNAs from these cells grown in 2,4-dithiouracil were labeled with thio-substrated UTP, purified, and analyzed by DNA microarray.[6] However, it is necessary to introduce this gene to the cell to be analyzed prior to DNA microarray analysis.

Bromouridine, which substitutes 5′ of uridine to bromine, is inexpensive and becomes incorporated into cells with very high efficiency. Additional steps, such as electroporation or lipofection, are not necessary for labeling mRNA with this compound. After its incorporation into cells, bromouridine is converted to Br-UTP. During transcription, converted Br-UTP is recognized as the same substrate as UTP and is incorporated into nascent RNAs.[7] This labeling method was applied in the immunocytochemical analysis by using anti-bromodeoxyuridine (BrdU) antibody in several studies.[8–11] That antibody was used also to immunoprecipitate this BrU-labeled nascent RNA.[12,13] The same reports analyzed transcriptional changes of some genes in small amounts (on the order of the picogram) of nascent RNAs.[12,13]

For a comprehensive analysis using DNA microarray, we have optimized the conditions for immunoprecipitation using BrdU antibody. We have improved the specificity of nascent mRNA isolation by orders of magnitude using adequate blocking agents in immunoprecipitation. As the amount of nascent RNA obtained was very small even under the optimized purification conditions, we sought to develop a method for amplifying small amounts of mRNA with little bias and have succeeded in this goal. Finally, we have performed transcriptional analysis of nascent RNAs using G_2 phase-synchronized mouse mammary carcinoma FM3A cells, and we have obtained several interesting insights into cell cycle regulation of the G_2 phase.

These results demonstrate that DNA microarray analysis with nascent RNAs obtained by our new method will provide valuable insights into the functions of genes in various types of cells.

2. Materials and Methods

2.1. Preparation of BrU-labeled eGFP cRNA and non-labeled luciferase cRNA

eGFP cRNA and luciferase cRNA were prepared by T7 *in vitro* transcription. DNA templates for each cRNA synthesis were constructed by PCR amplification from plasmid DNA containing eGFP or luciferase genes. These templates contain both T7 promoter sequence and polyA sequence. The DNA template of eGFP was amplified from pEGFP-c1 by PCR. The DNA template of luciferase was amplified from pTRE (Clontech, Mountain View, CA, USA) by PCR. Sequences of primers were described in 'Supplementary data'. The PCR products were purified from agarose gel by using Wizard SV gel and PCR purification kit (Promega, Madison, WI, USA).

cRNAs were transcribed with Br-UTPs (Sigma-Aldrich, St Louis, MO, USA) and NTPs using the MAXIscript® T7 kit. cRNAs were purified according to the RNA-cleanup protocol of the RNeasy Mini kit (Qiagen, Hilden, Germany).

2.2. Preparation of Mouse anti-BrdU IgG binding dynabeads

Two micrograms of mouse anti-bromodeoxyuridine antibody (Roche Diagnostics, Indianapolis, IN, USA) were incubated with 25 μl Dynabeads® Goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA) in 2.0 ml collection tubes containing 100 μl DEPC-treated phosphate-buffered saline (PBS)/0.1% bovine serum albumin (BSA) solution. The tubes were rotated at room temperature for an hour. After the dynabeads were collected by a magnet rack, the dynabeads were washed three times with 1 ml DEPC-treated PBS/0.1% BSA, and 100 μl DEPC-treated PBS /0.1% BSA was added.

2.3. Immunoprecipitation of BrU-labeled RNA by antibody beads

Following steps were conducted in the dark. Two hundred nanograms of BrU-labeled eGFP cRNA and 200 ng of non-labeled luciferase cRNA were denatured at 80°C for 10 min. As the blocking agent, 20 μg FM3A total RNA or 200 μg *Escherichia coli* 16S and 23S ribosomal RNA (rRNA) or uridine (final concentration of 0.3 M) was added. The denatured RNAs were added to the beads containing 225 U/ml RNasin® Plus RNase inhibitor (Promega). PBS(-)/0.1% BSA was added to this solution to a volume of 250 μl. The beads were rotated for 1 h at room temperature. After the beads were washed with 0.8 ml DEPC-treated PBS/0.1% BSA containing 45 U/ml RNasin® Plus RNase inhibitor (Promega), they were also washed with 0.8 ml DEPC-treated PBS three times. After the addition of 10 μl of RNase-free water, RNAs which bound to beads were eluted by denaturation at 80°C for 10 min, and then were kept cold on ice for 2 min.

2.4. Reverse transcription of cRNA and Real-time RT–PCR

2.4.1. Reverse transcription of cRNA Five microliters of the isolated RNAs were mixed with 1 μl of 50 μM oligo$(dT)_{20}$ primer and denatured at 65°C for 5 min. First-strand cDNAs were synthesized from the isolated RNAs using the SuperScript® III First-Strand Synthesis System for RT–PCR (Invitrogen).

After reverse transcription, 1 U of RNase H was reacted for 20 min at 37°C.

2.4.2. Real-time quantitative RT–PCR Real-time quantitative PCR was performed in an ABI prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using SYBR® Premix Ex Taq™ (Perfect Real Time) (Takara Bio, Otsu, Japan). Sequences of each primer were described in 'Supplementary data'. The PCR cycling program was set as follows: 95°C for 15 s followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. One of cDNAs diluted 100 times was used in a 50 μl reaction. A standard graph for eGFP cDNA and luciferase cDNA was simultaneously generated using 10^4, 10^5, 10^6, 10^7, and 10^8 copies of the pEGFP plasmid and the pTRE plasmid. Each cDNA was analyzed in triplicate. Three experiments were conducted, and the averages of the cDNA copy numbers as well as standard deviation were calculated.

2.5. Cell culture of FM3A cells and labeling nascent RNA with BrU

Mouse FM3A cells and tsFT210 cells, *Cdc2* temperature-sensitive mutant strain of FM3A cells,[14,15] were cultured in RPMI 1640 medium (Invitrogen) with 10% dialyzed bovine serum, 2 mM L-glutamine, 0.1 mM MEM non-essential amino acid (Invitrogen), and 50 U/ml penicillin–50 mg/ml streptomycin (Invitrogen) at 32°C in 5% CO_2.

After bromouridine was added to the medium to a final concentration of 100 μM, FM3A cells were cultured for an hour in the dark. FM3A cells were collected in 50 ml centrifuge tubes and washed twice with cold PBS, and total RNA was isolated by using an RNeasy midi kit (Qiagen). DNase I digestion was conducted during isolation of RNA.

2.6. Immunoprecipitation of nascent RNA from mouse FM3A cells

Twenty micrograms of total RNA from bromouridine-treated FM3A were immunoprecipitated with 200 μg of *E. coli* 16S and 23S rRNAs. The method of immunoprecipitation was described above. The amount of the eluted RNA was measured using the Quant-iT™ RiboGreen® RNA quantitation kit (Invitrogen). Five hundred nanometers of fluorescence of the RiboGreen reagent bound to RNA were measured using the fluorescence microplate reader ARVO (Perkin Elmer, Boston, MA, USA).

2.7. Spike-in experiment

Both BrU-labeled eGFP cRNA and non-labeled luciferase cRNA were added to 20 μg of total RNA from bromouridine-treated FM3A cells. We conducted three different kinds of experiments. Each experiment was performed with a different amount of cRNAs (2 ng, 200 pg, or 20 pg). These RNAs were immunoprecipitated with 200 μg *E. coli* 16S and 23S rRNAs as blocking agents.

2.8. Validation with RT–PCR of the DNA microarray data using each amplification method

Methods of experiences were described in 'Supplementary data'.

2.9. Preparation of DNA Microarray

Oligonucleotide probe sets of *Mus musculus* AROS Version 3.0 (Operon Biotechnologies, Huntsville, AL, USA) were dissolved at 10 μM in 3× SSPE solution (Invitrogen), and spotted on γ-amino propyl silane-coated UltraGAPS Coated Slides (Corning, New York, NY, USA) and UV cross-linked as described previously.[1]

2.10. cDNA synthesis and amplification

The amplification of RNA was performed using the Super SMART™ PCR cDNA Synthesis Kit (Clontech) as follows. We have modified the manufacture's protocol and optimized it for our DNA microarray system. Total RNA (20 ng) was mixed with 7 μl of 12 μM 3′ SMART CDS Primer II A and 7 μl of 12 μM SMART II A Oligonucleotide, incubated at 65°C for 2 min, and cooled to room temperature. To this mixture was added 20 μl of 5× First-Strand Buffer, 2 μl of 100 mM DTT, 10 μl of 10 mM of 50× dNTP, and 5 μl of PowerScript™ Reverse Transcriptase, and the reaction was incubated at 42°C for 90 min. After the reaction, 1 μl of 0.5 M EDTA was added. First-strand cDNA was purified with the NucleoSpin Extraction Kit (Clontech). For the amplification by long-distance PCR using the Advantage® 2 PCR Kit (Clontech), first-strand cDNA was mixed with 10 μl of 10× Advantage® 2 PCR Buffer, 2 μl of 10 mM 50× dNTP, 2 μl of 12 μM of 5′PCR Primer II A, and 2 μl of 50× Advantage® 2 Polymerase Mix. The PCR conditions consisted of a 95°C for 1 min, followed by appropriate number of cycles of amplification at 95°C for 15 s, 65°C for 30 s, and 68°C for 6 min. The appropriate number of cycling on which the PCR product will remain in the exponential phase of amplification was determined by the cDNA amplification results of the range of PCR cycles obtained in pre-experiments. The PCR product was purified with Wizard SV Gel and PCR Clean-Up System (Promega). For aminoallyl labeling, 1 μg cDNA was mixed with 15 μl of Random Primers Buffer Mixture (Invitrogen), incubated at 95°C for 5 min, and cooled on ice. Then, 5 μl of an aminoallyl–dNTP mixture which contained 5 mM aminoallyl–dUTP (Sigma), 5 mM dATP (GE healthcare, Uppsala, Sweden), 5 mM dGTP (GE healthcare), and 5 mM

dCTP (GE healthcare) was added. Next 9 U of Klenow Fragment (Invitrogen) was added and incubated at 37°C for 120 min. After the reaction, 5 μl of Stop Buffer (Invitrogen) was added. Aminoallyl-labeled cDNA was purified with ethanol precipitation following the first purification with Wizard SV Gel and PCR Clean-Up System. For the coupling reaction, aminoallyl-labeled cDNA was dissolved with 5 μl of 0.1 M $NaHCO_3$ and mixed with 5 μl of Cy3 Mono-reactive Dye (GE healthcare) or Cy5 Mono-reactive Dye (GE healthcare) in 50 μl Atlas Glass Approved DMSO (BD Bioscience), and incubated for 60 min at room temperature. After coupling, 15 μl of 4 M hydroxylamine–HCl (Sigma-Aldrich) was added and incubated at room temperature for 15 min. Cy-Dye labeled cDNA was purified with the MinElute Reaction Cleanup Kit (Qiagen).

2.11. Hybridization

These procedures were based on TF oligo microarray hybridization as described previously.[1] Some of the conditions and compositions of solutions were changed: the hybridization solution consisted of a mixture of Cy3- and Cy5-labeled cDNAs, 40% formamide, 5× SSC, 0.4% SDS, and 1.9 μg/μl acetylated BSA (Invitrogen). Hybridization was performed with a hybridization chamber (Agilent Technologies, Palo Alto, CA, USA) and a hybridization gasket slide (Agilent Technologies). The temperature in both the pre-hybridization and hybridization steps was 45°C. Hybridization images were scanned using an Agilent DNA microarray scanner (Agilent Technologies).

We hybridized four slides as self–self hybridization: Cy3-labeled cDNAs of nascent RNA from FM3A cells and Cy5-labeled cDNA of nascent RNA from FM3A cells.

In experiments using G_2 phase-arrested cells, we hybridized four slides: Cy3-labeled cDNAs of asynchronous cells and Cy5-labeled cDNAs of G_2 phase-arrested cells were hybridized in two slides, and dye-swapped cDNAs were hybridized in the other two.

2.12. DNA microarray analyses

Fluorescence intensities in spots were quantified using Feature Extraction software version GX (Agilent Technologies). Spots flagged as non-uniform outliners of either a feature (spot) or background were removed. In each array, genes whose expression levels were lower than those of the negative control spots under both conditions were removed. The ratios of signal intensities between the two cell types were calculated through Lowess normalization using GeneSpring GX (Agilent Technologies). We calculated the average and the standard deviation of the base 2 logarithm of the expression ratios of four spots and removed the expression ratios of genes whose standard deviation was >1. The expression ratios of genes whose average signal intensity was <50 were also removed.

2.13. Analyses of nascent RNA profiles in G_2-arrested cells and asynchronous cells

tsFT210 cells were arrested in the G_2 phase by incubation for 17 h at a non-permissive temperature (39°C). Bromouridine was added to the medium to a concentration of 100 μM at 16 h after the shift at 39°C. RNA was isolated using the RNeasy mini kit (Qiagen). DNase I digestion was conducted during isolation of RNA. The DNA microarray analyses are described above.

2.14. Cell cycle analysis

Cells were harvested, washed with PBS, and fixed in 70% ethanol at −20°C for at least 4 h. Fixed cells were washed twice with PBS and mixed with PBS containing 0.3% Triton X-100 (w/v) (Sigma-Aldrich). Cells were added with PBS (−) containing 50 μg/ml propidium iodide (Sigma-Aldrich) and 0.1 mg/ml RNase A (Sigma-Aldrich), and incubated for 20 min at 37°C in the dark before being filtered through a 41 μm nylon mesh. We analyzed the DNA contents by measuring the intensity of the fluorescence produced by propidium iodide using the FACSCalibur instrument (Beckon Dickinson, Mountain View, CA, USA) using instructions supplied by the manufacturer.

3. Results and Discussion

3.1. Experimental scheme of DNA microarray analysis for the nascent RNA

The new method for performing comprehensive analyses of nascent RNA is illustrated in Fig. 1. In the first step, bromouridine was added to the culture medium. This compound was incorporated into cells, which converted it to Br-UTP. As BrUTP is recognized as the same substrate as UTP, nascent mRNA is labeled by BrU[7] (Fig. 1). Total RNAs isolated from the BrU-treated cells were immunoprecipitated with anti-BrdU antibody beads.[12,13] The RNAs bound to the beads were eluted by heat denaturation after washing, and the eluted mRNAs were reverse-transcribed using oligo-dT primer. The cDNAs were linearly amplified using the template-switching (TS) PCR method. The amplified cDNAs labeled with Cy3/Cy5 were used for DNA microarray analysis (Fig. 1). Among these steps, the method for immunoprecipitating the BrU-labeled nascent RNAs was based on a method described previously[12,13] and optimized for DNA microarray analysis in the following manner.

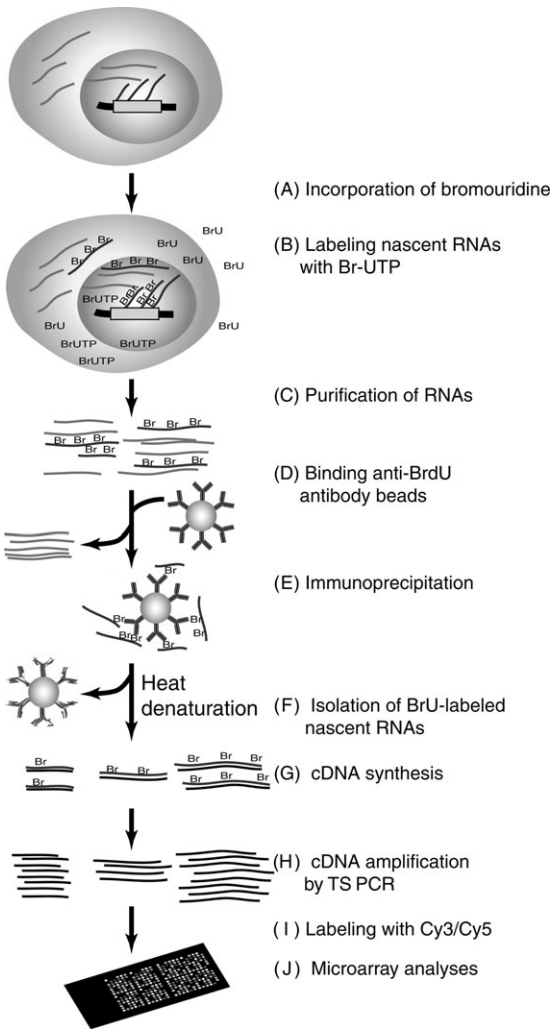

Figure 1. Overview of the isolation of nascent RNAs for microarray analysis.

Approximately 50 pg of the BrU-labeled RNA from mouse pre-implantation embryos was obtained and subjected to the same analysis as used in a previous study.[13] The RNA amount should be >20 ng in total RNA. This is $\sim$1000 times more than that in the previous study.

Therefore, it was necessary to develop a method for immunoprecipitating a large amount of BrU-labeled RNA with high specificity.

3.2. *Determination of optimum conditions for enriching nascent RNAs using cRNAs synthesized in vitro*

As the amount of BrU-labeled nascent RNAs is much smaller than that of non-labeled RNAs in the RNA fractions extracted from living cells, it was necessary to develop a method for obtaining BrU-labeled RNAs with high recovery as well as on a certain scale. In the first trial, a mixture of BrU-labeled eGFP cRNAs and non-labeled luciferase cRNAs prepared by T7 *in vitro* transcription was immunoprecipitated in the same tube. cRNAs eluted from the affinity beads were reverse-transcribed with oligo-dT primer, and the copy number of each cDNA was quantified by real-time quantitative PCR (Fig. 2A). We defined the ratio of the eGFP cDNA copy number to the luciferase cDNA copy number as the index of immunoprecipitation specificity. This index well reflects the enrichment efficiency of nascent mRNAs. We also examined the effects of adding blocking agents such as uridine, total RNA from FM3A cells, and *E. coli* 16S and 23S rRNAs. Although interferon-τ RNA was shown to work well as a blocker in a previous report,[13] we chose uridine, *E. coli* rRNA, and mouse total RNA as candidates for a blocking agent because we thought they would be more suitable for large-scale isolation of nascent RNAs. We evaluated the effects of these blocking agents with increasing amounts of cRNA. Under our conditions, the addition of uridine did not change the ratio. In contrast, the ratios observed in the experiments with *E. coli* rRNA or FM3A total RNA were approximately double those obtained in an experiment carried out without RNAs as the blocking agent (Fig. 2B). These data suggest that any kind of RNA can increase the specificity of the immunoaffinity purification of nascent RNAs. We thus chose *E. coli* 16S and 23S rRNAs as suitable blocking agents. These bacterial rRNAs are inexpensive and do not exist in mammalian cells. In addition, *E. coli* 16S and 23S rRNAs should not be reverse-transcribed when mRNA is reverse-transcribed by using oligo-dT primer.

3.3. *Conditions for labeling nascent RNAs with BrU*

Next, we treated living cells with bromouridine and immunoprecipitated BrU-labeled nascent RNAs from BrU-treated cells. We first tested and determined the appropriate concentration (100 μM) of bromouridine, i.e. that which did not affect proliferation and cell cycle progression in mouse FM3A cells (data not shown). Using DNA microarray analysis, we also confirmed that the gene expression profile of FM3A cells after treatment with 100 μM bromouridine for 4 h was extensively the same as that of untreated cells (data not shown). Approximately 20 μg total RNA was isolated from 2×10^6 FM3A cells that had been treated with 100 μM bromouridine for 1 h; this total RNA was then subjected to immunoaffinity purification. We used *E. coli* 16S and 23S rRNAs as blocking agents. The amount of eluted RNAs in the sample of BrU-treated cells was $\sim$30 ng, so we had successfully obtained a sufficient amount of BrU-labeled RNA for DNA microarray analyses. We then evaluated the specificity of immunoaffinity purification.

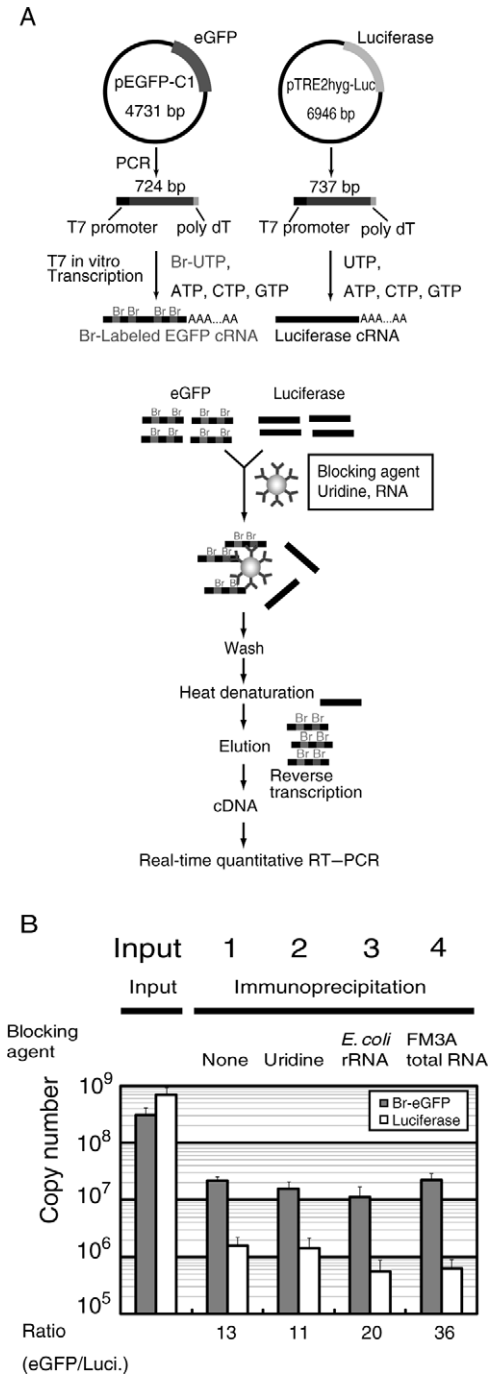

Figure 2. Binding specificity of BrU-labeled cRNA by *in vitro* transcription. (**A**) eGFP and luciferase gene were transcribed *in vitro* by T7 RNA polymerase. Br-UTP was used as the substrate instead of UTP during the transcription of eGFP. (**B**) Mixture of 200 ng of BrU-labeled eGFP and non-labeled luciferase cRNA was immunoprecipitated with addition to blocking agents. The eluted cRNAs were converted into cDNA, and then the cDNA copy number was measured using real-time quantitative PCR. The blocking agents used are as follows: (i) no addition, (ii) uridine, (iii) *E. coli* 16S and 23S RNAs, and (iv) total RNA from mouse FM3A cells. 'Ratio (eGFP/Luci.)' represents the ratio of the eGFP cDNA copy number to the luciferase copy number. Error bars represent standard errors of the means ($n = 3$).

3.4. *Evaluation of purification efficiency with spike-in control*

To more precisely estimate the efficiency with which nascent RNAs were purified, small amounts of T7 *in vitro* transcribed BrU-labeled eGFP cRNA and non-labeled luciferase cRNA were added to the RNAs obtained from BrU-treated FM3A cell samples as the 'spike-in' control. We immunoprecipitated these RNA mixtures and compared the cDNA copy numbers of eluted eGFP cDNA and luciferase cDNA. To determine whether or not the amount of the eluted BrU-labeled eGFP cRNA was proportional to that of the input eGFP cRNA, increasing amounts (20 pg, 200 pg, and 2 ng) of eGFP cRNA and luciferase cRNA were added to 20 μg of BrU-labeled total RNA obtained from FM3A cells (Fig. 3A). The ratio of eluted eGFP cDNA to eluted luciferase cDNA exceeded 83 under the conditions shown in Fig. 3B. In addition, the copy number of eluted BrU-labeled eGFP cRNA was proportional to that of the input eGFP cRNA. These results indicated that we were able to immunoaffinity-purify BrU-labeled RNA with high specificity and that the eluted RNA reflects the nascent RNA before immunoprecipitation. Taken together, these findings indicate that we have successfully optimized the conditions for immunoaffinity-purification with high specificity. It was already demonstrated that the enrichment process using anti-BrdU immunoaffinity beads does not have any bias.[13] As it is important to confirm that the cDNA amplification process does not have any significant bias, we performed the following experiments.

3.5. *DNA microarray analyses of nascent RNAs*

To carry out DNA microarray analysis, nascent RNAs of BrU-treated cells have to be amplified without bias. We previously developed a method for effectively amplifying small amounts of RNA using the TS-PCR reaction[16] (Fig. 4A). To examine whether or not this amplification method has any amplification bias, we compared the data obtained by DNA microarray analysis with those obtained by quantitative real-time PCR using RNA samples prepared from ES cells.[1] We considered that the efficiency of amplification would vary according to the differences in cellular RNA abundance. Genes with abundant RNA would be amplified without bias, but genes with low amounts of RNA might fail to be amplified accurately. As the expression levels of transcription-factor genes are generally much lower than those of genes in other categories, we focused on expression changes of the transcription factor genes. We used the TF oligo microarray, which can accurately determine expression changes of transcription factor genes.[1] As shown in Fig. 4B, the expression changes in TS-PCR

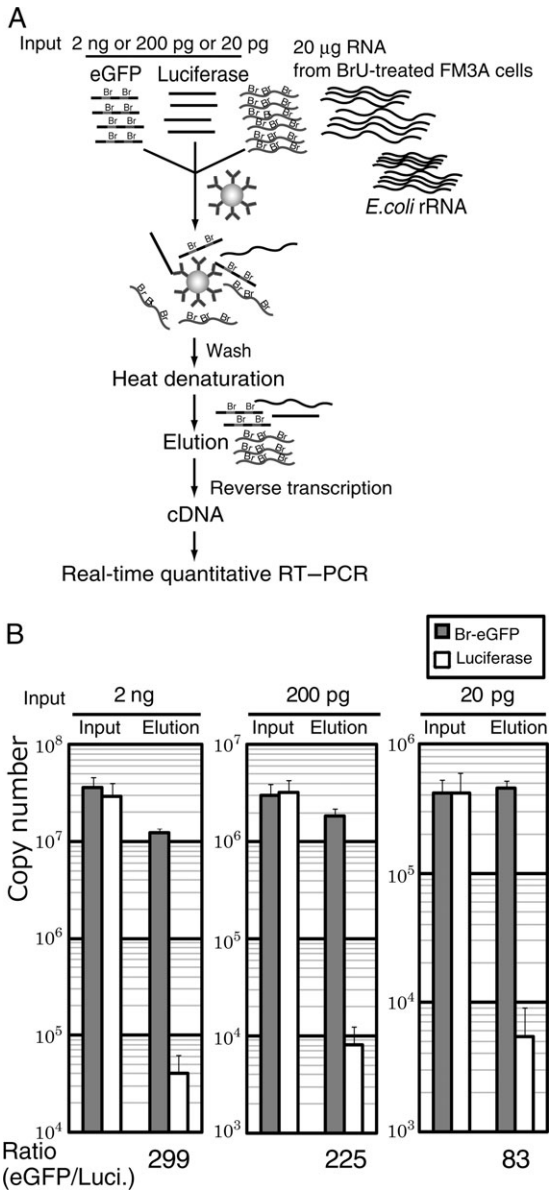

Figure 3. Binding specificity in immunoprecipitation with spike-in controls. (**A**) Overview of the experiment. (**B**) Br-eGFP and luciferase cRNA were prepared as spike-in controls. 'Ratio (eGFP/Luci.)' represents the ratio of the eGFP cDNA copy number to the luciferase copy number. Error bars represent standard errors of the means (n = 3).

amplification were detected accurately, without amplification bias, except for *Prdx1*, *Rara*. Especially, the fold changes of *Dcx*, *Meis1*, and *Ttyh1* obtained by TS PCR were not only quite similar to the data obtained without amplification, but were also similar to the data from quantitative real-time PCR, whereas amplification by *in vitro* transcription with T7 promoter exhibited some discrepancies. Thus, amplification by TS PCR has very little bias (Fig. 4B).

However, there was a possibility that amplification efficiency and the reverse-transcription efficiency of BrU-labeled mRNA were different from that of non-labeled mRNA, so we compared the agarose-gel electrophoresis pattern of amplified cDNA from BrU-labeled mRNA (nascent mRNA) with that from non-labeled mRNA (steady-state mRNA). The result obtained by agarose-gel electrophoresis experiment of amplified cDNA from BrU-labeled mRNA shows a smear pattern identical to or very similar to that of amplified cDNA from non-labeled mRNA (data not shown). This result suggests that both the reverse-transcription efficiency and the amplification efficiency were almost identical between BrU-labeled RNA and non-labeled RNA. A self–self hybridization experiment was conducted with amplified cDNA labeled with Cy3/Cy5, and we detected gene expression levels with broad signal ranges. The ratios of Cy5 to Cy3 in all genes were in the range of 0.5–2.0 (Fig. 4C), indicating that our DNA microarray analysis is highly reproducible.

3.6. *DNA microarray analysis of nascent RNA in mouse G_2 phase-arrested FT210 cells*

We then compared the expression profiles of nascent RNAs of 32 000 transcript products between asynchronous mouse FM3A cells and G_2 phase-synchronized FM3A cells. To obtain a highly synchronized population of G_2 phase cells, we chose tsFT210 cells, which are temperature-sensitive *Cdc2* mutant cells of mouse mammary carcinoma FM3A cells.[14,15] tsFT210 cells are grown at 32°C (the permissive temperature), but in the G_2 phase, they are arrested at 39°C (Fig. 5A). With this cell line, a highly synchronized population of cells is easily available by just shifting the culture temperature. It has already been shown that such synchronization is merely a result of the inactivation of just one protein (CDC2 protein).[15] We also analyzed steady-state RNA expression profiles of asynchronous cells and G_2-synchronized cells. Scatter plots of the expression level are shown in Fig. 5B. Considering the variation of DNA microarray data, we regarded the genes that showed changes exceeding twofold as either up- or down-regulated genes. Figure 5C shows Venn diagram analyses of genes whose expression was up- or down-regulated more than twofold in nascent RNA and steady-state RNA. There was no gene whose nascent RNA was up-regulated, whereas its steady-state RNA was down-regulated, and vice versa.

Among the genes whose nascent RNA levels changed more than twofold, cell cycle progression-related genes are listed in Table 1. Of the 18 genes listed, 14 genes exhibited fold changes in the analysis of nascent RNA but not in that of steady-state RNA.

We found interesting expression profiles in *Cdkn1a*. *Cdkn1a* (*p21*) was up-regulated in both analyses (Table 1). Although this gene was previously reported

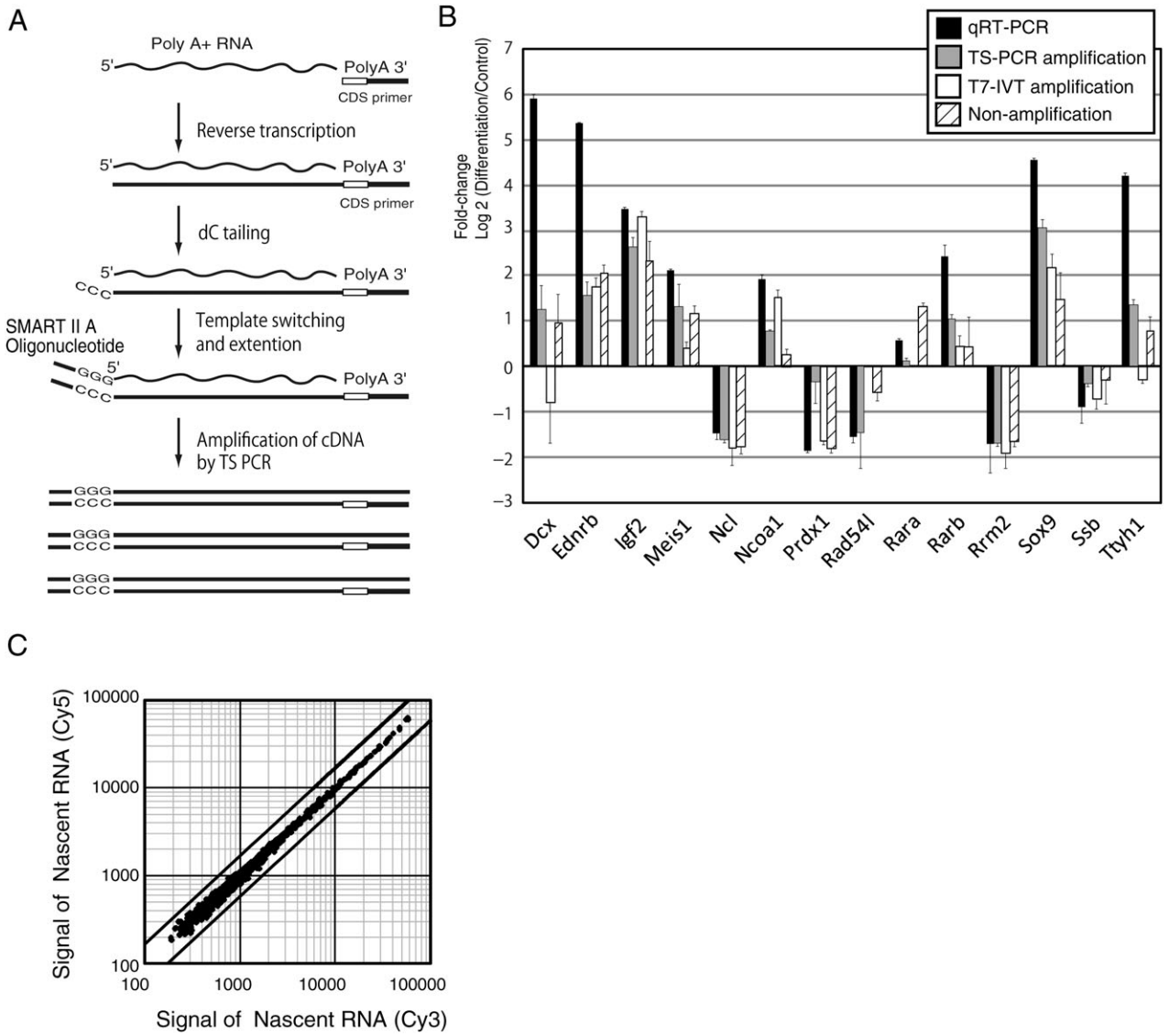

Figure 4. (**A**) Eluted RNAs were converted to cDNA, and cDNA was amplified by TS-PCR. (**B**) Validation with quantitative RT–PCR of the DNA microarray data using each amplification method. Genes expressed at various levels in the non-amplification method, TS-PCR amplification method, and T7 *in vitro* transcription (IVT) amplification method were confirmed using quantitative RT–PCR. Quantitative RT–PCR was performed using the total RNA of these transcription factors. *Gapd* was used as the control. The same total RNA was used for quantitative RT–PCR and DNA microarray experiments. The ratio of the total target RNA levels was normalized by using those of the *Tbp* (*Mus musculus* TATA box binding protein) gene, and the fold change for each gene in the differentiated ES cells compared to the control ES cells was calculated. Error bars represent standard deviations of log (base 2) of ratio. (**C**) Amplified cDNA labeled with Cy3/Cy5 underwent self-self hybridization.

to function as a signal transducer involved in the transition from the G_1 phase to the S phase or from the G_2 phase to the M phase,[17] the changes in the expression profiles of the gene at the G_2 phase were not analyzed. As up-regulation of the steady-state RNA and nascent RNA of this gene is clearly observed, it follows that this gene may have an important function in the transition from the G_2 phase to the M phase.

In addition to *Cdkn1a*, we have found that *Pak1* and *Pak2* also exhibited interesting expression profiles. The changes in expression levels of these genes were detected in the analysis of only the nascent RNAs. The PAK family is involved in microtubule and actin cytoskeleton reorganization.[18] PAK1 and PAK2 interact with centrosomal kinase Aurora-A, and activated PAK1 phosphorylates Aurora-A in mitosis.[19] On the other hand, the injection of 58 kDa active Pak1 into *Xenopus* oocytes inhibits cell cleavage by arresting G_2/M progression,[20] suggesting that these products have different role at these phases. These results of DNA microarray analysis indicate that mRNAs of these genes were not only synthesized but also degraded between the G_2 phase and the M phase. As it is likely that synthesis and degradation occur in

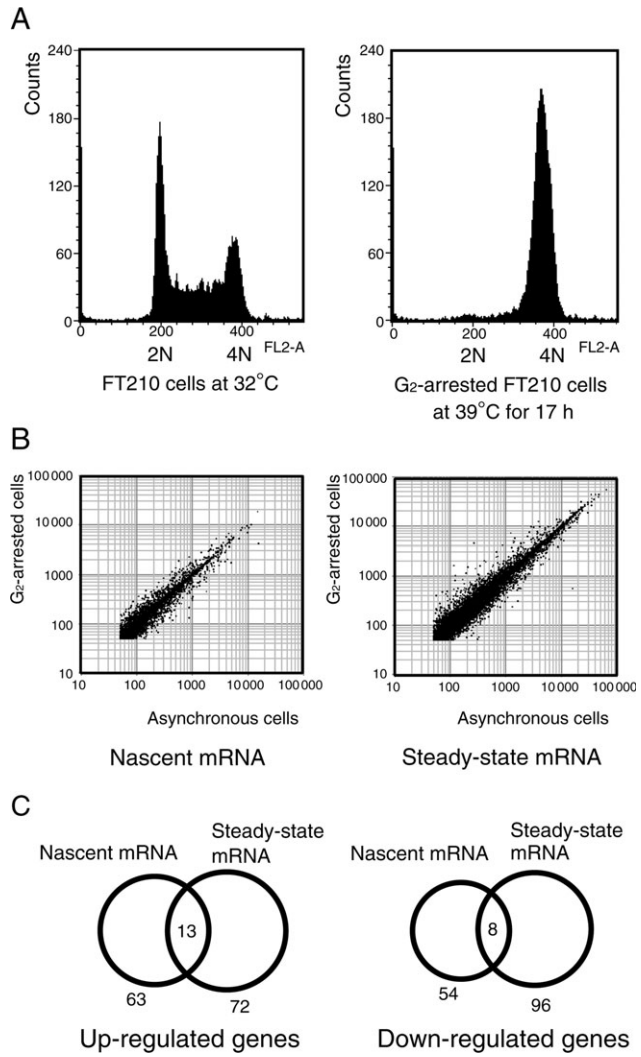

Figure 5. DNA microarray analyses of nascent RNAs. (**A**) FT210 cells were synchronized in the G_2 phase by incubation at 39 °C for 17 h. Cell cycle profiles of asynchronous cells and G_2-arrested cells were analyzed by propidium iodide staining and flow cytometry. (**B**) Scatter plots comparing the expression profiles of asynchronous cells with those of G_2-arrested cells in a nascent RNA profile (left panel) and a steady-state RNA profile (right panel). (**C**) The Venn diagrams show the overlap of genes whose expression levels changed more than twofold in the nascent RNA profile and the steady-state RNA profile.

Table 1. The partial lists of cell cycle related genes whose level of nascent RNAs changed more than twofold in G2 arrested cells

Gene symbol	Fold changes of nascent RNA	Fold changes of steady-state RNA	Description
Cdkn1a	2.00	3.29	Cyclin-dependent kinase inhibitor 1A (P21)
Pdgfa	2.41	3.02	Platelet derived growth factor, alpha
Ppm1b	2.15	1.10	Protein phosphatase 1B, magnesium dependent, beta isoform
Bin1	2.53	1.41	Bridging integrator 1
Cdkl2	2.16	1.68	Cyclin-dependent kinase-like 2 (CDC2-related kinase)
Arhgef5	2.26	0.90	Rho guanine nucleotide exchange factor (GEF) 5
Ppp1r15b	2.03	0.92	Protein phosphatase 1, regulatory (inhibitor) subunit 15b
Klf3	2.44	1.23	Krüppel-like factor 3 (basic)
Pak2	2.11	0.94	P21 (CDKN1A)-activated kinase 2
Pdpk1	3.30	1.04	3-phosphoinositide dependent protein kinase-1
E2F5	2.13	0.83	E2F transcription factor 5
Pak1	2.09	1.47	P21 (CDKN1A)-activated kinase 1
Gchfr	2.84	1.12	GTP cyclohydrolase I feedback regulator
Tk1	0.41	0.46	Thymidine kinase 1
Ak1	0.40	0.48	Adenylate kinase 1
Dusp1	0.38	1.52	Dual specificity phosphatase 1
Stk17b	0.38	0.60	Serine/threonine kinase 17b (apoptosis-inducing)
Pnrc2	0.29	0.89	Proline-rich nuclear receptor co-activator 2

the same period, it seems difficult to identify the changes in expression levels of these gene mRNAs just by analyzing the steady-state levels.

We also found that *Rplp2*, *Rpl27*, *Rps25*, *Rpl9*, and *Rps29*, which are categorized as ribosomal proteins, were down-regulated at the G_2 phase (Table 2). Interestingly, the changes in the expression of these genes were not detected in steady-state RNA, although they were detected in the nascent RNA analysis (Table 2). Changes in gene expression were not detected in other ribosomal proteins in both analyses. Therefore, it is possible that these five ribosomal proteins have distinct features in their gene expression regulatory mechanisms. A previous study predicted binding sites in transcriptional regulatory regions or promoters of mammalian ribosomal protein genes for transcriptional factors.[21] Those authors observed that genes for these five ribosomal proteins lack TATA-box and A/T-rich sequences similar to the TATA box, whereas two-thirds of the genes for ribosomal proteins have the TATA box or its homolog in their promoter regions. Another study found that a unique sequence (a tandem repeat of the TCTCGCGAGA motif) does exist among many human genes that lack the TATA box.[22] This sequence motif was found at especially high frequencies in the genes for cell cycle regulators as well as in human ribosomal

Table 2. The lists of ribosomal proteins whose level of nascent RNAs changed more than twofold in G2 arrested cells

Gene symbol	Fold change of Nascent RNA	Fold change of steady-state RNA	Description	TATA box	
Rplp2	0.36	0.63	Ribosomal protein, large P2	−	
Rpl9	0.49	0.98	Ribosomal protein L9	−	
Rps29	0.48	1.02	Ribosomal protein S29	−	
Rps25	0.41	0.99	Ribosomal protein S25	−	
Rpl27	0.49	1.00	Ribosomal protein L27	−	
Average of fold change of all ribosomal proteins	0.94	0.95		−	35% (28 genes)
				+	65% (51 genes)*

Presence or absence of TATA box and A/T rich similar sequence was based on the Perry et al.[21]
*Ribosomal protein genes which have TATA box or A/T rich tract in those upstream region.

proteins. This sequence motif was also found in the upstream regions of three genes (*Rpl9*, *Rps29*, and *Rps25*) out of the five genes that exhibit changes in gene expression in only the nascent RNA analysis. As translational control is also regarded as an important mechanism for cell cycle regulation, it is possible to obtain new insight into cell cycle regulation in the G_2 phase by performing functional analyses of these ribosomal proteins.

3.7. *Applications of comprehensive nascent RNA expression analyses*

These examples indicate that our DNA microarray analysis of nascent RNAs has advantages over DNA microarray analysis of steady-state mRNAs. One advantage is that it can provide numerous insights into the regulatory mechanism underlying gene expression. For example, as the changes in expression levels of many transcription factors are relatively small in the process of cellular differentiation, our new method will give us novel insights into the structure of the gene expression regulatory network, since this method can detect slight expression changes. Collecting time-course data on nascent RNAs will also provide important information.

Recently, Kenzelmann et al.[23] reported that nascent RNAs could be analyzed by DNA microarray. In their report, the nascent RNAs were mRNAs labeled using 4-thiol uridine and then purified using affinity beads whose surfaces were coated with a toxic organomercury compound.[23] In our method, precipitation was performed using Dynabeads, which are commonly used in classical immunoprecipitation and do not contain a heavy metal. This is another advantage of our method. Although the DNA microarray used in this study is an oligo-DNA microarray, this method is applicable to other platforms such as cDNA microarray or Affymetrix's GeneChip.

In summary, our study provides very useful techniques for isolating nascent RNAs with high specificity. By comparing the data between steady-state RNAs and nascent RNAs, we can analyze changes in gene expression with much higher sensitivity. We can also determine the kinetics of transcription by combining the DNA microarray study of nascent mRNAs with that of existing mRNAs. The application of such an analysis will provide important information in the field of transcriptome analysis. Microarray data of transcripts whose nascent RNAs labels changed more than twofold in G_2 arrested cells are showed in supplementary data.

Supplementary Data: Supplementary data are available online at www.dnaresearch.oxfordjournals.org.

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